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Matrix-assisted protein refolding

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For my parents

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2 Summary

The fast and efficient production of proteins is of great importance for both basic research and the biotech industry. Elucidation of numerous genome sequences enabled cloning and recombinant expression of thousands of DNA targets. Recombinant protein expression in *E. coli* is very popular, due to the high yield and low costs of the protein product. However overexpression of hetereologous proteins in *E. coli* often leads to the formation of large insoluble aggregates, the so called inclusion bodies. As formation of inclusion bodies has been discovered many years ago, several strategies have been developed to obtain native functional protein from IBs. Estimation of optimal refolding conditions involves choice of the appropriate refolding method, protein concentration, incubation time, temperature and refolding buffer.

The optimal refolding buffer is defined by parameters like basic buffer component, pH, ionic strength, redox potential, refolding promoting additives and their concentration, and has to be determined for each particular protein experimentally. Different approaches to identify the optimal refolding buffer by fractional factorial screening have been introduced by several companies and research groups; however, none of them offers the ultimate solution for the problem and guarantees a positive outcome. Additionally, all of the proposed screening procedures address refolding procedure by dilution.

At the same time optimization of refolding conditions for matrix-assisted refolding techniques remain underrepresented. In this work, IMAC (immobilized metal affinity chromatography) was applied to identify optimal buffer conditions for the refolding of his-tagged IBs protein. The experiments were carried out in 96 well format in HisMultiTrap plates (GE Healthcare). To minimize the number of buffer conditions in the screening procedure a stepwise approach was introduced. In the first step the basic buffer component and the pH of the buffer were determined, followed by the adjustment of the ionic strength of the buffers, in a second step. In the next steps, the influence of different additives on the efficiency of refolding was examined. The optimization of the refolding buffer was successful for six test proteins. Additionally, the efficiency of matrix-assisted refolding was compared with that of dilution-based methods. For all proteins tested, matrix-assisted refolding proved to be better or equal. Furthermore matrix-assisted refolding could be efficiently carried out at 1000 fold higher protein concentrations, compared to refolding in solution. Additionally analysis of refolding kinetics showed that refolding on-matrix was faster than in solution for all proteins tested.

Up-scaling of refolding under the determined optimal refolding condition was possible for all tested proteins and resulted in the similar refolding yields.

β-galactosidase (β-gal), a large tetrameric protein, was one of the proteins used in this work. Refolding of GdmCl denatured β-gal in solution was reported to be inefficient in the absence of molecular chaperones. Application of the stepwise optimization strategy combined with matrix-assisted refolding enabled identification of optimal refolding conditions for ß-gal and resulted in the increase of refolding efficiencies up to 30%. Subsequently, the mechanism of assembly and oligomerization of the ß-gal was addressed. The experiments reveal that correctly folded monomers of ß-gal are able to assemble in tetramers, in the matrix-bound state, if they are in an appropriate proximity. Folded monomers lacking interaction partners are still able to undergo assembly process during and after the elution process.

In summary, systematic analysis of matrix-assisted refolding revealed general reaction principles, which may be applied for a wide range of proteins. A generalized, rational strategy to optimize refolding conditions of IBs proteins proved efficient for the tested proteins. In the multi-factorial space of possible refolding buffers, the step-wise optimization allows to efficiently minimize the number of experiments. Additionally, up-scaling of the optimized refolding procedure is directly and easily possible. Analysis and comparison of refolding kinetics and concentration dependencies, showed the superiority of matrix-assisted refolding over folding in solution, which might suggest that it follows an alternative folding pathway.

3 Zusammenfassung

Die schnelle und effiziente Produktion von Proteinen ist sowohl für die Grundlagenforschung, als auch für die Industrie von zentraler Bedeutung. Rekombinante DNA-Technologie und Gentransfer eröffneten die Möglichkeit, Proteine heterolog in Wirtsorganismen zu exprimieren. Auf Grund der einfachen Handhabung, der hohen Proteinproduktion und den niedrigen Kosten, ist *E. coli* hierbei der am häufigsten eingesetzte Wirtorganismus. Jedoch führt die Überexpression von Fremdproteinen in *E. coli* häufig zur Bildung von großen, unlöslichen Aggregaten, den so genannten Einschlusskörperchen oder *inclusion bodies* (IBs).

Nach Entdeckung der IB-Bildung vor vielen Jahren, wurden mehrere Strategien zur Gewinnung des nativen funktionellen Proteins entwickelt. Zur Bestimmung der optimalen Rückfaltungsbedingungen müssen sowohl die Rückfaltungsmethode, als auch Proteinkonzentration, Inkubationszeit und Rückfaltungspuffer berücksichtigt werden. Der optimale Rückfaltungspuffer wird durch die Pufferkomponente, den pH-Wert, die Ionenstärke, das Redoxpotential und die Rückfaltung fördernde Additive und deren Konzentration definiert. Diese Parameter müssen experimentell an jedes Protein angepasst werden. Zur Bestimmung der optimalen Pufferbedingungen werden alle Teilfaktoren variiert. Die meisten bisher eingesetzten Verfahren setzen dabei „die Rückfaltung durch Verdünnung“ ein. Die Matrix-assistierte Optimierung der Rückfaltungsbedingungen bleibt dabei weitgehend unterrepräsentiert. In dieser Arbeit wurde Ni-Affinitätschromatographie eingesetzt, um die optimalen Pufferbedingungen für die Rückfaltung His-getagter inclusion body–Proteine zu ermitteln. Die Experimente wurden im 96-well-Format durchgeführt. Um die Anzahl der Pufferbedingungen im Screeningverfahren zu minimieren, wurde die Optimierung schrittweise ausgeführt. Im ersten Schritt wurden die Pufferkomponente und der pH-Wert bestimmt, gefolgt von der Anpassung der Ionenstärke im zweiten Schritt. Danach wurde die Auswirkung verschiedener Additive auf die Rückfaltungseffizienz ermittelt. In dieser Arbeit konnte der Rückfaltungspuffer für sechs getestete Proteine erfolgreich optimiert werden. Außerdem wurde die Effizienz der Matrix-assistierten Rückfaltungsmethode mit der Effizienz der Rückfaltung durch Verdünnung verglichen. Es stellte sich heraus, dass für alle getesteten Proteine die Matrix-assistierte Rückfaltung gleich oder besser geeignet und bei einer tausendfach höheren Proteinkonzentration möglich war. Zusätzlich zeigte die Analyse der Rückfaltungskinetik eine schnellere Rückfaltung aller Proteine auf der Matrix als in Lösung. Ein direkter Scale-up war für alle Proteine unter den ermittelten optimalen Pufferbedingungen möglich und ergab ähnliche Rückfaltungsausbeuten.

Eine der getesteten Proteine war die β-Galaktosidase (β-Gal) aus *E. coli*, ein großes tetrameres Protein. Bisher konnte keine effiziente Rückfaltung von chemisch denaturierten β-Gal in Abwesenheit von molekularen Chaperonen erzielt werden. In dieser Arbeit konnte mit Hilfe der schrittweisen Optimierungsstrategie und der Matrix-assistierten Rückfaltung eine 30%ige Rückfaltungsausbeute erlangt werden. Außerdem wurde der Mechanismus der Assemblierung und Oligomerisierung der β-Gal auf der Matrix näher untersucht. Es stellte sich heraus, dass die matrixgebundenen Monomere in der Lage sind zu Tetrameren zu assemblieren, solange sie sich in ausreihender Nähe zueinander befanden. Monomere, die keine Interaktionspartner auf der Matrix gefunden hatten, assemblierten darüber hinaus während oder nach der Elution.

Zusammenfassend lässt sich sagen, dass durch die systematische Analyse ein tieferes Verständnis für die grundlegenden Prinzipien der Matrix-assistierten Rückfaltung aufgeklärt werden konnte. Das konnte auf viele verschiedene Proteine effizient angewendet werden und stellte somit eine allgemeine Strategie zur Optimierung der Rückfaltungsbedingungen dar. Durch die schrittweise Optimierung des Rückfaltungspuffers konnte die große Zahl an möglichen Pufferzusammensetzungen auf eine übersehbare Zahl an Experimenten minimiert werden.

4 Introduction

Proteins are complex macromolecules that play an important part in almost all biological processes. The human genome is estimated to encode 28,913 distinct proteins, which is a great number of potential targets for structural and functional investigation as well as for pharmaceutical design (Trimpin and Brizzard, 2009). It is not surprising that the number of approved protein-based therapeutics is constantly growing, 50% of all new drugs are classified as biopharmaceuticals. Additionally, proteins are the main target of numerous pharmaceuticals themselves (Basu et al., 2011; Kamionka, 2011). Advances of modern technology enabled development of numerous powerful techniques that allow deep insight into the structure, mode of action and function of many proteins, however a sufficient quantity of a purified protein has to be obtained first. The technology of DNA recombination allows proteins to be produced on a large scale. Several expression systems are usually employed for this purpose, i.e. *E. coli*, yeast, insects and mammalian cells. Each of the expression systems offers its advantages; however, recombinant proteins are most commonly expressed in *E. coli*, as large amounts of protein product can be produced fast and at low costs.

4.1 Protein folding *in vivo* and molecular chaperones

Proteins are synthesized as unfolded linear polypeptide chains. To fulfill their biological functions they have to adopt a functional three-dimensional structure. The folding process is very complex and is driven by many weak non-covalent interactions, where hydrophobic interactions between amino acid side chains seem to play an important role (Walter and Buchner, 2002). This process can be described by the concept of a folding funnel: polypeptide chains explore the funnel-shaped rugged energy landscape towards the native structure, which is defined by a global minimum energy (Dill, 1990). Intermediates that are formed during the folding process might get kinetically trapped in local energy minima, when not able to overcome certain barriers within the energy landscape (Bartlett and Radford, 2009; Hartl and Hayer-Hartl, 2009).

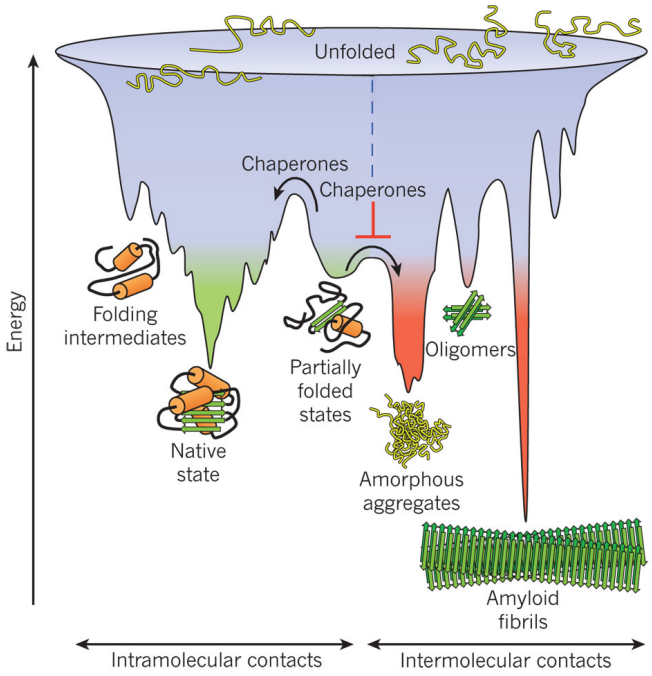


Figure 1 Scheme of the folding funnel: proteins explore the funnel-shaped rugged energy landscape as they move towards the native structure (green), which is defined by a global minimum energy

Folding intermediates might get kinetically trapped in the ruggedness of the free-energy and have to overcome the free-energy barriers to reach a favorable downhill path. *In vivo*, these steps might be facilitated by molecular chaperones. Concurrent folding of several molecules in the same compartment might result in overlapping of the free-energy surface of folding with that of intermolecular aggregation, resulting in the formation of aggregates, toxic oligomers or ordered amyloid fibrils (red). (Hartl et al., 2011)

These intermediates are usually transient, and may either aggregate, driven by hydrophobic forces, in the concentration dependent manner, or further remain in the on-pathway to the native structure (Bartlett and Radford, 2009; Hartl et al., 2011; Seckler and Jaenicke, 1992).

The native state of proteins is characterized by defined secondary, tertiary and quaternary structure. During the folding process of the nascent polypeptide chain, secondary structure elements α-helices and β-strands are formed first, followed by the formation of the three dimensional tertiary structure, and, in case of higher oligomeric state, subsequent assembly into the quaternary structure (Kim and Baldwin, 1982; Dill, 1990) .

As postulated by Anfinsen, the tertiary structure of the protein is defined alone by its amino acid composition, and can be reconstituted *in vitro* as a self-assembly process if appropriate conditions are given (Anfinsen, 1973). However, it has been shown that in the living cells protein factors are involved in the folding of the polypeptides, which are referred to as molecular chaperones (Seckler and Jaenicke, 1992; Walter and Buchner, 2002). As many of them have been discovered in correlation with the heat shock response, they are also called heat shock proteins or Hsps (Lindquist and Craig, 1988).

Molecular chaperones are a group of proteins related in their function. They do not provide any additional steric information for the folding of the target protein, their role is to suppress

nonproductive interactions, and in that way help the protein to acquire its native conformation (Hartl et al., 2011; Seckler and Jaenicke, 1992; Walter and Buchner, 2002)

Most chaperones can be defined by common functional features. In the first place, it is their ability to bind hydrophobic patches of unfolded, misfolded or partially folded polypeptides, and thus prevent aggregation process. Due to low specificity of the hydrophobic interaction, chaperones can bind to a very wide range of proteins. Secondly, some chaperones seem to be capable of initiation of conformational changes in the bound proteins, e.g. disruption of non-native contacts in the misfolded species. Third feature of molecular chaperones is their ability to release bound polypeptides in a controlled, usually ATP dependent manner (Walter and Buchner, 2002).

Molecular chaperones cover a wide range of cellular functions, i.e. de novo folding, refolding of stress denatured proteins, intracellular protein transport, oligomeric assembly and assistance in proteolytic degradation (Hartl and Hayer-Hartl, 2009). Chaperones can be divided in classes or families according to their molecular weight. Members of Hsp70 family primarily bind to hydrophobic patches exposed by the non-native proteins and thereby prevent their aggregation and promote their folding through an ATP-dependent cycle of binding and release (Richter et al., 2010). Hsp70 proteins represent a central part of a ubiquitous chaperone system that is present in most compartments of the eukaryotic cell, in eubacteria and archaea.

Chaperonins are large two ringed complexes; which can be divided in two groups. Representatives of the first group also called Hsp60s can be found in bacteria (GroEL), choloplats and mitochondria. They functionally cooperate with Hsp10s (GroES in bacteria), which form the lid of the folding cage. Chaperonins of the second group are found in archaea and in the cytosol of the eukaryotic cells. They are Hsp10-independent, with the lid being formed by the chaperonin rings itself. Protein processing by chaperonins is carried out in ATP dependent way, and involves protein encapsulation (Hartl et al., 2011; Walter and Buchner, 2002; Haslbeck et al., 2005b; Horwich et al., 2007).

Hsp90 is found in bacteria and eukaryotic cells, and represents a complex ATP driven machine, which is believed to include Hsp70s and comprises a large number of cofactors (co-chaperones). Hsp90 is required for the maturation of numerous client proteins, is important for the regulation of the cell cycle, protein degradation and maintenance of the structural integrity of the target proteins (Picard, 2002; Richter et al., 2010; Wandinger et al., 2008).

Members of Hsp100/Clp family were reported to dissolve protein aggregates with the assistance of Hsp70 system. Additionally they are involved in protein degradation process: they were shown to actively unfold target protein in the ATP dependent manner (Liberek et al., 2008; Sharma et al., 2009; Haslbeck et al., 2005b).

sHsps – small heat shock proteins are another class of molecular chaperones, represented in almost all organisms. They usually form large oligomeric complexes of 12 to 42 subunits. sHsps are able to bind a large number of non-native proteins, up to one target protein per subunit and do not require ATP for their mode of action. Release of protein substrate usually requires assistance of ATP-dependent chaperones, which suggests role of sHsps as a pool of non-native proteins for subsequent refolding (Haslbeck et al., 2005a; Laskowska et al., 2010).

Along with molecular chaperones, foldases play an important role in protein folding, as they accelerate potentially slow steps in the folding process and thereby prevent aggregation. There are two types of foldases, with different modes of action i.e. peptidyl-prolyl *cis* or *trans* isomerase (PPI), which catalyzes isomerization of prolyl peptide bonds; and protein-disulfide isomerase (PDI), which catalyzes formation and isomerization of disulfide bonds for proper folding (Gupta and Tuteja, 2011).

Protein folding is carried out in the highly crowded environment of the cell, at high protein concentration, with many proteins being synthesized simultaneously (Hartl and Hayer-Hartl, 2009). Under such conditions misfolding and aggregation seem to be unavoidable. The main difference between protein folding *in vitro* and *in vivo* is that in the cell, this process is regulated and facilitated by the network of molecular chaperones and folding helpers.

4.2 Protein Refolding

In contrast to *in vivo* protein folding, where all proteins have to gain their functional structure under the same conditions, folding conditions *in vitro* have to be determined for each protein empirically. Optimal *in vitro* folding conditions are defined by the specific characteristics of each particular protein and involve such parameters as protein concentration, temperature, incubation time and refolding buffer (Lange and Rudolph, 2005). Additionally, under *in vivo* conditions slow rate limiting reactions in the folding and association pathway are influenced by molecular chaperones and folding helpers, which prevent non-productive side reaction leading to misfolded protein and aggregation (Buchner and Rudolph, 1991; Kiefhaber et al., 1991).

Renaturation process can be initiated by the decrease of the high concentrations of the denaturant and reducing agents,used for denaturation. Aggregation of unfolded protein and misfolding of folding intermediates compete with the refolding reaction, which is a major complication during the renaturation process (**Fig 2**).

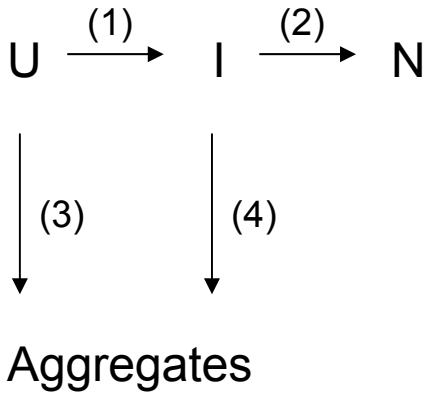


Figure 2 Simplified schematic illustration of folding and aggregation during protein refolding.

U – unfolded protein, I – folding intermediates, N – native protein. (1), (2) folding reactions leading to the native state. (3), (4) aggregation reactions, starting from different conformations during the renaturation process. [adapted from (Rudolph and Lilie, 1996)]

Folding intermediates, formed in the renaturation process, are believed to contain some elements of secondary structure and solvent-exposed hydrophobic regions, normally buried in the hydrophobic core of the native protein (Lange and Rudolph, 2005). These intermediates follow one of the two proposed reaction pathways. One includes a set of intramolecular reactions, follows first order kinetics and leads to the native structure. The second pathway involves intermolecular reactions of second and higher order kinetics and results in protein misfolding and aggregation (Eiberle and Jungbauer, 2010; De Bernardez et al., 1999; Mayer and Buchner, 2004; Rudolph and Lilie, 1996). Thus, the main objective of

the refolding process is to provide conditions that would minimize the aggregation and promote the reaction towards the renaturation pathway.

4.3 Inclusion Bodies

About 40% of all biopharmaceuticals are produced in *E. coli* cells (Eiberle and Jungbauer, 2010). *E. coli* is one of the most popular expression systems, as cultivation of this host organism is fast and inexpensive; moreover its well characterized genome enables various genetic operations to improve expression yield of a target protein. But often an overexpression of a recombinant protein in *E. coli* results in the accumulation of large insoluble and nonfunctional protein aggregates, also known as inclusion bodies (Prouty et al., 1975).

There is rather little information about the structure of inclusion bodies and the mechanism of their formation. Inclusion bodies are characterized as dense particles, which may span the whole diameter of the host cell (Figure 3).

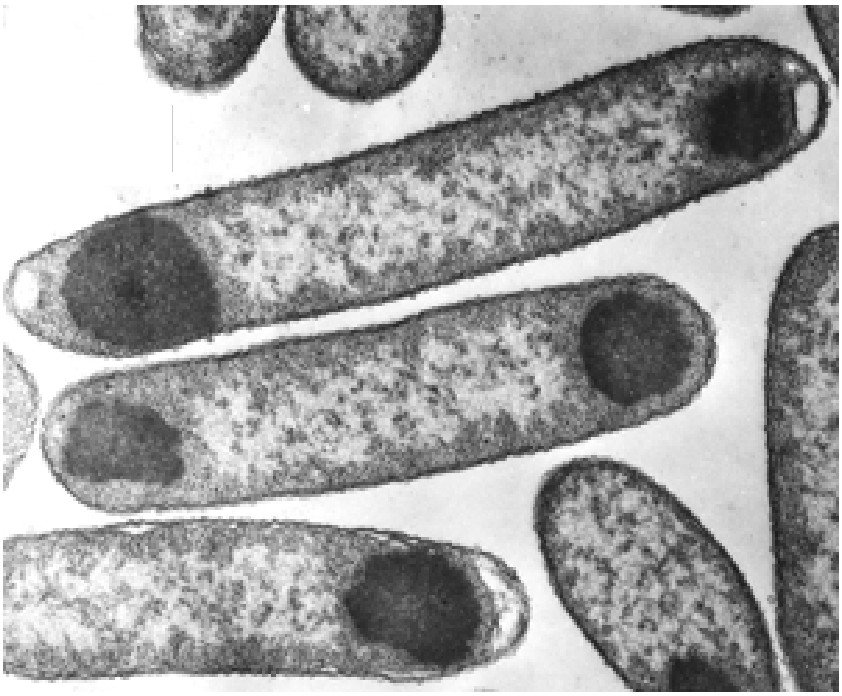


Figure 3 Inclusion bodies on the poles of *E. coli* cells. (Betts and King, 1998)

The recombinant protein is highly enriched and predominates in IBs, however some amount of host cell material is also present, for example inclusion body binding proteins (IbpA and IbpB), RNA polymerase, outer membrane proteins, some ribosome elements including ribosomal RNA etc. Some of these elements might have co-precipitated with recombinant

protein; however it is also possible that impurities found in the inclusion bodies preparations originate from the poor isolation and separation of the latter (Lange and Rudolph, 2005).

Formation of IBs is rather a rule than exception, especially in the case of eukaryotic proteins (Christendat et al., 2000; Mayer and Buchner, 2004; Service, 2002). High-throughput expression of *C. elegans* proteins in *E. coli* resulted only in 15% of soluble protein (Luan et al., 2004). In a study on human proteins, for example, only 13% of the recombinantly expressed proteins could be expressed and purified in soluble form (Braun et al., 2002). So despite the years of use of the *E. coli* system for protein production, there is still a large amount of proteins that can not be produced in their native functional form.

There are several theories and reasons for the formation of IBs: in the first place, increase of the concentration of recombinant protein in the cytosol *per se* is sufficient to evoke the aggregation process and accumulation of IBs. This fact yielded a kinetic model, where the extent of the soluble protein production depends on the rate of folding, aggregation and protein synthesis (Buchner and Rudolph, 1991; Kiefhaber et al., 1991). According to the model, upon the decrease of protein synthesis rate, yield of the native protein is increased, which was also experimentally confirmed by protein expression under suboptimal growth conditions. Decreased temperature, extreme pH etc. promoted soluble protein expression (Rudolph and Lilie, 1996). Formation of IBs could be also monitored during the overexpression of genuine *E. coli* proteins. In this context, it has been shown, that overproduction of the target protein correlates with an overexpression of molecular chaperones. Insufficient supply of the molecular chaperones during the folding process can also result in the accumulation of incorrectly folded polypeptides (Basu et al., 2011; Mayer and Buchner, 2004).

Expression of recombinant proteins containing disulfide bridges in their functional form also poses a challenge in the reducing environment of the bacterial cytosol. *In vitro* folding studies showed that the correct disulfide bond formation is possible under conditions mimicking those of the *E. coli* cytosol. However, in this case folding process is slowed down, which results in the increase of the inclusion body formation (Rudolph and Lilie, 1996)

Also *E. coli* rare codons may lead to an inefficient expression of heterologous genes in bacterial hosts. Expression of genes containing rare codons can lead to translational errors, like amino acid substitutions, frame shifting events or premature translational termination (Sorensen and Mortensen, 2005).

IBs have been discovered decades ago (Prouty et al., 1975) and numerous strategies have been developed to improve the bacterial expression system and overcome the problem of IBs formation: protein expression at lowered temperatures, co-expression of endogenous bacterial chaperones, optimization of rare codon usage, reduction of the activities of thioredoxin and gluthation reductases, introduction of fusion proteins etc (Sun et al., 2011;

Schlapschy and Skerra, 2011; Francis and Page, 2010; Francis and Page, 2010; Sahdev et al., 2008).

Co-expression of chaperones to guide the correct folding of recombinant proteins was shown to exhibit case-specific effects. Even in the presence of increased levels of molecular chaperones, formation of soluble aggregates, inhibition of cell growth and proteolytic product cleavage could not be avoided to the full extent (Basu et al., 2011). The efficiency of optimization of *E. coli* rare codons was also reported to be rather protein specific (Francis and Page, 2010; Vernet et al., 2011). Introduction of fusion proteins is accompanied with the increase of metabolic burden. Additionally inefficient downstream processing (cleavage), often results in decreased yields of protein product (Hewitt et al., 2011).

Despite numerous efforts to improve the solubility of the recombinant proteins produced in bacteria, there is no general strategy that would ensure soluble protein expression. Application of alternative expression systems such as yeast, insect or mammalian cells was shown to be efficient in some cases, however none of them seems to be the ultimate solution for recombinant protein expression. Difficulties of genetic manipulation, low productivity, long development times and increased costs restrict general applicability of these expression systems (Basu et al., 2011; Eiberle and Jungbauer, 2010; Lange and Rudolph, 2005; Rudolph and Lilie, 1996).

In this context, formation of IBs seems to be a deficiency of the approach just at first glance. IBs might be advantageous in several respects: the recombinant protein is usually highly enriched in IBs, it is protected from proteolytic degradation and shows minimized toxicity towards the host cell. Furthermore, IBs can be easily and effectively isolated (Basu et al., 2011; Greenshields et al., 2008; Jungbauer and Kaar, 2007; Rudolph and Lilie, 1996).

The high yield of protein product, obtained in a very short time, combined with the low production costs, makes the IB material attractive for both academic and industrial research. Therefore, there has been a lot of efforts to develop an efficient strategy for the reactivation of the recombinant proteins from IBs by *in vitro* folding.

4.3.1 Isolation and solubilization of inclusion bodies

IBs can be easily isolated by centrifugation, due to their rather high density (Mayer and Buchner, 2004). The IB containing cells are usually treated with lysozyme and disrupted by high pressure dispersion. Subsequently, DNAse is added to cleave the chromosomal DNA. To ensure complete removal of lipids and membrane proteins, the cell lysate is incubated with detergents such as Triton-X (0.1-4% w/v) or sodium deoxycholate (2%w/v). Addition of high salt concentration (e.g. 0.5 M NaCl) promotes solubilization of contaminating proteins.

Subsequent sedimentation steps separate IBs from cell debris and membrane proteins, resulting in highly enriched IBs in the pellet fraction. Detergents and other buffer components, which may potentially impair following solubilization and renaturation, can be removed by repeated resuspension and sedimentation (Cabrita and Bottomley, 2004; Lange and Rudolph, 2005; Mayer and Buchner, 2004).

Solubilization of IBs is usually achieved by the addition of high concentrations of denaturing agents such as 6 M guanidinium chloride (GdmCl) or 8 M urea. GdmCl is a stronger denaturant as urea, and allows solubilization of even urea-resistant IBs. Additionally, at alkaline pH urea decomposes to isocyanate, which can lead to the carbamylation of protein amino and sulfhydryl groups (Lange and Rudolph, 2005).

Aside from denaturants, a number of strong detergents such as SDS (1%) and sarkosyl (1-10% w/v) are used to solubilize IBs, due to their strong solubilization capacity, low cost and easy handling. However, application of surfactants is usually avoided, as their removal might be problematic and requires excessive purification steps (Kudou et al., 2011). Alternatively, denaturation can be achieved by the organic solvents such as n-propyl alcohol or isopropyl alcohol, or alkaline pH (Eiberle and Jungbauer, 2010).

It is very important that the solubilization buffer also contains a reducing agent (e.g. DTE/DTT, ß-mercaptoethanol), to keep cysteins reduced, even for protein lacking disulfide bridges in their native state but containing free cysteins. A chelating agent, such as EDTA or EGTA, can be added to the solubilization buffer to remove metal ions, which could cause unwanted oxidation reactions (De Bernardez et al., 1999). In other respects, solubilization can be performed in any buffer, for example Tris, Hepes, phosphate, generally with a neutral pH (pH 7 - 8). The solubilization time is dependent on the temperature and concentration of a target protein in IBs. Under normal conditions, 2 h incubation at room temperature is sufficient to denature a protein sample at a concentration of 50 mg/ml (Mayer and Buchner, 2004).

In some cases, inclusion bodies might require an additional purification step by column chromatography, which is usually performed after solubilization under denaturing conditions prior to the renaturation procedure. Successful refolding may depend on the purity of inclusion bodies, as it is assumed that protein contaminants can promote the aggregation and thus impair the renaturation efficiency (Cabrita and Bottomley, 2004).

4.4 Refolding methods

4.4.1 Refolding in solution

There are several approaches to remove the denaturant and reducing agents and thus to enable proteins to regain their native structure. The easiest and the most common way is a rapid dilution, where denatured protein is transferred directly into the refolding buffer, usually in 1:50 or 1:100 ratio; the method is also referred to as a “refolding in solution”.

As aggregation is an intermolecular reaction, an increase of the initial concentration of the denatured protein results in the decreased refolding yields. Therefore, protein concentrations during refolding are usually kept very low (10 – 50 µg/ml) to obtain efficient renaturation. This parameter is especially crucial for oligomeric proteins. Here suboptimal concentration may lead to a complete failure of refolding (De Bernardez et al., 1999; Mayer and Buchner, 2004; Rudolph and Lilie, 1996).

Such direct dilution of the denatured protein into the refolding buffer is also referred to as a batch dilution. This method is widely used, due to the relative ease of the procedure. However, large volumes of refolding buffer combined with low protein concentrations are required to overcome aggregation and obtain a reasonable protein yield. Additionally, downstream processing i.e. a protein concentration step is necessary.

Working with higher protein concentrations demands controlled addition of the denatured protein, which can be achieved by the fed-batch dilution (Fischer et al., 1992; Kiefhaber et al., 1991). Stepwise addition of denatured protein into the refolding buffer allows obtaining efficient refolding yields at higher protein concentrations, since correctly folded protein does not participate in aggregation side reactions. Time breaks between single feeds should be long enough to let the folding intermediates overcome aggregation-prone early stages on the refolding pathway. Under given conditions of stepwise protein addition at appropriate time points, the concentration of unfolded protein and folding intermediates is kept low, at any time point (Lange and Rudolph, 2005; Fischer et al., 1992; Kiefhaber et al., 1991).

Another type of refolding in solution is dialysis. In this case, denatured protein in the dialysis tube is transferred into a vessel with refolding buffer. This method is usually applied when the starting protein concentration is too low for further dilution into the refolding buffer, or a complete buffer exchange is required. The disadvantage of the method is protein loss to the dialysis membranes, and slow buffer exchange kinetics, which leads to the aggregation of folding intermediates. Also reproducibility and up-scaling are problematic (Leong and Middelberg, 2007; Rudolph and Lilie, 1996).

Buffer exchange can also be achieved by ultrafiltration, but, here, again slow removal of denaturant might result in irreversible precipitation of the protein and its sticking to the membrane.

An alternative approach is “reversed dilution”. In contrast to the abovementioned dilution methods, here, refolding buffer is gradually added to the denatured protein, which leads to the simultaneous decrease of denaturant and protein concentration (He et al., 2008).

4.4.2 Matrix-assisted refolding

The technique of matrix-assisted refolding is believed to be introduced by T. Creighton. He was able to obtain an efficient refolding of ovalbumin employing ion-exchange chromatography (Creighton, 1986). Since then, the method was not only improved, but was also extended to further types of chromatography (Eiberle and Jungbauer, 2010).

The basis of matrix-assisted refolding is the specific interaction of the protein with a solid phase, which should prevent unspecific interaction. However, there are several preconditions to consider. First, protein binding to the matrix of choice under denaturing conditions must be possible. Second, bound polypeptide should remain flexible enough to regain its native structure. Third, the protein–matrix interaction should be strong enough to prevent intermolecular interaction of individual polypeptide chains, which may result in aggregation (Lange and Rudolph, 2005).

There is a number of approaches applied for the matrix-assisted refolding: i) immobilization of the denatured protein on the solid phase and subsequent removal of the denaturant; ii) buffer exchange via size exclusion chromatography and iii) employing of folding catalyst attached to chromatography matrix prior to application of the protein to be refolded (Eiberle and Jungbauer, 2010).

Matrix-assisted refolding carried out by the means of chromatography columns is also known as on-column refolding, and is usually carried out using of one of the following chromatography types: size exclusion chromatography (SEC), ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and affinity chromatography (AC).

SEC (size exclusion chromatography). Size exclusion chromatography enables gradual removal of the denaturant. Notably, here the protein is not bound to the resin as it is the case with other chromatography principles. Additionally native protein, folding intermediates and aggregates are separated by their different properties in the process of chromatography performance. Unfolded protein is characterized by a higher thermodynamic radius and outruns the denaturant; upon the decrease of the denaturant concentration, the protein undergoes folding process, which is either completed and results in the formation of the

native structure, or leads to aggregation. The formed aggregates are then dissolved by the following denaturant and refolding process is initiated once again (Eiberle and Jungbauer, 2010; Lange and Rudolph, 2005). However, formation of large aggregates, which might clog the column, still cannot be excluded. Another disadvantage is that processing of only limited volumes of protein sample is possible per run. Also the refolding conditions have to be optimized very carefully, in terms of renaturation buffer, flow rate and gradient volume. Taken together all these factors limit application of SEC especially in larger scales (Eiberle and Jungbauer, 2010; Lange and Rudolph, 2005; Mayer and Buchner, 2004).

IEC (ion-exchange chromatography)**.** Refolding by IEC was first shown by Creighton in 1986. After urea denaturation, the target protein was adsorbed on an ion-exchange matrix, denaturant was removed gradually, inducing refolding, while the protein remained bound to the matrix, and elution was carried out by a salt gradient (Creighton, 1986). As ion-exchange chromatography is widely used in protein purification, the method could be easily employed for refolding processes. Since its introduction, the approach has been improved and modified to include for example concurrent pH, urea and salt gradients, which enabled efficient refolding of several proteins (Freydell et al., 2010). Compared to SEC, IEC offers higher loading capacities and the possibility to concentrate the protein product during elution.

AC (affinity chromatography). Here, in the majority of cases the interactions of immobilized bivalent metals (i.e. Ni^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}) with the polyhistidine tags introduced in the sequence of the protein of interest are used. As only the primary structure of the polypeptide chain is important for the high affinity complexes, this method enables processing of the target protein under denaturing conditions. Immobilized metal affinity chromatography is also frequently used in purification processes. The existing procedures can be easily adopted for the refolding procedures.

After denatured protein is bound to the column matrix, refolding procedure can be initiated either by a stepwise exchange of the buffer (direct application of the refolding buffer onto the column) or by refolding gradient (gradual application of the renaturation buffer and removal of the denaturant). Neither of the two approaches impairs the protein–matrix interaction, and protein remains bound to the column until elution is initiated by imidazole.

Since the denatured protein is only fixed to the matrix by an extension of one of the termini, protein folding should not be affected, due to the high freedom of the polypeptide chain. In this context it should be noted that many termini of the protein are solvent-exposed.

Additionally, this method enables processing of large protein volumes, offers the possibility of a purification step prior to the refolding procedure and enables product elution at high concentrations.

