

Effect of surface histidine mutations and their number on the partitioning and refolding of recombinant human granulocyte-colony stimulating factor (Cys17Ser) in aqueous two-phase systems containing chelated metal ions

Mindaugas Zaveckas^{a,*}, Aurelija Žvirblienė^a, Gintautas Žvirblis^b,
Valerija Chmieliauskaitė^b, Vladas Bumelis^b, Henrikas Pesliakas^b

^a Institute of Biotechnology, Graičiūno 8, LT-02241 Vilnius, Lithuania

^b SICOR Biotech UAB, Centre of Research and Development, Graičiūno 8, LT-02241 Vilnius, Lithuania

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Abstract

High-level expression of recombinant proteins in *Escherichia coli* frequently leads to the formation of insoluble protein aggregates, termed inclusion bodies. In order to recover a native protein from inclusion bodies, various protein refolding techniques have been developed. Column-based refolding methods and refolding in aqueous two-phase systems are often an attractive alternative to dilution refolding due to simultaneous purification and improved refolding yields. In this work, the effect of surface histidine mutations and their number on the partitioning and refolding of recombinant human granulocyte-colony stimulating factor Cys17Ser variant (rhG-CSF (C17S)) from solubilized inclusion bodies in aqueous two-phase systems polyethylene glycol (PEG)–dextran, containing metal ions, chelated by dye Light Resistant Yellow 2KT (LR Yellow 2KT)-PEG derivative, was investigated. Human G-CSF is a growth factor that regulates the production of mature neutrophilic granulocytes from the precursor cells. Initially, the role of His156 and His170 residues in the interaction of rhG-CSF (C17S) with Cu(II), Ni(II) and Hg(II) ions, chelated by LR Yellow 2KT-PEG, was investigated at pH 7.0 by means of affinity partitioning of purified, correctly folded rhG-CSF (C17S) mutants. It was determined that both His156 and His170 mutations reduced the affinity of rhG-CSF (C17S) for chelated Cu(II) ions at pH 7.0. His170 mutation significantly reduced the affinity of protein for chelated Ni(II) ions. However, histidine mutations had only a small effect on the affinity of protein for Hg(II) ions. The influence of His156 and His170 mutations on the refolding of rhG-CSF (C17S) from solubilized inclusion bodies in aqueous two-phase systems PEG-dextran, containing chelated Ni(II) and Hg(II) ions, was investigated. Reversible interaction of protein mutants with chelated metal ions was used for refolding in aqueous two-phase systems. Both histidine mutations resulted in a significant decrease of protein refolding efficiency in two-phase systems containing chelated Ni(II) ions, while in the presence of chelated Hg(II) ions their effect on protein refolding was negligible. Refolding studies of rhG-CSF variants with different number of histidine mutations revealed that a direct correlation exists between the number of surface histidine residues and refolding efficiency of rhG-CSF variant in two-phase systems containing chelated Ni(II) ions. This method of protein refolding in aqueous two-phase systems containing chelated metal ions should be applicable to other recombinant proteins that contain accessible histidine residues.

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1. Introduction

Overexpression of recombinant proteins in *Escherichia coli* often leads to the formation of inclusion bodies [1,2]. Inclusion

bodies are dense amorphous particles, which consist mainly of aggregated recombinant protein. To obtain biologically active protein from inclusion bodies, a refolding step is necessary. Upon *in vitro* folding, misfolding as well as aggregation competes with the correct folding pathway [3,4]. Correct protein folding follows first order kinetics, whereas aggregation reactions are second or higher order processes. Thus, at high protein concentrations aggregation dominates over folding reaction.

* Corresponding author. Tel.: +370 5 2602119; fax: +370 5 2602116.
E-mail address: mzaveck@ibt.lt (M. Zaveckas).

Refolding of solubilized inclusion body proteins is commonly performed by dilution or dialysis [5,6]. In order to slow down the aggregation process, protein concentration should be kept low, about 10–50 $\mu\text{g/ml}$. The yield of protein refolding at high protein concentrations may be improved by suppressing intermolecular interactions between aggregation-prone folding intermediates. For this purpose, various chromatographic methods have been proposed [7,8]. Techniques of protein refolding in reversed micelles [9,10] and in aqueous two-phase systems [11] have also been explored.

