Mindaugas Zaveckas

PARTITIONING AND REFOLDING OF RECOMBINANT HUMAN GRANULOCYTE-COLONY STIMULATING FACTOR IN AQUEOUS TWO-PHASE SYSTEMS CONTAINING CHELATED METAL IONS

Summary of Doctoral Dissertation
Technological Sciences, Chemical Engineering (05T),
Biotechnology (T490)

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Vilnius „Technika“ 2005

VILNIUS GEDIMINAS TECHNICAL UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY

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INTRODUCTION

Topicality of the problem. Expression of cloned genes in *Escherichia coli* is one of the most efficient ways to produce recombinant proteins. However, recombinant proteins in *E. coli* are often accumulated in insoluble inclusion bodies and the refolding step is necessary to obtain biologically active protein. Upon *in vitro* folding, misfolding as well as aggregation competes with the correct folding pathway (Kiefhaber et al, 1991). Protein aggregation can be inhibited by suppressing intermolecular interactions between aggregation-prone folding intermediates, thus improving the yield of correctly folded protein. For this purpose, various chromatographic methods have been proposed. Immobilized metal ion affinity chromatography (IMAC) was successfully used for the refolding of recombinant proteins from inclusion bodies, possessing $(His)_6$ tail at the C- or N-terminus (Etzerodt et al, 1995), naturally existing histidine residues or other metal binding sites (Rožėnaitė et al, 2001).

During recent years a lot of attention has been paid to the use of aqueous two-phase systems for protein refolding. In aqueous two-phase systems refolded protein may be separated from its denatured and aggregated forms (Forciniti, 1994; Umakoshi et al, 2000). To date aqueous two-phase systems containing chelated metal ions were not used for protein refolding. The studies of the dependence of protein refolding efficiency on the nature of metal ion and the content of surface-exposed cysteine and histidine residues could lead to a wider application of IMAC and aqueous two-phase systems, containing chelated metal ions, for protein refolding.

The use of these techniques for protein refolding requires knowledge which amino acid residues dominate protein binding to chelated metal ions. Protein interaction with chelated Cu(II) and Ni(II) ions is dominated by histidine residues (Hemdan et al, 1989; Arnold, 1991), however, it is thought that unpaired cysteine residues may also contribute to this interaction. It is supposed that unpaired Cys residue may participate in protein binding to chelated Hg(II) ions, but the direct evidence for this interaction was not obtained (Gelūnaitė et al, 2000).

The main goal of this study was to investigate the dependence of refolding efficiency of recombinant human granulocyte-colony stimulating factor (rhG-CSF) from inclusion bodies in aqueous two-phase systems PEG-dextran, containing chelated Ni(II) and Hg(II) ions, on the nature of the metal ion, surface-exposed Cys and His residues, and their number.

Towards this goal, ***the following tasks*** were formulated:

1. To evaluate the contribution of Cys17 and surface-exposed histidine residues in the interaction of rhG-CSF with Cu(II), Ni(II) and Hg(II) ions

chelated by LR Yellow 2KT-PEG by using site-directed mutagenesis and partitioning in aqueous two-phase systems. To develop techniques for the recovery and purification of rhG-CSF mutants from inclusion bodies.

2. To evaluate the process of rhG-CSF refolding from inclusion bodies in aqueous two-phase systems containing Ni(II) and Hg(II) ions chelated by LR Yellow 2KT-PEG.
3. To perform the comparative study of refolding efficiency of rhG-CSF, rhG-CSF (C17S), $(His)_6$-rhG-CSF and rhG-CSF (C17S) histidine mutants from inclusion bodies in aqueous two-phase systems containing chelated Ni(II) and Hg(II) ions.

Scientific novelty. The contribution of Cys17 and surface-exposed histidine residues in rhG-CSF interaction with Cu(II), Ni(II) and Hg(II) ions chelated by LR Yellow 2KT was evaluated for the first time. It was determined that His43, His52, His156 and His170 residues are involved in protein interaction with chelated Cu(II) ions. Protein interaction with chelated Ni(II) is governed by His52 and His170 residues, though Cys17 is also involved. The contribution of Cys17 side chain is dominant in the interaction between rhG-CSF and chelated Hg(II) ions. The direct interaction between chelated Hg(II) ions and the –SH group of protein was determined for the first time.

Based on the study of the interaction between rhG-CSF and chelated Ni(II) or Hg(II) ions, rhG-CSF was successfully refolded from inclusion bodies in aqueous two-phase systems containing chelated Ni(II) or Hg(II) ions for the first time. The refolding of rhG-CSF (C17S) in these systems was more effective compared to that of intact rhG-CSF. The dependence of refolding efficiency of rhG-CSF (C17S) in two-phase systems containing chelated metal ions on the number of histidine mutations was evaluated for the first time. It was determined that the refolding efficiency of protein in the systems containing chelated Ni(II) is inversely proportional to the number of histidine mutations. The affinity of purified rhG-CSF (C17S) and its histidine mutants for chelated Ni(II) ions was found to be directly proportional to their refolding efficiency in the systems containing chelated Ni(II). It was shown that the refolding efficiency of rhG-CSF (C17S) and its mutants in two-phase systems is also dependent on the nature of metal ion.

Practical value. The research results extend the knowledge about the mechanisms of protein binding to the chelated Ni(II) and Hg(II) ions, and the use of this binding for protein refolding from inclusion bodies. The developed method for the refolding and purification of rhG-CSF from inclusion bodies in aqueous two-phase systems containing chelated Ni(II) or Hg(II) ions may be used for the refolding and purification of rhG-CSF and other proteins, which possess surface histidine or unpaired cysteine residues.

Defended propositions

1. The contribution of Cys17 residue of rhG-CSF in the interaction of protein with Hg(II) ions, chelated by LR Yellow 2KT-PEG.
2. The contribution of surface-exposed histidine residues of rhG-CSF in the interaction of protein with Cu(II) and Ni(II) ions, chelated by LR Yellow 2KT-PEG.
3. Refolding process of rhG-CSF from inclusion bodies in aqueous two-phase systems PEG-dextran, containing Ni(II) or Hg(II) ions, chelated by LR Yellow 2KT-PEG.
4. The dependence of refolding efficiency of rhG-CSF and its mutants in aqueous two-phase systems, containing chelated metal ions, on the nature of metal ion.
5. The dependence of rhG-CSF (C17S) refolding efficiency in aqueous two-phase systems, containing chelated Ni(II) ions, on the number of histidine mutations.