HIC (hydrophobic interaction chromatography). This method involves the interaction between immobilized hydrophobic ligands and hydrophobic amino acids of the proteins. Thus, solvent-

exposed hydrophobic regions of the denatured protein can interact with the HIC matrix; such interactions prevent unspecific aggregation of polypeptide chains. However, successful protein folding also depends on the undisturbed intramolecular hydrophobic interactions, so protein binding to the matrix should not be too strong to enable flexibility of the polypeptide chain for efficient refolding. Otherwise the procedure will result in a rather low recovery of the product (Li et al., 2004).

Immobilized folding catalysts and chaperones. Folding catalyst, antibodies and molecular and artificial chaperones attached to the matrix are believed to mimic folding conditions *in vivo* and improve the efficiency of renaturation.

Lee et al (2009), for example, immobilized GroEL (a monomeric apical domain consisting of residues 191-345) and two foldases DsbA and human PPI on a NHS-activated Sepharose Fast Flow cation-exchange matrix. The folding machineries were additionally supplied with a C-terminal 10xArginine-tag, which ensured efficient immobilization. These refolding-facilitating matrices enabled efficient refolding of two cysteine- and proline-rich test proteins (Lee et al., 2009). Luo et al (2011) immobilized recombinant DsbA (disulfide bond formation protein A) onto NHS-activated Sepharose Fast Flow gel, then Sephadex G-100 gel was sequentially packed on the top of the DsbA Sepharose, to form a conjoint chromatography column, comprising SEC and immobilized folding helper. Application of the strategy resulted in enhanced refolding efficiencies of the test protein (Luo et al., 2011).

However, the method is not widely used, due to high expenses and low outcome, compared to other processes (Eiberle and Jungbauer, 2010; Li et al., 2004).

Zeolites. An alternative to matrix-assisted refolding by chromatography, represent zeolites (Chiku et al., 2006). Zeolites are crystalline porous aluminosilica compounds. The ratio of SiO_2/Al_2O_3 in the zeolite can be varied, with higher ratios indicating higher degree of hydrophobicity and lower ion-exchange capacity. Denatured proteins can be easily adsorbed onto the surface of the zeolite matrix; subsequently, denaturant is removed by a wash step, followed by the application of the renaturing buffer and subsequent desorbtion with detergent or polyethylene glycol (PEG). The method enabled large scale preparation and efficient refolding of 11 test proteins (Chiku et al., 2006).

4.4.3 Alternative refolding methods

4.4.3.1 High pressure induced refolding

The thermodynamics of pressure-induced protein transitions have been known for a long time. Application of high hydrostatic pressure (1–3 kbar) leads to the reversible dissociation of multimeric proteins. For complete unfolding, pressure of up to 8 kbar is required.

Application of moderate pressures, e.g. 1-2 kbar, to aggregates results in their dissociation (Eiberle and Jungbauer, 2010; Kim et al., 2006b; Lange and Rudolph, 2005).

High pressure was shown to be able to support the protein refolding processes. The basic idea of this application is to shift the competition between folding and aggregation towards the productive pathway. It has been reported, that high hydrostatic pressures disrupt hydrophobic and ionic interactions, but they do not considerably impair hydrogen bonds. Additionally, the phenomenon of the "refolding window" has been proposed, which is defined as pressure conditions where native conformations are thermodynamically favored, over higher-order aggregate structures (Qoronfleh et al., 2007).

The main advantages of the current method are simultaneous solubilization and refolding/disaggregation of a protein, without any denaturing agents, and fast and efficient processing of the protein product at high concentrations. Application of the method resulted in the successful refolding of a set of test proteins, and showed increased refolding efficiencies compared to conventional renaturation methods (Qoronfleh et al., 2007; Crisman and Randolph, 2009).

4.4.3.2 Temperature leap approach

In vitro refolding is usually carried out at temperatures lower than physiological, in the range of 5-15°C, as application of higher temperatures might result in the promotion of unfavorable hydrophobic interactions and lead to an increased rate of aggregation. However, there were several successful attempts to make use of elevated temperatures to improve the refolding efficiencies (Lange and Rudolph, 2005).

In the study by Sakamoto et al. (2004), denatured test protein was diluted into the refolding buffer at 98°C and incubated at the given temperature for 5 min and subsequently cooled down to 0°C and incubated for 24 h. Such an approach resulted in a three fold increase of refolding yield, compared to the protein sample diluted into the buffer at 20°C. It has been suggested that such a heat-induced unfolded state is more productive for folding, compared to that induced just by denaturation, as elevated temperatures might impair or prevent interactions of unproductive intermediates and increases the rate of disulfide exchange reactions, so that reshuffling of non-native disulfide bonds in the unproductive intermediates could proceed rapidly (Sakamoto et al., 2004; Shiraki and Kayano, 2010).

Xie et al (1996) developed "temperature leap tactic". Here refolding is initiated by the dilution of the denatured protein into the refolding buffer at 4°C, after 2h incubation, the solution was warmed up to 36°C, to finalize the process. As initial refolding proceeds at low temperature, early intermediates rearrange to late intermediates, which are less aggregation-prone, and can then be refolded at a higher rate at the higher temperature (Xie and Wetlaufer, 1996).

However, success and efficiency of the presented temperature leap techniques is still case specific and depends on the reaction pathway. Thus applicability and optimum folding temperatures need be determined for each single protein experimentally (Lange and Rudolph, 2005).

4.4.3.3 Enzyme mediated refolding

In this novel method for protein refolding, introduced by Okada et al (2009), removal of denaturant (urea), is carried out via an enzymatic reaction. Urease catalyzes the hydrolysis of urea to NH_3 and CO_2. As the reaction proceeds, urea is progressively removed from the solution and protein renaturation is initiated. In addition to the gradual and homogeneous removal of the denaturant, the method offers the advantage of protein refolding at high concentrations, as no dilution is required. Application of urease was shown to result in the efficient refolding of three test proteins (Okada et al., 2009).

4.5 Refolding conditions

The choice of an optimal refolding method is only a part of a successful refolding strategy. The efficiency of the procedure is also dependent on a whole range of factors: the concentration of the protein sample, temperature, incubation time and composition of the refolding buffer.

In vitro refolding is usually performed at low temperature, as this decreases the folding rate and hydrophobic interactions, which in most cases results in higher refolding yields and less aggregation (Mayer and Buchner, 2004). However, also the exceptions are possible (cf. 4.4.3.2) and efficient refolding temperatures should be determined for each protein empirically.

Optimum incubation times in the refolding procedure may vary from several seconds to several days, depending on the specific protein and given buffer conditions and should be determined experimentally for each particular case (Buchner and Rudolph, 1991; Jungbauer and Kaar, 2007; Rudolph and Lilie, 1996).

The protein concentration, at which the maximal refolding efficiency can be achieved, is a specific property of any given protein. Since unproductive aggregation competes with folding, increased initial concentrations of unfolded protein result in decreased refolding efficiency. Some proteins can be refolded at higher concentration, whereas others require an order of micrograms per milliliter to be efficiently refolded (Eiberle and Jungbauer, 2010; Mayer and Buchner, 2004; Rudolph and Lilie, 1996).

4.5.1 Refolding buffer

Successful refolding for some proteins can be achieved under rather simple buffer conditions, while for the majority the choice of a proper renaturation buffer is one of the most crucial parameters in the refolding procedure. Precise optimization of the refolding buffer conditions is unavoidable. Variation of just the basic buffer component under otherwise identical refolding conditions and with the same test protein results in a significant difference of refolding yields (Sakamoto et al., 2004; Dashivets et al., 2009).

The pH of the buffer solution is also an important factor. The pH range optimum for refolding is determined by the specific characteristics of the particular protein target. For the correct formation and reshuffling of disulfide bonds, pH should be kept alkaline. The pH used should also be different (at least one unit) to the pI (isoelectric point) of the protein, as values near pI may favor protein precipitation (Mayer and Buchner, 2004; Rudolph and Lilie, 1996; Vincentelli et al., 2004; Dashivets et al., 2009; Vincentelli et al., 2004). The ionic strength of the refolding buffer is also a case specific parameter and may vary dramatically depending on the properties of the given protein (Rudolph and Lilie, 1996).

4.5.2 Additives

Misfolding and aggregation are the main setback for the efficiency of refolding. Application of various additives in the refolding process has been shown to be an efficient strategy to overcome them, at least to some extent. Additives are defined as small molecules of different origins that are able to impair the aggregation process and improve renaturation: they may stabilize the native functional state, increase the solubility of folding intermediates or unfolded state. Additives can be usually easily removed from refolding buffer and do not impair subsequent purification steps (De Bernardez et al., 1999).

Denaturants The simplest additives, reported to enhance renaturation, are GdmCl and urea at nondenaturing concentrations. The positive impact of these substances can be explained by their denaturing characteristics, as they may stabilize intermediates and unfolded protein, thus increasing their solubility and decreasing aggregation.

Amino acids L-Arginine (L-Arg) is probably one of the most popular additives applied in protein refolding. Its positive effect on the renaturation efficiencies has been demonstrated for a wide range of proteins (Alibolandi and Mirzahoseini, 2011; Arakawa et al., 2006; Arakawa et al., 2007a; Buchner and Rudolph, 1991; Kudou et al., 2011; Tischer et al., 2010; Tsumoto et al., 2005; Buchner et al., 1992). However, the mechanism of action of L-Arg is still not fully understood. The latest model, proposed by Tischer and co-workers claims that L-Arg acts on the equilibrium solubility of the native state (Tischer et al., 2010). This is in contrast to the previously suggested role of Arg as a neutral crowder, which has an influence on the stability of the transition state of aggregation (Baynes et al., 2005).

In praxis, L-Arg was shown to positively influence refolding yield, when applied at concentrations of 0.2-1 M (Abe et al., 2009; Arakawa et al., 2006; Arakawa et al., 2007b; Basu et al., 2011; Baynes et al., 2005; Chen et al., 2009; Eiberle and Jungbauer, 2010; Tischer et al., 2010). However, L-Arg was also reported to impair activity and stability of several proteins. This effect was ascribed to the guanidinium group, which is also present in the denaturant GdmHCl, though it has been shown that Gdm and Arg vary in their mode of interaction with proteins (Alibolandi and Mirzahoseini, 2011; Arakawa et al., 2006; Baynes et al., 2005; Fan et al., 2008; Tischer et al., 2010).

Addition of proline or glycine in concentrations of up to 1M to the refolding buffer has also been reported to suppress aggregation and improve refolding efficiencies (Han et al., 2010; Ito et al., 2008; Kim et al., 2006a; Meng et al., 2001; Samuel et al., 2000). Other amino acids, reported to improve refolding, include glutamate, serine, lysine, alanine and sarcosine (Arakawa et al., 2007b; Ito et al., 2008; Ito et al., 2010a; Pradeep and Udgaonkar, 2004). Additionally, based on the initial success of amino acid mediated refolding, numerous amino acid derivatives such as glycine amid, arginine amid, L-arginine ethylester dihydrochloride, L-arginine methylester dihydrochloride etc have been applied to enhance refolding efficiencies (Hamada and Shiraki, 2007; Ito et al., 2010a; Ito et al., 2010b; Matsuoka et al., 2009).

Redox system Refolding of proteins with disulfide bridges, as well as those containing free cysteins, requires an introduction of an adequate redox system (Eiberle and Jungbauer, 2010; Lange and Rudolph, 2005; Mayer and Buchner, 2004). Disulfide bond formation has been shown to be a rate limiting step in the refolding process in many cases. Application of a system, comprised of thiol reagents in reduced and oxidized states, promotes disulfide formation as well as the reshuffling of the incorrectly formed bonds. The tripeptide Glutathione, in reduced and oxidized (GSH/GSSG) form, is most commonly used reagent. Alternatively cysteine/cystin, cysteamine/cystamine, or bis-ß-hydroxyethyl disulfide/ß-mercaptoethanol could be employed (Buchner and Rudolph, 1991; Lange and Rudolph, 2005; Thies et al., 2002). To attain a suitable redox potential for disulfide bridge formation and reshuffling, molar ratios of the reduced and oxidized agents are recommended in the range between 1:1and 5:1 (Lilie et al., 1998).

Proteins containing free cysteins are usually refolded under slightly reducing conditions, in this case only reducing agents such as DTT/DTE, TCEP, ß-mercaptoethanol or reduced glutathione are introduced into the refolding buffer, to prevent unwanted intra- and intermolecular disulfide bridge formation.

Polyol osmolytes Polyols are alcohols with the number of hydroxyl groups. Addition of polyols was shown to result in improved refolding and stabilized protein structure (Cleland et al., 1992; Dworeck et al., 2011; Kim et al., 2006a; Mishra et al., 2005). Within this group, glycerol is probably most frequently applied to enhance the refolding efficiency, and is

usually applied in a range of 10 - 50% v/v (Mishra et al., 2005; Tieman et al., 2001; Vagenende et al., 2009; Wang et al., 2009). It was proposed that glycerol prevents protein aggregation by inhibiting protein unfolding and by stabilizing the aggregation-prone intermediates through preferential interactions with hydrophobic surface regions that favor amphiphilic interface orientations of glycerol (Vagenende et al., 2009).

Polyethylene glycol (up to 0.5% w/v) is also widely used to improve refolding (Alibolandi and Mirzahoseini, 2011; Du et al., 2006; Dworeck et al., 2011; Lee and Lee, 1987). It increases the recovery of active protein by preventing aggregation. Analysis of solvent protein interaction showed that polyethylene glycols interacts favorably with hydrophobic side chains and thus influences the stability of the denatured protein, folding intermediates and misfolded forms in solution (Cleland et al., 1992; Holtz et al., 2007; Lange and Rudolph, 2005). Besides glycerol and PEG, polyols such as erythritol, xylitol and sorbitol were reported to positively influence the refolding process (Majumder et al., 2001; Mishra et al., 2005; Yu and Li, 2003).

Detergents Detergents represent another group of additives, which are believed to stabilize aggregation prone polypeptides by hydrophobic interactions. Examples are CTAB (cetyltrimethylammonium bromide), DMSO (dimethylsulfoxide), Triton-X -100, Tween-80, Laroylsarcosin, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), SDS (sodium dodecyl sulfate) and Brij 58P (Alibolandi and Mirzahoseini, 2011; Dong et al., 2009; Khodagholi et al., 2008; Laslo et al., 2009; Yamamoto et al., 2011b; Yazdanparast et al., 2008). Brij 58P has been reported to act as aggregation suppressor and refolding enhancer at very low concentrations (0.0001%) (Krause et al., 2002).

Sugars Cyclodextrins (CD) are cyclic oligosaccharides with the shape of a hollow truncated cone, which are characterized by a hydrophilic exterior and a hydrophobic inner cavity. Typically used CDs are α-cyclodextrin, ß- cyclodextrin, and γ – cyclodextrin, which consist of six, seven and eight glucose monomers respectively. CDs have bee reported to efficiently suppress aggregation by interaction with hydrophobic patches of aggregation-prone intermediates. Additionally to their role as refolding promoting additives, these substances are applied in artificial chaperone systems. This approach involves employment of the detergents to first capture non-native protein species, and the subsequent application of cyclodextrins to disrupt detergent-protein complex and initiate the refolding process (Aachmann et al., 2003). Enhanced refolding yields, upon application of CDs have been reported for numerous proteins (Aachmann et al., 2003; Dong et al., 2009; Krause et al., 2002; Sasaki and Akiyoshi, 2010; Yamamoto et al., 2011b; Yazdanparast et al., 2006).

Alginate is a natural linear polysaccharide, which consists of β-D-manuronic acid and α-L-guluronic acid, arranged in blocks. Alginate, was shown to initiate and propagate refolding, when applied in the artificial chaperone system instead of CDs. An advantage of alginate over CDs, is the absence of the hydrophobic cavity, which might be occupied by unwanted

molecules that could impair functionality. Additionally, its properties and number of Monomeric subunits in oligomer can be varied (Khodagholi et al., 2008).

Sucrose and glucose (up to 0.75 M), added into the refolding buffer, were also reported to enhance refolding efficiency (Akbari et al., 2010; Alibolandi and Mirzahoseini, 2011; Divsalar et al., 2006; Lin and Li, 2010; Monterroso and Minton, 2007).

Ionic liquids are organic salts with melting points below 100°C. They generally consist of combinations of organic cations, derivatives of N,N'-substituted imidazolium, N-substituted pyridinium, tetraalkylated ammonium and tetraalkylated phosphonium and either organic or inorganic anions. Thus, they represent a diverse group of organic solvents and co-solvents with different physico-chemical properties, which may be readily adjusted to the needs by changes in anion and cation substitution patterns (Buchfink et al., 2010).

Ionic liquids were reported to stabilize protein activity and structure and to improve refolding efficiencies. Dihydrogenphosphate-based ionic liquids were shown to be good and stabilizing solvents for functional proteins (Fujita et al., 2005); application of tetraethyl and tetrabutyl ammonium nitrate and 1-butyl-4-methyl-pyridinium tetrafluoroborate, N-ethyl, N-butyl and N-hexylpyridinium chlorides, and N-butyl-N-methylpyrrolidinium chloride resulted in increased refolding yields (Fujita et al., 2005; Lange et al., 2005; Summers and Flowers, 2000; Yamamoto et al., 2011a). Recently, analysis of different salts of N-methyl imidazolium revealed, that N-ethyl-N_-methyl imidazolium chloride was the most effective refolding enhancer (Buchfink et al., 2010).

Cofactors, chaperones and folding helpers. For some proteins cofactors are required for their structural integrity and biological function. The presence of the cofactors during the refolding procedure was reported to stabilize the native state of the proteins and accelerate refolding; additionally it was shown that cofactors are able to bind unfolded or partially folded protein states. In some cases application of the cofactor is indispensable for the efficiency of refolding (Bushmarina et al., 2006; Goedken et al., 2000; Lange and Rudolph, 2005; Aachmann et al., 2003; Rinas et al., 1990; Risse et al., 1992).

Metal ions such as copper, zinc, magnesium and calcium are the most commonly used cofactors. Other cofactors such as ATP, FADH, NAD(P)H might be also essential for the formation of native structure (Bushmarina et al., 2006; Lange and Rudolph, 2005; Wang and Engel, 2009).

Addition of molecular chaperones and other folding helpers, such as oxidoreductases, disulfide isomerases and peptidyl/prolyl cis/trans isomerases, was reported to be a potent tool to promote protein refolding and accelerate the rate of disulfide bond formation. In some cases, such additions were crucial for a gain of a native structure (Asayama et al., 2008; Buchner et al., 1991; de Marco A., 2011; Paul et al., 2007; Sahu et al., 2009; Tsumoto et al.,

2003). However, the use of these proteins is still limited especially in the industrial large scale processes, due to the high production expenses (Lange and Rudolph, 2005).
In the past decades of developing strategies to improve the efficiency of refolding process, numerous additives have been applied to promote renaturation and to suppress negative side reactions. A number of them were of a big success. However, there is no general recipe, and none of the additives seems to have exactly the same influence on every target protein. The success of refolding is determined by the chosen refolding method and by the given refolding conditions. Finding an optimal refolding buffer might be a very time-consuming procedure with a low success rate, since every single parameter i.e. buffer component, ionic strength, pH, additive and its concentration etc, has to be determined experimentally for each particular protein.

4.5.3 Optimization of refolding buffer

To overcome the problem of a lacking universal method to identify optimum refolding conditions high-throughput fractional factorial screens have been introduced, i.e. testing of several refolding buffers simultaneously.
Several commercial refolding kits have been introduced in the market by different companies. For instance the *Thermo Scientific Pierce Protein Refolding Kit* (by Thermo Fischer Scientific) consists of nine different basic refolding buffers and stock solutions of seven additives. There is a *QuickFoldKit* by Athena ES, comprising 15 refolding buffers. *The BioAssay™ Protein Refolding Kit* by Biomol is another kit consisting of 15 ready to use buffers. Hampton Research proposed a refolding kit *FoldIt* which suggests 16 refolding conditions. However, 9 to 16 buffer conditions barely cover the whole variety of possible buffer compositions, and the probability to find the optimum one is rather low. This probability increases with the number of refolding buffer conditions tested . Refolding systems introduced by Novagen – *iFOLD 1, iFOLD 2 and iFOLD 3* enable screening in 96-well format, each kit being supplied with 92 different refolding buffers, covering different pH ranges, buffer components (not *iFOLD1*), salt concentrations, and additives of different origins. Among the refolding kits on the market, the *iFOLD* system is probably the one that embraces the widest range of buffer conditions.
In addition to the industrial refolding strategies, several research groups addressed the question of high-throughput screening for optimal buffer conditions. Vincentelli et al developed a system usig 96-well format, with the similar of choice of buffers as described for the *iFOLD* kits (Vincentelli et al., 2004). A comparable strategy was proposed by (Cowan et al., 2008). In another screen Willis et al (2005) start with the set of 32 buffer conditions,

followed by the subsequent optimization of the buffers with a positive effect on the refolding efficiency from the primary screen.

Qoronfleh et al., (2007) suggested a novel approach where optimization of the buffer conditions is carried out in several steps with different refolding kits in the presence of the high hydrostatic pressure.

Dechavanne et al., (2011) combined the refolding screening process based on a 96-well assay format with a design of experiments (DOE) approach. After a first generic screen of refolding conditions the parameters that produced the best yield were further analyzed by the DOE software to determine the optimal concentrations of the critical additives. The optimal refolding conditions suggested by DOE were tested and resulted in an improved refolding, confirming reliability of the predictions.

A new strategy that combines screening and optimization of refolding yields with a genetic algorithm (GA) is proposed by Anselment et al. (2010). Here, the first set of 22 refolding buffers is randomly generated by the GA, based on given parameters. Subsequently, refolding efficiencies are analyzed by the GA and parameters of the best conditions are further optimized in the following generations. This strategy resulted in optimized refolding yields and activities for four model proteins.

Despite a number of already existing strategies, the search for an optimal strategy continues, and new screening procedures do not cease to be developed. There is no protocol that is generically applicable to any protein of interest. Therefore, fast and efficient methods of identification of optimal refolding conditions are still required. All the above mentioned screening procedures are aimed to determine buffer conditions, which would result in refolded target protein; however, none of them guarantees a successful outcome. The advantage of the described strategies is that they enable the parallel analysis of numerous buffer conditions, and since screening procedures are usually carried out in small scale, they do not require large amounts of the protein material. However, even with 96 refolding buffers they embrace rather a limited number of buffer compositions, and most of the described strategies do not address further optimization. In addition, dilution is the refolding method applied in all proposed optimization approaches, which makes protein concentration a critical success-defining parameter. Additional processing of protein samples is required prior to determining yields, as identical buffer conditions are required to compare the activities of the refolded samples. Thus, a large number of refolding buffers in the screening procedure increases not only the chances to identify a favorable condition, but also the number of samples to be processed and analyzed.

Matrix–assisted refolding methods are commonly not used in screens for the optimization of refolding buffer conditions. To the best of knowledge, solely Nara et al., (2009) proposed high-throughput refolding screening using zeolites. However, the method is relatively new

and not widely established and the efficiency of the zeolite-assisted refolding optimization was shown just for one test protein. Additionally, protein is eluted from the matrix by the refolding buffer, which, just like dilution-based refolding, requires additional processing step.

Hence, screening procedures using matrix-assisted refolding are currently highly underrepresented. At the same, this method does offer several advantages. In the first place, protein processing at higher concentrations might be possible without impairment of the refolding efficiency. Secondly, the method enables additional purification of the protein sample, as impurities in IB preparations might be critical for refolding efficiency. Additionally, the elution of the refolded protein can be carried out in the same buffer, which enables direct analysis and comparison of protein samples. IMAC is especially promising in this context, as introduction of polyhistidine tags to the protein of interest is a well established and very common tool in protein purification processes. Furthermore, protein binding to the matrix is possible under denaturing conditions, for both GdmCl and urea, and protein samples are eluted from the matrix in the same buffer.

Optimization of refolding conditions by the means of chromatography might be a very time consuming procedure, as the analysis of several refolding buffers in a parallel mode is rather limited. For IMAC, with the introduction of 96-well HisMultiTrap plates by GE Healthcare this limitation could be displaced. The 96-well HisMultiTrap plate was developed for high-throughput parallel purification screening and small scale purification of the histidine-tagged recombinant proteins. These plates can be easily employed for the purpose of optimization of refolding buffer conditions for the histidine-tagged proteins.

5 Objective

The aim of this work was to develop a high-throughput strategy for the optimization of refolding buffer conditions for insolubly expressed his-tagged proteins.

Although there are examples of the refolding of his-tagged proteins immobilized on chelating materials (Eiberle and Jungbauer, 2010) there is little information on general strategies and specific mechanisms. Systematic analysis of the matrix-assisted refolding performance of six different proteins should enable to gain an insight into the general principles of this strategy. To determine the competence of the strategy, the test proteins vary in their origin, molecular weights, quaternary structures and enzymatic activities.

Furthermore, comparative analysis of refolding yields, refolding kinetics and concentration dependencies of matrix-assisted and in-solution refolding, allowed determining the efficiency of the approach.

Special attention was paid to the analysis of the re-association of a large tetrameric protein β-galactosidase in the matrix-assisted refolding process.

6 Results

6.1 Test Proteins

To determine the efficiency and general applicability of matrix-assisted refolding, a set of test proteins was chosen. The set included proteins of different origin, size, activity and oligomeric state. Additionally, the chosen test proteins fulfilled three main requirements: first, each protein carried an N-terminal His6-tag; second, both soluble and insoluble expression of the test protein was possible; third, each protein could be characterized by activity assay (Table 1).

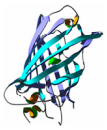
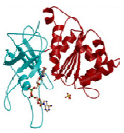
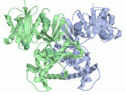
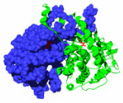
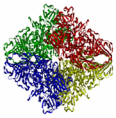
protein		organism	MW and oligomeric state	pI	Activity
enhanced Green Fluorescent Protein (eGFP)		*Aequorea victoria*	28 kDa monomer	5.67	intrinsic fluorescence
Ferredoxin NADP(H) Reductase (FNR)		*Arabidopsis thaliana*	35 kDa monomer	6.18	electron transfer between NADP(H) and ferredoxin (Fd)
Glucokinase (Glk)		*Escherichia coli*	35 kDa dimer	6.1	phosphorylation of glucose
Citrate Synthase (CS)		*Sus scrofa*	49 kDa dimer	8.12	Acetyl-CoA+ oxaloacetat to citrate + CoA
Beta – Galactosidase (β-gal)		*Escherichia coli*	116 kDa tetramer	5.28	hydrolysis of ß-D-galactosides
Dihydrofolate Reductase (DHFR)		*Mus musculis*	22. kDa monomer	8.56	NADP(H) dependent reduction of dihydrofalate to tetrahydrofolate

Table 1. Main characteristics of the test proteins.

Proteins are listed in the order of appearance.

6.2 Purification of test proteins

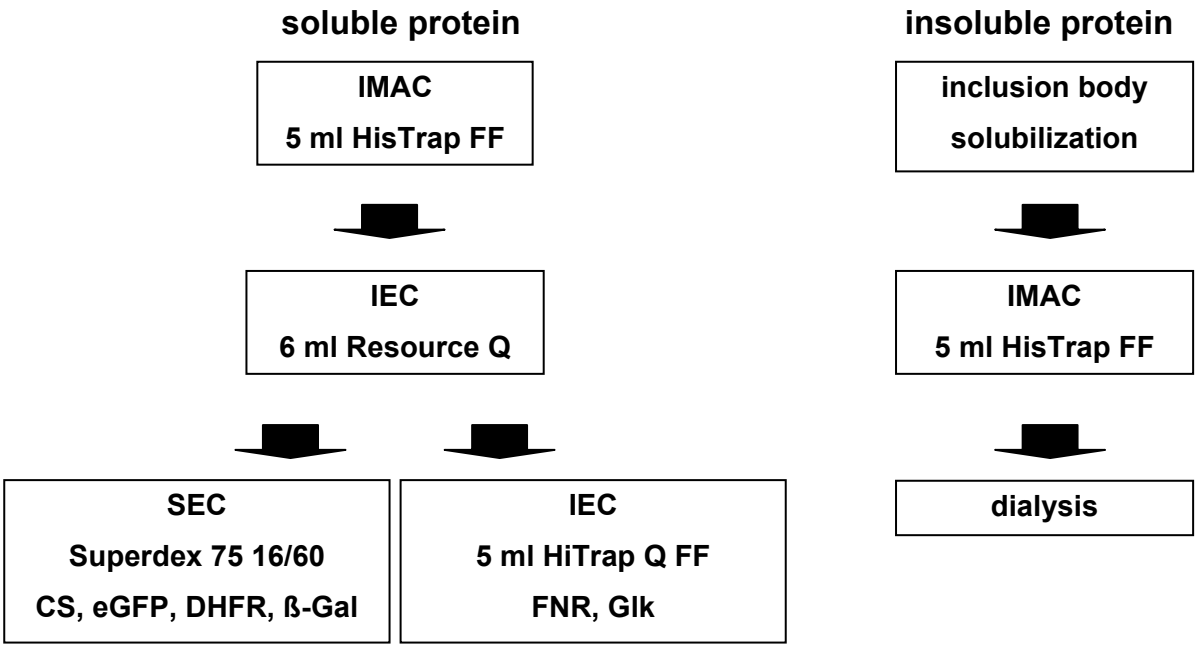


Figure 4 Schematic purification protocol of test proteins

Proteins were cloned and expressed as described in the material and method section (c.f. 8.6.5). All test proteins could be obtained in both soluble and insoluble form. Purification protocols were established as described in the flowchart (Figure 2). For soluble proteins immobilized metal affinity chromatography (IMAC) was used as a first purification step, followed by an ion-exchange chromatography (IEC), and subsequently by size exclusion chromatography (SEC) for eGFP, CS, DHFR, β-Gal. For FNR and Glk a second ion-exchange chromatography step with a different column was shown to be more effective. To obtain insoluble unfolded protein, IBs were solubilized in 6M GdmCl-containing buffer and purified by IMAC, followed by a dialysis step under denaturing conditions to remove imidazole.

6.3 Optimization of buffer conditions: outline

Matrix-assisted refolding experiments were performed in 96-well format by using 96-well HisMultiTrap FF plates, which enabled fast and parallel screening of refolding buffer conditions (c.f. 8.6.8.1).

Finding an optimal refolding buffer is a highly time- and labor-consuming process. There is or no general recipe or a set of optimal parameters applicable to any protein. Systematic variation of only 12 buffer parameters would require thousands of experiments (Mayer and

Buchner, 2004). Systematic analysis of the buffer parameters in a stepwise manner, however, minimizes the number of refolding conditions in the screening procedure. In this work, a novel approach of a stepwise optimization of the buffer conditions for the matrix-assisted refolding of different proteins was developed and analyzed (Figure 5).

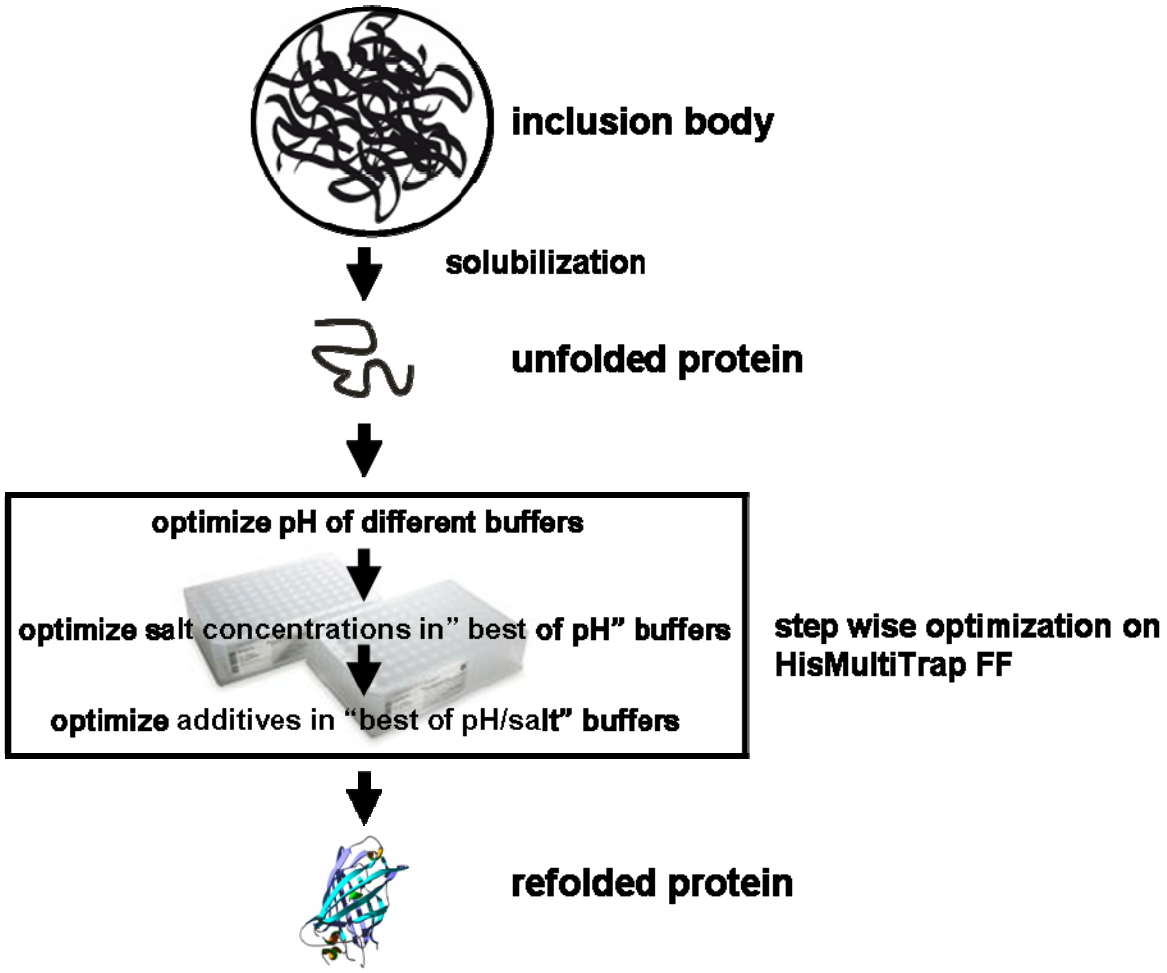


Figure 5 Schematic description of the stepwise optimization of the matrix-assisted refolding

The term "best of" refers to the buffer conditions, showing the highest refolding yields and chosen for the further optimization

In the first step, the basic buffer component and pH of the buffer were tested. Buffers with the highest refolding yields were chosen for the further optimization. In the second step, the optimal ionic strength was adjusted for these buffers. Subsequently, in the third and following steps, the impact of various additives on the refolding efficiency of the buffers from the previous step was examined. After each step, buffer compositions showing the best refolding efficiencies were used for the optimization of the next parameter. It should be mentioned, that all parameters tested in the screening procedures were adjusted to the requirements, in terms of chemical stability and compatibility, of the chromatography matrix material.

The set of the basic buffer components screened in the first step comprised 40 mM Na-P, 100 mM Tris/HCl, 100 mM Tris/AC, 100 mM K-P, 100 mM Hepes and 100 mM MOPS in the pH range 7.0 – 9.0. The chosen buffers are commonly used in protein chemistry and are

readily available. For the optimization of the ionic strength of the buffers, varying concentrations of sodium chloride in the range from 50 to 500 mM were tested in a second step.

Arginine and its derivatives have been shown to facilitate the refolding process, and it is probably one of the most widely used additives (Basu et al., 2011; Buchner and Rudolph, 1991; De Bernardez et al., 1999; Eiberle and Jungbauer, 2010; Jungbauer and Kaar, 2007; Lange and Rudolph, 2005; Mayer and Buchner, 2004; Rudolph and Lilie, 1996). However, commonly used L-Arg concentrations (100 mM – 1M) are not applicable in the matrix-assisted refolding. Abe et al showed that presence of L-Arg in concentrations higher than 100 mM results in an impaired binding of the target protein and in its elution from the matrix (Abe et al., 2009). Golovanov and Vedadi reported a stabilizing influence of the mixture of 50 mM Arg + 50 mM Gln on the proteins (Golovanov et al., 2004; Vedadi et al., 2006). Testing the mixture in on-matrix refolding experiments detected no loss of bound protein. In the third optimization step, different concentrations of the mixture in the equimolar ratio were tested. In the fourth step, further additives, which were shown to enhance the refolding efficiencies, were included in the screening: glycerol 2.5, 5 and 10 % v/v; PEG 6000 0.1%; sucrose 100, 200 and 300 mM; cyclodextrin 5 and 10 mM; TCEP 1, 2, 5 and 10 mM; DTE 1, 2 and 5 mM; $CaCl_2$ and $MgCl_2$ each 1mM.