Aqueous two-phase systems are widely used in biochemistry and biotechnology for the purification of biological materials [12,13]. During recent years, more attention has been given to their use for protein refolding. Aqueous PEG-salt systems containing moderate concentration of guanidinium chloride were used for the oxidative refolding of lysozyme [14]. T4-lysozyme mutants were selected as a model and the influence of protein conformation on protein partitioning was investigated in two-phase systems containing urea [15]. Recently, aqueous two-phase systems containing thermoseparating polymers were successfully used for protein refolding at moderate temperatures [16,17]. Thermoseparating polymers undergo phase separation in aqueous solution above a critical temperature and a system composed of polymer-rich phase and water phase is formed. Chymotrypsin inhibitor 2 was refolded with high yield in thermoseparating water–Breox (a random copolymer of ethylene oxide and propylene oxide) system [16]. The modification of upper PEG phase of PEG–dextran two-phase system with PEG bound to a thermo-responsive hydrophobic head (poly(propylene oxide)-phenyl group) allowed to suppress aggregate formation and enhance the refolding yield of bovine carbonic anhydrase at a temperature of 50–55 °C [17]. In aqueous two-phase systems refolded protein may be separated from its denatured and aggregated forms [11,14,16]. In this way, protein refolding in such systems could be an attractive alternative to classical refolding methods based on dilution or dialysis.

We reported that in aqueous two-phase systems PEG–dextran the interaction between recombinant human granulocyte-colony stimulating factor (rhG-CSF) from inclusion body extract and Ni(II) or Hg(II) ions chelated by LR Yellow 2KT-PEG induces the generation of correctly folded native protein [18]. Human G-CSF is a specific growth factor that regulates the production of neutrophilic granulocytes and enhances their maturation [19,20]. Recombinant human G-CSF can be produced through heterologous gene expression in *E. coli* and it is widely used clinically in the treatment of neutropenia. RhG-CSF contains unpaired cysteine at the position 17 and five histidine residues that may be involved in protein interaction with chelated metal ions [20]. Among amino acid residues, histidines are predominant ligands in the binding of proteins to chelated Ni(II), Zn(II), Co(II) and Cu(II) ions at nearby neutral pH [21,22]. The role of protein interaction with chelated metal ions is very important for successful protein refolding by means of affinity partitioning in aqueous two-phase systems or by immobilized metal ion affinity chromatography (IMAC). However, little is known about the influence of the interaction strength between

protein and chelated metal ion on refolding efficiency. Since histidine residues dominate this interaction, we have recently evaluated the influence of two surface histidines of rhG-CSF on protein interaction with chelated metal ions and refolding in aqueous two-phase systems [23]. RhG-CSF was selected as a model protein for these studies since the visualization of its NMR structure [24] with RasMol viewer [25] revealed that four histidine residues – His43, His52, His156 and His170 – are surface-exposed. The site-directed mutagenesis of more stable C17S variant of rhG-CSF was used for sequential replacing of His43 and His52 residues to alanine. We have found that both histidine mutations caused only a slight effect on partitioning and refolding of rhG-CSF (C17S) in two-phase systems containing Hg(II) ions [23]. However, the mutation of His52 resulted in a considerable decrease in both protein affinity to Ni(II) and its refolding efficiency in two-phase systems containing chelated Ni(II) ions. In this respect, it was of interest to investigate the effect of other two surface histidines of rhG-CSF and the influence of the number of histidines on protein refolding in two-phase systems containing Ni(II) ions. Such studies would serve to increase our understanding how the changes in metal chelate-protein interaction can influence protein refolding by immobilized metal ion affinity partitioning and IMAC techniques.