Methodology of research includes the isolation and solubilization of inclusion bodies, oxidative refolding and chromatographic purification of rhG-CSF mutants from inclusion bodies, synthesis of PEG-Light Resistant Yellow 2KT (PEG-LR Yellow 2KT) and its derivatives, partitioning and refolding of rhG-CSF and its mutants in aqueous two-phase systems, protein assays and the determination of the amount of correctly folded protein.

Aqueous two-phase systems were composed of polyethylene glycol (PEG) and dextran. Metal affinity partitioning experiments were performed by replacing part of PEG for PEG-LR Yellow 2KT-M(II). The partition coefficient for the protein (K) was defined as the ratio of protein concentration in the upper and lower phase. The protein affinity for immobilized metal ion was expressed in terms of $\Delta\log K$, given by $\Delta\log K = \log K_M - \log K_{dye}$, where K_M and K_{dye} are partition coefficients of the protein in the presence of metal-dye-PEG and in the presence of demetallized dye-PEG, respectively. All partitioning experiments were carried out in duplicate at 4^oC and the value of $\Delta\log K$ is given as the mean of two separate determinations.

A scheme for the refolding of rhG-CSF and its mutants from solubilized inclusion bodies in aqueous two-phase systems containing Ni(II) or Hg(II) ions chelated by LR Yellow 2KT-PEG is presented in Fig 2. The amount of correctly folded protein in the samples of rhG-CSF and its mutants was determined by reversed-phase HPLC analysis. The biological activity of rhG-CSF and its mutant samples was determined by measuring their proliferative effect on G-NFS-60 cells.

RESULTS AND DISCUSSION

1. Investigation of the contribution of cysteine and histidine residues in rhG-CSF binding to chelated metal ions

RhG-CSF contains unpaired Cys17 and five histidine residues (Nagata, 1994). Cys17 of rhG-CSF is partially surface-exposed (Arakawa et al, 1993). Visualisation of NMR resolved structure of rhG-CSF (Zink et al, 1994) by RasMol (version 2.6, R. Sayle, Glaxo Research and Development) revealed that His43, His52, His156 and His170 side chains are surface-exposed. Therefore, rhG-CSF was selected as a model protein for the evaluation of the role of cysteine and histidine residues in protein interaction with chelated metal ions.

RhG-CSF cysteine and histidine mutants were obtained by using the site-directed mutagenesis. The interaction of rhG-CSF, its cysteine and histidine mutants with Cu(II), Ni(II) and Hg(II) ions, chelated by LR Yellow 2KT was evaluated by immobilized metal ion affinity partitioning (IMAP). IMAP is successfully employed to detect metal-binding sites on the protein surface (Otto and Birkenmeier, 1993), to probe their microenvironment and to measure metal-protein binding constants (Suh et al, 1991).

1.1. Refolding, purification and characterization of rhG-CSF (C17S) histidine mutants

RhG-CSF (C17S) histidine mutants, like rhG-CSF, were expressed in *E. coli* as inclusion bodies. Correctly folded and highly purified rhG-CSF mutants were necessary for the partitioning studies. For this purpose, a scheme for the recovery and purification of rhG-CSF mutants from *E. coli* inclusion bodies has been designed. Inclusion bodies were solubilized in guanidine hydrochloride solution and oxidative refolding was performed with Cu^{2+} ions. Since chelated Cu(II) ions bind proteins with even one available histidine quite strongly, Sepharose-LR Yellow 2KT-Cu(II) adsorbent was selected for the initial purification of rhG-CSF mutants according to the methodology of IMAC. Cation-exchange chromatography was chosen for the further purification of rhG-CSF mutants. Homogeneity of purified rhG-CSF (C17S) histidine mutants was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing gel electroforesis. The purity of rhG-CSF mutants was 92-99%, as determined by RP HPLC. Thus, the proposed scheme allowed to obtain correctly folded and highly purified rhG-CSF (C17S) histidine mutants.

Purified rhG-CSF mutants exhibited biological activity with the magnitude comparable to that of intact rhG-CSF. Further confirmation that rhG-CSF (C17S) histidine mutants possess the structure similar to that of wild-type rhG-CSF is evident from fluorescence emission spectra and immunoblotting results.

1.2. Immobilized metal ion affinity partitioning of rhG-CSF, rhG-CSF (C17S) and $(His)_6$-rhG-CSF

For IMAP experiments, reactive dye Light Resistant Yellow 2KT (LR Yellow 2KT) was used as a chelator (Fig 1). LR Yellow 2KT form relatively stable chelates with Cu^{2+}, Ni^{2+}, Zn^{2+} and even Hg^{2+} ions within the studied pH range 5.0-7.0 and chelated metal ions possess free coordination sites for protein binding (Zaveckas, 1998). The increase of demetallized LR Yellow 2KT-PEG concentration in the two-phase system caused only a slight alteration in the partition coefficient of rhG-CSF with respect to the dye-free system.

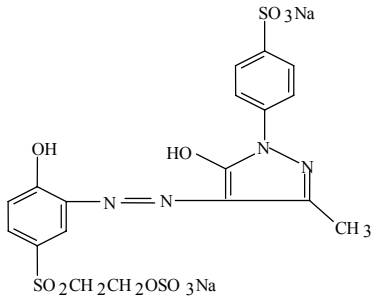

Fig 1. Structural formula of reactive dye Light Resistant Yellow 2KT. The azobond and two adjacent hydroxyl moieties are involved in complexation with d group metal ions

RhG-CSF, rhG-CSF (C17S) and $(His)_6$-rhG-CSF were effectively extracted into the upper phase at pH 7.0, when Cu(II), Ni(II) or Hg(II) ions chelated by the LR Yellow 2KT-PEG derivative were introduced into the two-phase system (Table 1). The extraction power of chelated Cu(II) ions towards all three protein variants at pH 7.0 was of the same range. Similar Δlog K values of rhG-CSF (C17S) and rhG-CSF raised doubts as to the involvement of Cys17 of rhG-CSF in the interaction with chelated Cu(II) ions.