In all refolding experiments the same amount of the native protein sample was applied as a reference in at least three wells, and treated equally to the samples to be refolded. The average of activities of the native protein was set to 100%.

Additionally, refolding efficiencies of all tested buffers were determined with a dilution based refolding method in parallel, to compare with the efficiency of matrix-assisted refolding (c.f. 8.6.8.3). Again, at least three native samples were applied as a reference.

6.4 Optimization of refolding buffer conditions

6.4.1 Optimization of refolding buffer conditions for eGFP

Enhanced green fluorescent protein (eGFP) was the first test protein to examine the applicability of the strategy. The fluorophore of eGFP is formed from three residues in the nascent polypeptide (Ser65-Tyr66-Gly67) which undergo an autocatalytic intramolecular cyclization. The formed fluorophore is enclosed within the protein (Chudakov et al., 2010). Denatured eGFP is nonfluorescent and changes its color from neon green to light yellow. However, upon refolding, the fluorescence of eGFP and its color are recovered (Paige et al., 2011). Application of eGFP allowed not only the direct monitoring of the refolding process, but also offered the possibility to track the chromatography performance of the protein in all steps of the refolding experiment. Initial experiments showed complete binding of loaded the eGFP sample to the chromatography matrix, as no traces of the protein could be detected in

the flow through. Application of the refolding buffer did not cause any loss of protein material and eGFP remained bound to the matrix till eluted. Additionally, recovery of the fluorophore upon incubation in the refolding buffer could also be observed directly on the matrix.

6.4.1.1 Matrix-assisted refolding of eGFP

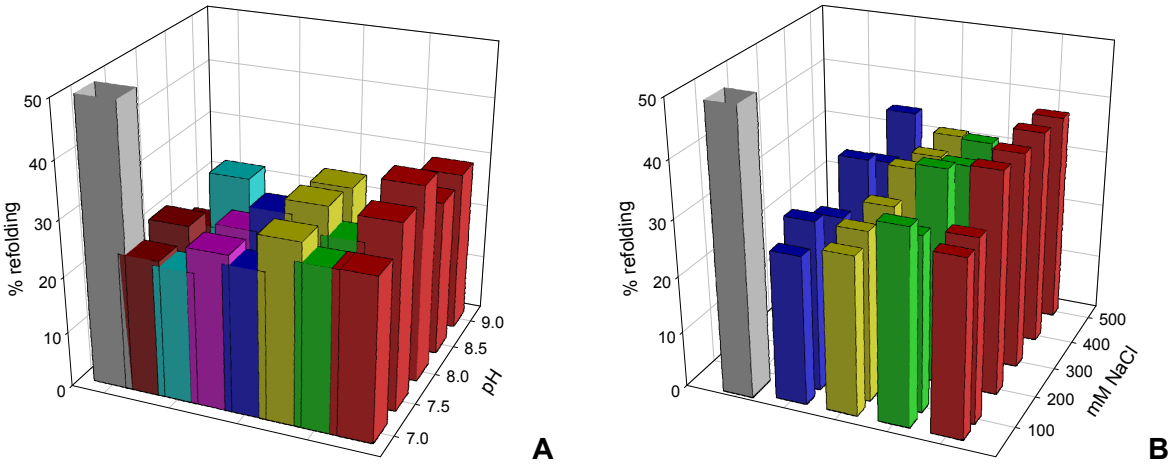


Figure 6 Step-wise optimization of matrix-assisted refolding of enhanced GFP.

Efficiencies of refolding after incubation for 1 h at 20°C in the respective buffers were determined according to the intrinsic GFP fluorescence at 508 nm. Equally treated, native GFP as reference is depicted in grey. The fluorescence of the native protein was cut at 50 %
A: First step, initial screening for optimal buffer substances and pH. Buffers: 100 mM HEPES (red), 100 mM MOPS (green), 40 mM Na-P (blue), 100 mM Tris/Ac (pink), 100 mM Tris/HCl (cyan), 100 mM Na-P (dark red), 100 mM K-P (dark green).
B: Second step, screening of salt concentrations in the best buffers selected in A. Buffers: 40 mM Na-P, pH 7.5 (red) or pH 8.0 (green), 100 mM Tris/HCl, pH 7.5 (blue) or pH 8.0 (cyan).

In the first step, the optimal buffer component and pH of the buffer were determined. Each of the tested buffer conditions resulted in refolded eGFP, with varying refolding efficiencies, ranging from <5% up to 30%. It should also be noted that the buffers with pH 7.5 - 8 showed overall the highest refolding yield (Figure 6A).

Four buffer conditions, with the highest refolding efficiencies were chosen, for the optimization of the next parameter, namely ionic strength. NaCl concentrations from 50 mM to 500 mM were tested. Analysis of the activities showed an increase of the average refolding yield and increased refolding efficiencies at salt concentrations of 200 - 300 mM NaCl. Higher salt concentrations did not result in a further improvement of the refolding yield. The compositions 100 mM TrisHCl 300 mM NaCl pH 7.5 or 8.0, and 40 mM Na-P 200 mM NaCl pH 7.5 or 8.0 showed the highest refolding yield (~35%) and were chosen for further optimization (Figure 6B).

Subsequently, in the third optimization step different concentrations of Arg and Gln (from 10 to 60 mM in equimolar ratios) were tested. Third step resulted in a further growth of the overall refolding yield. The optimum concentration of the tested amino acid mixture was determined to be 40 mM, with the refolding yield of ~40% and was included in the optimum buffer compositions (Figure 7A). In the following optimization step, a number of additional additives, which might further improve the refolding efficiency, was tested. This screening step revealed a positive influence only of a reducing agent; moreover, the presence of some other additives resulted in the drop of the activity of eGFP (Figure 7B). To further examine the influence of the reducing agent on the eGFP refolding, in the subsequent optimization step, several reducing agents in different concentrations were tested in all four optimum buffers.

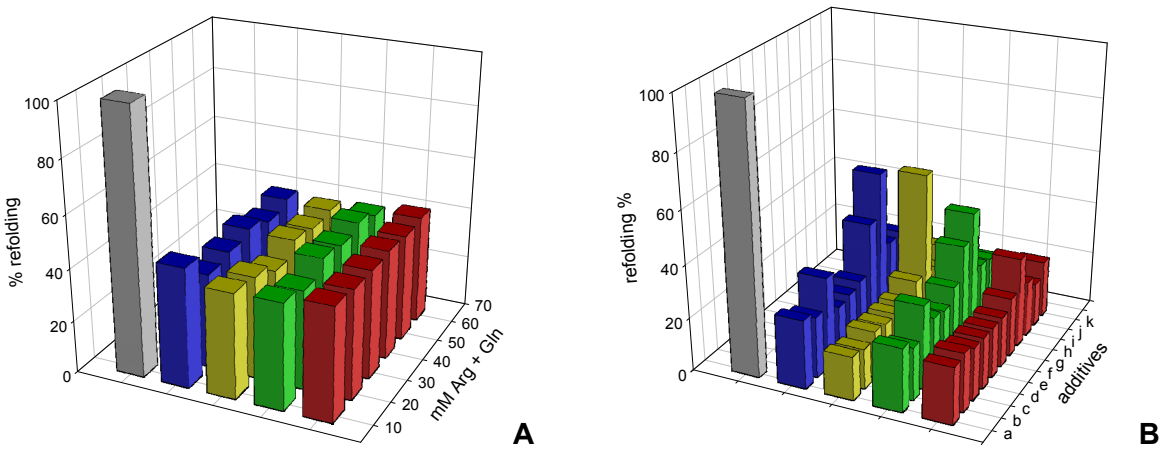


Figure 7 Step-wise optimization of matrix-assisted refolding of enhanced GFP.

Efficiencies of refolding after incubation for 1 h at 20°C in the respective buffers were determined according to the intrinsic GFP fluorescence at 508 nm. Equally treated, native GFP as reference is depicted in grey.
A: Third step, screening of different concentrations of an L-Arginine / L-Glutamine mixture. Buffers: 40 mM Na-P 200, mM NaCl, pH 7.5 (red) or pH 8.0 (green), 100 mM Tris/HCl, 300 mM NaCl, pH 7.5 (yellow) or pH 8.0 (blue).
B: Fourth step, screening of different refolding additives: Glycerin, 2.5% (a), 5% (b), 10% (c); Sucrose (c), 100 mM (d), 200 mM (e), 300 mM (f); TCEP, 1 mM (g) 2 mM (h); PEG, 0.01% (i); CaCl2, 1 mM (j); MgCl2, 1 mM (k) 40 mM Na-P 200, mM NaCl, pH 7.5 (red) or pH 8.0 (green), 100 mM Tris/HCl, 300 mM NaCl, pH 7.5 (yellow) or pH 8.0 (blue).

The presence of the reducing agent appeared to be essential for the optimal refolding of eGFP, and refolding yields of up to 100% could be achieved (Figure 8).
In this context, it should be mentioned, that the chromophore of eGFP has to be oxidized correctly for the determination of the activity and it seems that especially buffers containing reducing agents are potent to decrease wrong or to fast oxidation during refolding. Additionally, eGFP has two free cysteines in its amino acid composition, which emphasizes the importance of a suitable redox potential for the efficient refolding.

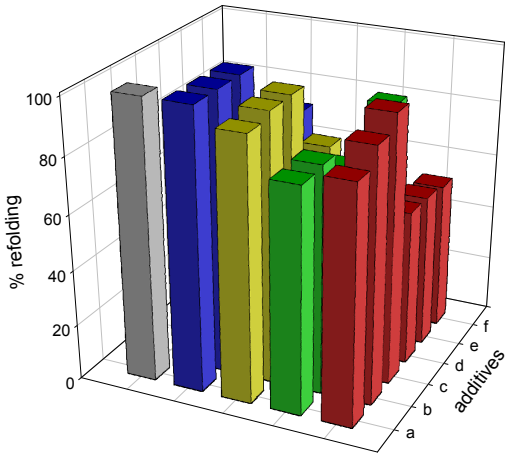


Figure 8 Step-wise optimization of matrix-assisted refolding of enhanced GFP.

Efficiencies of refolding after incubation for 1 h at 20°C in the respective buffers were determined according to the intrinsic GFP fluorescence at 508 nm. Equally treated, native GFP as reference is depicted in grey.
Fifth step, screening of reducing conditions. Buffers: 40 mM Na-P, 200 mM NaCl, 40 mM Arg, 40 mM Gln, pH 7.5 (red) or pH 8.0 (green), 100 mM Tris/HCl, 300 mM NaCl, 40 mM Arg, 40 mM Gln, pH 7.5 (yellow) or pH 8.0 (blue). Reducing agents: DTE, 1 mM (a), 2 mM (b), 5 mM (c); TCEP, 2 mM (d), 5 mM (e), 10 mM (f).

In summary, the buffer compositions resulting in the highest refolding yields comprised 100 mM TrisHCl and 40 Na-P with a pH either 7.5 and 8.0, 300 mM or 200 mM NaCl respectively, 40 mM Arg+Gln mixture and 2 mM and 5 mM DTE (Table 2).

buffer composition	refolding yield
100 mM Tris/HCl, 300 mM NaCl, 40 mM Arg, 40 mM Gln, 5 mM DTE pH 7.5	90 - 95%
100 mM Tris/HCl, 300 mM NaCl, 40 mM Arg, 40 mM Gln, 2 mM DTE pH 8.0	95 – 99%
100 mM Tris/HCl, 300 mM NaCl, 40 mM Arg, 40 mM Gln, 5 mM DTE pH 8.0	95-99%
40 mM Na-P, 200 mM NaCl, 40 mM Arg, 40 mM Gln, 5 mM DTE pH 7.5	90-95%

Table 2. Buffers resulting in the highest refolding yield in matrix-assisted refolding of eGFP

6.4.1.2 In-solution refolding of eGFP

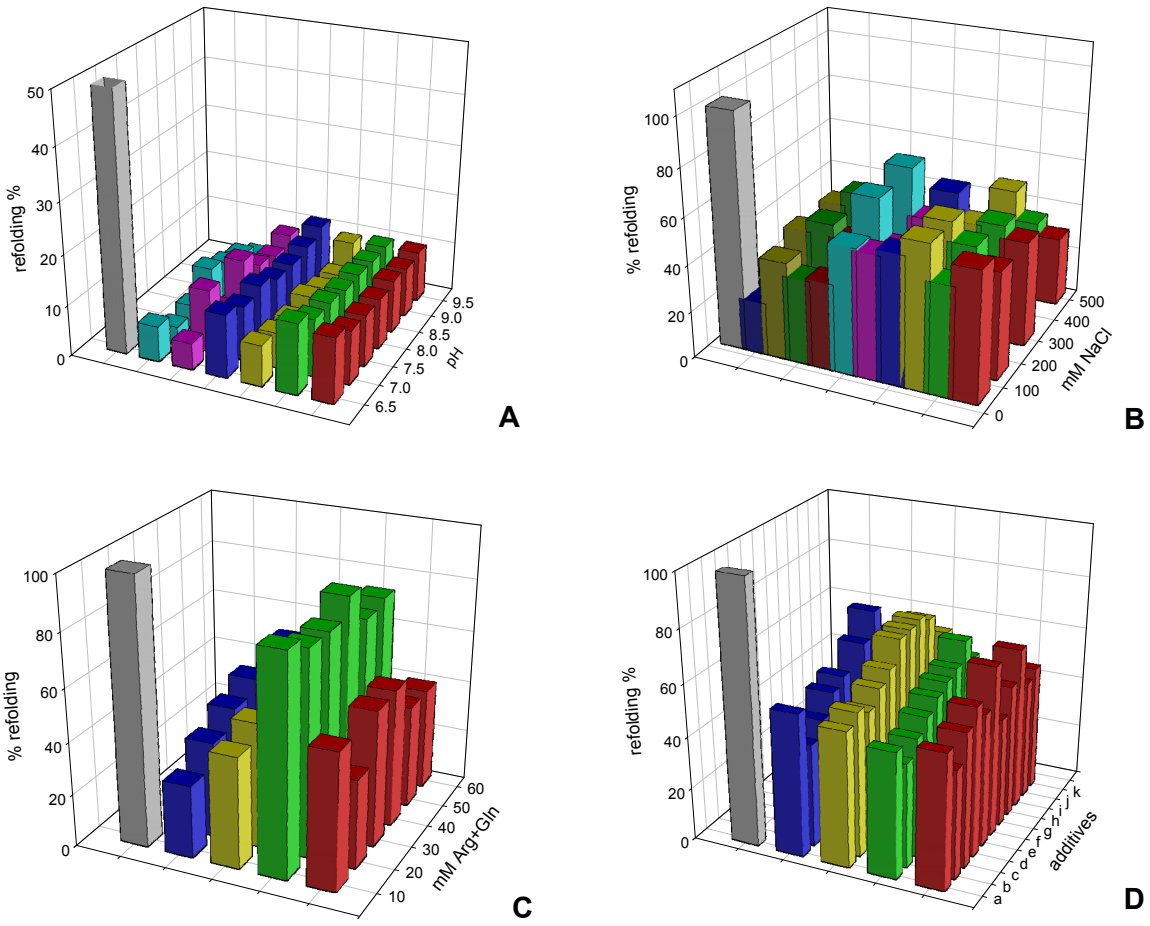


Figure 9 Refolding in solution of enhanced GFP.

Efficiencies of refolding in the respective buffers. Native GFP as reference is depicted in grey. Activity of native protein was cut at 50 % (A) for better visualization.
A: First step, initial screen of buffer substances and pH: 100 mM Tris/HCl (red), 100 mM Tris/Ac (green), 40 mM Na-P (yellow), 100 mM HEPES (blue), 100 mM MOPS (pink), 100 mM K-P (cyan).
B: Second step, screening of salt concentrations in the best buffers selected in A. Buffers: 40 mM Na-P, pH 7.0 (red) or pH 7.5 (green) or pH 8.0 (yellow), 100 mM Tris/HCl, pH 7.0 (blue) or pH 7.5 (pink) or pH 8.0 (cyan), 100 mM MOPS, pH 7.0 (dark red) or pH 8.0 (dark green), 100 mM HEPES, pH 7.0 (dark yellow) or pH 8.0 (dark blue).
C: Third step, screening of different concentrations of an L-Arginine / L-Glutamine mixture. Buffers: 40 mM Na-P, 200 mM NaCl, pH 7.5 (red) or pH 8.0 (green), 100 mM Tris/HCl, 300 mM NaCl, pH 7.5 (yellow) or pH 8.0 (blue).
D: Fourth step, screening of different refolding additives: Glycerin, 2.5% (a), 5% (b), 10% (c); Sucrose, 100 mM (d), 200 mM (e), 300 mM (f); TCEP, 1 mM (g) 2 mM (h); PEG, 0.01% (i);$CaCl_2$, 1 mM (j); $MgCl_2$, 1 mM (k) in 40 mM Na-P, 200 mM NaCl, pH 7.5 (red) or pH 8.0 (green), 100 mM Tris/HCl, 300 mM NaCl, pH 7.5 (yellow) or pH 8.0 (blue).

The presented optimization strategy was also applied to classical refolding in solution, which enabled to test and compare the refolding efficiencies of the two approaches.

Similarly to matrix–assisted refolding, the initial step resulted in low refolding yields (Figure 9A).

It should also be noted that during the first step, no clear results for the buffer component and pH could be obtained; consequently much more buffer compositions were tested in the following step. The refolding efficiency gradually increased in the next optimization steps

(Figures 9B-D), and also here a maximal refolding yield of 100 % could be finally achieved. Again the redox potential was determined to be a crucial parameter in refolding (Figure 10).

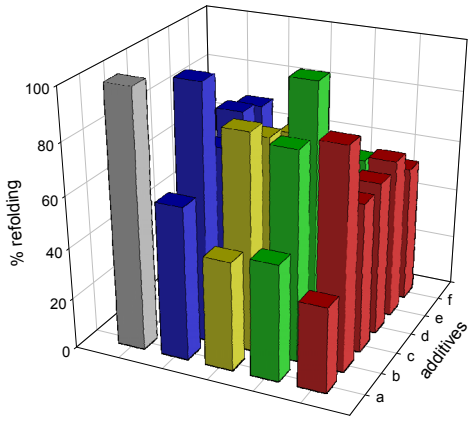


Figure 10 Refolding in solution of enhanced GFP.

Efficiencies of refolding in the respective buffers. Native GFP as reference is depicted in grey.
Fifth step, screening of reducing conditions. Buffers: 40 mM Na-P, 200 mM NaCl, 40 mM Arg, 40 mM Gln, pH 7.5 (red) or pH 8.0 (green), 100 mM Tris/HCl, 300 mM NaCl, 40 mM Arg, 40 mM Gln, pH 7.5 (yellow) or pH 8.0 (blue). Reducing agents: DTE, 1 mM (a), 2 mM (b), 5 mM (c); TCEP, 2 mM (d), 5 mM (e), 10 mM (f)

However, there were also some differences compared to the on-matrix refolding screen. Refolding in solution resulted in higher overall yields during the optimization process, for example an average of ~ 50% (Figure 9B) could be obtained during the second optimization step, compared to ~35% of on-matrix refolding, the same tendency could be observed during the additive screen (55% vs 30%) (Figure 9C-D).

The composition of the refolding buffers with the highest yield for in-solution refolding, is very similar to those obtained in on-matrix refolding i.e. 100 mM TrisHCl and 40 mM Na-P pH 7.5 and 8.0, with the salt concentrations of 200-300 mM, 40 mM Arg+Gln and 2mM and 5 mM DTE (Table 3).

buffer composition	refolding yield
100 mM Tris/HCl, 300 mM NaCl, 40 mM Arg, 40 mM Gln, 2 mM DTE pH 8.0	95 - 99%
100 mM Tris/HCl, 300 mM NaCl, 40 mM Arg, 40 mM Gln, 2 mM DTE pH 7.5	80 – 85%
40 mM Na-P, 200 mM NaCl, 40 mM Arg, 40 mM Gln, 5 mM DTE pH 8.0	95 - 99%
40 mM Na-P, 200 mM NaCl, 40 mM Arg, 40 mM Gln, 2 mM DTE pH 7.5	80 - 85%

Table 3. Buffers resulting in the highest refolding yield for refolding in solution of eGFP

Taken together, these results show that the stepwise optimization approach was efficient for both matrix-assisted refolding and refolding by dilution, and in both cases refolding yields of 100 % could be obtained.

6.4.2 Optimization of refolding buffer conditions for FNR

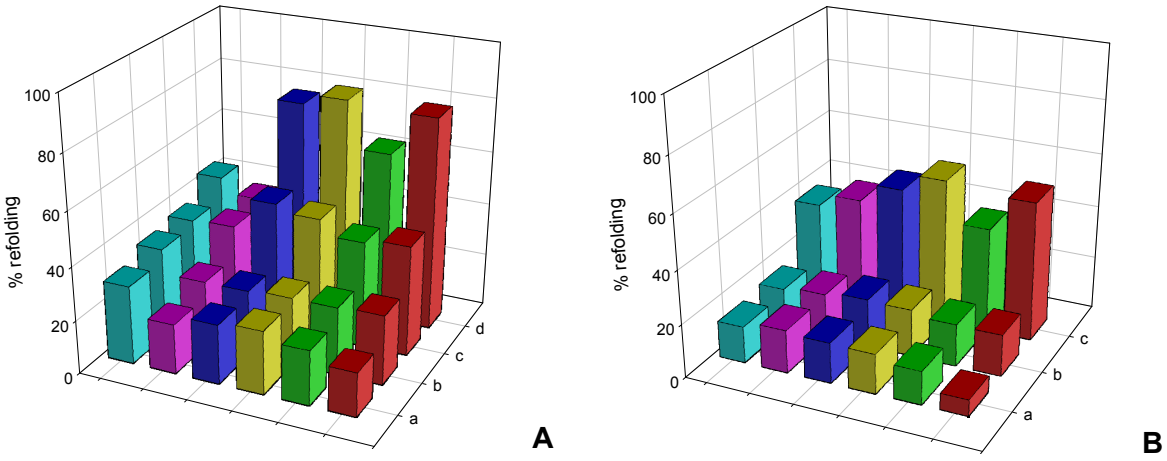


Figure 11. Summary of the optimization procedure for FNR.

Buffers resulting in the highest refolding efficiencies after each step are shown.
A. Matrix-assisted refolding. **B**. In-solution refolding
(a) first step, initial screening for optimum buffer substances and pH. Buffers: 40 mM Na-P pH7.5 (red), 40 mM Na-P pH 8.0 (green), 100 mM Tris/HCl pH 7.5 (yellow), 100 mM TrisHCl pH 8.0 (blue), 100 mM K-P pH 7.5 (pink), 100 mM K-P pH 8.0 (cyan).
(b) second step, screening of salt concentrations in the best buffers selected in (a): 200 mM NaCl in 40 mM Na-P pH 7.5 (red), pH 8.0 (green), 300 mM NaCl in 100 mM TrisHCl pH7.5 (yellow), pH 8.0 (blue), 100 mM K-P pH 7.5 (pink), pH 8.0 (cyan).
(c) third step, screening of different concentrations of an L-Arginine / L-Glutamine mixture, 50 mM for all buffers: 40 mM Na-P 200 mM NaCl pH 7.5 (red), pH 8.0 (green), 100 mM TrisHCl 300 mM NaCl pH7.5 (yellow), pH 8.0 (blue), 100 mM K-P 300 mM NaCl pH 7.5 (pink), pH 8.0 (cyan).
(d) fourth step, screening of different refolding additives: 5 mM TCEP for all buffers: 40 mM Na-P 200 mM 50 mM Arg+Gln NaCl pH 7.5 (red), pH 8.0 (green), 100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln pH7.5 (yellow), pH 8.0 (blue), 100 mM K-P 300 mM NaCl 50 mM Arg+Gln pH 7.5 (pink), pH 8.0 (cyan).

Ferredoxin NADP(H) reductase (FNR) was the second monomeric test protein applied to examine the applicability of the developed strategy. FNR is a 35 kDa flavoenzyme that catalyzes reversible electron transfer between NADP(H) and ferredoxin. The activity of the refolded samples was determined as described in material and methods (8.6.11.4).

The first two steps of the optimization of on-matrix refolding were characterized by relatively low refolding efficiencies (~ 15% and ~20 % respectively), resulting in six buffer conditions that were further optimized in the following steps. The refolding yield increased almost twofold upon the introduction of the Arg+Gln mixture. Subsequent screening of the different additives resulted in an overall increase of refolding yields, however addition of the reducing agent improved the refolding most efficiently and the maximum achieved refolding yields reached approximately 80% (Figure 11A).

Like for on-matrix refolding, the initial steps in solution were characterized by low refolding yields, with an average refolding efficiency after the second step of about 20%. Subsequent addition of the Arg+Gln mixture increased the refolding yield up to an average of 30% and a maximum of 55% (Figure 11B). Further screening was not possible, since the determination of the activity of the refolded samples was influenced by most of the additives, used in the refolding buffers. So an additional desalting step prior to an activity measurement would have been required to eliminate any influences of the test buffers. This finding highlights one of the advantages of the matrix-assisted method: all refolded samples are eluted in the same buffer and can be directly compared with each other, as influences of the different refolding buffers can be excluded.

In summary, the developed approach enabled efficient optimization of the refolding conditions. Although the matrix-assisted technique was more suitable for the FNR renaturation and yielded much higher recoveries, improvement of the refolding yields was also clear in every step of the in-solution refolding.

buffer composition	**refolding yield**
100 mM TrisHCl, 300 mM NaCl, 50 mM Arg+Gln, 5 mM TCEP pH 7.5	75 – 80%
100 mM Tris/HCl, 300 mM NaCl, 40 mM Arg, 40 mM Gln, 2 mM DTE pH 7.5	75 – 80%
100 mM TrisHCl, 300 mM NaCl, 50 mM Arg+Gln, 5 mM TCEP pH 8.0	75 – 80%
40 mM Na-P, 200 mM NaCl, 50 mM Arg+Gln, 2 mM TCEP pH 7.5	60 - 65%

Table 4. Buffers resulting in the highest refolding yield in on-matrix refolding of FNR

The refolding buffers with the highest refolding yields on-matrix and in-solution are summarized in Tables 4 and 5 respectively. The optimal basic buffer component, the pH and ionic strength are very similar for both refolding approaches and are in agreement with the parameters optimal for the eGFP refolding. For on-matrix refolding, the optimum concentration of the Arg+Gln mixture was determined to be 50 mM, for in-solution refolding lower concentrations of 20-30 mM were sufficient to improve the refolding yield. Additionally, for the matrix-assisted refolding the presence of the reducing agent in the refolding buffer was the factor that considerably increased the refolding efficiencies (up to 80%) (Figure 11A). Analysis of the influence of the reducing agent and other additives on the in-solution refolding was hindered and maximum refolding after the third optimization step was shown to be 55% (Figure 11B).

buffer composition	**refolding yield**
100 mM TrisHCl, 300 mM, 40 mM Arg+Gln pH 7.5	50 – 55%

100 mM TrisHCl, 300 mM, 30 mM Arg+Gln pH 8.0	45 – 50%
40 mM Na-P, 200 mM NaCl, 20 mM Arg+Gln pH 7.5	45 – 50%
40 mM Na-P, 200 mM NaCl, 30 mM Arg+Gln pH 8.0	45 - 50%

Table 5. Buffers resulting in the highest refolding yield for refolding in solution of FNR

6.4.3 Optimization of refolding buffer conditions for Glk and CS

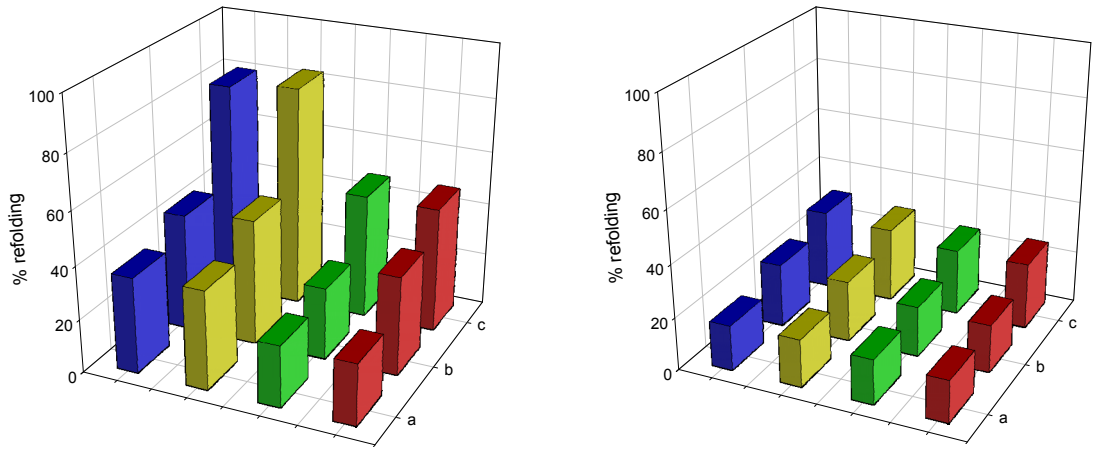


Figure 12 Summary of the optimization procedure of Citrate Synthase

Buffers resulting in the highest refolding efficiencies after each step are shown.
A. Matrix-assisted refolding. **B**. In-solution refolding
(a) first step, initial screening for optimal buffer substances and pH. Buffers: 40 mM Na-P pH7.5 (red), 40 mM Na-P pH 8.0 (green), 100 mM Tris/HCl pH 7.5 (yellow), 100 mM TrisHCl pH 8.0 (blue)
(b) second step, screening of salt concentrations in the best of buffers selected in (a): 200 mM NaCl in 40 mM Na-P pH 7.5 (red), pH 8.0 (green), 300 mM NaCl in 100 mM TrisHCl pH7.5 (yellow), pH 8.0 (blue)
(c) third step, screening of different concentrations of an L-Arginine / L-Glutamine mixture, 40 mM Arg+Gln for all buffers: 40 mM Na-P 200 mM NaCl pH 7.5 (red), pH 8.0 (green), 100 mM TrisHCl 300 mM NaCl pH7.5 (yellow), pH 8.0 (blue).

Refolding of the oligomeric proteins is a multi step process: in the first place monomers should adopt an association-competent conformation, followed by their assembly to a functional oligomer (Jaenicke, 1981). To examine the applicability of the stepwise optimization strategy and the on-matrix refolding to oligomeric proteins, the refolding performances of two dimeric proteins citrate synthase (CS) and glucokinase (Glk) were examined. CS from *Sus scrofa* is a homodimeric enzyme, consisting of 49 kDa subunits, that catalyze the first step of the citric acid cycle. *E. coli* Glk consists of the two identical subunits of 35 kDa and catalyzes the phosphorylation of glucose.

For both proteins tested, the applied systematic refinement of the refolding conditions on-matrix was very efficient, and improvement of the refolding yield could be observed after each optimization step. After the final step of optimization, reactivation of up to 70-80% for both dimeric test proteins could be achieved (Figure 12A, 13A). For CS, the addition of the

Arg+Gln mixture was the key component of the optimum refolding buffer. In the case of Glk, the redox potential was the most crucial parameter that improved the refolding yield.

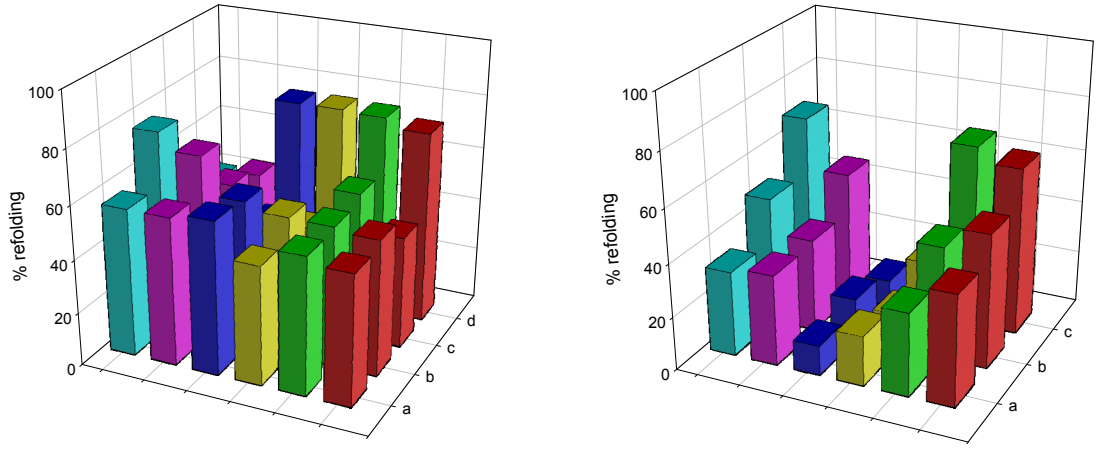


Figure 13 Summary of the optimization procedure of Glucokinase

Buffers resulting in the highest refolding efficiencies after each step are shown.
A. Matrix-assisted refolding. **B**. In-solution refolding
(a) first step, initial screening for optimal buffer substances and pH. Buffers: 40 mM Na-P pH7.5 (red), 40 mM Na-P pH 8.0 (green), 100 mM Tris/HCl pH 7.5 (yellow), 100 mM TrisHCl pH 8.0 (blue), 100 mM K-P pH 7.5 (pink), 100 mM K-P pH 8.0 (cyan).
(b) second step, screening of salt concentrations in the best of buffers selected in (a): 200 mM NaCl in 40 mM Na-P pH 7.5 (red), pH 8.0 (green), 300 mM NaCl in 100 mM TrisHCl pH7.5 (yellow), pH 8.0 (blue), 100 mM K-P pH 7.5 (pink), pH 8.0 (cyan).
(c) third step, screening of different concentrations of an L-Arginine / L-Glutamine mixture, 50 mM for all buffers: 40 mM Na-P 200 mM NaCl pH 7.5 (red), pH 8.0 (green), 100 mM TrisHCl 300 mM NaCl pH7.5 (yellow), pH 8.0 (blue), 100 mM K-P 300 mM NaCl pH 7.5 (pink), pH 8.0 (cyan).
(d) fourth step, screening of different refolding additives: 5 mM TCEP for 40 mM Na-P 200 mM 50 mM Arg+Gln NaCl pH 8.0 (green), 100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln pH 7.5 (yellow)and pH 8.0 (blue), 100 mM K-P 300 mM NaCl 50 mM Arg+Gln pH 7.5 (pink), pH 8.0 (cyan); 2 mM DTE for 40 mM Na-P 200 mM 50 mM Arg+Gln) pH 7.5 (red).

Comparison of the refolding efficiencies of in-solution and on-matrix refolding showed that the matrix-assisted strategy resulted in higher refolding yields for both proteins through the entire screening procedure (Figure 12B, 13B).