This work had two objectives. The first is to study the effect of His156 and His170 mutations on the interaction of rhG-CSF (C17S) primarily with chelated Ni(II) ions as well as with Cu(II) and Hg(II) for comparison, and refolding in aqueous two-phase systems containing chelated Ni(II) and Hg(II) ions. The second objective is to evaluate the refolding efficiency of rhG-CSF mutants, containing different number of histidine residues, in the presence of chelated metal ions. We examined the partitioning of purified, correctly folded rhG-CSF (C17S, H43A, H170A) and (C17S, H43A, H52A, H156A) mutants and their refolding from inclusion bodies in two-phase systems containing chelated metal ions. Partitioning and refolding results for these mutants were further compared with those for rhG-CSF (C17S, H43A) and (C17S, H43A, H52A) from our previous work [23]. We report that surface histidine residues of rhG-CSF play a critical role in the interaction of protein with Ni(II) and refolding in aqueous two-phase systems, containing chelated Ni(II) ions.

2. Experimental

2.1. Materials

Sepharose CL-6B, SP-Sepharose Fast Flow and Sephadex G-25 Medium were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). MES (2-[*N*-morpholino]ethanesulfonic acid) and imidazole (buffer substance) were purchased from Merck (Darmstadt, Germany). HEPES (N-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]), Trizma Base (Tris[hydroxymethyl]aminomethane) and isopropyl β -D-thiogalactopyranoside (IPTG) were purchased from Sigma (St. Louis, MO, USA). Polyethylene glycol (PEG) 6000 was obtained from Fluka (Buchs, Switzerland) and dextran 60,000

from Clinical Preparations (Saransk, Russian Federation). Reactive dye Light Resistant Yellow 2KT-Cu(II) (LR Yellow 2KT) was obtained from a local textile factory (Alytus, Lithuania) and was purified as described in Ref. [26]. Sepharose CL-6B-LR Yellow 2KT-Cu(II) adsorbent was prepared according to the method for coupling Cibacron Blue F3GA to Sephadex G-200 [27]. PEG 6000-LR Yellow 2KT derivative was synthesized by coupling of reactive dye LR Yellow 2KT to PEG 6000 [28]. PEG 6000-LR Yellow 2KT-Ni(II) and Hg(II) complexes were prepared from demetallized PEG-dye and relevant metal salt [23].

Triton X-100 and Tween 80 were purchased from Ferak (Berlin, Germany). Phenylmethylsulfonyl fluoride (PMSF), 2-mercaptoethanol, maleic acid (all of puriss grade) and guanidine hydrochloride (purum and BioChemika MicroSelect grades) were purchased from Fluka (Buchs, Switzerland). Lysozyme (chicken egg white), EC 3.2.1.17, and dialysis tubing (types 8/32, 20/32 and 27/32) were obtained from Serva (Heidelberg, Germany). All other chemicals were of analytical reagent or puriss grade and obtained from Fluka, Merck or Sigma.

2.2. Preparation of purified and correctly folded rhG-CSF (C17S) histidine mutants from inclusion bodies

The genes for hG-CSF-Ser17-Ala43-Ala170 and hG-CSF-Ser17-Ala43-Ala52-Ala156 protein production were obtained by the modification of hG-CSF-Ser17-Ala43 and hG-CSF-Ser17-Ala43-Ala52 genes, respectively. Cloning, DNA preparation, transformation and expression of modified hG-CSF variants in *E. coli* were performed similarly as described in [23]. Isolation of inclusion bodies from harvested *E. coli* cells, refolding and purification of rhG-CSF mutants were performed according to [23], with several modifications. Three grams of inclusion bodies were solubilized in 100 ml, 10 mM Tris-HCl buffer (pH 7.0), containing 7 M GdmHCl by stirring overnight at 4 °C. The solution was centrifuged at 40,000 × *g* for 25 min. The supernatant was adjusted to the 1 mg/ml concentration of total protein (determined according to Bradford [29]), 6 M GdmHCl, 10 mM Tris-HCl (pH 7.0) and CuSO₄ solution was added to a final concentration of 20 μM. The mixture was stirred for 1 h at room temperature, EDTA solution was added to the 10 mM, pH was adjusted with 1 mM HCl to 5.0 or 4.8 for rhG-CSF (C17S, H43A, H170A) and (C17S, H43A, H52A, H156A), respectively, and centrifuged. The supernatant was applied onto a glass column (2.6 cm × 100 cm, Pharmacia, Uppsala, Sweden) packed with 500 ml of Sephadex G-25 Medium and elution was performed with 25 mM Tris-maleic acid-NaOH buffer, pH 6.5, 0.25 M Na₂SO₄.