In two-phase systems, containing Ni(II)-LR Yellow 2KT-PEG (Table 1), the Δlog K value of rhG-CSF (C17S) was notably lower, compared to that of rhG-CSF. Therefore, the involvement of Cys17 in rhG-CSF interaction with

chelated Ni(II) is possible. Comparison of Δlog K values determined for $(His)_6$-rhG-CSF and rhG-CSF clearly shows that the $(His)_6$ tag may play an essential role in the interaction of $(His)_6$-rhG-CSF with Ni(II)-LR Yellow 2KT-PEG.

Table 1
Partitioning of purified rhG-CSF, rhG-CSF (C17S) and $(His)_6$-rhG-CSF in the presence of metal ions chelated by LR Yellow 2KT-PEG at pH 7.0[a]

M(II)	rhG-CSF		rhG-CSF (C17S)		$(His)_6$-rhG-CSF	
	log K	Δlog K	log K	Δlog K	log K	Δlog K
-	0.16	-	0.11	-	0.21	-
Cu(II)	2.38	2.22	2.49	2.38	2.41	2.20
Ni(II)	1.72	1.56	1.10	0.99	2.47	2.26
Hg(II)	2.35	2.19	1.40	1.29	2.31	2.10

[a]Two-phase system (4 g) contained 5% (w/w) PEG 6000, 8% (w/w) dextran 60,000, 0.8 mg of protein, 0.25 M Na_2SO_4, 50 mM HEPES-NaOH buffer, pH 7.0. M(II)-LR Yellow 2KT-PEG concentration was 800 μmol/kg.

As seen from Table 1, the Δlog K value of rhG-CSF (C17S) in the presence of chelated Hg(II) ions at pH 7.0 was considerably lower compared to that of rhG-CSF and $(His)_6$-rhG-CSF. This reflects the contribution of the –SH group of Cys17 to the rhG-CSF extraction power by the Hg(II)-LR Yellow 2KT-PEG complex, and evidences that the direct interaction between chelated Hg(II) and unpaired Cys residue of rhG-CSF is possible.

Table 2
Partitioning of purified rhG-CSF, rhG-CSF (C17S) and $(His)_6$-rhG-CSF in the presence of metal ions chelated by LR Yellow 2KT-PEG at pH 5.0[a]

M(II)	rhG-CSF		rhG-CSF (C17S)		$(His)_6$-rhG-CSF	
	log K	Δlog K	log K	Δlog K	log K	Δlog K
-	0.20	-	0.20	-	0.10	-
Cu(II)	1.98	1.78	1.93	1.73	2.34	2.24
Ni(II)	0.48	0.28	n.d.	n.d.	n.d.	n.d.
Hg(II)	2.05	1.85	1.10	0.90	2.26	2.16

[a]Composition of the two-phase system was similar to that in Table 1, except for the buffer, 50 mM MES-NaOH, pH 5.0.
n.d. - not determined.

In the presence of chelated Ni(II) ions at pH 5.0, the Δlog K value of rhG-CSF (Table 2) decreased drastically compared to that at pH 7.0 (Table 1). Such a decrease may be related to the protonation of the imidazole group of His residue at pH 5.0. In two-phase systems containing chelated Cu(II) ions at pH 5.0, Δlog K values of rhG-CSF and rhG-CSF (C17S) were of similar magnitude

(Table 2), and lower compared to that at pH 7.0 (Table 1). The pH-dependent decrease in Δlog K of protein may be related to the protonation of surface His residues. In two-phase systems containing Hg(II)-LR Yellow 2KT at pH 5.0 (Table 2) the Δlog K value of rhG-CSF (C17S) (0.90) was twice lower compared to that of rhG-CSF (1.85). Thus, Cys17 residue plays a major role in rhG-CSF interaction with chelated Hg(II) ions at both pH 7.0 and pH 5.0.

Metal affinity partitioning studies of rhG-CSF and its variants demonstrated that the role of histidine residues is important in rhG-CSF interaction with chelated Cu(II) and Ni(II) ions at pH 7.0, and Cys17 residue predominates in rhG-CSF interaction with chelated Hg(II) ions.

1.3. The effect of low molecular weight ligands on rhG-CSF, rhG-CSF (C17S) and $(His)_6$-rhG-CSF partitioning

The specificity of the interaction between protein and ligand coupled to the polymer in aqueous two-phase systems may be investigated by adding interfering ligands to the two-phase system (Kopperschlager, 2000). The interaction of rhG-CSF with metal ions chelated by LR Yellow 2KT-PEG derivative was studied when selected low molecular weight compounds were present in the two-phase systems. Among various compounds, imidazole and 2-mercaptoethanol were the most effective in reducing the binding of rhG-CSF to chelated Ni(II) and Hg(II) ions. Imidazole was a more effective competing ligand than 2-mercaptoethanol in the interaction of all three protein variants with chelated Ni(II) ions (Table 3). In contrast, the effect of 2-mercaptoethanol on the partitioning of rhG-CSF variants was more pronounced in two-phase systems containing chelated Hg(II) ions.

Table 3
The effect of low molecular weight ligands on partitioning of purified rhG-CSF, rhG-CSF (C17S) and $(His)_6$-rhG-CSF in aqueous two-phase systems containing M(II)-LR Yellow 2KT-PEG, pH 7.0[a]

M(II)	Ligand[b]	Δlog K reduction (%)		
		rhG-CSF	rhG-CSF(C17S)	$(His)_6$-rhG-CSF
Ni(II)	Imidazole	84	65	69
	2-mercaptoethanol	73	1	46
Hg(II)	Imidazole	81	60	22
	2-mercaptoethanol	98	77	98

[a]Both the system composition and M(II)-LR Yellow 2KT-PEG concentration were similar to those in Table 1.
[b]Ligand concentration 10 mM.