For CS, the parameters of the optimal refolding buffers were shown to be identical for on-matrix and in-solution refolding, however, the refolding efficiencies varied significantly with 80% on-matrix vs. 30% in solution. Optimum buffer parameters i.e. buffer component, pH, ionic strength, were very similar to those determined for eGFP and FNR, but in the final optimization step the 100 mM TrisHCl based buffers showed higher refolding efficiencies, compared to the 40 mM Na-P based buffers (Figure 12A, B, Table 6,7).

buffer composition	**refolding yield**
100 mM TrisHCl 300mM NaCl 40 mM Arg +Gln pH 7.5	75 – 80%
100 mM TrisHCl 300mM NaCl 40 mM Arg +Gln pH 8.0	75 – 80%

Table 6. Buffers resulting in the highest refolding yield in on-matrix refolding of CS

buffer composition	refolding yield
100 mM TrisHCl 300mM NaCl 40 mM Arg +Gln pH 7.5	25 – 30%
100 mM TrisHCl 300mM NaCl 40 mM Arg +Gln pH 8.0	25 – 30%

Table 7. Buffers resulting in the highest refolding yield for refolding in-solution of CS

For Glk, comparison of the parameters of the optimum refolding buffers for the two refolding approaches showed that 100 mM TrisHCl and 40 mM Na-P based buffers were shown to result in the highest refolding efficiencies on-matrix, whereas for the refolding in-solution phosphate-based buffers (Na-P and K-P) appeared to be preferential (Figure 13A,B). The optimum concentration of the Arg+Gln mixture was shown to be 50 mM. The presence of the six free cysteines in the polypeptide chain of Glk indicates the importance of the redox potential during the refolding and might explain the improvement of the refolding yield by the addition of reducing agent (2mM DTE or 5 mM TCEP) (Figure 13A, Table 8). For the Glk refolding in-solution, the activity determination of the refolded samples in the fourth step was not possible, since similar to FNR, the assay was influenced by the additives in the refolding buffers (Figure 13B, Table 9).

buffer composition	refolding yield
40 mM Na-P 200 mM NaCl 50 mM Arg+Gln 2 mM DTE pH 7.5	75 – 80%
40 mM Na-P 200 mM NaCl 50 mM Arg+Gln 5 mM TCEP pH 8.	75 – 80%
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 5 mM TCEP pH 7.5	75 – 80%
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 5 mM TCEP pH 8.0	75 – 80%

Table 8.Buffers resulting in the highest refolding yield in on-matrix refolding of Glk

buffer composition	refolding yield
40 mM Na-P 200 mM NaCl 50 mM Arg+Gln pH 7.5	65 - 70%
40 mM Na-P 200 mM NaCl 50 mM Arg+Gln pH 8.0	60 – 65%
100 mM K-P 300 mM NaCl 50 mM Arg+Gln pH 7.5	45 – 50%

100 mM K-P 300 mM NaCl 50 mM Arg+Gln pH 8.0	65 – 70%

Table 9. Buffers resulting in the highest refolding yield for refolding in-solution of Glk

6.4.4 Optimization of refolding buffer conditions for β-galactosidase

Since the stepwise optimization could be successfully applied to refold two dimeric proteins, refolding of a protein of a higher oligomeric state was tested next.

β-galactosidase from *E.coli* is a tetrameric protein, with identical subunits of 116 kDa, and is one of the largest enzymes known. There has been a number of studies on the structure and mechanism of action of β-gal and it has been shown, that the assembly of a tetramer is a prerequisite for the enzymatic activity of the protein (Juers et al., 2000; Matsuura et al., 2011; Nichtl et al., 1998).

β-gal has been shown to be a rather demanding protein in terms of refolding, and application of the dilution-based refolding methods for the GdmCl denatured β-gal resulted in almost no active protein. Only in the presence of molecular chaperones refolding yields of 20-40% could be achieved (Ayling and Baneyx, 1996; Freeman and Morimoto, 1996).

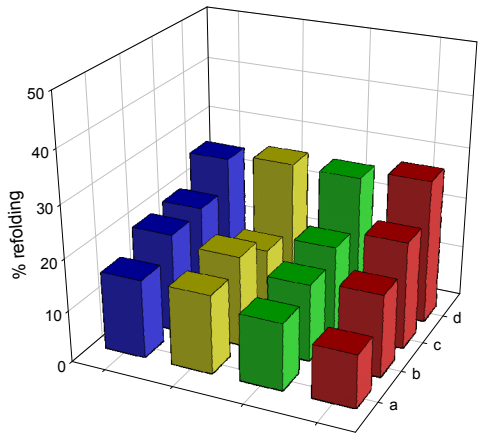


Figure 14 Summary of the optimization procedure for β-gal.

Buffers resulting in the highest refolding efficiencies after each step for Matrix-assisted refolding are shown.
(a) first step, initial screening for optimal buffer substances and pH. Buffers: 40 mM Na-P pH7.5 (red), 40 mM Na-P pH 8.0 (green), 100 mM Tris/HCl pH 7.5 (yellow), 100 mM TrisHCl pH 8.0 (blue),
(b) second step, screening of salt concentrations in the best of buffers selected in (a): 200 mM NaCl in 40 mM Na-P pH 7.5 (red), pH 8.0 (green), 300 mM NaCl in 100 mM TrisHCl pH7.5 (yellow), pH 8.0 (blue).
(c) third step, screening of different concentrations of an L-Arginine / L-Glutamine mixture, 50 mM for all buffers: 40 mM Na-P 200 mM NaCl pH 7.5 (red), pH 8.0 (green), 100 mM TrisHCl 300 mM NaCl pH7.5 (yellow), pH 8.0 (blue).
(d) fourth step, screening of different refolding additives: 5 mM DTE for 40 mM Na-P 200 mM 50 mM Arg+Gln NaCl pH 7.5 (red) and pH 8.0 (green); 2 mM TCEP for 100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln pH 7.5 (yellow)and pH 8.0 (blue).

Applying the stepwise optimization approach allowed determining buffer conditions for the successful on-matrix refolding of β-gal, and refolding yields of up to 30% (Figure 14) could be obtained. It should be mentioned that also for β-gal every optimization step resulted in improved refolding efficiency of the target protein. Interestingly, in consistence with literature, none of the in-solution refolding experiments performed in parallel resulted in noteworthy refolding yields.

In the optimization procedure, 40 mM Na-P and 100 mM TrisHCl based buffers with of pH 7.5 or 8.0 showed again the best refolding performance. The optimum salt concentration was determined to be 200 - 300 mM NaCl and the mixture of 50 mM Arg+Gln was shown to improve the refolding yield most efficiently. The addition of the reducing agent was shown to further improve the refolding efficiency. The polypeptide chain of β-gal contains 16 free cysteines, which indicates that a suitable redox potential was required for efficient refolding (Table 10).

buffer composition	refolding

	yield
40 mM Na-P 200 mM 50 mM Arg+Gln NaCl 5 mM DTE pH 7.5	25 - 30%
40 mM Na-P 200 mM 50 mM Arg+Gln NaCl 5 mM DTE pH 8.0	25 – 30%
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 2 mM TCEP pH 7.5	20 – 25%
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 2 mM TCEP pH 8.0	25 – 30%

Table 10. Buffers resulting in the highest refolding yield in on-matrix refolding of β-gal

Additionally, several buffer conditions, applied to refold β-gal in the presence of molecular chaperones described in literature, were tested for both matrix-assisted and in-solution techniques. In-solution refolding experiments showed only negligible refolding yields, as reported in the respective studies (Ayling and Baneyx, 1996; Freeman and Morimoto, 1996; Nichtl et al., 1998; Takayama et al., 1997), whereas the matrix-assisted approach under the same buffer conditions was very efficient with the refolding yields around 30% (Figure 15). Remarkably, for the buffer condition described by Ayling and Baneyx similar refolding yields (~20%) could be achieved only in the presence of a twofold molar excess of GroEL (Ayling and Baneyx, 1996). Comparison of the buffer compositions from the literature with the refolding buffers derived from the optimization procedure revealed rather little similarities, however, all the buffers tested were in the similar pH range and included reducing agents.

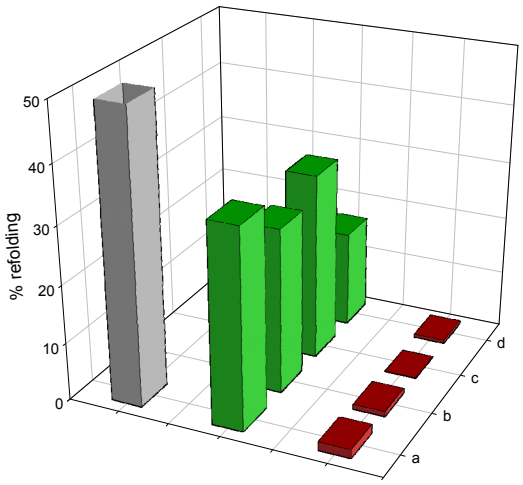


Figure 15 Comparison of refolding efficiencies on-matrix (green) and in-solution (red).

Refolding experiments were performed under identical buffer conditions. The native reference is shown grey, the activity of the native reference is cut at 50%.
(a).100 mM Na-P, 1mM $MgCl_2$, 5 mM DTE, 1.4 M Urea pH 7.5 (Nichtl et al., 1998)
(b) 100 mM TrisHCl, 10 mM KCl, 10mM $MgCl_2$, 2.5 mM DTE, pH 7.5 (Ayling and Baneyx, 1996)
(c) 25 mM Hepes, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTE, pH 7.5 (Takayama et al., 1997)
(d) 100 mM Na-P, 1 mM $MgCl_2$, 10 mM KCl, 50 mM β-mercaptoethanol, pH 7.5 (activity assay buffer (Miller J.H., 1972))

The results of the optimization procedure for the five test proteins showed that the proposed stepwise strategy for on-matrix refolding proved efficient and allowed to determine optimal

refolding buffer conditions in all cases. Surprisingly, the optimal refolding buffer compositions for all proteins tested appeared to be rather similar; moreover, buffer component, pH and ionic strength of the buffers correlate well with those recommended for the used chromatography type, which might indicate that a good chromatography performance is a precondition for the matrix-assisted refolding *per se*.

6.4.5 Definition of a minimum buffer set and application for refolding of DHFR

The narrow range of the optimal refolding buffers for the tested proteins enables to propose a minimum set of the refolding buffer conditions, which might be generally applicable for the matrix-assisted protein refolding. With regards to the buffer conditions resulting in the highest refolding yields for the proteins tested, a minimum buffer set was established (Table 11).

buffer composition
40 mM Na-P 200 mM 50 mM Arg+Gln NaCl 2 mM DTE pH 7.5
40 mM Na-P 200 mM 50 mM Arg+Gln NaCl 5 mM DTE pH 7.5
40 mM Na-P 200 mM 50 mM Arg+Gln NaCl 2 mM TCEP pH 7.5
40 mM Na-P 200 mM 50 mM Arg+Gln NaCl 5 mM TCEP pH 7.5
40 mM Na-P 200 mM 50 mM Arg+Gln NaCl 2 mM DTE pH 8.0
40 mM Na-P 200 mM 50 mM Arg+Gln NaCl 5 mM DTE pH 8.0
40 mM Na-P 200 mM 50 mM Arg+Gln NaCl 2 mM TCEP pH 8.0
40 mM Na-P 200 mM 50 mM Arg+Gln NaCl 5 mM TCEP pH 8.0
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 2 mM DTE pH 7.5
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 5 mM DTE pH 7.5
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 2 mM TCEP pH 7.5
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 5 mM TCEP pH 7.5
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 2 mM DTE pH 8.0
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 5 mM DTE pH 8.0
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 2 mM TCEP pH 8.0
100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 5 mM TCEP pH 8.0

Table 11. Minimum set of buffers proposed for the matrix-assisted refolding

To examine whether the proposed minimum buffer set would result in successful renaturation of dihydrofolate reductase (DHFR), refolding experiments on-matrix and in-solution were carried out.

All of the tested refolding buffers resulted in refolded DHFR with varying refolding yields. Refolding on-matrix appeared to be more efficient and refolding yields of up to 90% could be achieved, whereas a parallel in-solution approach resulted only in renaturation yields of up to 60% (Figure 16). High refolding efficiencies on-matrix could be achieved under the respective buffer conditions without any preceding optimization, which seems to confirm the proposed hypothesis of the chromatography-defined restriction of the buffer conditions.

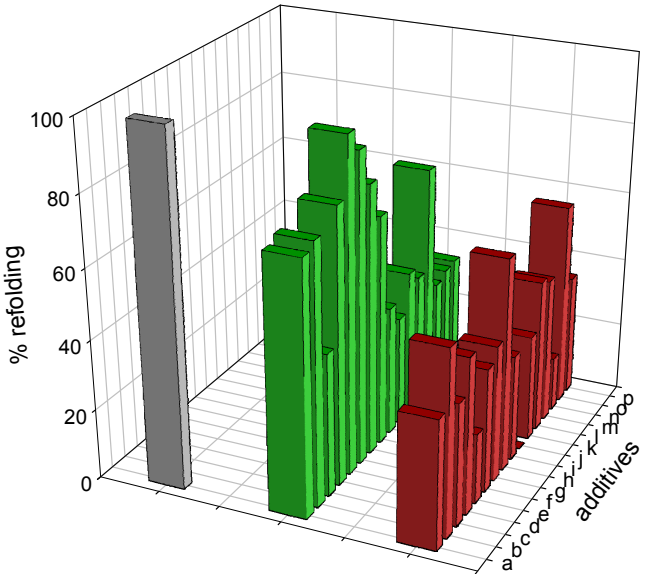


Figure 16 Comparison of refolding efficiencies of DHFR in solution (red) and on-matrix (green) in different buffers.

The native reference is depicted in grey.
40 mM Na-P 200 mM NaCl 50 mM Arg+Gln, 2 mM DTE (a), 5 mM DTE (b), 2 mM TCEP (c), 5 mM TCEP (d) pH 7.5; 40 mM Na-P 200 mM NaCl 50 mM Arg+Gln, 2 mM DTE (e), 5 mM DTE (f), 2 mM TCEP (g), 5 mM TCEP (h) pH 8.0; 100 mM Tris/HCl 300 mM NaCl 50 mM Arg+Gln, 2 mM DTE (i), 5 mM DTE (j), 2 mM TCEP (k), 5 mM TCEP (l) pH 7.5; 100 mM Tris/HCl 300 mM NaCl 50 mM Arg+Gln, 2 mM DTE (m), 5 mM DTE (n), 2 mM TCEP (o), 5 mM TCEP (p) pH 8.0.

6.5 Transfer from HisMultiTrap to scalable columns

The introduced stepwise optimization strategy enabled to determine the optimal refolding buffer conditions for the proteins tested. To examine the efficiency of the refolding procedure on a large scale under the obtained refolding conditions, matrix-assisted refolding was carried out on 1ml HisTrap FF column by the means of Äkta Explorer chromatography system.

The up-scaling procedure was kept similar to that applied for the HisMultiTrap FF plate: solubilized protein was loaded on the pre-equilibrated column, washed with the denaturing

buffer, treated with refolding buffer (5 column volumes (CV)= 5ml), left to incubate for 1h and treated once again with refolding buffer (5CV). Elution was performed by a linear gradient of 0 - 500 mM imidazole. Eluted fractions were pooled together and the activity of the refolded sample was determined with the respective assay. Prior to the refolding experiment, the same amount of the native protein, as a reference, was loaded onto the column and treated equally. Figure 17 demonstrates the refolding procedure for Glk.

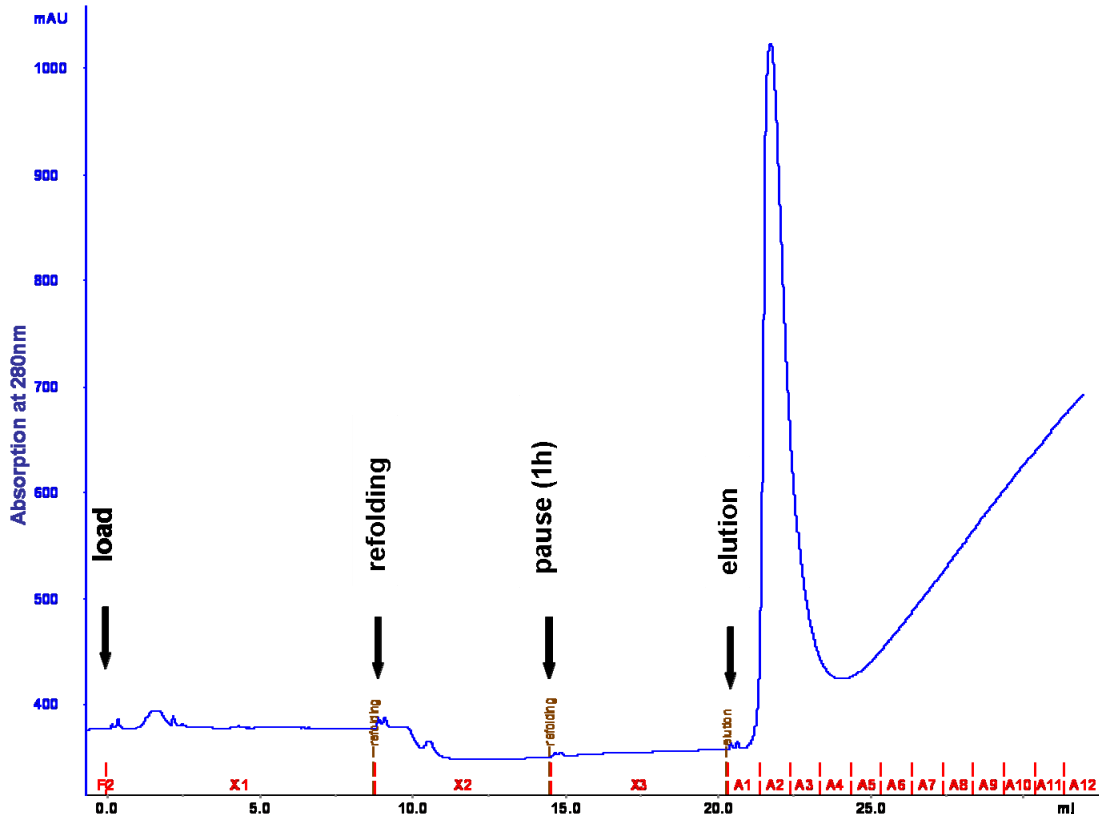


Figure 17. Chromatogram of the refolding procedure of Glucokinase: 1 ml HisTrap FF column, Äkta Explorer

Refolding experiment was carried out in 40 mM Na-P, 200 mM NaCl, 50 mM Arg+Gln, 5 mM TCEP, pH 8.0, at 0.5 ml/min at 20°C.The single steps of the refolding procedures are indicated with arrows. A1 – A12 - elution fractions

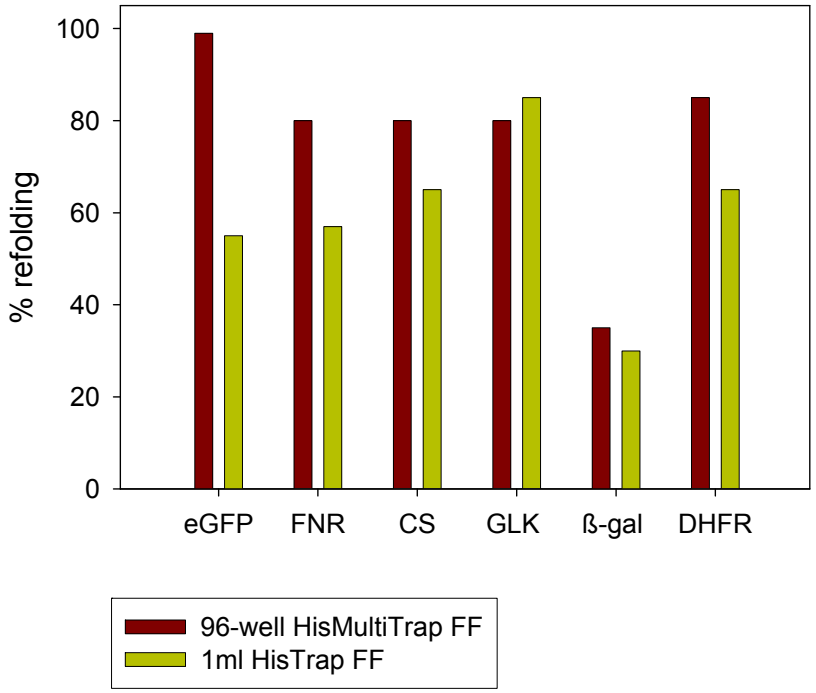


Figure 18. Comparison of refolding efficiencies of matrix-assisted refolding on HisMultiTrap FF and 1ml HisTrap FF. Incubation for 1h at 20 °C.

Respective optimum buffers applied:
eGFP: 40 mM Na-P, 200 mM NaCl, 40 mM Arg, 40 mM Gln, 5 mM DTE pH 7.5
FNR: 100 mM TrisHCl, 300 mM NaCl, 50 mM Arg+Gln, 5 mM TCEP pH 8.0
CS: 100 mM TrisHCl 300 mM NaCl 40 mM Arg+Gln pH 8.0
GLK: 100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 5 mM TCEP pH 7.5
β-gal: 100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln 2 mM TCEP pH 7.5
DHFR: 40 mM Na-P 300 mM NaCl 50 mM Arg+Gln, 5 mM DTE pH 8.0

Up-scaling refolding experiments were carried out for all test proteins with the respective optimum refolding buffer. Refolding efficiencies of the two matrix-assisted approaches are summarized in Figure 18.

The presented results show that direct up scaling of the optimized refolding procedure was possible for all proteins tested, without any further optimization of any parameters. Comparison of the refolding efficiencies of two approaches showed slight differences, however, they were comparable for all tested targets, except for eGFP (c.f. 7.2).

6.6 Refolding kinetics

Matrix-assisted refolding was shown to be more efficient for most of the tested proteins. Next, the kinetics of the on-matrix and in-solution refolding was studied to examine whether there are any approach specific differences. To do so, denatured test protein immobilized on the matrix was incubated in the refolding buffer for different periods of time - 5, 10, 20, 30, 45, 60 and 120 min. In case of the in-solution approach, the test protein was diluted into the refolding buffer and the activity of the sample was measured at the respective time points. Both refolding approaches were carried out under identical buffer conditions.

6.6.1 Refolding kinetics of FNR

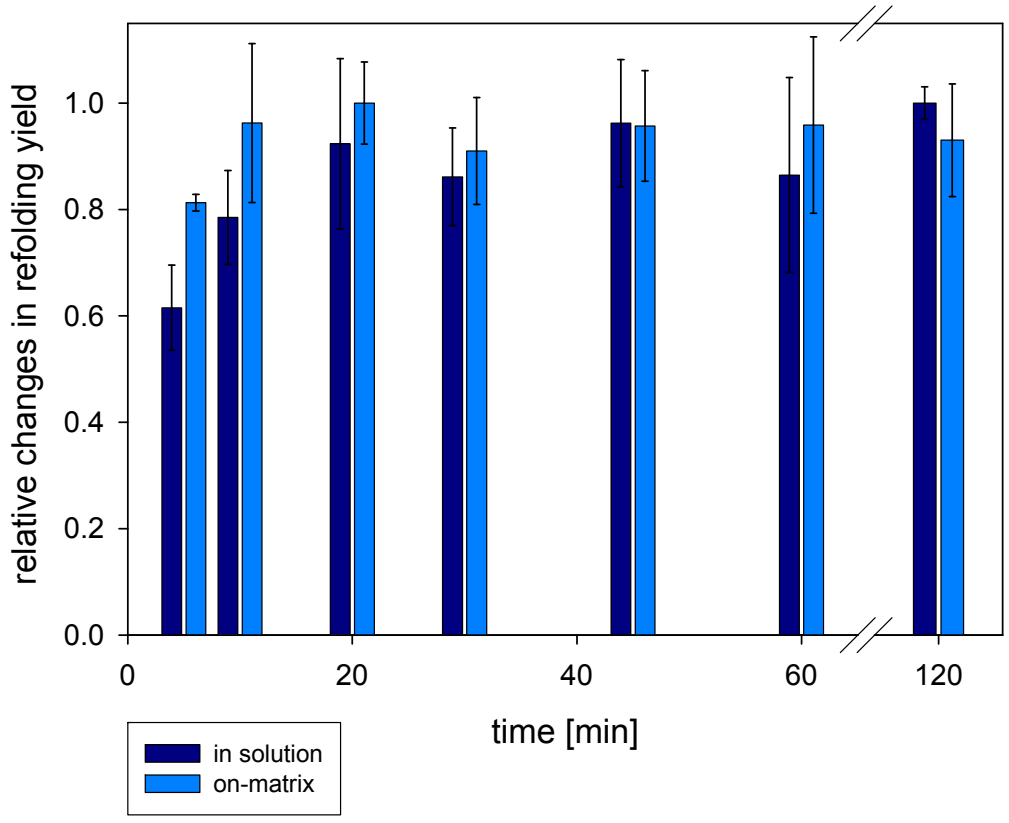


Figure 19 Comparison of refolding kinetics of FNR in solution (dark blue) vs. on-matrix (light blue)

Refolding experiments were carried out in 100 mM TrisHCl, 300 mM NaCl, 50 mM Arg+Gln, 5 mM TCEP, pH 8.0. The maximum refolding yield under the respective buffer condition was set to 1.

Analysis of the refolding times in correlation to renaturation yields showed that for FNR, immobilized on matrix, an incubation time of 10 min was sufficient to regain maximal yield. No further improvement of refolding yield could be monitored upon longer incubation on-

matrix. In-solution refolding procedures showed slower refolding kinetics, compared to refolding on-matrix, to reach the refolding optimum. Longer incubation times also did not result in the increased renaturation efficiency (Figure 19)

6.6.2 Refolding kinetics of CS

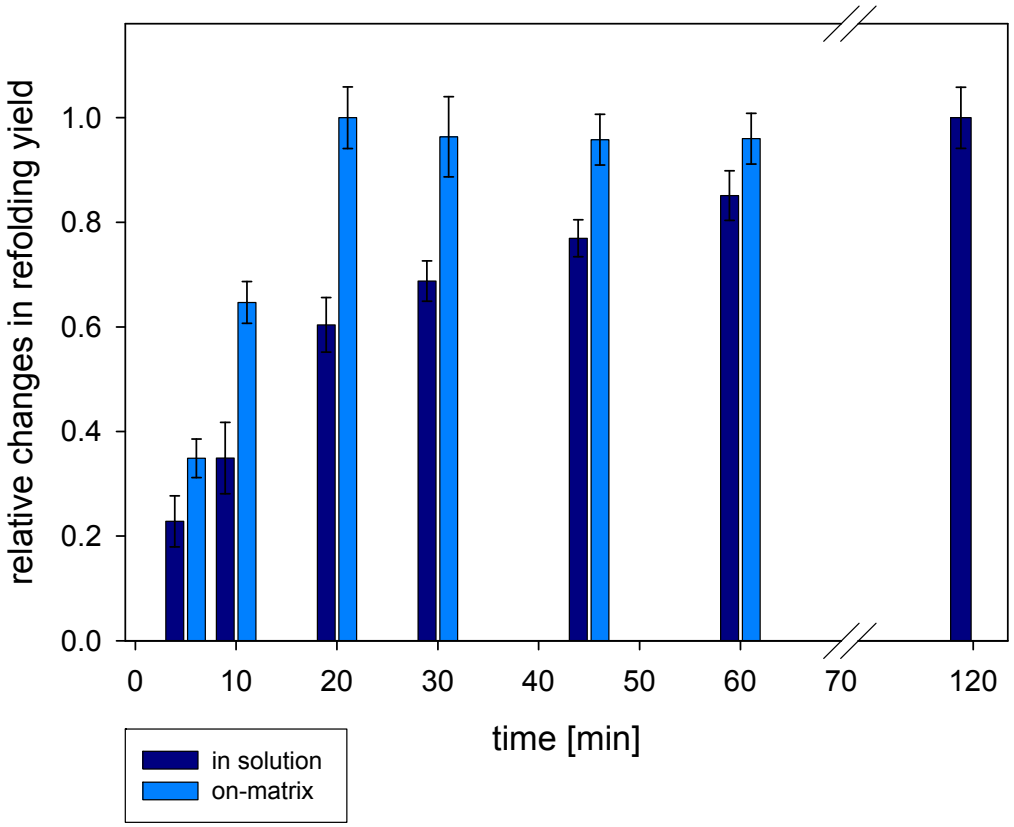


Figure 20 Comparison of refolding kinetics of CS in solution (dark blue) vs. on-matrix (light blue)

Refolding experiments were carried out in 100 mM TrisHCl, 300 mM NaCl, 40 mM Arg+Gln, pH 8.0. The maximum refolding yield under the respective buffer condition was set to 1

Comparison of the refolding kinetics of CS showed that for in-solution refolding, incubation times of more than one hour were required to reach the refolding maximum. The matrix-assisted approach resulted in an accelerated renaturation compared to the in-solution strategy, and a shorter refolding time of ~20 min was sufficient to obtain the maximum refolding yield (Figure 20).

6.6.3 Refolding kinetics of Glk

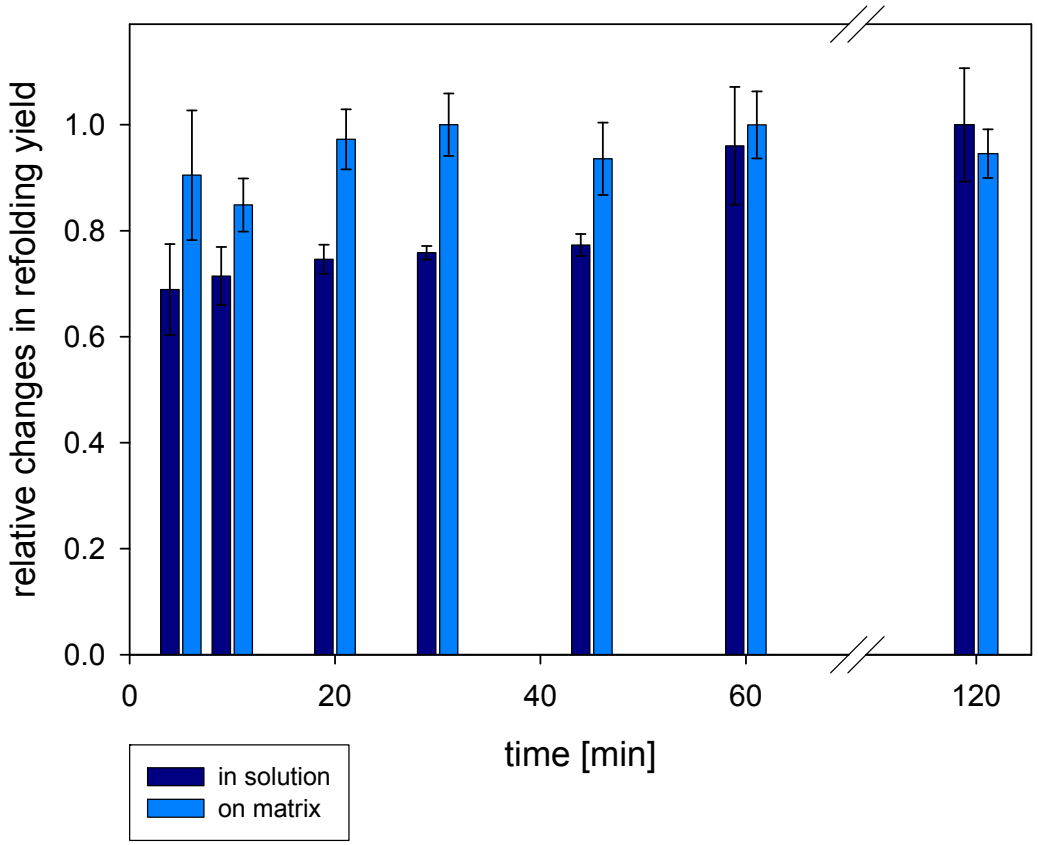


Figure 21 Comparison of refolding kinetics of Glk in solution (dark blue) vs. on-matrix (light blue)

Refolding experiments were carried out in 40 mM Na-P 200 mM NaCl, 50 mM Arg+Gln, 5 mM TCEP pH 8.0. The maximum refolding yield under the respective buffer condition was set to 1

For the matrix-assisted refolding of Glk, the maximum refolding yield could be obtained on-matrix after incubation for only 5 min and longer incubation times did not result in any further improvement. For the in-solution refolding, maximal refolding could be obtained after 60 min of incubation in the respective refolding buffer (Figure 21).

6.6.4 Refolding kinetics of β-gal

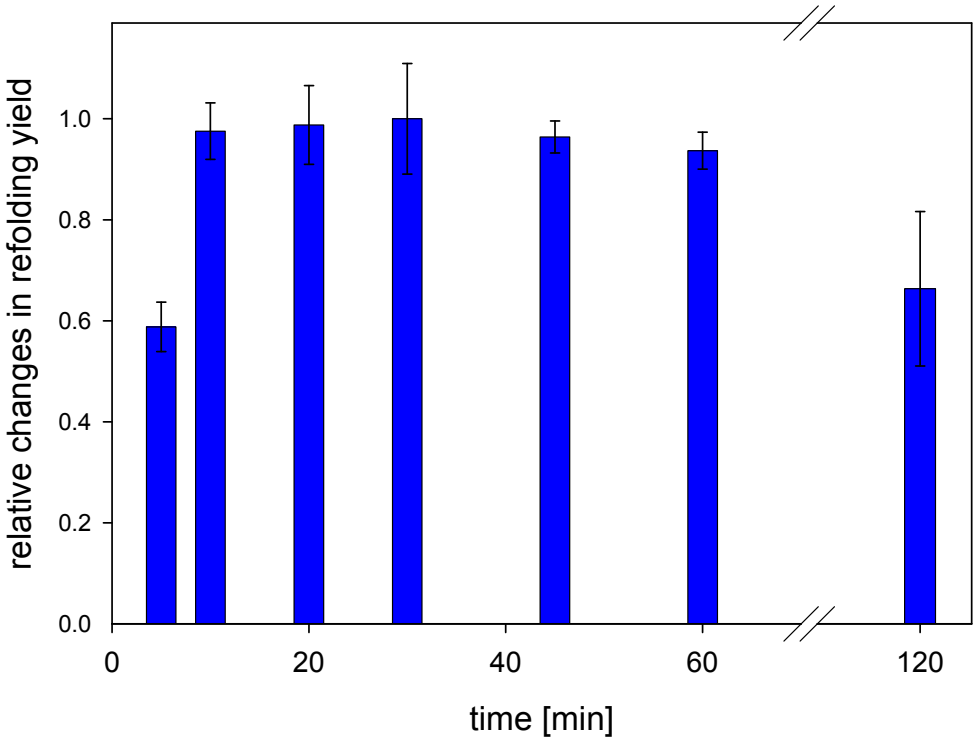


Figure 22 Refolding kinetics of β-gal on-matrix

Refolding experiments were carried out in 40 mM Na-P 200 mM NaCl 50 mM Arg+Gln 2 mM TCEP pH 7.5. The maximum refolding yield under the respective buffer condition was set to 1

No differences in the refolding efficiencies could be observed after incubation of immobilized β-gal in the refolding buffer for 10, 30, 45 or 60 min (Figure 22), which suggests that the renaturation process was rather fast, with the maximum refolding achieved on-matrix after incubation for only 10 min. Surprisingly, a longer incubation time did not result in any additional positive impact on the refolding efficiency of β-gal, moreover, incubation for two hours resulted even in decreased refolding yields.
Analysis of the refolding kinetics of β-gal in solution was not possible, as none of the tested buffer conditions resulted in refolded protein.

Taken together, the analysis and comparison of the refolding kinetics of the two refolding approaches showed that the renaturation of the proteins tested was in general a rather fast process. Nevertheless, matrix-assisted refolding still resulted in shorter renaturation times. On-matrix incubation in the respective refolding buffer for 20 min or less was sufficient to obtain maximal refolding yield for the respective buffer condition. In all cases longer refolding times didn't improve the refolding efficiencies and, in case of β-gal even caused a decrease in the refolding yield.

6.7 Concentration dependency of the refolding yield

There is no general rule for the optimum protein concentration during refolding, and it has to be experimentally determined for each single test protein (Mayer and Buchner, 2004). For in-solution refolding, the concentration of the target protein is usually kept very low (ng/ml-µg/ml) to obtain efficient renaturation yields. The concentration is especially crucial for oligomeric proteins (Lilie et al., 1998). The concentration dependence of the matrix-assisted refolding was not reported previously. To test the efficiency of matrix-assisted and in-solution refolding in this context, a comparative analysis of the dependency of the refolding yield on the concentration of test proteins sample was performed.