Protein fractions after gel permeation chromatography were pooled and loaded on a glass column (2.6 cm × 24 cm, Pharmacia, Uppsala, Sweden) packed with 50 ml Sepharose CL-6B containing covalently attached dye LR Yellow 2KT-Cu(II) (4.2 μmol/ml). The column was washed with a loading buffer, 25 mM Tris-maleic acid-NaOH, pH 6.5, 0.25 M Na₂SO₄, and rhG-CSF mutant was eluted by the linear 0–300 mM imidazole gradient. Protein fractions from the imidazole gradient were

pooled, dialysed at 4 °C against 25 mM Na-acetate buffer, pH 5.0 or 4.8 for rhG-CSF (C17S, H43A, H170A) and rhG-CSF (C17S, H43A, H52A, H156A), respectively, and centrifuged at 40,000 × *g* for 25 min. For the next purification step, 40 ml of SP-Sepharose was packed into a glass column (2.6 cm × 24 cm) and equilibrated with 25 mM Na-acetate buffer (pH 5.0 for rhG-CSF (C17S, H43A, H170A) or 4.8 for (C17S, H43A, H52A, H156A)). The elution of bound protein was achieved by the linear 0–500 mM NaCl gradient. Fractions containing rhG-CSF mutant were pooled, dialysed against 10 mM Na-acetate buffer, pH 4.0 at 4 °C, centrifuged and filtered through a 0.22 μm sterile filter.

A single path monitor UV-1 (Pharmacia, Uppsala, Sweden) was used for the detection of absorbance at 280 nm during chromatography. Additionally, the protein concentration in eluate was determined by Bradford assay at 595 nm [29]. Identity and purity of the protein after each purification step was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [30] and by RP-HPLC analysis (see Section 2.9). Purified proteins were also analysed by isoelectric focusing gel electrophoresis [31].

2.3. Immunoblotting

RhG-CSF mutants were electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) in buffer 25 mM Tris, 150 mM glycine, 10% (v/v) methanol at 20 mA for 40 min. Membranes were blocked by incubation for 30 min. at room temperature in 20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4–0.1% Tween 20 (PBS-T) containing 1% gelatine. Afterwards membranes were incubated with monoclonal antibodies against G-CSF (1:1000 dilution), generated at the Institute of Biotechnology (Vilnius, Lithuania) for 2 h at room temperature, washed with PBS-T and incubated with peroxidase conjugate against mouse IgG (Amersham NXA 931, 1:1000) for 1 h at room temperature. Membranes were then washed with PBS-T for 7–8 times and developed with TMB-blotting substrate (Fermentas UAB, Vilnius, Lithuania).

2.4. Biological activity assay

Mouse myeloid leukemia G-NFS-60 cells (National Institute for Biological Standards and Control, United Kingdom) were incubated with serial dilutions of standard rhG-CSF and rhG-CSF mutant samples for 48 h and then incubated for additional 4 h with the tetrazolium salt, MTS (Promega, USA). Cell growth was quantitated spectrophotometrically by measuring the relative quantity of formazan produced in each well at 492 nm. The activity of a rhG-CSF mutant sample was determined by comparing the dilution yielding 50% maximal stimulation to the dilution of a standard rhG-CSF sample yielding 50% maximal stimulation [32]. The differences between mean activity values for intact rhG-CSF and its mutants were tested for significance using Student's *t*-test (Microcal Origin software, Northampton, MA, USA). A difference was accepted as statistically significant at *p* < 0.05.

2.5. Two-phase systems

Two-phase systems (4 g) were prepared by weighing stock solutions of polymers in water, 40% (w/w) PEG 6000 and 20% (w/w) dextran 60,000. The final concentrations of PEG and dextran in the aqueous two-phase systems were 5% (w/w) PEG and 8% (w/w) dextran.

All necessary ingredients – buffer, water, protein samples and selected agents – were mixed with polymer solutions to give necessary final concentration as indicated in tables (see Section 3). IMAP experiments were performed by replacing part of PEG for PEG–dye–metal ion complex. The amount of metal ion–dye–PEG derivative is expressed as metal ion concentration ($\mu\text{M}/\text{kg}$) per kg of two-phase system.