Histidine side chain contains imidazole ring, while 2-mercaptoethanol, analogously to cysteine side chain, contains –SH group. Considering this, the observed dependence of competing effect of imidazole and 2-mercaptoethanol on the type of chelated metal ion suggest that chelated Ni(II) ions exhibit higher affinity to protein histidine residues compared to that to cysteine, while chelated Hg(II) ions display higher affinity to protein cysteine residues.

1.4. Immobilized metal ion affinity partitioning of rhG-CSF (C17S) histidine mutants

Partitioning data of rhG-CSF (C17S) histidine mutants in two-phase systems containing metal ions chelated by the LR Yellow 2KT-PEG at pH 7.0 are summarized in Table 4. It is seen from Table 4, that in two-phase systems containing chelated Cu(II) ions each histidine mutation resulted in the decrease of Δlog K of respective rhG-CSF (C17S) histidine mutant compared to the protein variant without this mutation. Therefore, all mutated His residues – His43, His52, His156 and His170 – are surface-exposed and contribute to rhG-CSF interaction with chelated Cu(II) ions.

Table 4
Partitioning of purified rhG-CSF (C17S) histidine mutants in aqueous two-phase systems containing M(II)-LR Yellow 2KT-PEG, pH 7.0[a]

rhG-CSF mutant	M(II)						
	-	Cu(II)		Ni(II)		Hg(II)	
	log K	log K	Δlog K	log K	Δlog K	log K	Δlog K
C17S	0.11	2.49	2.38	1.10	0.99	1.40	1.29
C17S, H43A	0.28	2.34	2.06	1.29	1.01	1.72	1.44
C17S, H43A, H170A	0.34	1.68	1.34	0.84	0.50	1.83	1.49
C17S, H43A, H52A	0.38	1.87	1.49	0.79	0.41	1.75	1.37
C17S, H43A, H52A, H156A	0.40	1.55	1.15	0.81	0.41	1.73	1.33

[a]Two-phase system (4 g) contained 5% (w/w) PEG 6000, 8% (w/w) dextran 60,000, 0.8 mg of protein, 0.25 M Na_2SO_4, 50 mM HEPES-NaOH buffer, pH 7.0. M(II)-LR Yellow 2KT-PEG concentration was 800 μmol/kg.

The effect of individual histidine mutations on rhG-CSF (C17S) partitioning in two-phase systems containing chelated Cu(II) ions was different (Table 4). Accordingly, His52 and His170 side chains dominate in rhG-CSF interaction with chelated Cu(II), while the contribution of His43 and His156 is lower. The different contributions of individual histidine residues in this

interaction may result from different their steric accessibility and pK_a. It was shown that the binding strength of proteins, which display single histidyl residue, on the Cu(II)-IDA column is inversely proportional to the pK_a value of histidyl residue (Hemdan et al, 1989). The protein affinity for chelated metal ion is also dependent on steric accessibility of the histidine imidazole nitrogen (Arnold, 1991).

As seen from Table 4, His52 and His170 mutations led to the marked decrease in protein Δlog K in two-phase systems containing chelated Ni(II) ions. These results suggest that His52 and His170 residues dominate in rhG-CSF (C17S) interaction with chelated Ni(II) ions. Since mutations of His43 and His156 residues had no effect on rhG-CSF (C17S) partitioning in the presence of chelated Ni(II), these residues do not contribute to protein interaction with chelated Ni(II) ions.

In two-phase systems containing chelated Hg(II) ions Δlog K values for rhG-CSF (C17S) histidine mutants were similar and very close to that for rhG-CSF (C17S) (Table 4). A negligible effect of histidine mutations on rhG-CSF (C17S) partitioning in the presence of Hg(II)-LR Yellow 2KT suggests that surface-exposed histidine residues are not important in rhG-CSF interaction with chelated Hg(II) ions.

The partitioning study of rhG-CSF (C17S) histidine mutants in two-phase systems containing chelated metal ions confirmed that at pH 7.0 the mechanism of protein interaction with chelated Hg(II) ions is different from that of chelated Cu(II) or Ni(II). It was shown that surface-exposed histidine residues predominate in rhG-CSF interaction with chelated Cu(II) and Ni(II) ions. However, these residues do not contribute to protein interaction with chelated Hg(II) ions.

2. Refolding of rhG-CSF and its mutants from inclusion bodies in aqueous two-phase systems containing chelated metal ions

The effect of a nature of chelated metal ion and the mutations of surface-exposed cysteine and histidine residues on protein refolding efficiency in aqueous two-phase systems, containing Ni(II) and Hg(II) ions, chelated by LR Yellow 2KT-PEG, was investigated by using rhG-CSF as a model protein. As it was shown in the first part of this work, surface-exposed Cys17 and histidine residues of rhG-CSF are involved in the interaction with chelated metal ions. Furthermore, rhG-CSF accumulates in *E. coli* as inclusion bodies, and the refolding procedure is required to obtain biologically active protein.

A scheme for the refolding and purification of rhG-CSF and its variants from inclusion bodies in aqueous two-phase systems containing chelated metal ions is represented in Fig 2. In these systems, rhG-CSF from solubilized inclusion bodies interact with chelated metal ions and is extracted into the upper phase, while the majority of *E. coli* proteins are partitioned in the lower phase (Fig 2, 1). The upper phase, containing chelated metal ions and extracted rhG-CSF, is separated from the lower dextran phase, containing *E. coli* proteins and transferred on the dextran phase of the two-phase system without metal chelate and protein (Fig 2, 2). Finally, refolded rhG-CSF is desorbed to the lower dextran phase by imidazole or 2-mercaptoethanol (Fig 2, 3).