6.7.1 Refolding of FNR at different protein concentrations

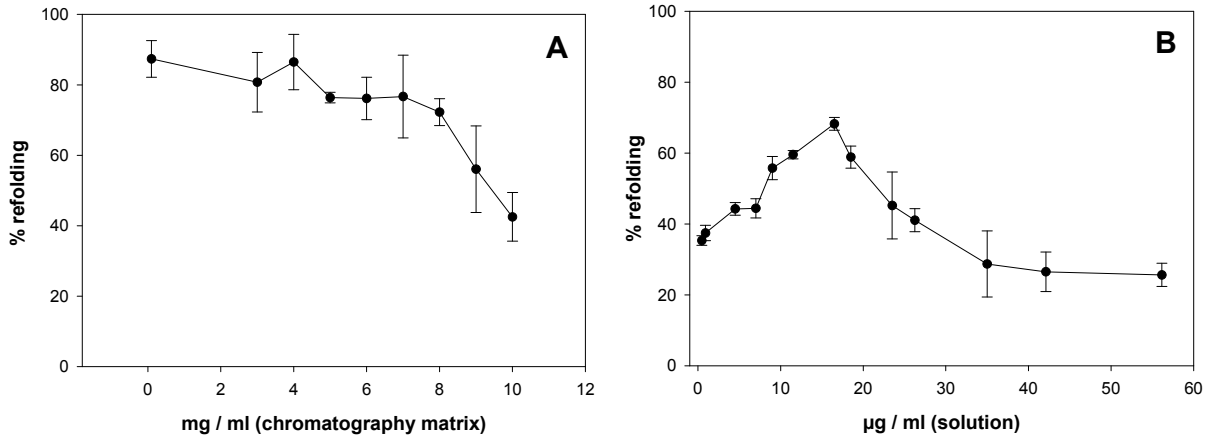


Figure 23 Comparison of the concentration dependencies of the refolding yields of FNR on-matrix (A) and in solution (B).

Refolding experiments were carried out in 100 mM TrisHCl, 300 mM NaCl, 50 mM Arg+Gln, 5 mM TCEP, pH 8.0

As shown in Figure 23B optimum concentrations for the refolding of the FNR in solution were between 10 and 20 µg/ml. Refolding yield decreased three fold at concentrations higher than 35 µg/ml, although some renaturation of the protein could still be achieved. On the other hand, the matrix-assisted approach resulted in the efficient refolding at concentrations of up to 8 mg protein per ml, with no significant decrease of the refolding yield. The drop of the refolding efficiency could be monitored at higher protein concentrations, but refolding yields of 40-50% could still be achieved (Figure 22A).

6.7.2 Refolding of CS at different protein concentrations

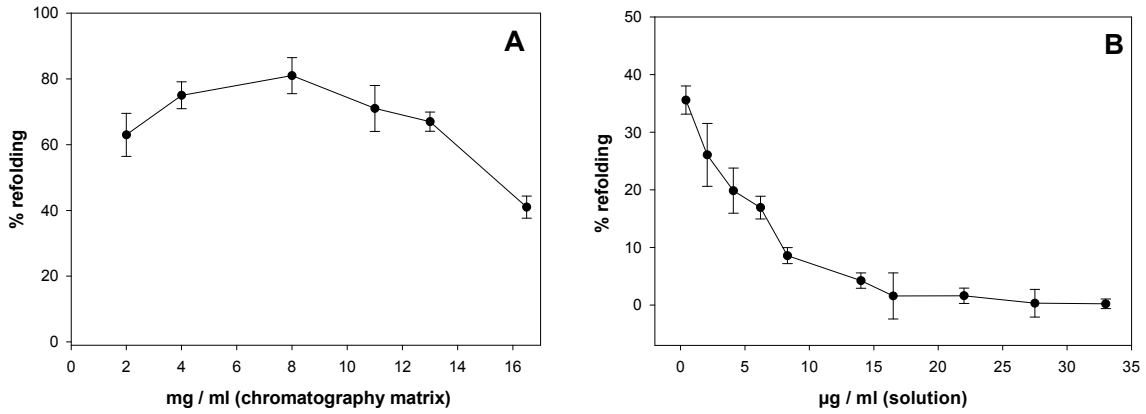


Figure 24 Comparison of the concentration dependencies of the refolding yields of CS on-matrix (A) and in solution (B).

Refolding experiments were carried out in 100 mM TrisHCl, 200 mM NaCl, 40 mM Arg+Gln, pH 8.0

Efficient on-matrix refolding of CS was possible at concentrations of up to 12 mg protein sample per ml without any noteworthy loss of refolding efficiency. The efficiency of the in-solution refolding, in contrast, was highly concentration-dependent. The highest refolding yields could be obtained at protein concentrations lower than 5 µg/ml; samples with the concentration higher than 15 µg/ml resulted in no detectable refolding.

6.7.3 Refolding of Glk at different protein concentrations

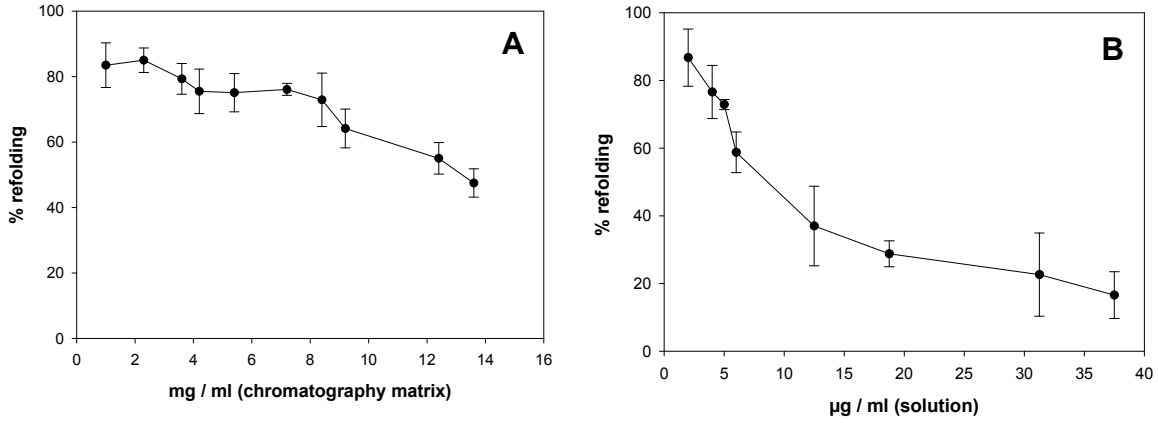


Figure 25 Comparison of the concentration dependences of the refolding yields of Glk on-matrix (A) and in solution (B).

Refolding experiments were carried out in 40 mM Na-P, 200 mM NaCl, 50 mM Arg+Gln, 5 mM TCEP, pH 8.0

The refolding efficiency of Glk was only weakly affected by increasing the protein concentration in the case of matrix-assisted refolding, and reactivation yields decreased only slightly at concentrations higher than 10 mg/ml. In comparison to the matrix-assisted approach, in-solution refolding appeared to be extremely concentration dependent, with most efficient refolding at concentrations lower than 5 µg/ml and a fourfold drop of the activity at concentrations higher than 25 µg/ml.

6.7.4 Refolding of β-gal at different protein concentrations

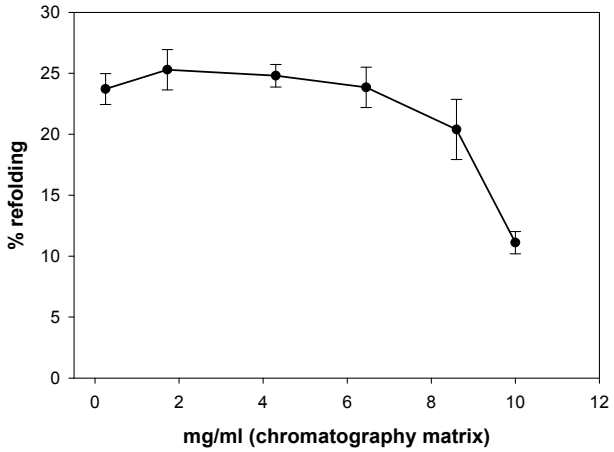


Figure 26 Concentration dependence of the refolding yield of β-gal on-matrix

Refolding experiments were carried out in 40 mM Na-P, 200 mM NaCl, 50 mM Arg+Gln, 2 mM TCEP, pH 7.5

Since β-gal renaturation in-solution resulted in no detectable refolding under any of the tested buffer conditions, a direct comparison of the concentration dependency of refolding efficiencies of the two approaches was not possible. However, for the matrix-assisted approach, an increase of the protein concentration during refolding procedure up to 7 mg/ml did not cause a noteworthy drop in the protein activity (Figure 26).

In summary, the analysis of the concentration dependencies of the refolding yield for all tested proteins showed that matrix-assisted refolding at high protein concentration was possible. An increase of the protein concentration up to 8-12 mg/ml in the refolding procedure did not significantly affect refolding efficiencies of the tested proteins. On the other hand, in-solution, refolding was optimal at concentrations in 5-10 µg/ml range for the dimeric proteins CS and Glk and 10-25 µg/ml for monomeric FNR. Moreover, an increase of the concentration resulted in all cases in a considerable decrease or even no regained activity.

6.8 Downstream processing of the refolded sample

The refolding step is usually followed by the separation of the refolded protein from the unfolded or misfolded and aggregated fraction. To address this question, three different chromatography types were tested, evaluating their separation properties for the correctly folded protein: SEC, IEC and HIC. These chromatography methods vary in their mode of action; however, each of them could in principle be a potent tool for the separation procedure. SEC restricts available pore size and volume for the different forms of the protein in the chromatography matrix, thus promoting the separation of correctly folded and aggregated species. Differences in the net charge and in the properties of hydrophobicity of correctly and incorrectly folded proteins define their chromatography performance in IEC and HIC, respectively, making the separation of folded and misfolded protein possible. However, an appropriate and efficient separation method must be determined for each particular case empirically.

In the following experiments the separation of the correctly folded protein fraction was carried out for five test proteins by SEC, IEC and HIC. All protein-containing fractions were analyzed by SDS PAGE and in terms of the specific protein activity to determine the efficiency of each separation method.

6.8.1 Separation of refolded FNR

FNR was refolded on a 1ml HisTrap FF in 100 mM TrisHCl, 300 mM NaCl, 50 mM Arg+Gln, 5 mM DTE, pH 7.5. Protein-containing fractions were pooled and the activity of the refolded sample was measured subsequently. Refolding experiment resulted in 50% refolding yield. The refolded sample was directly applied onto a SEC Superdex 200 16/60 PG column for separation. Fractions corresponding to the chromatogram peaks were analyzed by SDS PAGE. Additionally, the specific protein activity in the respective fractions was determined (Figure 27). The analysis showed that the correctly folded protein with 100% activity corresponds to the chromatogram peak comprising fractions C3-C5. The aggregated protein and misfolded species, which correspond to the peaks comprising fractions B10-C2, could be efficiently separated from the folded protein fraction in the chromatography procedure. Fractions D10 –D2 correspond to the imidazole peak, and fractions D12 and E3-E4 to the peaks containing salts and/or additives present in the loaded sample.

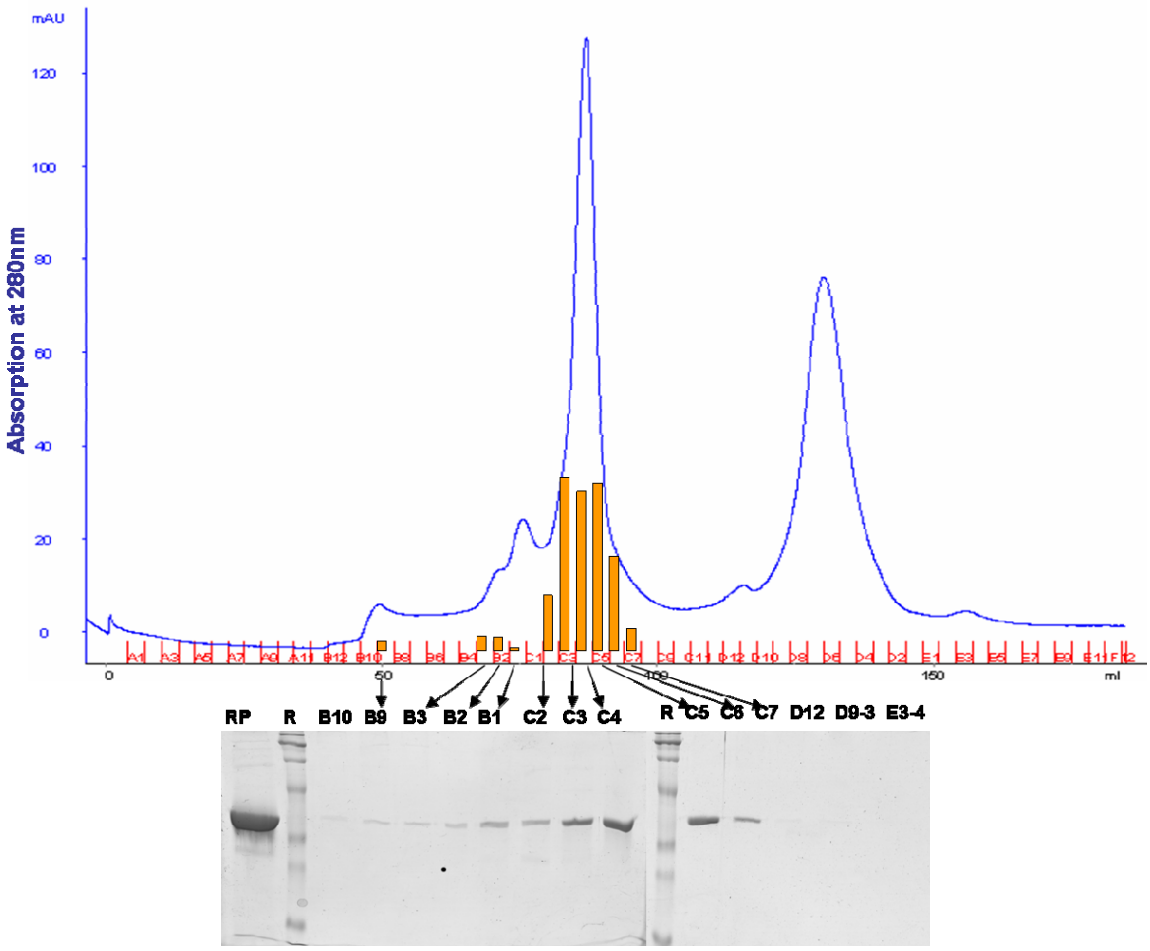


Figure 27 Separation of refolded FNR by SEC.

Separation was carried out on a Superdex 200 16/60 PG in PBS. Orange bars represent activity of the protein in the respective fractions. 12.5 % SDS-PAGE gel: RP refolded protein sample prior to separation, R-Rotimark prestained; B10–E4 analyzed fractions

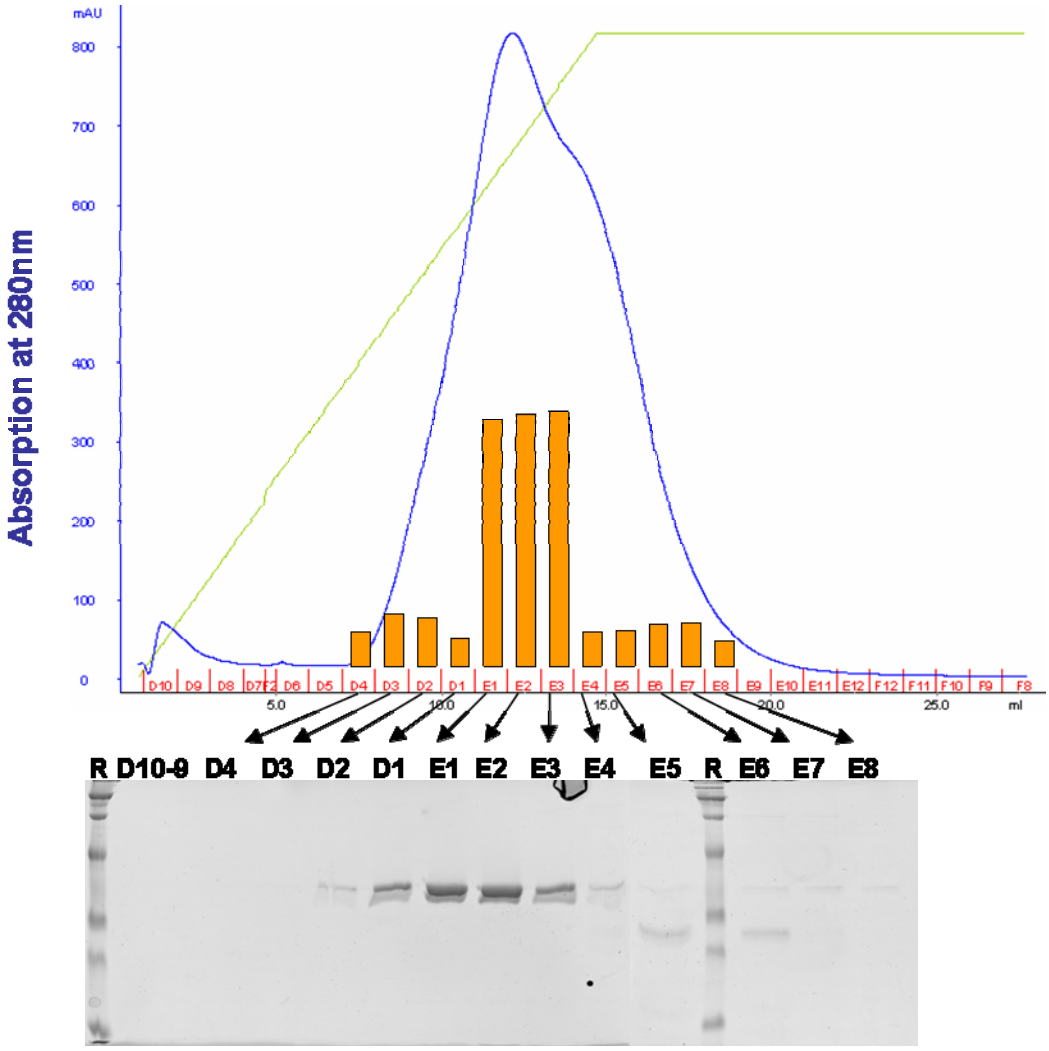


Figure 28 Separation of refolded FNR by HIC.

Separation was carried out on 1ml HiTrap Butyl HP. The elution gradient from 0 – 100% buffer B is indicated in green. Orange bars represent the activity of the protein in the respective fractions. 12.5 % SDS gel: R-Rotimark prestained; D10–E8 analyzed fractions

The separation performance of hydrophobic interaction chromatography (HIC) was studied next. Refolding of FNR was performed as described for the SEC separation. The chromatography column was equilibrated in 40 mM Na-P, 300 mM NaCl, 1M $(NH_4)_2SO_4$, pH 7.8. Prior to the application on the HIC column an equal volume of 40 mM Na-P, 300 mM NaCl, 2M $(NH_4)_2SO_4$, pH 7.8 was added to the protein sample, to ensure efficient binding. Elution was carried out by a gradient from 0 to 100% buffer B (40 mM Na- pH 7.8) over 15 ml (c.f. 8.5.1.4). Analysis of the chromatogram showed no clear separation of the protein sample as the elution resulted in only one major peak. Solely the right peak shoulder suggested the presence of different species (Figure 28). However, the analysis of single fractions revealed protein samples with a different range of activities, and fractions E1-E3 appeared to contain FNR with 100 % specific activity.

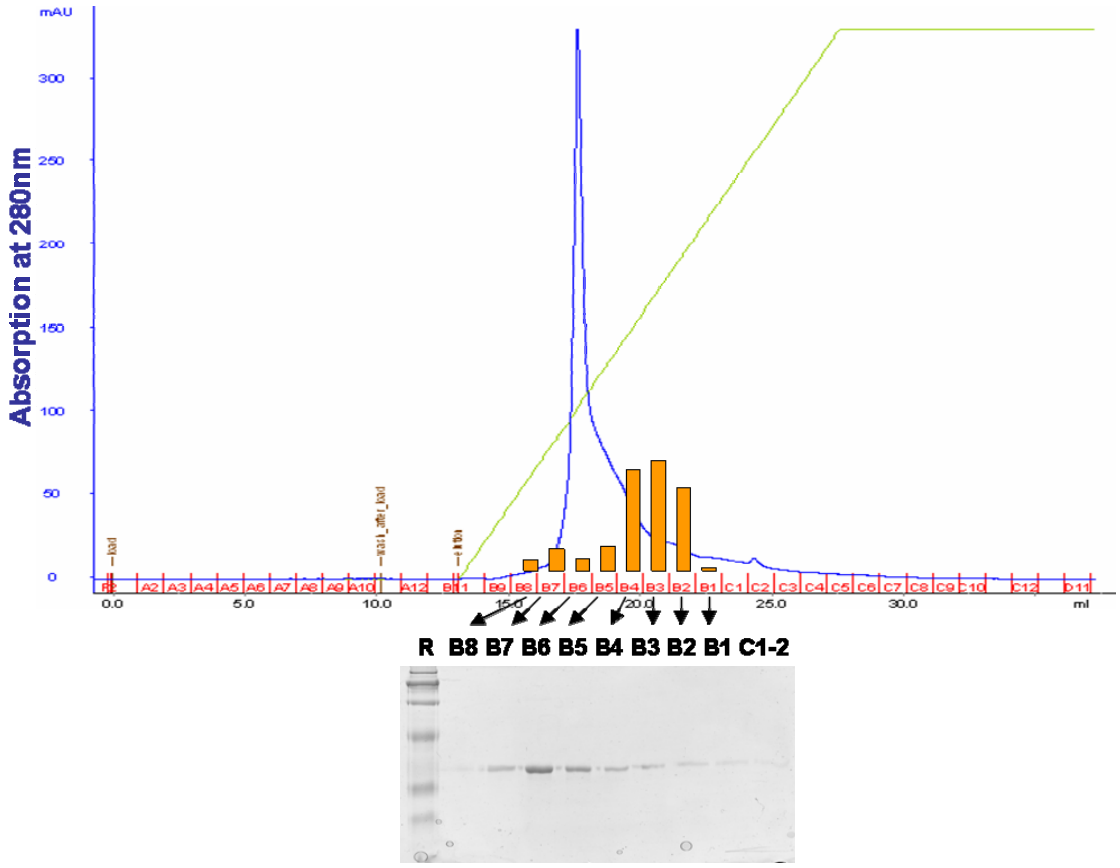


Figure 29 Separation of refolded FNR by IEC.

Separation was carried out on 1ml Resource Q. Orange bars represent activity of the protein in the respective fractions. The elution gradient from 0 – 100% buffer B is indicated in green 12.5 % SDS gel: R-Rotimark prestained; D10–E8 analyzed fractions

To determine the efficiency of the separation by ion exchange chromatography (IEC), a 1 ml resource Q column was used. As refolded FNR is eluted with high concentration of imidazole in the refolding chromatography, the sample was desalted prior to loading onto the IEC column. Desalting was carried out using the desalting column HiPrep 26/10 in 20 mM TrisHCl pH 7.5.

The protein sample was loaded onto the resource Q column and subsequently eluted by increasing the salt concentration from 0 to 1 M NaCl in a linear gradient over 15 ml (c.f. 8.5.1.2).

The elution profile revealed one single peak with a tailing shoulder (Figure 29). Surprisingly, analysis of the single fractions showed that the peak shoulder (fractions B3-B1) corresponded to the correctly folded protein fraction with 100% activity, which indicated rather poor recovery, in terms of total protein amount.

Taken together, the comparison of the applied chromatography methods for the separation of the correctly refolded FNR from the misfolded or aggregated species showed that the most

efficient separation could be achieved by SEC. Moreover application of this chromatography method did not require any additional processing of the refolded protein sample prior to separation.

The same separation procedures were carried out for the other four test proteins. All experiments were performed according to the protocol described for FNR. Chromatography conditions for each chromatography type were kept identical for all proteins tested (c.f. Apendix). Refolding of the test proteins was carried out under the respective optimal buffer conditions. Efficiencies of the separation performance of the applied chromatography methods are summarized in table 12.

	chromatography type		
test protein	**SEC**	**HIC**	**IEC**
eGFP	**+**	**+**	**+**
Glk	**+**	**-**	**-**
β-gal	**+**	**+**	**-**
FNR	**+**	**+**	**+**
DHFR	**+**	**-**	**-**

Table 12. Comparison of the separation efficiencies of different chromatography types for the five tested proteins

(+) separation of the protein fraction with 100% activity was possible
(-) separation of the protein fraction with 100% activity was not possible
Recommended method highlighted in green

The obtained results show that SEC is an efficient separation method for all proteins tested. For eGFP and FNR, either chromatography type enabled separation of the correctly folded protein. DHFR showed poor chromatography performance in IEC and HIC and further optimization of the chromatography conditions was required.
The chromatography method that was shown to be the most efficient for separation, in terms of chromatography performance and protein recovery, is stated as “recommended” in table 12.

6.9 On–matrix assembly of β-galactosidase

β-galactosidase was reported to be a challenging protein in terms of refolding, and various attempts to refold GdmCl-denatured β-gal in the absence of molecular chaperones yielded rather poorly (Ayling and Baneyx, 1996; Freeman and Morimoto, 1996; Nichtl et al., 1998).

The strategy of the stepwise improvement of the refolding buffer conditions and the matrix-assisted refolding approach developed here enabled refolding of β-gal with the renaturation yields of up to 30%. Further analysis of the refolding procedure of β-gal also showed that on-matrix refolding seems to be a fast process, and incubation on-matrix for just 10 min resulted in maximum refolding yields. Additionally, refolding experiments could be carried out at high protein concentrations without any significant loss of refolding efficiency.

Refolding at high protein concentrations is one of the advantages of the matrix-assisted refolding approach. Here, single polypeptide chains are to some extent spatially separated, which might at least partially decrease unfavorable interactions and prevent the formation of aggregation-prone intermediates. In the case of β-gal, however, tetramer assembly is indispensable for protein activity, and interaction of single monomers is therefore a precondition.

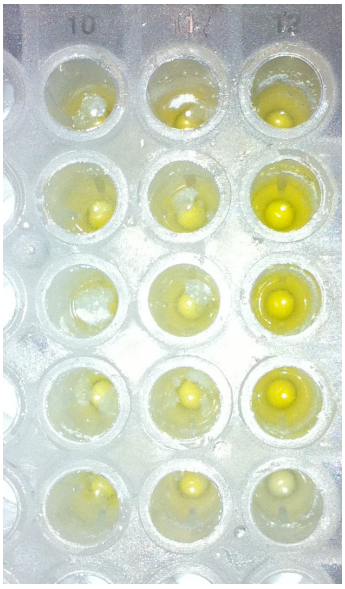
For the on-matrix assembly of the tetramer, the proximity of four correctly folded monomers is essential. This could only be achieved under appropriate chelator density and/or sufficient size and flexibility of the spacer, which links the chelator to the matrix. In this, work all experiments were carried out on HisTrap FF material. According to the manufacturer, GE Healthcare, this material complies with all of the above-mentioned requirements (personal communication Ake Danielson and co-workers). Therefore, there are several possibilities for on-matrix tetramer assembly: either four chelators with coordinated metal ion and adsorbed polypeptide chains are close enough to enable the association on one bead, or several chelator carrying beads stand in sufficient vicinity, allowing an interaction of single monomers. Thus, on-matrix tetramer assembly is theoretically possible. To examine whether this assumption was true for β-gal, or if the protein associates upon or after elution from the column, the set of experiments, described in the next sections, was carried out.

6.9.1 On-matrix activity of β-gal

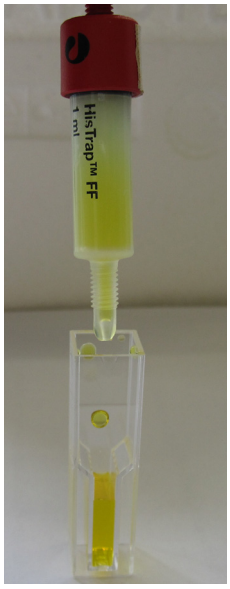
β-galactosidase catalyzes the hydrolysis of β-galactosides into monosaccharides. o-nitrophenyl-β-D-galactopyraniside (ONPG) is often used as substrate to monitor the activity of β-gal *in vitro* (Miller J.H., 1972). Upon ONPG hydrolysis, o-nitrophenol is released; the latter has a characteristic yellow color, visible to the naked eye, and can also be monitored at 420 nm in a spectrophotometer. ONPG was also employed here to determine, whether refolded matrix-bound β-gal was active.

Refolding experiments were performed in 96-well HisMultiTrap plates, which enabled parallel analysis of several samples. Denatured β-gal in different concentrations was applied to the columns, treated with the refolding buffer, and incubated for 1h at room temperature. Subsequently, the substrate solution containing ONPG was applied to the β-gal containing wells. In all cases, release of the o-nitrophenol could be observed, which indicates the correct assembly β-galactosidase tetramer on-matrix (Figure 30A).

To rule out the possible desorbtion of β-gal upon substrate binding and processing, the substrate/product containing flow through was examined by SDS PAGE (data not shown). No traces of β-gal could be observed in any of the analyzed samples, which proved that β-gal remains bound on the matrix till specifically eluted with imidazole. These results could be reproduced also in the larger scale of the 1ml HisTrap FF column (Figure 30B).



A



B

Figure 30 On-matrix activity of refolded β-gal.

A. 96-well HisMultiTrap FF B. 1 ml HisTrap FF
Refolding experiments on-matrix were carried out in 100 mM TrisHCl 300 mM NaCl 50 mM Arg+Gln, 2 mM DTE pH 7.5. Subsequently ONPG containing substrate was applied onto refolded matrix-bound β-gal.) Upon hydrolysis of ONPG o-nitrophenol (yellow) is released.

6.9.2 Refolding of β-gal in the presence of eGFP

To further examine the on-matrix assembling process of β-gal during refolding, β-gal was refolded in the presence of a second his-tagged protein bound to the column. Such an approach enabled competition of two proteins for binding sites and thus increased the distance between β-gal monomers on the affinity matrix, which consequently would impair their ability to associate to functional tetramers.

To examine this hypothesis, refolding experiments of β-gal in the presence of eGFP were carried out. Denatured β-gal and eGFP were mixed prior to the application on-matrix and refolding in different ratios, β-gal (50 µg) : eGFP 1:0, 1:1, 1:2, 1:3, 1:4, 1:5 and 1:6. The standard refolding procedure with one hour on-matrix incubation in the refolding buffer was applied.

Comparison of the activities of the refolded samples showed that yield of active β-gal decreased with increasing amounts of eGFP present, which was consistent with the hypothesis (Figure 31A).

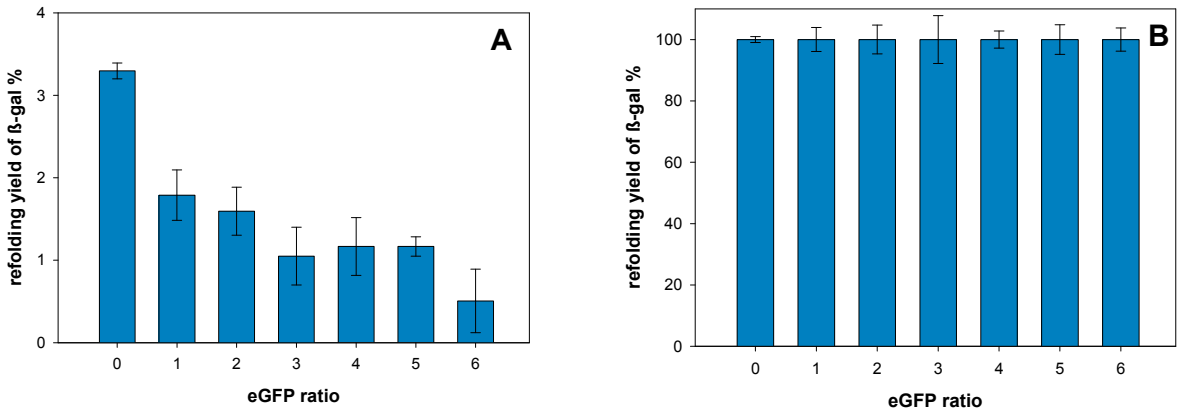


Figure 31 Matrix assisted refolding of β-gal in the presence of eGFP

A. Bars indicate refolding yields of the β-gal samples refolded in the presence of different amounts of eGFP. Refolding experiments were carried out in 100 mM TrisHCl, 300 mM NaCl, 50 mM Arg+Gln, 2 mM DTE, pH 7.5.The activity was measured directly after elution.
B Activities of the native β-gal samples incubated on-matrix in the presence of the respective eGFP amounts, applied as a reference

The presence of eGFP did not have any impact on the activity of native β-gal, which was applied as a reference (Figure 31B). This showed that the drop in the refolding yield was not due to destabilizing influences of increasing amounts of eGFP.

The obtained results showed that the presence of eGFP resulted in the separation of the β-gal monomers on the chromatography matrix and thus hindered their on-matrix association, which resulted in the impaired refolding yields.

6.9.3 Off-matrix assembly of β-gal

To test whether β-gal assembles exclusively on-matrix or if an association to an active tetramer is possible off-matrix, the activity of the on-matrix refolded sample was determined at different time points after elution.

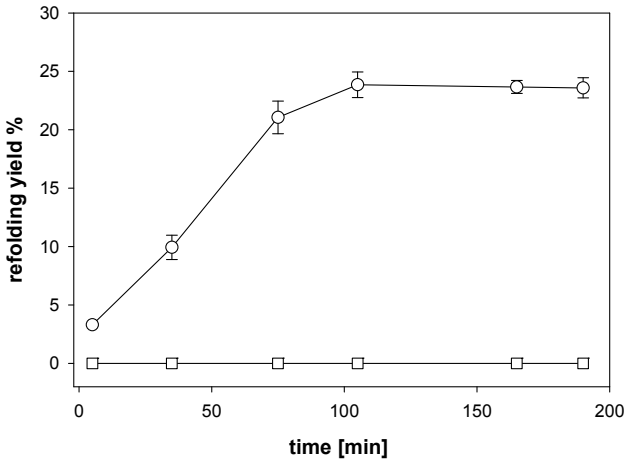


Figure 32 Comparison of the activities of the on-matrix refolded eluted β-gal (circles) and β-gal refolded in the elution buffer (squares).

On-matrix refolding was carried out in 100 mM TrisHCl 300 mM NaCl 50 mM Arg + Gln, 2 mM DTE pH 7.5 for 1 h at 20°C. Activities of the refolded eluted β-gal (shown in circles) and denatured β-gal diluted into the elution buffer (shown in squares) were determined at the respective time points

Analysis of the refolded sample directly after elution showed a rather low degree of regained activity. Surprisingly, the activity measurement 35 min later revealed a three fold increase in the activity of refolded sample. A further increase of the activity could be monitored at the later time points. The maximum activity was reached ~2 h after elution (Figure 32). Subsequently, the refolded sample was analyzed 16 h later, but no further gain of activity could be detected (data not shown).

As a control, denatured β-gal was diluted into the elution buffer and incubated; here no protein activity could be detected at any time point, which eliminates the possibility that the reactivation of β-gal is caused by the elution buffer alone.

The gradual increase in activity of the eluted refolded sample indicates the presence of not only correctly assembled tetramers, but also different assembly-competent species, which undergo association upon or after elution.

To examine if the presence of eGFP during refolding of β-gal influences not only on-matrix but also the subsequent off-matrix assembly, kinetic studies of β-gal samples refolded in the presence of different eGFP amounts were carried out (Figure 32).

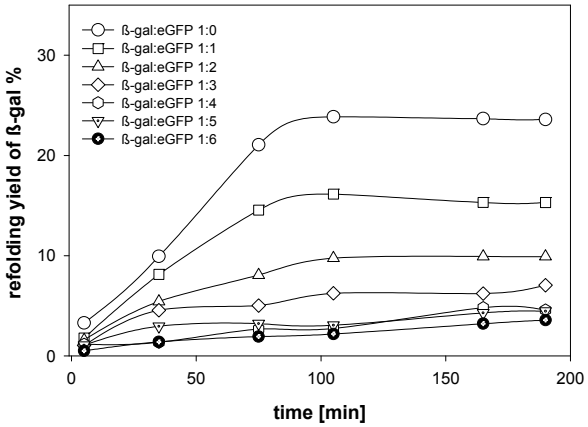


Figure 33 Comparison of the off-matrix assembly performances of β-gal refolded in the presence of different eGFP amounts.