2.6. Partitioning of purified rhG-CSF (C17S) histidine mutants

For the partitioning experiments, the solution of respective rhG-CSF mutant was dialysed against either 20 mM MES–NaOH buffer, pH 5.0 or 20 mM HEPES–NaOH buffer, pH 7.0. Subsequently, the sample containing 0.8 mg of the respective mutant was introduced into the two-phase system, the obtained mixture was shaken gently for 15–20 s, kept at 4 °C for 10 min, and then centrifuged at $3000 \times g$ for 5 min to complete the phase separation. The samples of known volume were withdrawn from each phase and protein concentration was determined by the method of Bradford [29]. Since both demetallized LR Yellow 2KT and LR Yellow 2KT-M(II) complexes absorb in the visible region and give residual absorbance in a protein assay at 595 nm, similar two-phase systems, but without a protein were prepared for each partitioning experiment. Samples withdrawn from these systems were equal in volume to those with a protein and were used to prepare the reference solutions for a protein assay.

The partition coefficient for the protein (K) was defined as the ratio of protein concentration in the upper phase to that in the lower phase. The protein affinity for immobilized metal ion was expressed in terms of $\Delta \log K$, defined as the difference between the logarithmic partition coefficient of protein in the presence of metal–dye–PEG (K_M) and the logarithmic partition coefficient in the presence of demetallized dye–PEG (K_{dye}) ($\Delta \log K = \log K_M - \log K_{\text{dye}}$). The alteration of protein affinity for immobilized metal ion, when selected agents were introduced into the two-phase systems, was expressed as a percentage of the initial value of $\Delta \log K$ in the presence of metal–dye–PEG and in the absence of the agent.

All partitioning experiments were carried out in duplicate at 4 °C and the value of $\Delta \log K$ is given as the mean of two separate determinations.

2.7. Preparation of crude inclusion body extracts of rhG-CSF (C17S) histidine mutants

Inclusion body extracts were prepared fresh before each partitioning experiment. Inclusion bodies (0.25 g) were homogenized in 10 ml of 20 mM HEPES–NaOH buffer, pH 7.0. The pH of

homogenizate was adjusted to 12–13 with 2 M NaOH solution. After 2 min the homogenizate was titrated with 0.25 M H_2SO_4 up to pH 7.0 and centrifuged at $40,000 \times g$ for 25 min.

2.8. Partitioning of rhG-CSF (C17S) histidine mutants from inclusion bodies

Partitioning of rhG-CSF (C17S) histidine mutants from their inclusion body extracts was investigated in two-phase systems composed of 5% (w/w) PEG 6000–8% (w/w) dextran 60,000 in 50 mM HEPES–NaOH buffer, pH 7.0, containing 0.25 M Na_2SO_4 and 800 $\mu\text{M}/\text{kg}$ of immobilized Ni(II) or Hg(II) ion. The amount of total protein was 0.8 mg.

For refolding of rhG-CSF (C17S) histidine mutants from inclusion body extract, three two-phase systems, which contained PEG-LR Yellow 2KT-Ni(II) or -Hg(II) complex were prepared. The extract of inclusion bodies containing approximately 1.5 mg of total protein was introduced into the two of these systems. Into the third one, the buffer instead of a protein was added. In parallel, three two-phase systems free of both the extract of inclusion bodies and PEG–dye–metal complex were prepared. After mixing and phase separation, the upper phase of the latter systems was replaced with that from two-phase systems containing the protein and (or) metal complex. The selected desorption agent – imidazole – was then added into the “mixed” two-phase systems. After mixing and phase separation, samples of known volume were withdrawn from each phase for a protein assay. The film-like layer between the phases was collected, dissolved in 1.0 ml of 6 M GdmHCl solution in 10 mM Tris–maleic acid–NaOH buffer, pH 6.5 and centrifuged at $24,500 \times g$ for 10 min. The samples of the initial extract of inclusion bodies and of the dextran phase of two-phase systems, which contained imidazole, and the sample of the interface layer were taken and analysed by reversed-phase HPLC to detect the conformation changes of rhG-CSF mutants in the two-phase systems. SDS-PAGE [30] of the samples was performed to assess purity of rhG-CSF mutants.