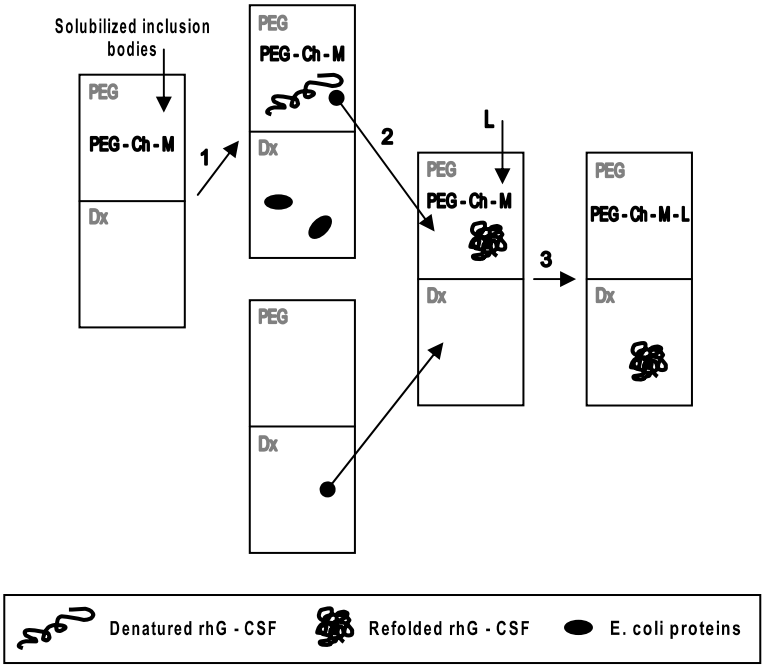

Fig 2. A scheme for the refolding and purification of rhG-CSF in polyethylene glycol (PEG) - dextran (Dx) two-phase systems, containing Ni(II) or Hg(II) ions chelated by LR Yellow 2KT-PEG. Ch-M – LR Yellow 2KT-Ni(II) or -Hg(II) chelate; L – imidazole or 2-mercaptoethanol. 1 – partitioning of rhG-CSF inclusion body proteins; 2- separation of rhG-CSF from *E. coli* proteins and rhG-CSF refolding; 3 - desorption of refolded rhG-CSF to the lower dextran phase by using imidazole or 2-mercaptoethanol solution

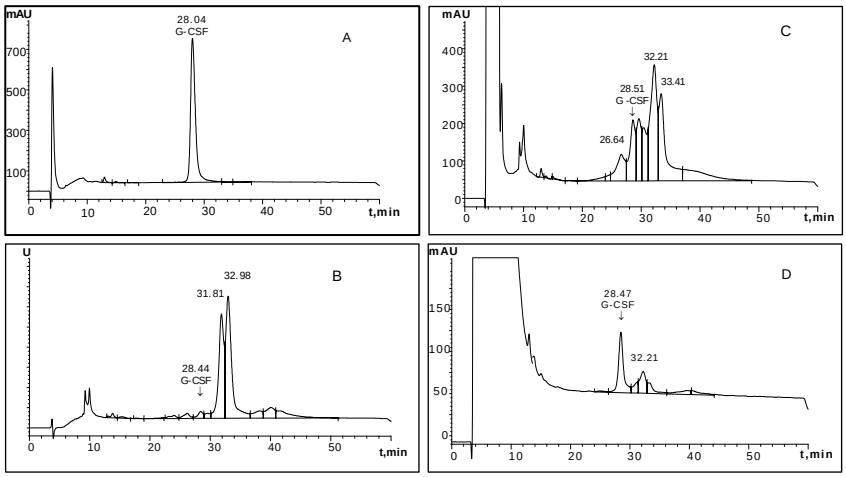

Fig 3. RP-HPLC analysis of rhG-CSF samples. A – standard (1.042 mg/ml), injection volume 40 µl; B – inclusion body extract (2.25 mg/ml), 40 µl. C, D – samples from the two-phase system containing chelated Ni(II) ions. C – interface layer solubilized in 6 M GdmHCl (0.29 mg/ml), 300 µl; D – sample from the dextran phase (0.06 mg/ml), 500 µl. The composition of the two-phase system is indicated in Table 5

The samples taken from both the interface and the dextran-rich phase of two-phase systems were analysed by RP-HPLC, typical chromatograms are shown in Fig 3. In the initial inclusion body extract of rhG-CSF, folding intermediates with one reduced disulphide bond, as previously described (Lu et al, 1992), clearly predominate and the relative amount of correctly folded protein conformation is negligible (Fig 3, B). Misfolded and/or aggregated protein forms tend to assemble at the interface layer of the two-phase system with some properly folded protein (Fig 3, C). In the dextran phase of two-phase system, correctly folded form of rhG-CSF predominates (Fig 3, D).

The samples drawn from the dextran phase after partitioning of rhG-CSF inclusion body extract in two-phase systems exhibited rhG-CSF biological activity, which magnitude correlates well with the relative amount of correctly folded protein determined by RP-HPLC (Table 5). Thus, the results of RP-HPLC analysis and biological activity data revealed that the interaction between the rhG-CSF from inclusion body extract and chelated metal ion in aqueous two-phase systems induces the generation of a correctly folded native protein possessing rhG-CSF activity. It was also demonstrated by using RP HPLC and SDS-PAGE that partial purification of the target protein in two-phase systems was performed.

2.1. Refolding of rhG-CSF, rhG-CSF (C17S) and $(His)_6$-rhG-CSF

The amounts of correctly folded rhG-CSF and its variants in samples from two-phase systems, calculated from RP-HPLC chromatograms are presented in Table 5. The amounts of correctly folded rhG-CSF (C17S) in two-phase systems were higher compared to that for rhG-CSF, irrespective of the chelated metal ion and selected desorption agent (Table 5). Due to the Cys17 mutation, rhG-CSF (C17S) conformation which contains two –SH groups may predominate in the initial inclusion body extract, while the analogous rhG-CSF conformation contains three –SH groups. Consequently, the possibility of incorrect disulphide bond formation during rhG-CSF (C17S) refolding is lower and therefore higher refolding yield of this protein variant may be obtained.