On-matrix refolding of β-gal in the presence of different amounts of eGFP was carried out in 100 mM TrisHCl, 300 mM NaCl, 50 mM Arg + Gln, 2 mM DTE, pH 7.5 for 1 h at 20°C .Activity measurements were carried out at the respective time points after elution.

The initial activity measurement, directly after elution showed decreased reactivation upon increasing eGFP amounts, consistent with the previous results (Figure 33). After 35 min, the activity of all samples increased three-fold. At the next time point, 75 min after elution, a two-fold increase of the activity could be monitored for the samples with the lower eGFP amounts (1:1 and 1:2 ratios). For the sample with the higher eGFP amounts, the increase of the activity at the respective time points appeared to be only marginal. Approximately 2 h after elution, the maximum activity was reached for all samples and no further activity increase was observed. Interestingly, none of the β-gal samples, refolded in the presence of eGFP reached the refolding yield of β-gal refolded alone. Moreover, increasing amounts of eGFP resulted in a decreased off-matrix regain of activity. These results indicate that the presence of eGFP during the refolding of β-gal impairs both, on-matrix and off-matrix assembly of β-gal.

6.9.4 Oligomerization of refolded β-gal

To examine the oligomerization state of the on-matrix refolded β-gal and to further study the off-matrix assembly process, analysis of the refolded sample was carried out by the means of analytical SEC.

Refolding of the β-gal was performed according to the standard protocol (8.6.8.1). The refolded sample was centrifuged for 5 min @ 10,000, to remove possibly present aggregates. Analysis of the sample was carried out at different time points after elution. Additionally, the activity of the sample was determined at the respective time points.

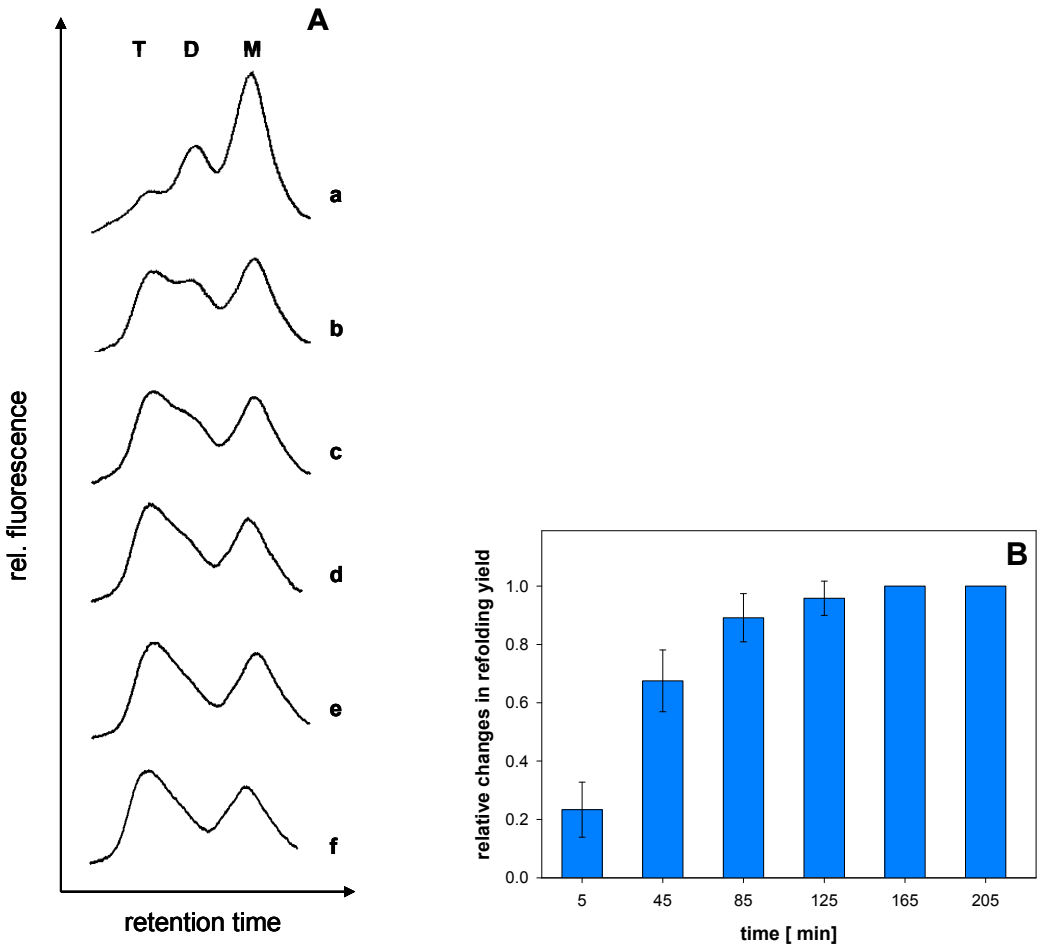


Figure 34 Off-matrix oligomerization of on-matrix refolded β-gal

A. Retention profiles of the refolded β-gal. Experiments were performed on TSK 3000 SW column, at 0.7 ml/min, in 40 mM Na-P 300 mM NaCl pH 7.8. Analysis of the sample was carried out 5 min (**a**), 45 min (**b**), 85min (**c**), 125 min (**d**), 165 min (**e**) and 205 min (**f**) after elution from the chromatography matrix. T, D, M indicate tetramer, dimer and monomer respectively (c.f. respective retention profiles in Appendix)
B. Activity of the refolded β-gal measured at the respective time points after elution. Maximal activity was set to 1.

As shown in Figure 34Aa, 5 min after elution, the monomeric intermediates predominate in the spectrum, followed by the dimeric intermediates. But also the tetramers are already

present, which is consistent with the previous results and the observed activity directly after elution and on-matrix (Figure 34B).

After 45 minutes, a decrease of monomers and an increase of dimers and tetramers could be monitored (Figure 34Ab). Consistently the protein activity increased three-fold (Figure 34B). Kinetic measurements demonstrated further the formation of the dimers and their association to tetramers, resulting in the concomitant increase of activity (Figure 34Ac,d, and 34B). After 165 min, the assembly process seemed to be completed; the activity of the refolded β-gal reached its maximum and no further gain could be monitored (Figure 34A e,f and 34B). At this time point, only tetramers and monomers could be detected in the refolded sample. The monomeric species seem to represent misfolded or partially folded but soluble intermediates, with an impaired ability to undergo assembly.

Taken together, the presented results show that in the refolding process, first monomeric species regain their native structure, but only a part of the monomers is association-competent. Further assembly involves dimer formation and their subsequent association into tetramers. Tetramer assembly on-matrix is possible, when correctly folded monomers are in appropriate proximity. Some of the reactivated monomeric species are still able to undergo association upon and after elution from the chromatography matrix.

7 Discussion

7.1 Optimization of refolding buffer conditions

Recombinant protein production is widely employed for basic research and biotech industry. *E. coli* expression systems are very popular, as large protein amounts can be expressed very quickly and at low costs. However, overexpression of recombinant protein often results in the accumulation of insoluble protein in amorphous aggregates i.e. inclusion bodies (IBs). Several strategies have been developed to obtain correctly folded functional protein from IBs. Traditional refolding methods include dilution, dialysis, diafiltration and liquid chromatography. However, the choice of an optimum refolding method and the estimation of the optimum refolding conditions are still a case-specific matter. Thus, a universal technology for the refolding of proteins, expressed as IBs, is still a major bottleneck in protein production. Such a technology must be economical, scalable, easy to automate and applicable for a broad range of proteins.

In this work, a strategy for the optimization of the refolding buffer conditions for matrix-assisted refolding of his-tagged proteins was developed. A novel approach of a stepwise optimization of the refolding buffer was introduced and its efficiency was tested for several proteins.

The strategy of the systematic stepwise optimization of different parameters of the refolding buffer was tested with the panel of different model proteins. To determine whether a generic application of the strategy was possible, the panel of the test proteins included proteins of different origins, sizes, oligomeric states and activities.

The applied approach enabled efficient optimization of the refolding buffer conditions for two monomeric proteins eGFP and FNR, two dimeric proteins Glk and CS, and finally for a large tetrameric protein, β-gal. Improvement of the refolding efficiencies could be monitored after each single optimization step, and in final screening steps refolding yields of at least 80% could be achieved for the greater part of the proteins tested. Refolding yields of eGFP were higher or similar compared to other studies (Anselment et al., 2010; Dong et al., 2009; Shi et al., 2007), refolding efficiencies of Glk were in a comparable range with the results of Anselment et al., (2010). For CS, the achieved refolding yields were up to four times higher than previously reported (Krause et al., 2002; Melkani et al., 2010; Yamaguchi et al., 2010; Zhi et al., 1992; Daugherty et al., 1998).

Finally, β-gal, denatured in GdmCl, had been reported to refold poorly by the conventional dilution method as long as molecular chaperones were absent (Ayling and Baneyx, 1996;

Freeman and Morimoto, 1996). However, the stepwise optimization strategy and matrix-assisted refolding presented here gave refolding yields of up to 30%.

Interestingly, the formulations of the optimum refolding buffers appear to be in a very narrow range, concerning composition and pH value, and correlate with conditions optimal for the used chromatography type. This assumption suggests the restriction of the buffer parameters by the chromatography requirements. Based on the results obtained in this study a set of the refolding buffers, acquired in the optimization procedure, could be suggested for generic application in the on-matrix refolding of his-tagged proteins. As a proof of principle, this buffer set was successfully employed for the matrix-assisted refolding of dihydrofolate reductase (DHFR) and refolding yields of up to 90% were obtained. These results suggest that the set of buffers might have a broad utility for the on-matrix refolding of different his-tagged proteins. Moreover, the proposed buffer set might be further minimized to 40 mM Na-P, 200 mM NaCl, 50 mM Arg+Gln, pH 7.5 or 8.0 and 100 mM TrisHCl, 300 mM NaCl, 50 mM Arg+Gln, pH 7.5 or 8.0, where the redox potential and / or additives could be adjusted according to the specific requirements of the respective target protein.

96-well HisMultiTrap plates allowed a parallel screening of the refolding buffers in a small scale, so no large amounts of protein were required for the optimization procedure. Moreover, refolded protein samples were obtained in the identical buffer condition after elution, allowing direct analysis and comparison of protein activities and refolding efficiencies.

Comparison of the refolding performances of the in-solution and on-matrix methods, showed a clear superiority of the matrix-assisted refolding in terms of refolding yields, for the majority of the proteins tested (Figure 35). Additionally, analysis of the samples refolded in solution was hindered for several test proteins in the final optimization steps, as the additives in the refolding buffers affected the activity assays. Therefore an additional processing step, i.e. buffer exchange, would be required, prior to activity assay.

Matrix–assisted refolding approach and optimization of refolding conditions by genetic algorithm (Anselment et. al., 2010) might be a potent tool to determine the optimum refolding conditions. Such approach would combine an alternative optimization strategy and all the advantages of the on-matrix refolding.

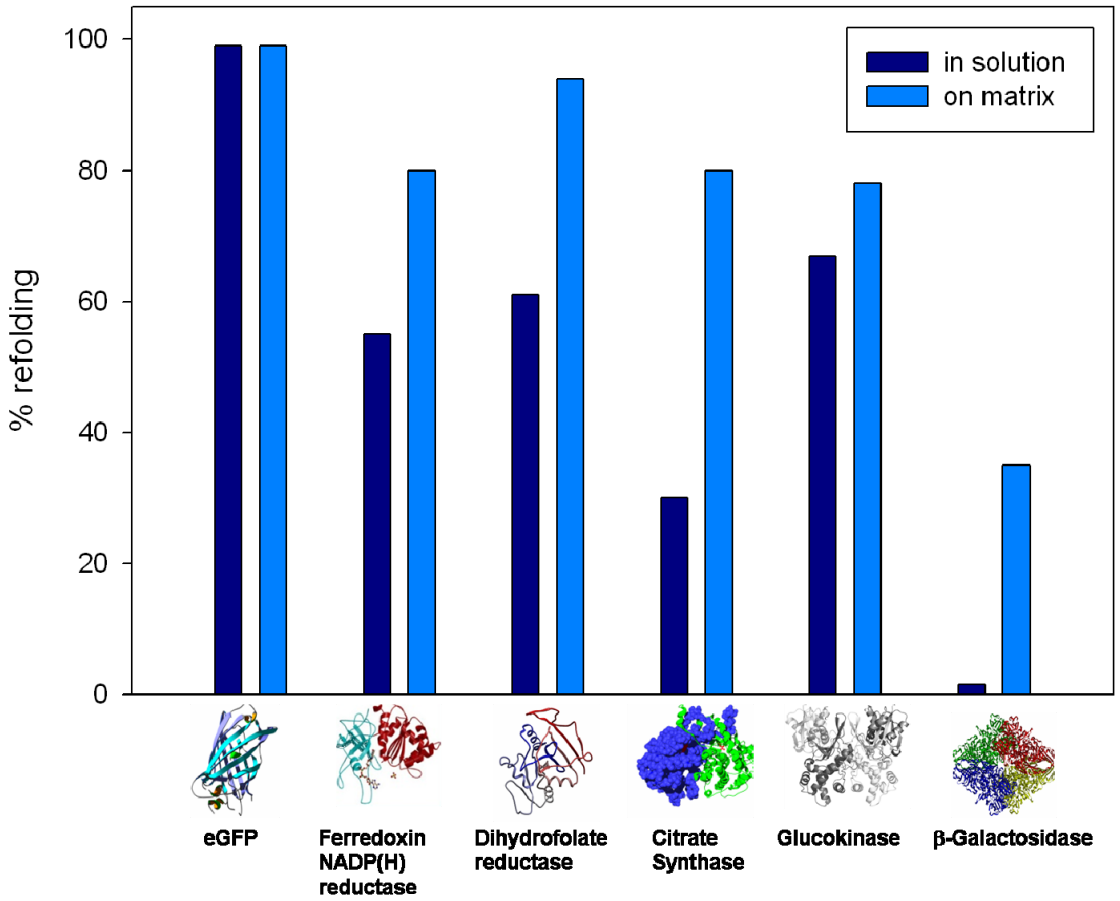


Figure 35 Comparison of refolding efficiencies.

The yields of the respective optimum buffer for on-matrix (light blue) and in-solution (dark blue) refolding is shown for the six proteins tested

A well established assay for protein activity was one of the characteristics of the chosen test proteins, which enabled to determine the refolding yields and the efficiency of the refolding strategy. However, a generic method for determining the refolding yield and analysis of the quality of the refolded protein with unknown or not detectable activity is still required. Several techniques are usually used to overcome this problem i.e. light scattering, SDS–PAGE, size exclusion chromatography (SEC) and RP-HPLC chromatography, however most of these are time and cost consuming (Dechavanne et al., 2011). The Thermofluor Stability Assay (TSA) (Ericsson et al., 2006) might be a potent tool for determining refolding efficiencies as it can be easily combined with the optimization strategy presented here. Here, a fluorescent dye SYPRO orange is added to the refolded protein sample. This dye interacts with the hydrophobic stretches of a polypeptide chain which greatly enhances its fluorescence. This effect is exploited to monitor thermal denaturation when the hydrophobic core residues become solvent-exposed upon unfolding (Ericsson et al., 2006; Pantoliano et al., 2001). Conveniently, a real-time PCR machine can be used to heat up the sample plate gradually, whilst detecting fluorescence emissions from.

Trial experiments with the refolded β-gal showed a difference in the thermal transitions depending on the refolding yield of the analyzed sample (Figure 36). Additionally, this method is fast and can be carried out in 96-well format, does not require large proteins amounts (1-5 µg protein per assay) and can be directly applied to the refolded protein samples.

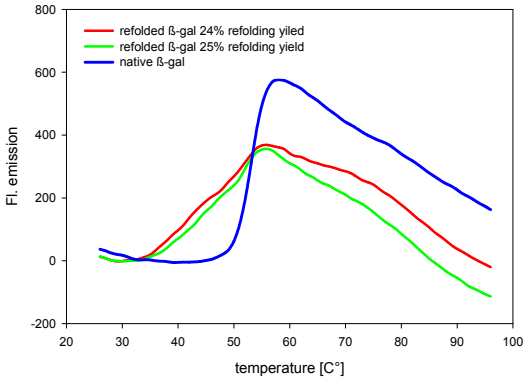


Figure 36 Comparison of thermal transitions of native and refolded β-gal

Significant differences could be observed in melting points and in the shapes of melting curves between the native protein sample and sample with the fraction of folded protein of 24 and 25 %.

One of the bottlenecks of potent renaturation process lies in minimizing the number of steps (to increase the overall yield) and reducing time and cost requirements. The stepwise optimization performed here allowed minimizing the number of experiments in the multifactorial space of possible buffers and thus a rapid and potent screening process. Of more than 10,000 possible buffer compositions in the optimization procedure only 150-200 buffers per target protein were tested to determine the respective optimal buffer. The developed here strategy of the systematic analysis and improvement of the refolding conditions for the matrix-assisted approach proved efficient for the set of the test proteins.

7.2 Up-scaling of the refolding procedure

Having determined optimal refolding conditions for the respective target protein, scaling-up of the refolding procedure was carried out using an automated chromatography system with prepacked columns. The refolding protocol for the HisMultiTrap plates could be easily adopted for the chromatography system, and comprised the same steps: load, refolding with one hour on-matrix incubation and elution of the target protein.

The scaling-up of the refolding procedure could be directly carried out for all proteins tested. No additional improvement or modification of the refolding protocol was necessary. The obtained refolding efficiencies were similar to those shown for the HisMultiTrap plates. The only exception was eGFP, where the maturation of the fluorophore is a prerequisite of an active protein. The fluorophore is formed from three residues in the nascent protein, (Ser65-Tyr66-Gly67) that undergo an autocatalytic intramolecular cyclization. This reaction requires

molecular oxygen (Barondeau et al., 2003; Chudakov et al., 2010). This precondition is accomplished more efficient in HisMultiTrap plates, due to the greater exposure to air throughout the procedure. In comparison, the chromatography system is air-free, which might explain the differences in the refolding efficiencies.

7.3 Refolding kinetics

Comparison of the refolding times, i.e. incubation time in the refolding buffer on-matrix or in-solution, required to reach the maximal refolding yield under the optimal buffer condition, demonstrated that matrix-assisted refolding results in accelerated renaturation compared to folding in-solution. For all proteins tested, short on-matrix incubation, of 20 min or less, was sufficient to reach the maximum refolding yield. This observation is especially surprising for oligomeric proteins, since, in these cases, regaining activity is a multi step process of first refolding and subsequent association. However, for CS, β-gal and Glk and on-matrix incubation times of only 20, 10 and 5 min respectively were required to regain the maximal activity. Refolding in-solution, compared to matrix-assisted refolding, appeared to require longer incubation times, e.g. maximum refolding yields for Glk and CS were reached after one and two hours of incubation respectively.

Immobilization of the denatured protein on matrix usually results in separation between the molecules. Such spatial separation is believed to provide each protein with its own space to refold and to limit, at least to some extent, unfavorable intramolecular interactions; moreover misfolded species might also be spatially constrained during the refolding process, which might as well prevent aggregation. However, one site immobilization on the chromatography matrix leaves the protein a certain extent of freedom and flexibility to regain its native structure. Moreover, the distances between binding sites enable high local concentrations of the polypeptides on the chromatography resin, which makes on-matrix oligomer formation possible. The results presented here show that the on-matrix assembly of the β-gal tetramer was possible, which confirms the flexibility of the bound polypeptide chains and their ability to associate.

In refolding by dilution, residual concentrations of the denaturant are still present; this might additionally slow down the renaturation process. The matrix-assisted refolding was carried out with the stepwise exchange from denaturing to the refolding buffer, thus providing denaturant-free refolding conditions. Taken together, all these factors might serve to accelerate the refolding process.

7.4 Concentration dependency of the refolding yield

The possibility of processing protein at high concentrations is another advantage of the matrix-assisted refolding method. The recommended protein concentrations for on-matrix refolding are in the range of 1-5 mg protein per ml of chromatography matrix (Oganesyan et al., 2005; Swietnicki, 2006). The performed refolding experiments showed that an efficient refolding was possible even at protein concentrations of up to 8-10 mg per ml of the chromatography matrix, without any significant drop in the refolding yield.

On the chromatography column, the protein sample distributes gradually from the top to the bottom (Figure 37). This might suggest that the protein concentration is in the same range at any point of the column, which would explain the more or less constant refolding yield upon the increase of the protein concentrations.

For in-solution refolding, the optimal protein concentrations, in terms of refolding yield, were very low, e.g. for the CS <1µg/ml and for the Glk 1-2 µg/ml. Efficient refolding of FNR was possible at the protein concentrations between 10 and 20 µg/ml. For all the proteins tested, and especially for the oligomeric ones, an increasing protein concentration resulted in a significant decrease of the refolding yield. In the case of CS, for example, at protein concentrations higher than 15 µg/ml refolding was not possible anymore. Refolding of β-gal was not achievable in-solution at any concentration tested. In-solution, the yield of correct oligomer assembly, might be limited by the kinetic competition of the correct subunit folding and aggregate formation, or by the aggregation at intermediate denaturant concentrations. Additionally, reactivation of the protein (or oligomer assembly) and aggregation were shown to be strongly correlated with the increasing protein concentrations (Jaenicke and Rudolph, 1986).

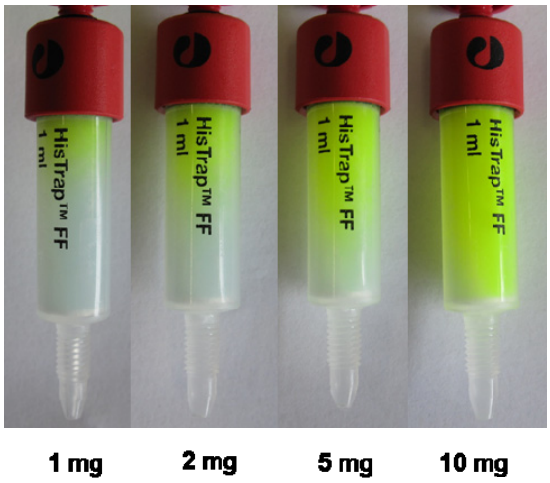


Figure 37 Distribution of eGFP on the chromatography column 1ml HisTrap FF

Loaded eGFP is shown to distribute gradually from top to the bottom on chromatography column, suggesting comparable protein concentrations at any point of the column

7.5 Downstream processing of the refolded sample

The refolding procedure is usually followed by the separation of the folded protein fractions from unfolded or aggregated content. To address this question, three chromatography types SEC, HIC and IEC for five refolded target proteins were tested and compared.

SEC appeared to be an efficient separation method for all proteins tested. Refolded protein samples could be directly applied onto the column and no additional processing was required. Another advantage of SEC is that the mobile phase can be chosen over a wide range and accordingly to the specific requirements of the target protein. However, the eluted protein samples are highly diluted, so an additional concentration step might be required.

On the other hand, IEC and HIC enable protein elution at higher concentrations, but to ensure efficient binding, the refolded protein sample should be desalted prior to on-column application in case of IEC or the salt concentration of the protein sample should be increased in the case of HIC. Standard protocols for HIC and IEC did not work for the preparation of DHFR (no binding of the protein could be achieved) and only poorly for Glk (inefficient separation). The chromatography conditions for both proteins had to be optimized experimentally. On the other hand, for eGFP and FNR either chromatography type enabled efficient separation of the correctly folded protein product under standard conditions. The refolded β-gal could be separated either by SEC or HIC.

Taken together, the obtained results from the optimization or the refolding conditions, up-scaling of the refolding procedure and subsequent separation of the correctly folded protein product suggest a general protocol for protein production from the IBs to purified protein with 100% specific activity (Figure 38).

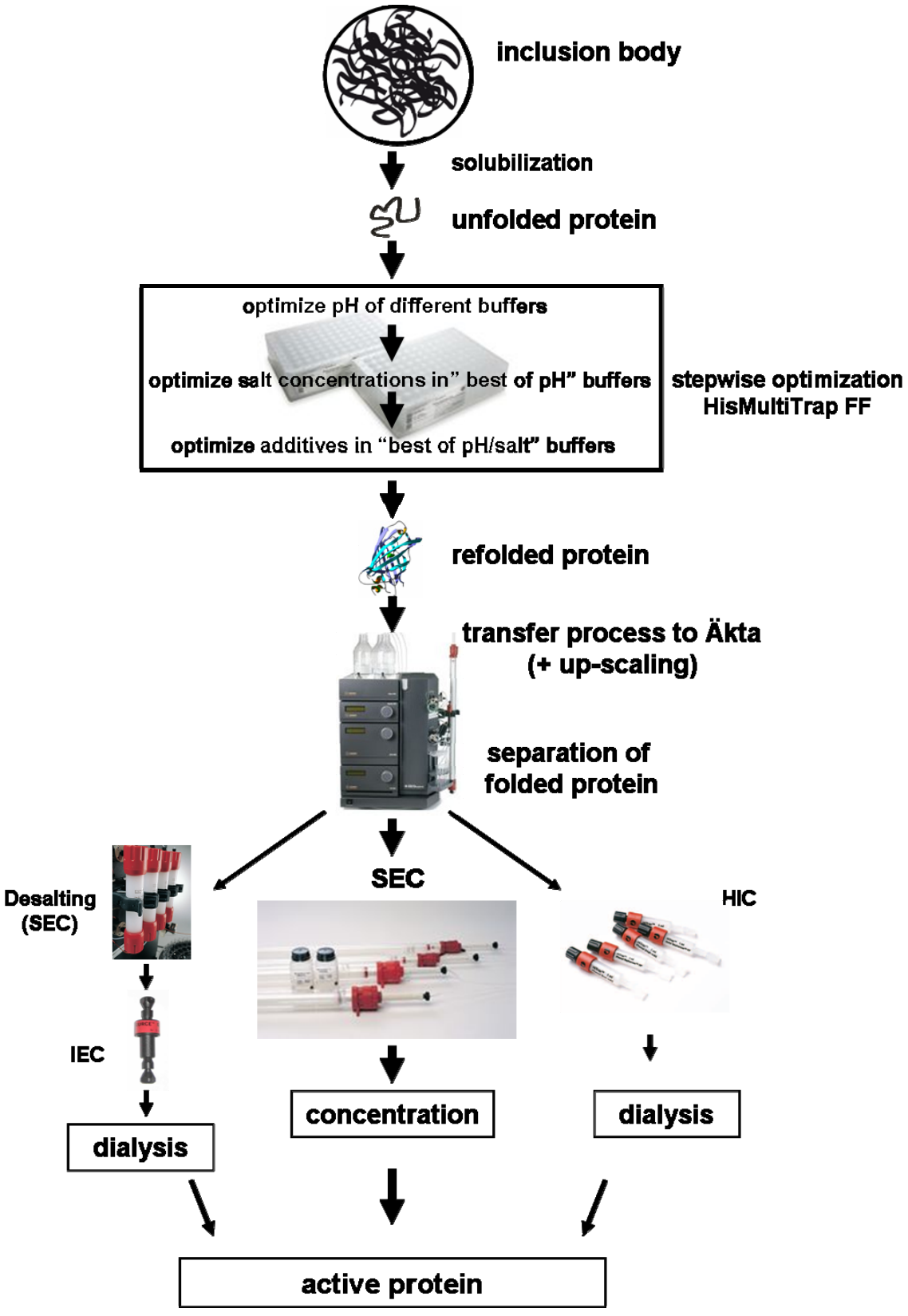


Figure 38 Schematic description of the processing steps: from IBs to correctly folded protein.

Stepwise optimization of the refolding buffer is carried out with 96-well HisMultiTrap plates, followed by the up-scaling of the refolding procedure under determined optimum conditions on the automated system. The separation of the folded protein fraction is performed by one of the suggested chromatography types with the respective processing steps.

7.6 Refolding and assembly of β-galactosidase

β-galactosidase (β-gal) from *E. coli* is one of the largest known tetrameric enzymes, with identical subunits of 116 kDa. It had been shown that the assembly of the tetramer is required for the enzymatic activity of the protein (Juers et al., 2000).

Tetramer assembly is preceded by the formation of correctly folded monomers and their subsequent association into oligomerization-competent dimers and can be described by the following model (Nichtl et al., 1998):

$$\mathbf{4Mu \rightarrow 4M \rightarrow 2D \rightarrow T}$$

M – monomer, Mu – unfolded monomer, D - dimer , T – tetramer

The same study revealed that inactivation, dissociation and unfolding are simultaneous processes.

β-gal has been shown to be a rather challenging protein when it comes to refolding. Application of a standard refolding method via dilution for GdmCl-denatured β-gal resulted in almost no active protein, as long as no molecular chaperones were present (Ayling and Baneyx, 1996; Freeman and Morimoto, 1996; Nichtl et al., 1998)

Stepwise optimization of the refolding buffer conditions enabled to determine refolding buffers for the successful matrix-assisted refolding of the his-tagged β-gal with refolding yields of up to 30%. Consistent with the above mentioned studies, none of the refolding experiments by dilution performed for the β-gal resulted in the reactivation of the protein.

Furthermore, experiments, performed in this work, show that refolded matrix-bound β-gal regained its enzymatic activity, which indicates the formation of functional tetramers and the ability of β-gal monomers to assemble in the matrix-bound state. This suggestion was verified by the introduction of different amounts of eGFP in the refolding process. This enabled the spatial separation of the β-gal monomers on the affinity matrix and impaired their ability to assemble. Increasing amounts of eGFP introduced in the samples resulted in decreased refolding efficiencies of β-gal.

Additionally, the analysis of the refolded β-gal sample by the means of the analytical SEC directly after elution revealed the presence of different oligomeric species: predominantly monomers, followed by dimers and tetramers. Further analysis of the protein sample showed association of the monomers into dimers and subsequent tetramer assembly, which was confirmed by the observed increasing protein activity (c.f. 6.9.4).

Taken together, the obtained results showed that in the on-matrix refolding process the monomeric species fold first. If a sufficient proximity of the four correctly folded subunits is

given, they undergo assembly into dimers, followed by tetramer formation. Correctly folded monomers, lacking interaction partners on-matrix, can assemble during or after elution. Additionally, there was a fraction of soluble monomeric species, apparently misfolded or partially folded, with an impaired ability to associate.

Usually, the advantage of the matrix-assisted refolding is that the single polypeptide chains are spatially restrained on the matrix, but in the case of β-gal the regained protein activity and thus tetramer assembly was shown on-matrix, which points out an interaction of single monomers. This may also apply for the tested dimeric proteins CS and Glk, as in both cases yield and rate of matrix-assisted refolding was higher, compared to in-solution refolding.

Studies of Kiho and Rich (1964) indicated that β-gal folds, assembles into tetramers, and exhibits activity on the ribosome, this was confirmed by Matsuura et al. (2011) by *in vitro* translation/transcription system. Moreover, Matsuura et al. suggested that β-gal, which already has certain elements of the native conformation on the ribosome, quickly assembles into dimers, and subsequently into tetramers. This process was described as a first-order reaction with the lack of a rate-limiting steps in the assembly process (Matsuura et al., 2011). Matsuura and co-workers compared their results to the kinetic analysis of the refolding of urea-denatured β-gal by Nichtl et al. (1998) , where the authors suggest a bi-uni-molecular mechanism of folding and association with two rate limiting steps: monomer to dimer assembly and structural rearrangements of the dimers to the association competent state (Nichtl et al., 1998). Matsuura et al also showed that the assembly rate on the ribosome is 50 fold higher than that during refolding, which can be explained by the differences in the folding pathway to the dimer of the native and denatured protein, or by the fact that the ribosome might provide appropriate conditions for tetramer assembly. Additionally, the authors suggest that the assembly occurs exclusively in the ribosome-bound state (Matsuura et al., 2011). Immobilization of the β-gal on matrix might to some extent mimic the ribosome-bound state. One point binding to the solid phase allows retaining the flexibility and under the given condition of four correctly folded monomers in the appropriate distance, tetramers are formed. Tetramer assembly on-matrix was rather fast: on-matrix incubation for just 5-10 min resulted in the maximum activity regained (c.f.6.6.4). On the other hand, the tetramer assembly of the folded monomers, which were not able to undergo association on-matrix, seems to follow the mechanism proposed by Nichtl et al (1998).

8 Materials and methods

Methods are adopted from standard methods developed at the chair of biotechnology, TU München.

8.1 *E. coli* strains

Strains	Geno- / Phenotype	Source / Reference
E. coli XL1 Blue	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F'proAB *lac*IqZDM15 Tn10 (*Tet*R)]	Stratagene, La Jolla, USA
E. coli BL21 (DE3)	F– *omp*T hsdS(rB– mB–) *dcm*+ *Tet*r *gal* l (DE3) *end*A *Hte* [*arg*U *ile*Y *leu*W *Cam*R]	Stratagene, La Jolla, USA

8.2 Bacterial vectors

Name	Vector	Origin
Glucokinase (glk)	pet28a	This work
β-galactosidase (ß-gal)	pet28a	This work
citrate Synthase (CS)	pIVEX2.3	Martin Haslbeck
dihydrofolate reductase (DHFR)	pQE40	Qiagen, Hilden Germany

The expression plasmids for eGFP and FNR were kind gifts from S. Töpell and F. Rohdich respectively

8.3 Primer

Name	Sequence	Restriction site
β-gal Fw	gagatcgctagcatgaccatgattacggattcactg	Nhe1
β-gal Rev	gagatcggatccttatttttgacaccagaccaactg	BamH1
Glk Fw	gagatcgctagcatgacaaagtatgcattagtcggt	Nhe1
Glk Rev	gagatcggatccttacagaatgtgacctaaggtctg	BamH1

8.4 Chemicals

Acrylamide solution (38% with 2% bisacrylamide)	Roth, Karlsruhe, Germany
Adenosin-5´-diphosphate (ADP), disodium salt	Roche, Mannheim, Germany
Adenosin-5´-triphosphate (ATP), disodium salt	Roche, Mannheim, Germany
Agarose, ultra-pure	Roth, Karlsruhe, Germany
Albumin from bovine serum	Sigma, St. Louis, USA
Ammoniumperoxodisulfate (APS)	Roche, Mannheim, Germany
Ammoniumsulfate	Merck, Darmstadt, Germany
Ampicillin	Roth, Karlsruhe, Germany
Bacto Peptone	Difco, Detroit, USA
Bacto Tryptone	Difco, Detroit, USA
Bromphenol blue S	Serva, Heidelberg, Germany
Coomassie Brilliant Blue G-250	Serva, Heidelberg, Germany
Coomassie Protein Assay Reagent	Pierce, Rockford, USA
Dithiothreitol	Roth, Karlsruhe, Germany
ECL+plus Western Blotting Detection System	Amersham, Uppsala, Sweden
Ethanol, p.a.	Roth, Karlsruhe, Germany
Ethidiumbromide	Sigma, St. Louis, USA
Glycerine, 99%	ICN, Irvine, USA
Guanidinium hydrochloride, p.a.	ICN, Irvine, USA
Kanamycin	Roth, Karlsruhe, Germany
2-Mercaptoethanol, pure	Sigma, St. Louis, USA
N-(2-Hydroxyethyl)-piperazine-N'-2-ethansulfonic acid (Hepes)	ICN, Irvine, USA
N,N,N',N'-Tetramethylethylendiamin (TEMED)	Roth, Karlsruhe, Germany
Protease inhibitor mix HP	Serva, Heidelberg, Germany
Sodiumdodecylsulfate (SDS)	Roth, Karlsruhe, Germany
Tris-(hydroxymethyl)-aminomethan (Tris)	ICN, Irvine, USA
Tetracyclin	Roth, Karlsruhe, Germany
Titriplex (EDTA)	Serva, Heidelberg, Germany
Isopropyl ß-D-1-thiogalactopyranoside (IPTG)	Merck, Darmstadt, Germany

All other chemicals were purchased from the company Merck (Darmstadt, Germany) and were of grade p.a. if not stated otherwise. For the preparation of buffers double distillated water was used.