Table 5
Dependence of the relative amount of correctly folded protein in two-phase systems containing metal ions chelated by LR Yellow 2KT-PEG on the nature of protein variant, metal ion and desorption agent at pH 7.0[a]

Protein variant	M(II)	Desorption agent (mM)	Relative amount of correctly folded protein (%)	
			Interface	Dextran phase
rhG-CSF	Ni(II)	Imidazole, 50 mM	12	46 ($6.45 \cdot 10^7$)[c]
rhG-CSF (C17S)			27	56
$(His)_6$-rhG-CSF			b	60
rhG-CSF	Hg(II)	Imidazole, 200 mM	26	17
rhG-CSF (C17S)			90	99
rhG-CSF	Hg(II)	2-mercaptoethanol, 10 mM	31	43 ($5.40 \cdot 10^7$)
rhG-CSF (C17S)			65	91

[a]Two-phase system (4 g) contained 5% PEG 6000, 8% dextran 60,000, 1.5 mg of protein, 800 μmol/kg M(II)-LR Yellow 2KT-PEG, 0.25 M Na_2SO_4 and 50 mM HEPES-NaOH buffer, pH 7.0.
[b]Protein concentration was less than detection limit.
[c]hG-CSF biological activity (IU/mg). rhG-CSF standart exhibited biological activity $1 \cdot 10^8$ IU/mg.

In two-phase systems containing chelated Hg(II) ions, the higher amount of correctly folded rhG-CSF was obtained when 2-mercaptoethanol was used as a desorption agent (Table 5). Low concentration of 2-mercaptoethanol may initiate reshuffling of incorrect protein disulphide bonds, which in turn leads to the generation of more energetically stable native disulfide bonds. However, the effect of a desorption agent on the relative amount of correctly folded rhG-CSF (C17S) was negligible.

After the release of protein from chelated metal ions with imidazole, the amount of correctly folded rhG-CSF was higher in the dextran phase of two-phase systems containing chelated Ni(II) ions, while the amount of correctly folded rhG-CSF (C17S) was higher in two-phase systems containing chelated Hg(II) ions (Table 5).

2.2. Refolding of rhG-CSF (C17S) histidine mutants

The relative amounts of correctly folded rhG-CSF (C17S) histidine mutants in samples from two-phase systems, determined by using RP-HPLC analysis, are presented in Table 6. The amounts of correctly folded rhG-CSF mutants in dextran phase of two-phase systems containing chelated Ni(II) ions (Table 6) decreased in the order (C17S) ≈ (C17S, H43A) > (C17S, H43A, H170A) ≈ (C17S, H43A, H52A) > (C17S, H43A, H52A, H156A). Δlog K values of purified rhG-CSF (C17S) histidine mutants (except for (C17S, H43A, H52A, H156A)) in two-phase systems containing chelated Ni(II) ions at pH 7.0 decreased in the similar order (Table 6). Consequently, the direct correlation between the affinity of rhG-CSF mutant for chelated Ni(II) ions and its refolding efficiency in two-phase systems containing chelated Ni(II) ions exists.

Table 6
Dependence of the relative amount of correctly folded rhG-CSF (C17S) histidine mutants in two-phase systems containing metal ions chelated by LR Yellow 2KT-PEG on histidine mutations and the nature of metal ion[a]

rhG-CSF mutant	Relative amount of correctly folded protein in the dextran phase[b] (%)	
	Ni(II)	Hg(II)
C17S	56 (0.99)[c]	99
C17S, H43A	67 (1.01)	94
C17S, H43A, H170A	28 (0.50)	92
C17S, H43A, H52A	39 (0.41)	76
C17S, H43A, H52A, H156A	8 (0.41)	91

[a]Two-phase system (4 g) contained 5% PEG 6000, 8% dextran 60,000, 1.5 mg of protein, 800 μmol/kg M(II)-LR Yellow 2KT-PEG, 0.25 M Na_2SO_4 and 50 mM HEPES-NaOH buffer, pH 7.0.
[b]Imidazole concentration was 50 mM in the systems containing chelated Ni(II) ions, and 200 mM in those containing chelated Hg(II) ions.
[c]For comparison, Δlog K values of purified rhG-CSF mutants in two-phase systems containing chelated Ni(II) ions are presented.

According to this correlation, the mutations of histidine residues, which contribute to rhG-CSF (C17S) interaction with chelated Ni(II) ions, led to the decrease in refolding efficiency of protein. Partially folded form of rhG-CSF mutant, which contains more surface-exposed histidine residues, bind a higher number of Ni(II)-LR Yellow 2KT-PEG molecules and this may prevent protein aggregation more efficiently in comparison to the analogous protein forms which contain less histidine residues. Furthermore, chelated metal ions may stabilize nascent correctly folded protein conformation and shift the equilibrium between the correct folding and aggregation pathways towards the correct folding. Arnold and coworkers had demonstrated that proteins which contain His-X_3-His site can be stabilized effectively by Cu(II)-IDA complex (Arnold and Haymore, 1991). Mutations of surface-exposed histidine residues in rhG-CSF (C17S) lead to weaker protein binding to chelated Ni(II) ions and the decrease in the stabilizing effect, which result in lower refolding efficiency of protein in two-phase systems containing chelated Ni(II) ions.

In the dextran phase of two-phase systems containing chelated Hg(II) ions, the amounts of correctly folded rhG-CSF (C17S) and its histidine mutants (except for (C17S, H43A, H52A)) were similar (Table 6). These data suggest that the effect of histidine mutations on rhG-CSF (C17S) refolding efficiency in two-phase systems containing chelated Hg(II) ions was negligible. This may be explained by the fact that histidine residues do not contribute to the interaction of protein with chelated Hg(II) (see 1.4.).

The refolding efficiency of rhG-CSF (C17S) and its histidine mutants in two-phase systems was higher in the presence of chelated Hg(II) ions compared to that in the presence of Ni(II) ions (Table 6). The different effect of histidine mutations on the refolding efficiency of rhG-CSF (C17S) in two-phase systems containing chelated Ni(II) and Hg(II) ions, and the dependence of protein refolding efficiency on the nature of metal ion may result from the different mechanisms of protein interaction with chelated Ni(II) and Hg(II) ions.

In this work aqueous two-phase systems PEG-dextran, containing chelated Ni(II) and Hg(II) ions were applied for the refolding of rhG-CSF and its mutants from solubilized inclusion bodies. It was determined that protein refolding efficiency in these systems may be dependent on the cysteine or histidine composition of protein and the nature of metal ion. Aqueous two-phase systems containing chelated metal ions should also be applicable for the refolding of recombinant proteins such as interferons, granulocyte macrophage-colony stimulating factor, interleukins etc, which contain surface-exposed histidine or cysteine residues.

CONCLUSIONS

1. The effective scheme for the recovery and purification of rhG-CSF (C17S) histidine mutants from inclusion bodies, based on oxidative refolding of protein in solution and purification by immobilized metal ion affinity and ion-exchange chromatography, was developed.

2. Partitioning studies of rhG-CSF, rhG-CSF (C17S) and rhG-CSF (C17S) histidine mutants in aqueous two-phase systems containing Cu(II), Ni(II) and Hg(II) ions chelated by LR Yellow 2KT-PEG demonstrated that:

i. rhG-CSF binding to chelated Cu(II) and Ni(II) ions at pH 7.0 is dominated by histidine side chains.

ii. Each of His43, His52, His156 and His170 side chains is important in rhG-CSF binding to chelated Cu(II) ions, while His52 and His170 dominate protein interaction with chelated Ni(II).

iii. Cys17 side chain contribute to rhG-CSF binding to chelated Ni(II) ions (pH 7.0), but its contribution to protein interaction with chelated Cu(II) was not observed.

iv. rhG-CSF interaction with chelated Hg(II) ions at pH 7.0 and pH 5.0 is dominated by Cys17 side chain, and the contribution of surface-exposed histidine residues in this interaction was not detected. The direct interaction between –SH group of protein and chelated Hg(II) ions was detected for the first time.

3. The evaluation of the effect of various low molecular weight ligands on the partitioning of rhG-CSF, rhG-CSF (C17S) and $(His)_6$-rhG-CSF in aqueous two-phase systems containing metal ions chelated by LR Yellow 2KT-PEG at pH 7.0 demonstrated that the interaction between chelated Ni(II) ions and all three rhG-CSF variants was reduced more effectively by a competing ligand imidazole compared to 2-mercaptoethanol and conversely, 2-mercaptoethanol was a more effective competing agent in the interaction between chelated Hg(II) ions and rhG-CSF variants.

4. Aqueous two-phase systems PEG/dextran containing Ni(II) and Hg(II) ions chelated by LR Yellow 2KT-PEG were applied for the refolding of rhG-CSF from inclusion bodies for the first time. Protein refolding in these systems was combined with partial purification.

5. Refolding of rhG-CSF (C17S) in two-phase systems containing chelated Ni(II) and Hg(II) ions was more effective compared to that of rhG-CSF. After

the partitioning of solubilized inclusion-bodies in two-phase systems and the release of protein from chelated metal ions by the addition of a competing ligand, relative amounts of correctly folded rhG-CSF form in the dextran phase were 46% and 17%, and those of rhG-CSF (C17S) – 56% and 99%, when two-phase systems contained Ni(II) and Hg(II) ions, respectively. The lower refolding efficiency of rhG-CSF is determined by its Cys17 side chain, which may be involved in protein misfolding and aggregation events.

6. The nature of a competing ligand used to disrupt the interaction of protein with chelated metal ion had effect on rhG-CSF refolding efficiency in two-phase systems containing chelated Hg(II) ions. After the addition of 2-mercaptoethanol (10 mM) and imidazole (200 mM), the amounts of correctly folded rhG-CSF form in the dextran phase of two-phase systems reached 43% and 17%, respectively.

7. Refolding efficiency of rhG-CSF (C17S) and its histidine mutants from inclusion bodies in aqueous two-phase systems containing chelated metal ions was dependent on the nature of a metal ion. The amounts of correctly folded form of rhG-CSF mutants in the dextran phase of two-phase systems containing chelated Ni(II) ions reached from 8% to 67%, and in those containing chelated Hg(II) ions – from 76% to 99%. The dependence of refolding efficiency on the nature of metal ion may be determined by different mechanisms of protein binding to chelated Ni(II) and Hg(II) ions.

8. Increase in a number of histidine mutations on the rhG-CSF (C17S) surface resulted in a decrease of protein refolding efficiency in two-phase systems containing chelated Ni(II) ions, the amounts of correctly folded protein form in the dextran phase decreased from 67% for rhG-CSF (C17S, H43A) to 8% for rhG-CSF (C17S, H43A, H52A, H156A) mutant. The effect of histidine mutations on rhG-CSF (C17S) refolding efficiency in the systems containing chelated Hg(II) was negligible, the amounts of correctly folded protein form for rhG-CSF mutants in the dextran phase reached from 76% to 94%.

9. Refolding efficiency of rhG-CSF (C17S) and its histidine mutants (except for rhG-CSF (C17S, H43A, H52A, H156A)) in two-phase systems containing chelated Ni(II) ions is directly proportional to the affinity of purified rhG-CSF mutants to chelated Ni(II) ions.

Published works on the topic of the dissertation
In the acknowledged editions

1. **Zaveckas, M.;** Baškevičiūtė, B.; Lukša, V.; Žvirblis, G.; Chmieliauskaitė, V.; Bumelis, V. and Pesliakas, H. (2000) Comparative studies of recombinant human granulocyte-colony stimulating factor, its Ser-17 and $(His)_6$-tagged forms interaction with metal ions by means of immobilized metal ion affinity partitioning. Effect of chelated nickel and mercuric ions on extraction and refolding of proteins from inclusion bodies. *J. Chromatogr. A* **904**:145-169.
2. **Zaveckas, M.;** Lukša, V.; Žvirblis, G.; Chmieliauskaitė, V.; Bumelis, V. and Pesliakas, H. (2003) Mutation of surface-exposed histidine residues of recombinant human granulocyte-colony stimulating factor (Cys17Ser) impacts on interaction with chelated metal ions and refolding in aqueous two-phase systems. *J. Chromatogr. B* **786**:17-32.