8.4.1 Size markers and kits

1 kb DNA ladder molecular weight standard	Peqlab, Erlangen, Germany
Calibration proteins for HPLC	Serva, Heidelberg, Germany
Wizard® Plus SV Mini-Preps DNA purification kit	Promega, Madison, USA
High Pure PCR Product Purification Kit	Promega, Madison, USA
High-Range-molecular weight marker (HMW for SDS-PAGE)	BioRad, München, Germany
Low-Range-molecular weight marker (LMW for SDS-PAGE)	BioRad, München, Germany
Roti-Mark prestained	Roth, Karlsruhe, Germany,
Wizard Plus Gel Extraction Kit	Promega, Madison, USA
Wizard Plus SV Minipreps DNA Purification System	Promega, Madison, USA

8.4.2 Proteins

Alkaline phosphatase	Roche, Mannheim, Germany
Pwo-DNA polymerase	Roche, Mannheim, Germany
Restriction enzymes	Promega, Madison, USA
T4-Ligase	Promega, Madison, USA

8.4.3 Chromatography material

HisTrap FF 1ml, 5 ml	GE Healthcare, Uppsala, Sweden
HisMultiTrap FF	GE Healthcare, Uppsala, Sweden
Resource-S	GE Healthcare, Uppsala, Sweden
Resource-Q	GE Healthcare, Uppsala, Sweden
Superdex 75 Prep Grade	GE Healthcare, Uppsala, Sweden
Superdex 200 Prep Grade	GE Healthcare, Uppsala, Sweden
Superdex 200 HR	GE Healthcare, Uppsala, Sweden
HiTrap Q FF 5 ml	GE Healthcare, Uppsala, Sweden
HiTrap S FF 5ml	GE Healthcare, Uppsala, Sweden
HiTrap Butyl HP 1ml	GE Healthcare Uppsala, Sweden
HiPrep 26/10	GE Healthcare, Uppsala, Sweden

8.4.4 Additional material

Centricon 3, Centricon 10, Centricon 30	Amicon, Witten, Germany
Millipore Ultra-15 device	Millipore, Bedford, USA
Dialysis bags Spectra/Pore 6000-8000 Da	Spectrum, Houston, USA
Sterile filters 0,2 µm	Zefa, München, Germany
PE tubes (50/15 ml)	Greiner & Söhne, Nürtingen, Germany
pH-indicator strips	Roth, Karlsruhe, Germany
Disposable cuvettes, 1,5 ml, halfmicro	Zefa, München, Germany
Ultrafiltration membranes YM3, YM10, YM30	Millipore, Bedford, USA
Silica glass Suprasil cuvettes	Hellma, Jena, Germany

8.4.5 Equipment

Balances

Analysis balance BP 121 S	Sartorius, Göttingen, Germany
Halfmicro balance BL 310	Sartorius, Göttingen, Germany

Centrifuges

Avanti J 25 with JA-10 and JA-25.50 rotors	Beckman, Wien, Austria
Eppendorf table-top centrifuge 5415 C	Eppendorf, Hamburg, Germany
Rotina 46 R coolable centrifuge	Hettich, Tuttlingen, Germany
Universal 32 R coolable centrifuge	Hettich, Tuttlingen, Germany

Chromatographic machines

Äkta Explorer	Amersham, Uppsala, Sweden
FP-1520 fluorescence detector	Jasco, Groß-Umstadt, Germany
GradiFrac system	Amersham, Uppsala, Sweden
HighLoad system	Amersham, Uppsala, Sweden
LG-980-02S gradient unit	Jasco, Groß-Umstadt, Germany
PU-1580 HPLC Pump	Jasco, Groß-Umstadt, Germany
Super loop 150 ml	GE Healthcare, Uppsala, Sweden
Super loop 10 ml	GE Healthcare, Uppsala, Sweden
UV-1575 UV-VIS detector	Jasco, Groß-Umstadt, Germany

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Gelelectrophoresis and blotting devices

Hoefer Mighty Small II gelelectrophoresis unit	Amersham, Uppsala, Sweden
RHU10X	Roth, Karlsruhe, Germany

Spectrophotometers

Biotech Ultrospec 3000 UV-VIS-spectrophotometer	Amersham, Uppsala, Sweden
Cary 50 Bio UV-VIS-spectrophotometer	Varian, Palo Alto, USA
J-715 spectropolarimeter with PTC 343 peltier unit	Jasco, Groß-Umstadt, Germany tempering
Novaspec II visible	Amersham, Uppsala, Sweden

Voltage sources

LKB-GPS 200/400	Amersham, Uppsala, Sweden
EPS 3500, 301 und 1001	Amersham, Uppsala, Sweden

Further equipment

Air circulation incubator	New Brunswick Scientific, Nürtingen, Germany
Cell disruption machine Basic Z	Constant Systems, Warwick, England
Culture shaker Certomat S	Braun Biotech, Melsungen, Germany
Digital thermometer with thermosensor	Keithley, Cleveland, USA
Eppendorf thermomixer	Eppendorf, Hamburg, Germany
Icemachine	Ziegra, Isernhagen, Germany
Magnetic stirrer Heidolph MR 2000	Heidolph, Kelheim, Germany
Metal thermo block TB 1	Biometra, Göttingen, Germany
pH-meter	WTW, Weilheim, Germany
Thermocycler Primus	MWG, Ebersberg, Germany
Test tube roller	Heidolph, Kelheim, Germany
Sonic cell disruption device Sonifier B-12	Branson, Danbury, USA
Sonic water bath Sonsorex RK 100H	Bandelin, Berlin, Germany
Varioklav steam autoclave EP-Z	H+P, Oberschleißheim, Germany
Water bath Haake F6-K	Haake, Karlsruhe, Germany

8.4.6 Computer software

Adobe Photoshop 6.0	Adobe Inc., San Jose, USA
Adobe Reader 5.0	Adobe Inc., San Jose, USA
Borwin	Jasco, Groß-Umstadt, Germany
ProtParamTool	ExPasy (http://expasy.hcuge.ch/)
Reference Manager 12	ISI, Philadelphia, USA
Sigma Plot 2001	SPSS, Chicago, USA

Molecular methods

8.4.7 Molecular biological buffers

TAE (50×):	Tris/Acetate pH 8,0	2 M
	EDTA pH 8,0	50 mM
Gel loading buffer (10×):	Glycerin	50% (v/v)
	EDTA pH 8,0	10 mM
	Bromphenole blue	0,2% (w/v)
	Xylencyanole	0,2% (w/v)
1% Agarose solution:	Agarose	1 g
	TAE (1×)	100 ml
	Ethidiumbromide solution	1 µl
dNTP-Mix:	dATP	10 mM
	dGTP	10 mM
	dCTP	10 mM
	dTTP	10 mM
Solution A:	3 M NaAc (pH 5,5)	13 ml
	1 M $CaCl_2$	100 ml
	2.8 M $MnCl_2$	25 ml
	H_2O sterile filtered	862 ml
Solution A - Glycerin:	Glycerin (87%)	69 ml
	Lösung A	331 ml

8.4.8 Media and solutions

The following media were used for growth of *E. coli*:

LB_0	10 g/l Tryptone
	5 g/l Yeast extract
	5 g/l NaCl
	For plates: 15 g Bacto-Agar

Antibiotic concentrations used for growth *of E. coli*:

Ampicillin	100 µg/ml
Kanamycin	35 µg/ml

8.4.9 Cultivation and storage of *E. coli*

E. coli was cultivated either in liquid LB (Luria-Bertani broth) medium or on the LB plates, containing respective antibiotics, in a thermostated incubator at 30 or 37°C. The growth of bacterial cells in liquid cultures was monitored by measuring OD at 600 nm (OD_{600nm} = 1 corresponding to approx. $8x10^8$ cells/ml) (Sambrook J and Maniatis T, 1989)

For the long term storage 5 ml of cells in exponential growth phase, were centrifuged for 3 min at 5000 x g, sediment was resuspended in 700 µl LB medium, subsequently 300µl of 50 % glycerol were added. Cultures were frozen with liquid nitrogen and stored at – 80°C.

8.4.10 Polymerase chain reaction (PCR)

For cloning genomic DNA from *E. coli* was used as template and the respective primers. The Pwo polymerase with the provided buffer containing $MgCl_2$ was used for amplification of DNA. PCR products were analyzed by a 1 % agarose gel electrophoresis containing 0,001% ethidiumbromide.

Reaction mix:

dNTPS (100 mM)	2 µl
Pwo polymerase	1 µl
Primer	2 µl
Pwo buffer	10 µl
Template	0.5 µl
H_2O, sterile	84.5 µl

Program	Temperature	Time	Number of cycles
hot start	95°C	2 min	1x
denaturation annealing polymerisation	95°C 55°C 70°C	30 s 30 s 1 min per 1kb of product	35x
Storage	4°C	∞	1x

8.4.11 Purification and storage of DNA

For the purification of PCR products and digested DNA fragments the Wizard Plus Gel Extraction Kit was used, purification was carried out according to the supplied centrifugation protocol.

For the plasmid isolation the Wizard Plus SV Minipreps DNA Purification System was used. DNA was stored in sterile H_2O at -20 °C.

8.4.12 Restriction and ligation protocols

Preparative restriction:

PCR product or plasmid	50 µl
Enzyme 1	2 µl
Enzyme 2	2 µl
10x buffer	6 µl

Analytical restriction:

PCR product or plasmid	10 µl
Enzyme 1	0.5 µl
Enzyme 2	0.5 µl
10x buffer	2 µl
H_2O sterile	6 µl

Mixtures were incubated for 2 h at 37°C. Analysis was carried out by the means of agarose gel electrophoresis

Ligation:

Insert (digested PCR product)	3 µl
Vector (digested plasmid)	1 µl
Ligase	2 µl
Ligase buffer	1 µl
H_2O sterile	3 µl

Ligation preparations were incubated ON at 4°C

8.4.13 Preparation of competent *E. coli* cells

(Sambrook J and Maniatis T, 1989)

Respective *E. coli* cells were inoculated in 100 ml LB medium and incubated at 37°C till OD_{600} of 0.5-1 was reached. 2 ml of 1M $MgCl_2$ were added and the culture was incubated for the further 10 minutes. Subsequently, the culture was cooled down on ice for the next 60 min, and then centrifuged for 5 min at 4000x g. Sediment was resuspended in 20 ml of solution A and incubated on ice for further 60 min. After the next centrifugation step for 5 min at 4000x g, sediment was resuspended in 2 ml solution A + glycerine, aliqouted to 200 µl, frozen with liquid nitrogen and stored at -80°C.

8.4.14 Transformation of competent *E. coli* cells and cultivation

200 µL of chemical competent *E. coli* cells were mixed with 50 ng of plasmid DNA, and incubated on ice for at least 30 min, then transferred for 60 sec to 42°C for a heat step, and cooled down on ice for another 5 min. After the addition of 1ml LB_0, cells were gently shaken at 37°C for at least 45 min, which enabled transcription of resistance genes and synthesis of the antibiotics processing enzymes. Subsequently, cells were centrifuged at 4000x g for 5 min and either plated or inoculated in to the liquid media, with the respective antibiotics. *E. coli* cultures were bred ON at 37°C.

8.4.15 Amplification of plasmid DNA

For amplification of plasmid DNA, single colonies were transferred into 3 ml of LB containing the respective antibiotics and grown in a rotary shaker at 37°C ON. Wizard Plus SV Minipreps DNA Purification System was used for plasmid preparation.

8.5 Protein chemical methods

8.5.1 Methods for protein purification

The following chromatographic methods were applied for protein purification during this work. The purification success was controlled after every step by SDS-PAGE.

8.5.1.1 Affinity chromatography

Affinity chromatography makes use of a specific and reversible binding of the target molecule to the affinity ligand, coupled to an inert chromatography matrix.

IMAC (immobilized metal affinity chromatography) is a typical example, it is based on the formation of chelate complex between certain protein residues (histidines, cysteins and to some extent tryptophanes) and matrix-bound transition metal cations like Ni^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+}. IMAC is used for the purification of recombinant proteins carrying a polyhistidine tag,

latter is a sequence of usually 6 or 10 histidines on either N- or C- terminal of the target protein. Due to its characteristics in terms of selectivity and loading capacity Ni^{2+} is the most often used ion. The his-tag binds to nickel ions with micromolar affinity. The target protein is usually eluted with 100-500 mM Imidazole, which competes with the histidine-tag for the binding ligands, however, low concentrations of imidazole (5-20mM) in the washing buffer contribute to the purification efficiency, as all interactions of unspecifically bound proteins with the metal ion are abolished.

The choice of pH of the chromatography buffer is also of great importance, as at low pH (<5 for nickel binding) histidine is protonated and does not bind to the metal ion anymore. A pH shift is also an alternative elution method, if pH stability of the target protein allows such application.

Another advantage of the method is that it also enables purification of target proteins under denaturing conditions, as only the primary structure is important for the mode of action.

In this work IMAC was employed for the purification of soluble and insoluble 6xHis-tagged proteins, as well as for the refolding experiments.

All experiments were carried out on HisTrap FF material. Briefly, this media is highly cross-linked spherical agarose pre-charged with Ni^{2+}, also characterized by its high loading capacity, low Ni^{2+} leakage and stability against different chemical substances (red/ox agents, detergents etc.)

For the purification of target proteins pre-packed 5 ml columns were used. Screening for the optimal refolding buffer was performed in 96-well HisMultiTrap plates. Up-scaling of refolding experiments was carried out on 1 ml columns.

IMAC buffers:

Buffer A (native)	40 mM Na-P
	300mM NaC
	10 mM Imidazole
	pH 7.8
Buffer B (native)	40 mM Na-P
	300mM NaC
	500 mM Imidazole
	pH 7.8
Buffer A (denaturing)	50 mM TrsHCl
	100 mM NaCl
	6 M GdmHCl
	10 mM Imidazole
	pH 8.0
Buffer B (denaturing)	50 mM TrsHCl

100 mM NaCl
6 M GdmHCl
500 mM Imidazole
pH 8.0

Procedure	Volume
Equilibration (Buffer A)	5 CV
Sample load	5 – 150 ml
Wash after load (Buffer A)	10 CV
Elution gradient (0 – 100 % Buffer B)	15 CV

8.5.1.2 Ion exchange chromatography

Ion exchange chromatography (IEC) relies on the reversible adsorption of a charged molecule to the ion exchange groups with the opposite charge immobilized on matrix.
IEC can be subdivided into cation exchange chromatography, in which positively charged ions bind to a negatively charged resin; and anion exchange chromatography, where immobilized functional group is positively charged and the binding molecules are negative.
Net charge of a protein can be positive or negative; it depends on amino acid composition and pK of their side chains and pH of the chromatography buffer. pI (isoelectric point) of a protein is defined as the pH, at which protein looses any charge. At pH lower than pI protein adopts net positive charge, at pH higher than pI – net negative charge.
Depending on the chosen chromatography material (cation or anion) and taking in consideration the pI of a target protein, buffer with the desired condition should be applied.
Elution of the target protein is carried out by changing to conditions unfavorable for ionic bonding, which is achieved either by change of the pH or by increase of ionic strength of elution buffer.
In this work protein purifications were carried out on anion exchangers (6ml resource Q and 5 ml HiPrep Q FF). Separation of folded protein was carried out on 1 ml resource Q. Proteins were eluted with an increasing salt concentration

Buffer A 20 mM TrisHCl pH 7.5
Buffer B 20 mM TrisHCl pH 7.5
1M NaCl

Procedure	Volume
Equilibration (Buffer A)	5 CV
Sample load	5 – 10 ml

Wash after load (Buffer A)	5 CV
Elution gradient (0 – 100 % Buffer B)	15 CV

8.5.1.3 Size exclusion chromatography (SEC)

By the means of size exclusion chromatography proteins can be separated according to their hydrodynamic radius. The stationary phase is composed of porous beads with a defined pore size. Proteins larger than a pore do not fit in the beads and are the first ones to be eluted from column. Smaller proteins however are able to enter the pores, and thus their movement is impeded which results in their later elution compared to bigger protein molecules.

The separation range of a size exclusion column is defined by the pore size of the stationary phase. Generally, buffers with higher ionic strength are used, which enables the suppression of unspecific ionic interactions between proteins and the matrix.

Additionally to separation, SEC can also be applied to change the buffer of the protein sample. In this work size exclusion chromatography was applied for both, analytical and preparative purposes. Superdex 75 Prep Grade and Superdex 200 Prep Grade were used depending on the required separation range.

Buffer 1: 40 mM Na-P pH 7.8
300 mM NaCl

Buffer 2: PBS
16 mM K_2HPO4
4 mM NaH_2PO4
116 mM NaCl
pH 7.2 – 7.4

Preparative SEC:

Procedure	Volume
Equilibration	2 CV
Sample load	5 – 10 ml
Elution	1.5 CV

This method can also be applied in an analytical scale to obtain molecular size of a protein. Moreover, it also enables determination of oligomeric state of the target protein.

Analytical SEC was used in this work to study oligomerization process of the ß–galactosidase. All experiments were carried out on a Jasco HPLC system with UV and fluorescence detection. β-gal was analyzed on TSK 3000 SW XL column with Buffer 1 and a flow rate of 0.7 ml/min at room temperature. First, selected standard proteins were used to

create a calibration curve; here the retention time was plotted against the log of the molecular weight. Oligomeric state of β-gal was determined according to this curve.

8.5.1.4 Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography uses properties of hydrophobicity to separate proteins. Here stationary phase carries hydrophobic groups such as phenyl, octyl or butyl, which interact with and bind hydrophobic amino acid side chains of target protein. Addition of salt, usually ammonium sulfate, increases such hydrophobic interaction. Elution is usually achieved by the graduate decrease of salt concentration, alternatively mild organic modifiers or detergents can be used to elute protein.

Protein separation experiments were carried out on the HiTrap Butyl HP chromatography columns. Prior to application onto the column, equal volume of sample buffer was added to the protein sample.

Sample buffer: 40 mM Na-P pH 7.8
300 mM NaCl
2 M $(NH_4)_2SO_4$

Buffer A : 40 mM Na-P pH 7.8
300 mM NaCl
1 M $(NH_4)_2SO_4$

Buffer B : 40 mM Na-P pH 7.8

Procedure	Volume
Equilibration (Buffer A)	5 CV
Sample load	5 – 10 ml
Wash after load (Buffer A)	5 CV
Elution gradient (0 – 100 % Buffer B)	15 CV

8.5.1.5 Concentration of proteins

To concentrate protein solutions Millipore Ultra concentrators, with the respective cut off 3 kDa, 10 kDa or 30 kDa, depending on protein size, were used. Volumes less than 15 ml were concentrated with Ultracel 4 ml, volumes larger than 15 ml were concentrated with Ultracel 15 ml. Concentration was achieved by centrifugation at 3000 x g at 8°C, here protein solution is pressed through a membrane with a defined pore size and molecular weight cut-off and the proteins are concentrated at the membrane.

8.5.1.6 Protein dialysis

Dialysis was applied, alternatively to size exclusion chromatography, to change buffer composition of the protein sample. Protein was applied into the dialysis bag and dialyzed against 100 to 1000 fold volume of a target buffer. Dialysis was carried out for at least 4 h, but usually ON, at 4 °C for soluble protein samples and at room temperature for insoluble.

8.6 Working techniques in protein analytics

8.6.1 Solutions in protein chemistry

Running buffer (10x):	Tris	0,25 M
	Glycin	2 M
	SDS	1% (w/v)
5x-Laemmli loading buffer:	SDS	10% (w/v)
	Glycerin	50% (w/v)
	Tris	300 mM
	Bromphenol blue	0,05% (w/v)
	2-Mercaptoethanol	5% (v/v)
Transfer buffer:	Glycine	150 mM
	Tris	25 mM
	Methanol	500 ml
	SDS	0,3% (w/v)
	H_2O	2,5 l
PBS (-T):	NaCl	5,84 g
	Na_2HPO_4	11,5 g
	NaH_2PO_4	2,96 g
	H_2O	ad 1 l
	(Tween-20	1 ml)

8.6.2 SDS-polyacrylamide electrophoresis

Discontinuous SDS-Polyacrylamide gelelectrophoresis (SDS-PAGE) was applied to analyze protein samples and was performed according to the protocol of Laemmli (Laemmli, 1970). In this work 12.5% and 10% SDS-PAGE gels were used.

Separation gel: 12.5%:
3.125 ml 40% Acrylamide (40% w/v, Acrylamide/Bisacrylamide 38:2)
2.5 ml 4 x SDS-Buffer (0.8% SDS, 1.5 M Tris/HCl, pH 8.8)
4.375 ml bidest. H_2O
10%:
2.5 ml 40% Acrylamide (40% w/v, Acrylamide/Bisacrylamide 38:2)
2.5 ml 4 x SDS-Buffer (0.8% SDS, 1.5 M Tris/HCl, pH 8.8)
5 ml bidest. H_2O

Stacking gel: 0.625ml 40% Acrylamide (40% w/v, Acrylamide/Bisacrylamide 38:2)
2.5 ml 2 x SDS-Buffer (0.4% SDS, 0.25 M Tris/HCl, pH 6.8)
1.875 ml bidest. H_2O

Polymerization reaction was started by the addition of TEMED (10 µl) and APS (100µl). Electrophoresis was carried out at constant current, with 30 mA per gel for 60 min.

Protein samples were mixed with 5x Laemmli loading buffer and heated at 95 °C for 5 min prior to loading on the gel. Depending on the size of the analyzed protein, either LMW (low molecular weight) marker, HMW (high molecular weight) or a prestained marker was applied as a size reference.

8.6.3 Coomassie staining of SDS gels

After SDS-PAGE, gels were stained according to modified protocol by Fairbanks and others (Fairbanks et al., 1971). Fairbanks Solution A was used to stain SDS-polyacrylamide gels; distaining was achieved with Fairbanks Solution D, Fairbanks Solutions B and C were omitted. Gels were heated up in the staining and distaining solutions to shorten incubation times.

Fairbanks Solutions:

Solution A: 25% (v/v) Isopropanol, 10% (v/v) technical grade acetic acid, 0.05% Coomassie Blue R

Solution D: 10% acetic acid

8.6.4 Determination of protein concentration with Bradford

Determination of protein concentration according to Bradford relies on binding of a chromophore to basic and aromatic amino acids of proteins (Bradford, 1976). Such binding

results in a shift of the absorption maximum of the chromophore from 465 nm to 595 nm, and can be monitored spectroscopically. Chromophore solution (Coomassie Protein Assay Reagent, Pierce, Rockford, USA) was diluted according to the manufacturer before use.
BSA samples with defined concentration were measured first, to create a calibration curve, as this method implies that chromophore binding to the target protein is in comparable range with BSA. Prepared samples were incubated for 10 min at RT prior to absorption measurement at 595 nm.

8.6.5 Protein expression, harvest and disruption of *E.coli* cells

All proteins were expressed in *E. coli* BL21 (DE3) COD+ RIL cells (Qiagen, Hilden, Germany). The cells were cultivated in Luria-Bertani broth (LB) supplemented with 100 µg/ml ampicillin or 35 µg/ml kanamycin, respectively, at 37°C until an OD_{600}=0.8 was reached. The expression was induced subsequently with 1 mM IPTG and the cells were shifted to 30°C for 16 hours. Expression of proteins was run on 4 l scale.
Cells were harvested by centrifugation at 3000xg for 20 min. The harvested cells were resuspended in respective buffer and processed using a Basic Z cell disruption system at 1.8 kbar. (Constant systems, Warwick, UK).

8.6.6 Purification of soluble proteins

Cell pellets of 4 l cultures were resuspended in 100 ml buffer A containing 1 ml of Protease Inhibitor Mix HP and disrupted. To separate soluble fraction from insoluble and cell debris, cell lysate was centrifuged at 20000xg and 4°C for 35 min. The supernatant was loaded onto a pre-equilibrated 5 ml HisTrap Fast Flow (GE Healthcare) Ni-affinity chromatography column.
To purify the target proteins to homogeneity, ion exchange chromatography on a 6 ml Resource Q column followed by preparative size exclusion chromatography on a 16/60 Superdex 75 pg column was applied for CS, GFP, DHFR and ß-Gal. FNR and GLK were further purified by ion exchange chromatography on 5 ml HiTrap Q FF columns. After every chromatography step the protein containing fractions were analyzed by SDS-PAGE.

8.6.7 Purification of insoluble proteins and preparation of inclusion bodies

Insoluble target proteins were further isolated from insoluble cell fraction, described in previous section. Pellets were resuspended in the denaturing buffer, and stirred for 2 h at RT. Subsequently, solution was centrifuged for 20 min 20000xg. Supernatant was affinity-purified on 5 ml HisTrap columns under denaturing conditions and dialyzed into denaturing

buffer (50 mM Tris/HCl, 100 mM NaCl, 10 mM DTE (GLK, FNR, ß-Gal), 20 mM DTE (CS) or without DTE (eGFP) , pH 8.0 containing 6 M GdmCl) subsequently after elution. All chromatography steps were performed on an Äkta Explorer 10 system (GE Healthcare, Uppsala, Sweden) equipped with an 8 column vent system.

For the inclusion body preparation, harvested cells were resuspended in an appropriate volume of inclusion body (IB) preparation buffer, containing inhibitor mix HP. Subsequently, the cells were disrupted, 2.5% of Triton X-100 was added, and the solution was stirred for 30 min at 4°C. After centrifugation (20 min at 20000xg), inclusion bodies were washed three times with the IB preparation buffer. IB pellet was either immediately dissolved in denaturing buffer or stored at -20°C.

IB preparation buffer	100 mM TrisHCl pH 7.5 100 mM NaCl 1 mM EDTA
Denaturing Buffer	50 mM Tris/HCl pH 8.0 100 mM NaCl, 10 mM DTE (GLK, FNR, ß-Gal)/20 mM DTE (CS)/0 DTE (eGFP) 6 M GdmCl
Chromatography buffer A	50 mM TrisHCl pH 8.0 100 mM NaCl 10 mM Imidazole 6 M GdmCl
Chromatography buffer B	50 mM TrisHCl pH 8.0 100 mM NaCl 500 mM Imidazole 6 M GdmCl

8.6.8 Protein refolding

Refolding buffers

Buffer	pH	mM NaCl	mM L-Arg	mM L-Gln
40mM Na-P	6.5 - 9.0	100 – 500	10 – 100	10 – 100
100 mM Na-P	6.5 - 9.0	100 – 500	10 – 100	10 – 100
50 mM TrisHCl	6.5 - 9.0	100 - 500	10 – 100	10 – 100

100 mM TrisHCl	6.5 - 9.0	100 – 500	10 – 100	10 – 100
100 mM Hepes	6.5 - 9.0	100 – 500	10 – 100	10 – 100
100 mM MOPS	6.5 - 9.0	100 – 500	10 – 100	10 – 100
100mM TrisAc	6.5 - 9.0	100 – 500	10 – 100	10 – 100
100 mM K-P	6.5 - 9.0	100 – 500	10 – 100	10 – 100

additive	**concentration**
DTE/DTT	1 – 10 mM
TCEP	1 – 10 mM
glycerine	2.5 – 10 %
PEG 6000	0.01 %
α-cyclodextrin	5 – 15 mM
sucrose	100 – 300 mM
$CaCl_2$	1 mM
$MgCl_2$	1 mM

8.6.8.1 Optimization of refolding buffer conditions on-matrix

Denatured protein samples were applied to HisMultiTrap FF plates HisMultiTrap plates were handled according to the manufacturer's protocols, pre-equilibrated in denaturing buffer and 50 µg of test protein per well were applied. 500 µl of the respective refolding buffer were added subsequently to each well and the plates were centrifuged for 2 min at 50xg followed by the application of further 500 µl refolding buffer to each well and incubated for 1 h at 20°C. The refolded proteins were eluted with 200 µl of 40 mM Na-P, 300 mM NaCl, 500 mM imidazole, pH 7.8. For reference, in each plate 3 wells were loaded with the same amount of the respective native protein. These wells were treated under non-denaturing conditions (without GdmCl) following identical processing conditions. The activity of the native sample was set as 100%. The refolding yields in the various buffers were normalized accordingly. All data shown represent the average of at least 3 individual experiments.

8.6.8.2 Up-scaling of the refolding procedure on-matrix

Refolding experiments were performed on 1 ml HisTrap FF column on an Äkta Explorer at 20°C. Depending on the experiment, different amounts (0.5 -5 mg) of the denatured protein were applied onto the column. The single steps of the refolding procedure are summarized in the following table. Refolding was started by the stepwise exchange to the respective refolding buffer. Elution was carried out with a linear gradient of 0-100% buffer B. Subsequently, activity of the protein containing fractions was determined.

Procedure	Volume
Equilibration (buffer A)	5 CV
Sample load	5 – 15 ml
Wash after load (buffer A)	5 CV
Refolding buffer	5 CV
Pause (1h)	
Refolding buffer	5 CV
Elution gradient (0 – 100 % Buffer B)	15 CV

Chromatography buffer A	50 mM TrisHCl pH 8.0
	100 mM NaCl
	10 mM Imidazole
	6 M GdmCl
Chromatography buffer B	40 mM Na-P pH 7.8
	300 mM NaCl
	500 mM Imidazole

8.6.8.3 Refolding in solution

For each refolding experiment, a total of 5 µg denatured protein were diluted 100-fold into the respective buffer. The samples were mixed immediately, gently but vigorously, and incubated at 20°C for 16 h prior to activity determination. For reference, 3 dilutions of the respective native protein in 50 mM Tris/HCl, 100 mM NaCl, pH 8.0 were prepared and incubated at 20°C for 16 h. Data analysis was performed as described for the matrix-assisted procedure.

8.6.9 Refolding kinetics

Analysis of the kinetics of the matrix-assisted refolding was carried out on HisMultiTrap plates. Denatured test protein (50µg) was immobilized on-matrix, treated with the refolding buffer and incubated for 5, 10, 20, 30, 45, 60 and 120 min. In case of the in-solution approach the test protein was diluted into the refolding buffer to a final concentration of 5 µg/ml and the activity of the sample was measured at the respective time points. For each test protein both refolding approaches were carried out under identical respective buffer conditions. In each experiment the same amount of native protein was applied as a reference. All the data represent the average of at least three individual experiments.

8.6.10 Concentration dependence

Respective amounts of the target protein were loaded onto the column or diluted into the refolding buffer. During on-matrix refolding experiments the target protein was incubated in the refolding buffer for 1h at 20°C. In-solution refolding experiments were carried out for 16 h at 20°C. Equal amounts of the native protein sample were applied as a reference in all experiments. Experiments were carried out at least three times.

8.6.11 Determination of protein activity

8.6.11.1 Enhanced green fluorescent protein eGFP.

The activity of eGFP F64L S65T was determined according to the fluorescence emission of the folded and oxidized protein. Fluorescence was analyzed in a SPEX II fluorescence spectrometer. The excitation wavelength was set to 395 nm and fluorescence emission was scanned from 430-550 nm. After baseline subtraction, the signal intensities of refolded samples at 508 nm were compared to the intensity of equally treated native eGFP.

8.6.11.2 Glucokinase (Glk).

Activity of Glk samples was determined according to Darrow et al (Darrow R.A. and Colowick S.P., 1962)

Princple of the enzymatic activity assay:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Glk}} \text{Glucose 6-Phosphate} + \text{ADP} + \text{H+}$$

$$\text{Cresol Red} + \text{H}^{+} \rightarrow \text{Reduced Cresol Red}$$

Reaction buffer:
- 5.00 ml of 100 mM ATP
- 6.60 ml of 0.01% Cresol Red 128 mM MgCl2
- 5 ml of 100 mM Glycine-glycine pH 8.5
- 33.4 ml of bidest water

All components were mixed together; pH of mixture was adjusted to 8.5.

For the activity measurement 800 µl of reaction buffer were mixed with 200 µl of 200 mM glucose (in bidest. water), the reaction was started by the addition of Glk. Reduction of Cresol Red (decrease ob absorbance) was spectrophotometricaly monitored at 560 nm, at 25°C for 3 min.

The amount of Glk in the reaction depended on the origin of the sample. For Glk refolded on HisMultiTrap FF plates, ~25 µg protein (in 100 µl) were applied, for Glk refolded on 1ml HisTrap FF (using Äkta Explorer) - ~25 µg (150µl), for Glk refolded in solution ~1 µg (in 200 µl).

8.6.11.3 Beta-galactosidase (β-gal)

Modified from (Miller J.H., 1972)

β-Galactosidase hydrolyzes β-D-galactosides. This enzyme facilitates growth on carbon sources like lactose by cleaving it into glucose and galactose, which can be catabolized by the cells. In the assay o-nitrophenyl-β-D-galactopyraniside (ONPG) was used to determine the activity of the β-gal. Cleavage of the ONPG, results in the release o-nitrophenol, which is characterized by an intensive smell and yellow color and can be monitored at 420 nm.

Reaction buffer (buffer Z): 100 mM Na-P
10 mM KCl
50 mM β-mercaptoethanol
1 mM $MgSO_4$
pH 7.0

ONPG was dissolved in 100 mM Na-P pH 7.0 to the final concentration of 4 mg/ml.

50 ml buffer Z were mixed with 14 ml ONPG, aliquoted and stored at -20°C.

For the activity measurements, 800µl of buffer Z+ ONPG mixture were mixed with the β-gal sample. For the β-gal refolded on HisMultiTrap FF plates, ~10-15 µg protein (in 50 µl) were applied, for β-gal refolded on 1ml HisTrap FF (using Äkta Explorer) - ~25 µg (150µl), for β-gal refolded in solution ~1 µg (in 200 µl). Increase of 420 nm absorbance was monitored for 1 min.

8.6.11.4 Ferredoxin NADP(H) reductase (FNR)

Ferredoxin NADP(H) reductase catalyzes NADPH dependent reduction reduction of potassium ferricyanide. To determine FNR activity, decrease in absorbance of potassium ferricyanide was monitored at 419 nm. The reaction was performed at 25°C in 100 mM Tris/HCl, pH 8.0 containing 1 mM potassium ferricyanide and varying concentrations of NADPH (200 – 400 µM).

For the activity measurements different amounts of the FNR were added to the 600 µl of the reaction mixture: ~20-25 µg protein (in 100 µl) refolded on HisMultiTrap FF plates, were applied, ~25 µg FNR (150 µl) refolded on 1ml HisTrap FF (using Äkta Explorer), ~1 µg (in 200 µl) of FNR refolded in solution.

8.6.11.5 Citrate Synthase (CS)

By (Buchner et al., 1998)

Citrate synthase catalyses the reaction of oxaloacidic acid (OAA) and acetyl-coenzyme A (Ac-CoA) to citrate and CoA. The enzyme activity is determined by a colorimetric test using DTNB. According to Buchner et al DTNB reacts with the free thiol-groups of the reaction product CoA. This reaction can be monitored in a spectrophotometer at 412 nm.

Solutions:

TE-buffer : 50 mM Tris/HCl, 2 mM EDTA, pH = 8.0

OAA-solution; 10 mM, disolve in 50 mM Tris no pH adjust to pH 8.0

OAA-solution; 100 mM, dissolve in 50 mM Tris no pH, adjust to pH 8.0

DTNB-solution; 10 mM, dissolve in TE-buffer, pH 8.0

Ac-CoA-solution, 5 mM, dissolve in TE-buffer, pH 8.0

The reaction mixture for the activity assay consists of 930 µl TE, 10 µl OAA-solution, 10 µl DTNB-solution and 30 µl Ac-CoA-solution. The reaction is started by the addition of CS and the change in absorbance is monitored for 1 min in a UV spectrophotometer at 412 nm: 10-15 µg in 50 µl refolded on the HisMultiTrap FF plates, 25 µg CS (150 µl) refolded on 1ml HisTrap FF (using Äkta Explorer), ~0.5 µg (in 100 µl) of CS refolded in solution.

8.6.11.6 Dihydrofolate reductase (DHFR)

DHFR, is an enzyme that reduces dihydrofolic acid to tetrahydrofolic acid, using NADPH as electron donor.To determine activity of DHFR, reduction of dihydrofolate was monitored at 340 nm (NADPH uptake). Reaction was carried out at 25°C in 50 mM TrisHCl pH 7.5 containing 100 µM NADPH, 50 µM DHF (dihydrofolate). The reaction was started by addition of DHFR: : 10-15 µg in 50 µl refolded on the HisMultiTrap FF plates, 25 µg DHFR (150µl) refolded on 1ml HisTrap FF (using Äkta Explorer), ~1 µg (in 200 µl) of DHFR refolded in solution.

8.7 Spectroscopic methods

8.7.1 UV-absorption spectroscopy

UV spectroscopy was employed to determine protein concentrations.

Disulfide bond and aromatic amino acids tyrosyne, tryptophane and phenylalanine are capable of absorbtion of the UV light at 250 – 300 nm (see table), additionally amid groups forming peptide bond absorb at 180 - 240.

	λ_{max} (nm)	ε_{max} (M^{-1} cm^{-1})
Trp	280	5700
Tyr	274	1400
Phe	257	200
disulphide bond	250	300

Lambert-Beer law correlates measured absorbance and protein concentration:

$$A = \varepsilon \times c \times d$$

where *A* Absorbance, ε molar extinction coefficient (M^{-1} cm^{-1}), *c* protein concentration (M) and *d* cell length.

Molar extinction coefficients of the target proteins were determined by the means of *protparam* tool provided by www.expasy.org. Protein samples were measured in UV Quartz cuvettes, all spectra were buffer corrected.

8.7.2 Fluorescence spectroscopy

Fluorescence is a spectrochemical method of analysis where the analyzed molecules are excited at a certain wavelength and emit radiation of a different wavelength. Upon excitation by the light of an appropriate wavelength, the electronic state of the molecule changes from the ground state to one of many vibrational levels in one of the excited electronic states. The excited electronic state is usually the first excited singlet state. From the excited state relaxation to the ground state can occur via several processes. Fluorescence corresponds to the relaxation of the molecule from the singlet excited state to the singlet ground state with emission of light

In this work fluorescence spectroscopy was applied to determine the activity of eGFP, the protein was excited at 395 nm, emission at 508 nm was monitored.

9 Declaration

I, Tetyana Dashivets, hereby declare that this thesis was prepared by me independently and using only the references and resources stated here. The work has so far not been submitted to any audit commission. Parts of this work have been published in scientific journals.

Hiermit erkläre ich, Tetyana Dashivets, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die Arbeit wurde bisher keiner Prüfungskommission vorgelegt. Teile dieser Arbeit wurden in wissenschaftlichen Journalen veröffentlicht.

_____

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10 Publications

Dashivets,T., Wood,N., Hergersberg,C., Buchner,J., and Haslbeck,M. (2009). Rapid matrix-assisted refolding of histidine-tagged proteins. Chembiochem. *10*, 869-876.

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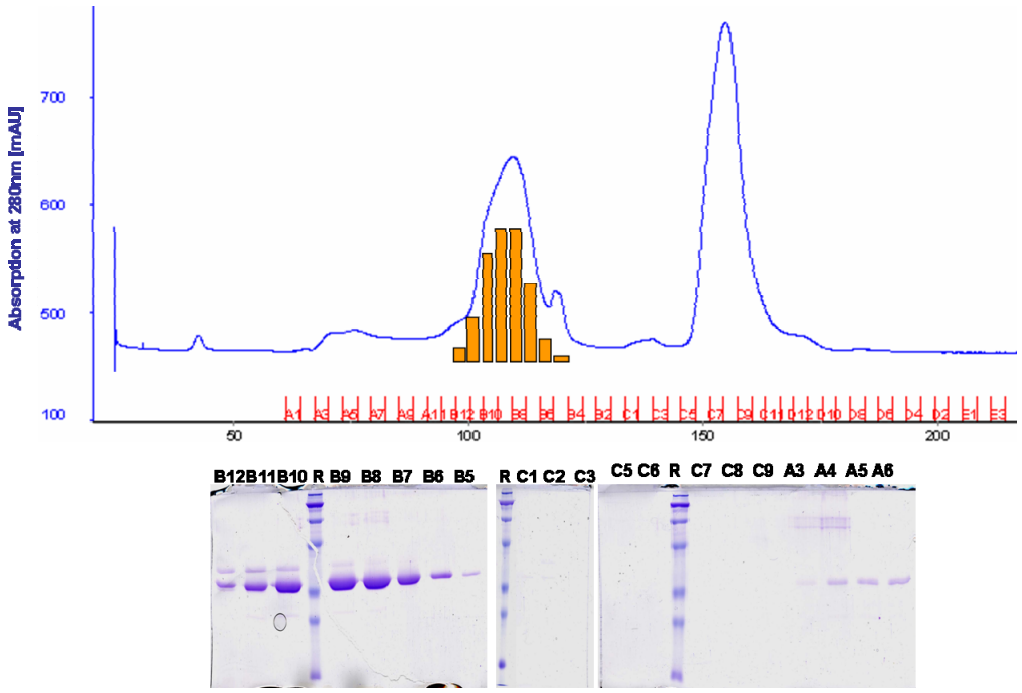
13 Appendix

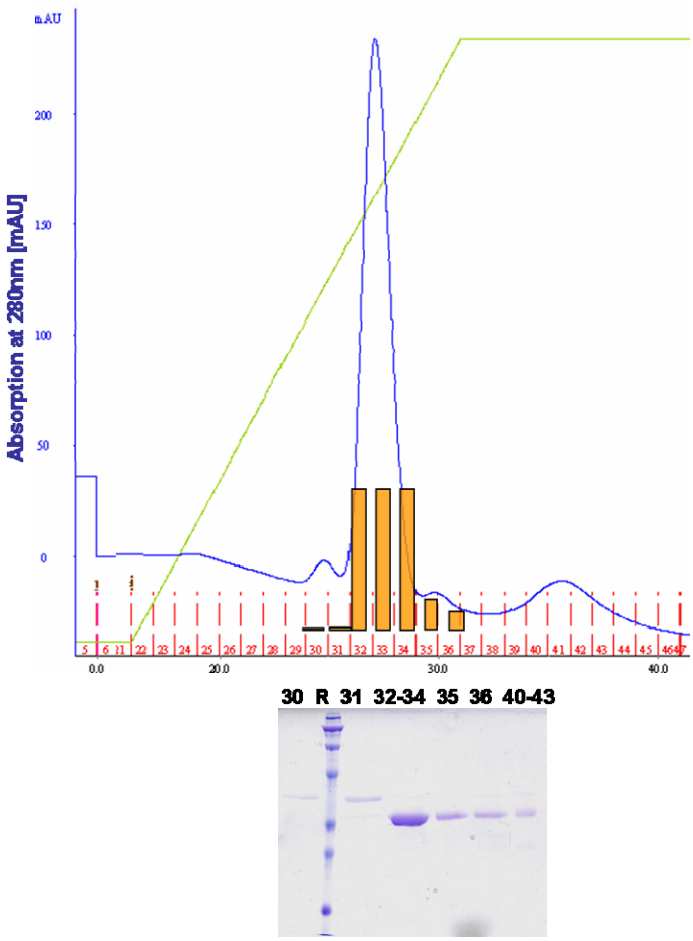
13.1.1 Downstream processing of the refolded proteins

13.1.2 eGFP



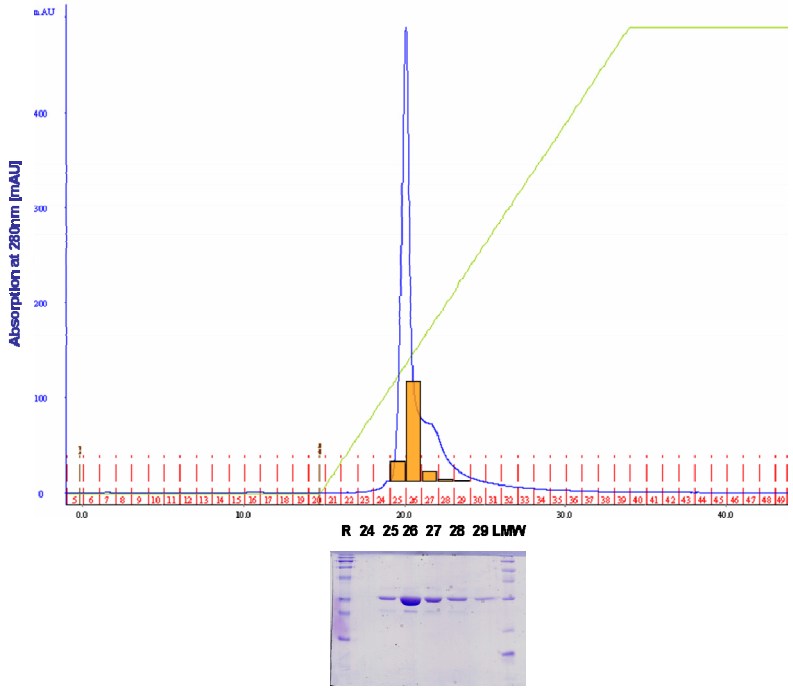
Separation of the refolded eGFP by SEC

Separation carried out on a Superdex 200 16/60 PG in PBS. Orange bars represent activity of the protein in the respective fractions. 12.5 % SDS-PAGE gel: R-Rotimark prestained; A3–C9 analyzed fractions



Separation of the refolded eGFP by HIC

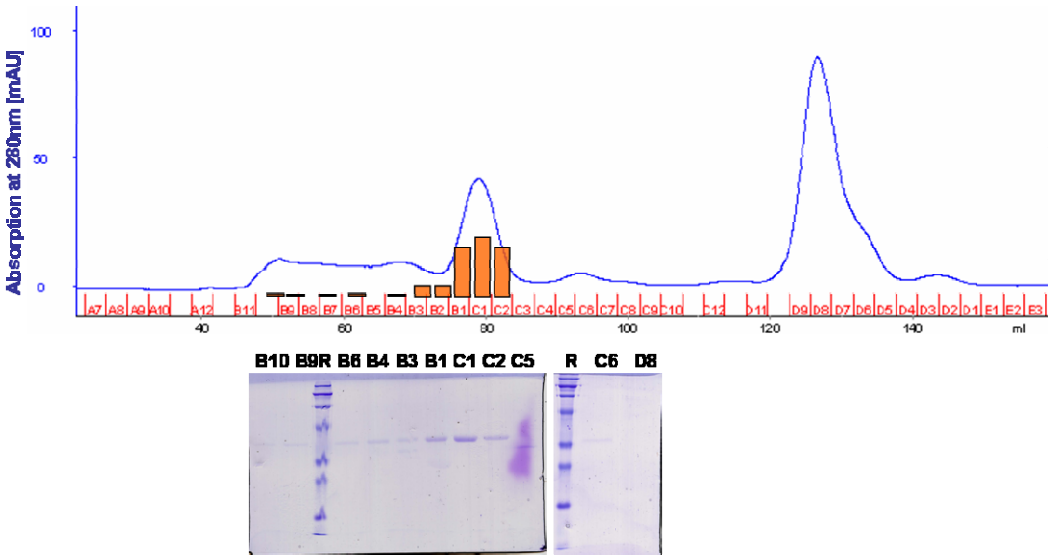
Separation was carried out on 1ml HiTrap Butyl HP. Buffer A: 40 mM Na-P 300 mM NaCl, 1M $(NH_4)_2SO_4$ pH 7.8; Buffer B: 40 mM Na-P pH 7.8. Elution gradient from 0 – 100% buffer B is indicated in green. Orange bars represent the activity of the protein in the respective fractions. 12.5 % SDS gel: R-Rotimark prestained; 30-43 analyzed fractions. Fractions 7 - 20 are not shown



Separation of the refolded eGFP by IEC

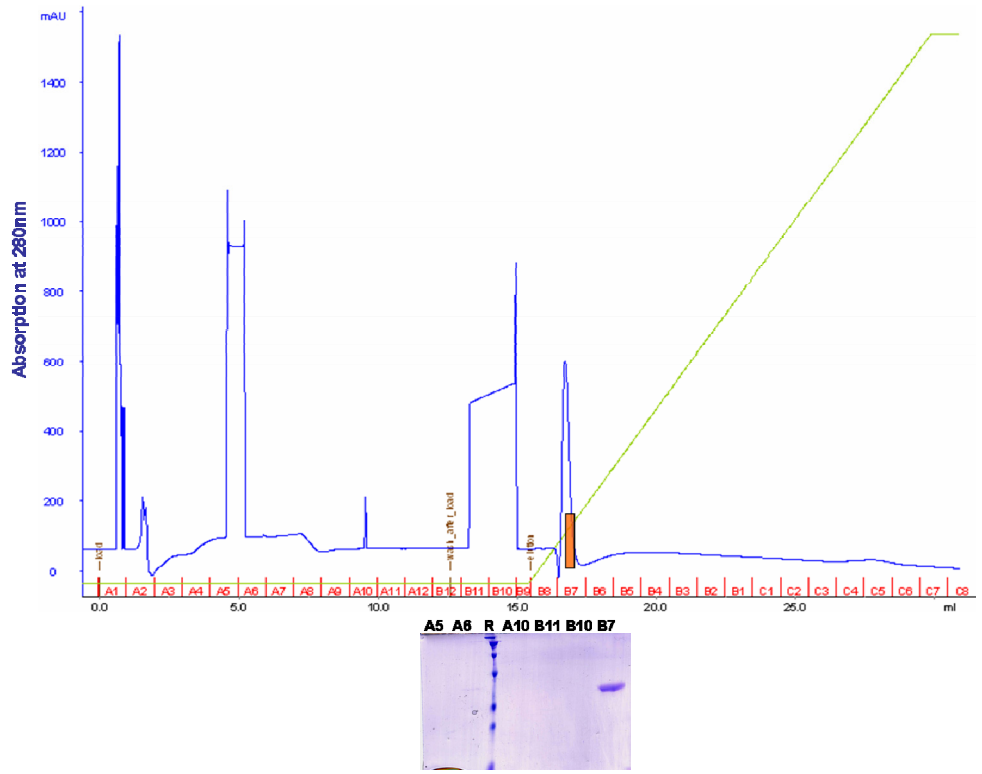
Separation was carried out on 1ml Resource Q. Orange bars represent activity of the protein in the respective fractions. Buffer A: 20 mM TrisHCl pH 7.5. Buffer B: 20 mM TrisHCl 1M NaCl pH 7.5. Elution gradient from 0 – 100% buffer B is indicated in green 12.5 % SDS gel: R-Rotimark prestained; LMW – low range molecular weight marker; 24-29 analyzed fractions

13.1.3 Glk



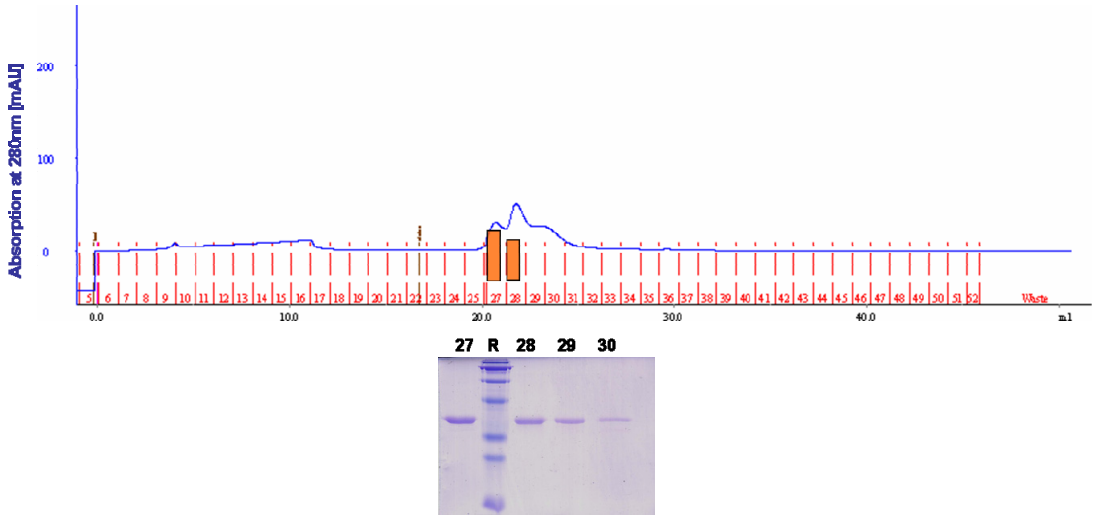
Separation of the refolded Glk by SEC

Separation carried out on a Superdex 200 16/60 PG in PBS. Orange bars represent activity of the protein in the respective fractions. 12.5 % SDS-PAGE gel: R-Rotimark prestained; B10–D8 analyzed fractions



Separation of the refolded Glk by HIC

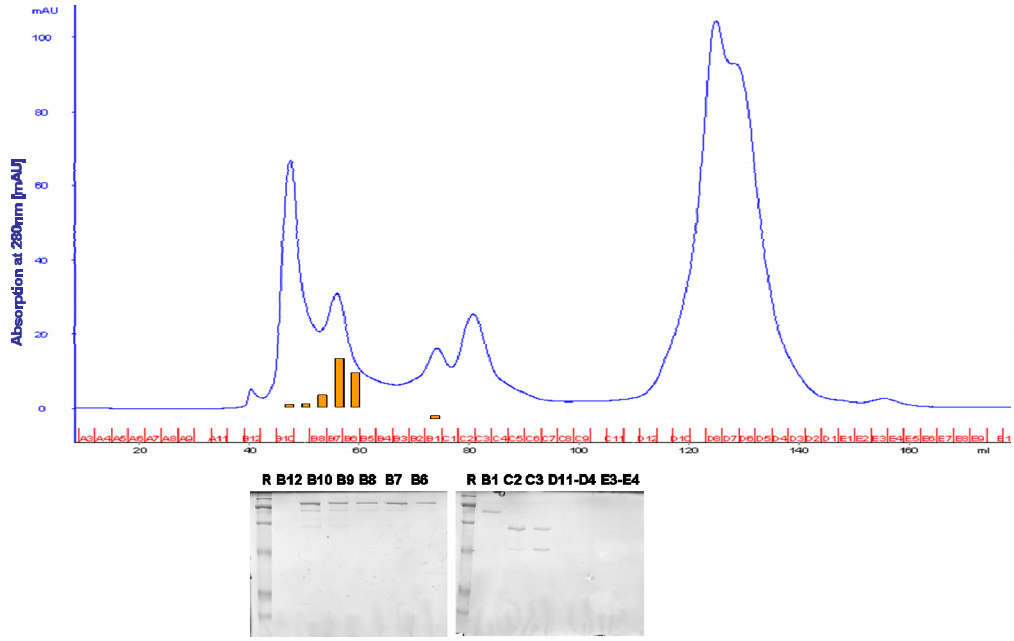
Separation was carried out on 1ml HiTrap Butyl HP. Buffer A: 40 mM Na-P 300 mM NaCl, 1M $(NH_4)_2SO_4$ pH 7.8; Buffer B: 40 mM Na-P pH 7.8. Elution gradient from 0 – 100% buffer B is indicated in green. Orange bars represent the activity of the protein in the respective fractions. 12.5 % SDS gel: R-Rotimark prestained; A5-B7 analyzed fractions.



Separation of the refolded Glk by IEC

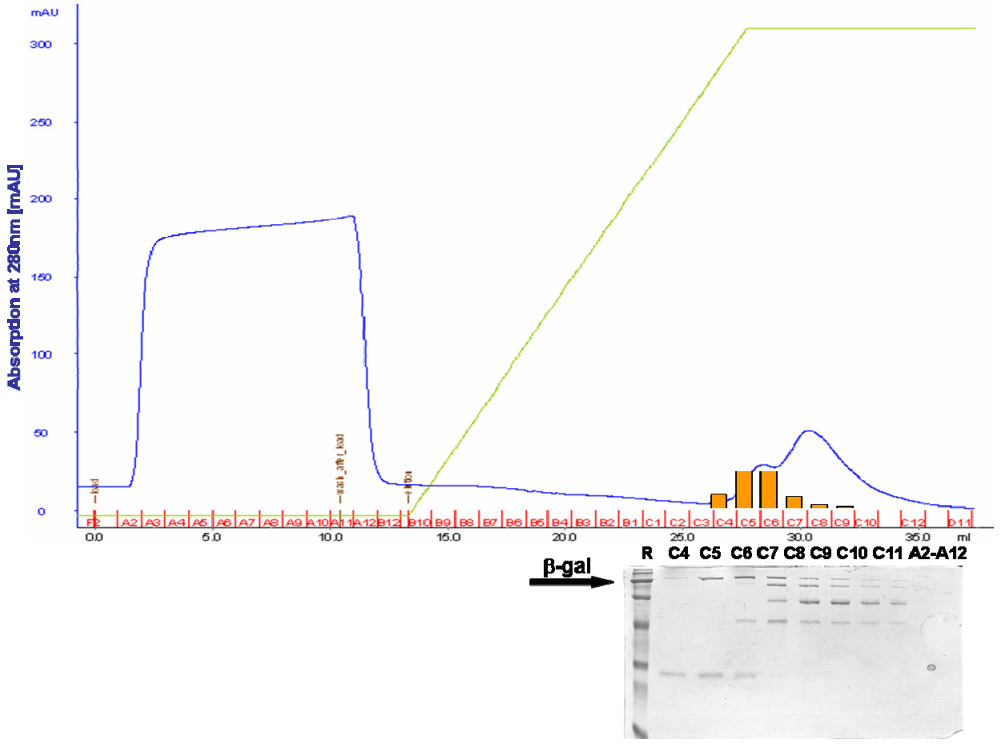
Separation was carried out on 1ml Resource Q. Orange bars represent activity of the protein in the respective fractions. Buffer A: 20 mM TrisHCl pH 7.5. Buffer B: 20 mM TrisHCl 1M NaCl pH 7.5. Elution gradient from 0 – 100% buffer B is indicated in green 12.5 % SDS gel: R-Rotimark prestained; 27 - 30 analyzed fractions

13.1.4 β-gal



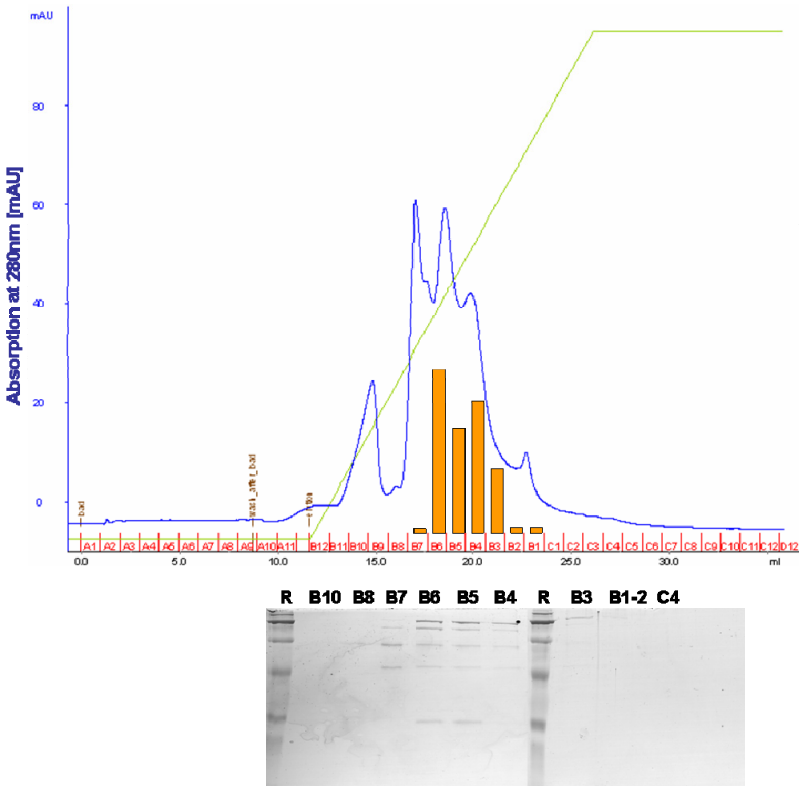
Separation of the refolded β-gal by SEC

Separation carried out on a Superdex 200 16/60 PG in PBS. Orange bars represent activity of the protein in the respective fractions. 12.5 % SDS-PAGE gel:, R-Rotimark prestained; B12 – E4 analyzed fractions



Separation of the refolded β-gal by HIC

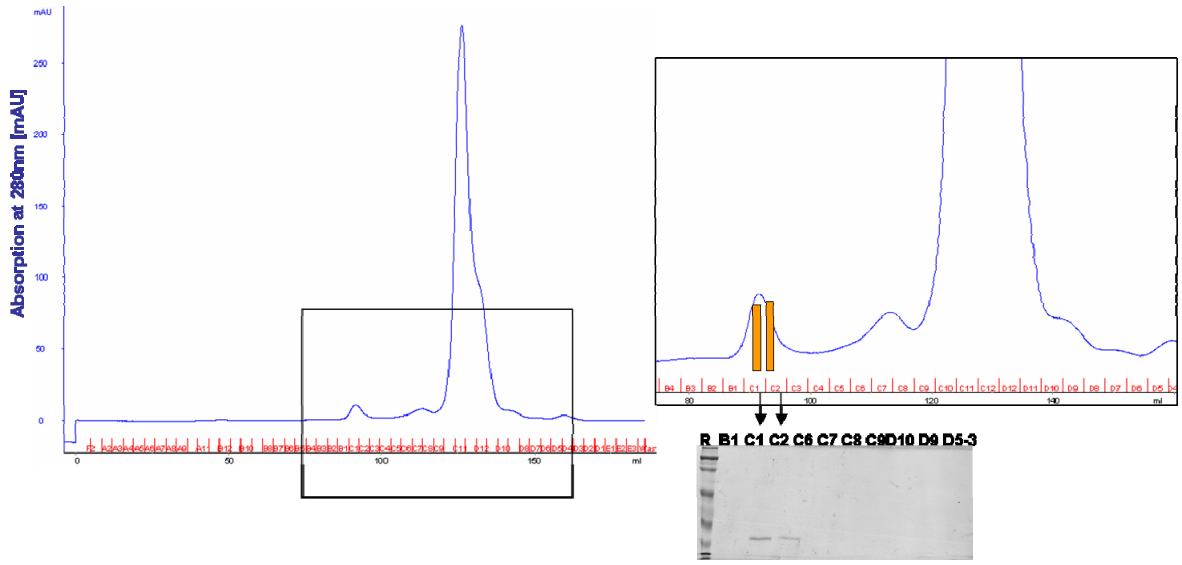
Separation was carried out on 1ml HiTrap Butyl HP. Buffer A: 40 mM Na-P 300 mM NaCl, 1M $(NH_4)_2SO_4$ pH 7.8; Buffer B: 40 mM Na-P pH 7.8. Elution gradient from 0 – 100% buffer B is indicated in green. Orange bars represent the activity of the protein in the respective fractions. 12.5 % SDS gel: R-Rotimark prestained; A2 – C12 analyzed fractions.



Separation of the refolded β-gal by IEC

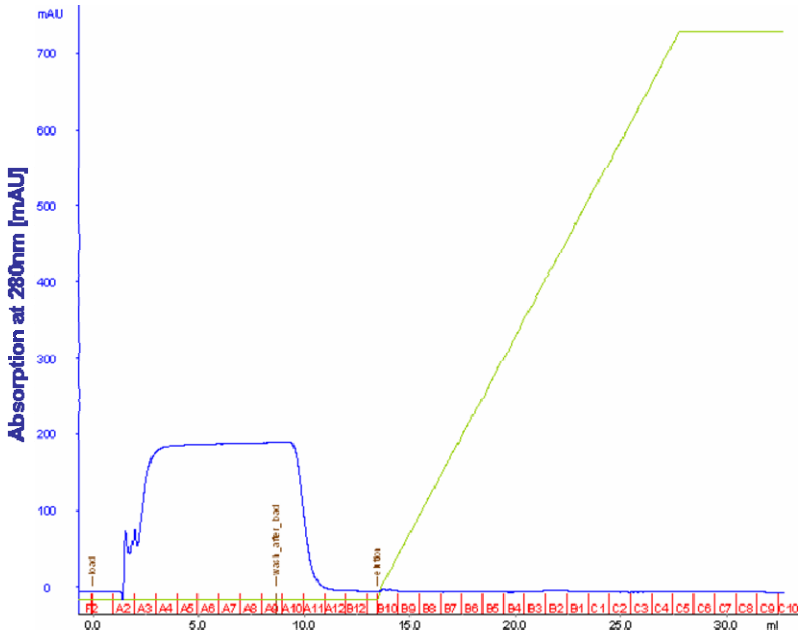
Separation was carried out on 1ml Resource Q. Orange bars represent activity of the protein in the respective fractions. Buffer A: 20 mM TrisHCl pH 7.5. Buffer B: 20 mM TrisHCl 1M NaCl pH 7.5. Elution gradient from 0 – 100% buffer B is indicated in green 12.5 % SDS gel: R-Rotimark prestained; B10 – C4 analyzed fractions.

13.1.5 DHFR



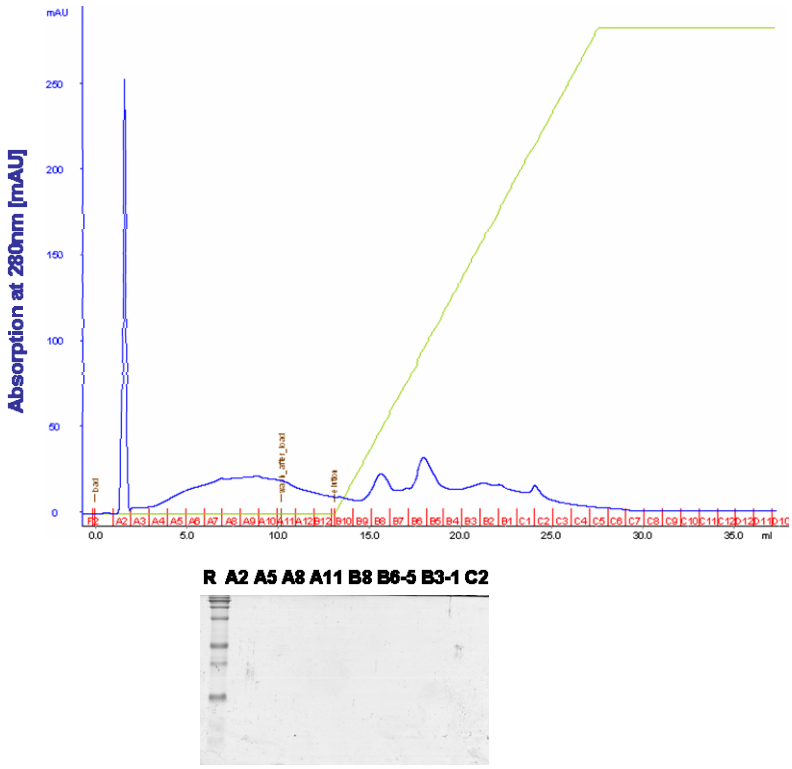
Separation of the refolded DHFR by SEC

Separation carried out on a Superdex 200 16/60 PG in PBS. Analyzed part of the chromatogram is enlarged. Orange bars represent activity of the protein in the respective fractions. 12.5 % SDS-PAGE gel:, R-Rotimark prestained; B1 – D3 analyzed fractions



Separation of the refolded DHFR by HIC

Separation was carried out on 1ml HiTrap Butyl HP. Buffer A: 40 mM Na-P 300 mM NaCl, 1M $(NH_4)_2SO_4$ pH 7.8; Buffer B: 40 mM Na-P pH 7.8. Elution gradient from 0 – 100% buffer B is indicated in green. No protein binding could be achieved under standard protocol conditions



Separation of the refolded DHFR by IEC

Separation was carried out on 1ml Resource Q. Orange bars represent activity of the protein in the respective fractions. Buffer A: 20 mM TrisHCl pH 7.5. Buffer B: 20 mM TrisHCl 1M NaCl pH 7.5. Elution gradient from 0 – 100% buffer B is indicated in green 12.5 % SDS gel: R-Rotimark prestained; A2 – C2 analyzed fractions. No protein or protein activity could be detected

13.1.6 Off-matrix assembly of the on-matrix refolded β-gal: retention profiles

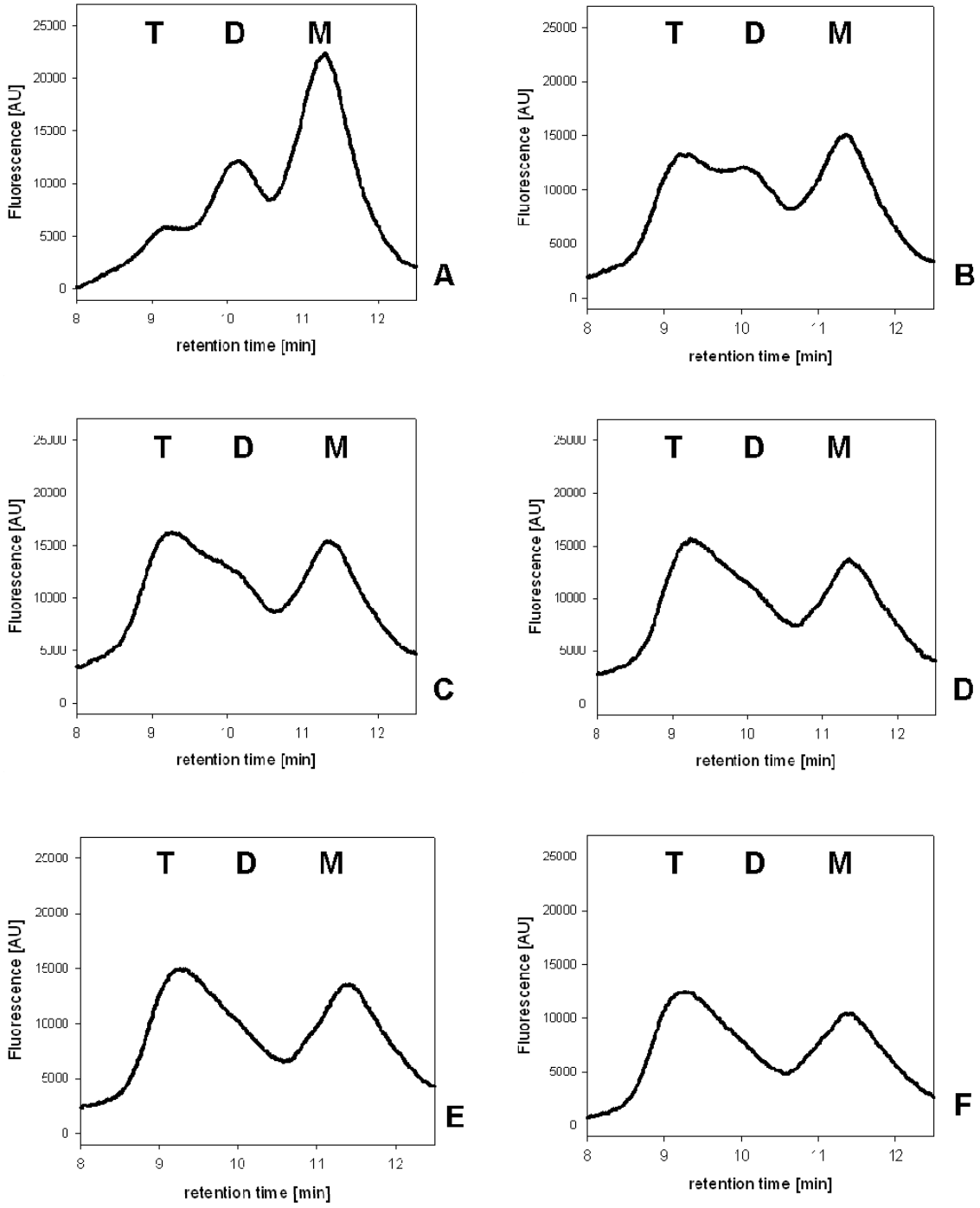


Figure 39. Off-matrix oligomerization of the on-matrix refolded β-gal: Retention profiles

Experiments were performed on TSK 3000 SW column, at 0.7 ml/min, in 40 mM Na-P 300 mM NaCl pH 7.8. Analysis of the sample was carried out 5 min (**A**), 45 min (**B**), 85min (**C**), 125 min (**D**), 165 min (**E**) and 205 min (**F**) after elution from the chromatography matrix. T, D, M tetramer, dimer and monomer respectively.