In the other editions

3. **Zaveckas, M.;** Lukša, V.; Žvirblis, G.; Chmieliauskaitė, V.; Bumelis, V. and Pesliakas, H. Influence of surface-exposed histidines mutation of recombinant human granulocyte-colony stimulating factor (Cys17Ser) on interaction with chelated metal ions and refolding in aqueous two-phase systems. In: Abstracts of European workshop „From Gene to Functional Protein“ on cloning, expression, refolding and purification of recombinant proteins, in conjunction with Contest for Young Scientists, Paris, France, June 10-11, 2002, p.78.
4. **Zaveckas, M.;** Lukša, V.; Žvirblis, G.; Chmieliauskaitė, V.; Bumelis, V. and Pesliakas, H. Mutation of surface-exposed histidine residues of recombinant human granulocyte-colony stimulating factor (Cys17Ser) impacts on interaction with chelated metal ions and refolding in aqueous two-phase systems. In: Abstracts of EMBO Lecture Course “The biology of heat shock proteins and molecular chaperones”, Warsaw, Poland, September 25-29, 2002, p.69.

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REKOMBINANTINIO ŽMOGAUS GRANULOCITŲ KOLONIJAS STIMULIUOJANČIO FAKTORIAUS PASISKIRSTYMAS IR RENATŪRACIJA VANDENS DVIFAZĖSE SISTEMOSE, DALYVAUJANT CHELATUOTIEMS METALŲ JONAMS

Santrauka

Rekombinantiniai baltymai *E. coli* bakterijose dažnai ekspresuojami netirpių įterptinių kūnelių pavidalu, todėl norint iš jų gauti tirpų ir biologiškai aktyvų baltymą, reikalinga renatūracijos stadija. Didesnę baltymo renatūracijos išeigą galima pasiekti, užkirtus kelią dalinai susisukusių jo molekulių tarpusavio sąveikai, sukeliančiai agregaciją. Šiam tikslui naudojami įvairūs chromatografiniai metodai ir dvifazės vandeninės sistemos. Dvifazėse sistemose su metalų chelatiniais kompleksais baltymų renatūracija iki šiol nebuvo atliekama. Norint baltymo renatūracijai panaudoti metodus, pagrįstus jo sąveika su metalo chelatiniu kompleksu, yra svarbu žinoti, kokių aminorūgščių liekanos dominuoja šioje sąveikoje. Baltymų sąveikoje su Cu(II) ir Ni(II) chelatiniais kompleksais dominuoja histidino liekanos (Arnold, 1991), bet manoma, kad šioje sąveikoje galimas ir cisteino liekanų indėlis. Spėjama, kad su Hg(II) kompleksu gali sąveikauti nesuporuota baltymo cisteino liekana (Gelūnaitė ir kt., 2000), tačiau tiesioginių tokios sąveikos vyksmo įrodymų nebuvo gauta. Pagrindinis šio darbo tikslas buvo ištirti rekombinantinio žmogaus granulocitų kolonijas stimuliuojančio faktoriaus (rhG-CSF) renatūracijos iš įterptinių kūnelių vandens dvifazėse sistemose PEG-dekstranas, dalyvaujant chelatuotiems Ni(II) ir Hg(II) jonams, efektyvumo priklausomybę nuo metalo jono prigimties ir baltymo paviršiuje eksponuotų Cys bei His liekanų, ir pastarųjų skaičiaus.

Šiame darbe įvertintas rhG-CSF paviršiaus Cys17 ir histidino liekanų indėlis sąveikoje su Cu(II)-, Ni(II)- ir Hg(II)-Geltono ŠA 2KT-PEG chelatiniais kompleksais. Panaudojant kryptingos mutagenezės ir pasiskirstymo dvifazėse sistemose metodus nustatyta, kad rhG-CSF sąveikoje su Cu(II) kompleksu (pH 7.0) dalyvauja His43, His52, His156 ir His170 liekanos. Baltymo sąveiką su Ni(II) kompleksu (pH 7.0) nulemia His52 ir His170 liekanos, taip pat galimas ir Cys17 indėlis. RhG-CSF sąveikoje su Hg(II) kompleksu (pH 7.0 ir pH 5.0) dominuoja Cys17 liekana. Tiesioginė baltymo cisteino liekanos –SH grupės sąveika su Hg(II) chelatiniu kompleksu nustatyta pirmą kartą.

Pirmą kartą sėkmingai atlikta rhG-CSF renatūracija iš įterptinių kūnelių dvifazėse vandeninėse sistemose, dalyvaujant Ni(II)- ir Hg(II)-Geltono ŠA 2KT-PEG kompleksams. RhG-CSF (C17S) mutanto renatūracija šiose sistemose buvo efektyvesnė negu rhG-CSF. Įvertinta histidino mutacijų įtaka

rhG-CSF (C17S) renatūracijos efektyvumui dvifazėse sistemose su metalų chelatiniais kompleksais ir nustatyta, kad sistemose su Ni(II) kompleksu baltymo renatūracijos efektyvumas ženkliai mažėjo, mažėjant histidino liekanų skaičiui jo paviršiuje. Pastebėta tiesioginė priklausomybė tarp išgryninto rhG-CSF (C17S) bei jo mutantų su pakeistomis histidino aminorūgštimis gimiringumo Ni(II) kompleksui ir jų renatūracijos iš įterptinių kūnelių efektyvumo sistemose su Ni(II) kompleksu. Nustatyta, kad rhG-CSF ir jo mutantų renatūracijos dvifazėse sistemose efektyvumas priklauso ir nuo chelatuoto metalo jono prigimties. Tikėtina, kad dvifazėse vandeninėse sistemose su Ni(II) ir Hg(II) chelatiniais kompleksais gali būti efektyviai renatūruojami ir kiti baltymai, paviršiuje turintys histidino ar cisteino liekanų.

Mindaugas Zaveckas

PARTITIONING AND REFOLDING OF RECOMBINANT HUMAN GRANULOCYTE-COLONY STIMULATING FACTOR IN AQUEOUS TWO-PHASE SYSTEMS CONTAINING CHELATED METAL IONS

Summary of doctoral dissertation
Technological Sciences, Chemical Engineering (05T), Biotechnology (T490)

Mindaugas Zaveckas

REKOMBINANTINIO ŽMOGAUS GRANULOCITŲ KOLONIJAS STIMULIUOJANČIO FAKTORIAUS PASISKIRSTYMAS IR RENATŪRACIJA VANDENS DVIFAZĖSE SISTEMOSE, DALYVAUJANT CHELATUOTIEMS METALŲ JONAMS

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