2.9. RP-HPLC analysis

Samples of rhG-CSF (C17S) histidine mutants were analysed by RP-HPLC using a C_4 (Hi-Pore RP-304, 250 mm \times 4.6 mm I.D., 30 nm, Bio-Rad, Hercules, CA, USA) reversed-phase column similar to that described in [33]. Solvent A: 0.1% trifluoroacetic acid (TFA) in water, solvent B: TFA–water–acetonitrile (0.1:9.9:90) was used for gradient elution. The column was initially equilibrated with 90% A/10% B at a flow rate of 1 ml/min. The separation was performed with a linear gradient of 10–57% B in the first 10 min, followed by 57–63% B in the next 42 min, and 63–75% B in the last 24 min on a HP 1100 liquid chromatographic system (Hewlett-Packard, Palo Alto, CA, USA) equipped with an autosampler and a photodiode array detector. Protein peaks were detected at 215 nm. The amount of correctly folded protein form was determined according to its retention time (t_R) and relative absorbance at 215 nm. If necessary separate samples of purified rhG-CSF or its mutants were used as references.

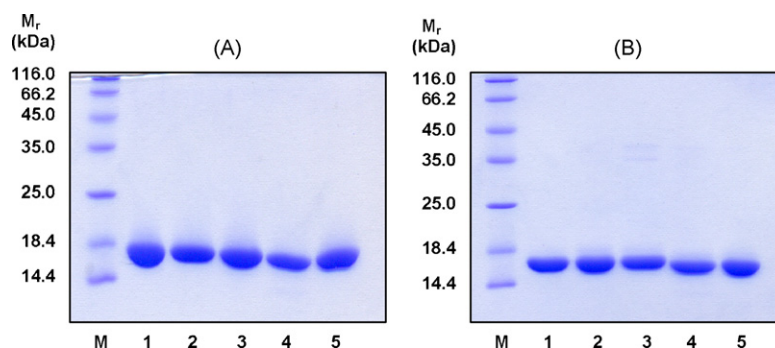


Fig. 1. SDS-PAGE analysis of purified rhG-CSF (C17S) histidine mutants in 12% polyacrylamide gel, stained with Coomassie Brilliant Blue R-250. (A) Reducing conditions, (B) non-reducing conditions. M: Molecular mass markers. Lanes: (1) rhG-CSF standard; (2) rhG-CSF (C17S, H43A); (3) rhG-CSF (C17S, H43A, H52A); (4) rhG-CSF (C17S, H43A, H52A, H156A); (5) rhG-CSF (C17S, H43A, H170A). In each lane 10 μ g of protein was loaded.

3. Results and discussion

3.1. Refolding and purification of rhG-CSF (C17S) histidine mutants

RhG-CSF (C17S, H43A, H170A) and (C17S, H43A, H52A, H156A) mutants, like rhG-CSF, were expressed in *E. coli* as inclusion bodies. These mutants were recovered from inclusion bodies and purified according to the protocol developed for rhG-CSF (C17S, H43A) and (C17S, H43A, H52A) [23], but with some modifications. The oxidative refolding of rhG-CSF mutants from solubilized inclusion bodies with Cu^{2+} ions was initially performed and the RP HPLC purity of rhG-CSF (C17S, H43A, H170A) and (C17S, H43A, H52A, H156A) after this step reached 68% and 64%, respectively.

Sepharose-LR Yellow 2KT-Cu(II) adsorbent was used for the initial purification of rhG-CSF mutants according to the methodology of IMAC. Since chelated Cu(II) ions quite strongly coordinate protein macromolecules with even one available histidine [21,34], the use of this adsorbent might cover purification of all rhG-CSF mutants which contain at least one surface histidine residue. The elution profiles of rhG-CSF (C17S, H43A, H170A) and (C17S, H43A, H52A, H156A) from Sepharose-LR Yellow 2KT-Cu(II) column were nearly identical, peak elution occurred at an imidazole concentration of approximately 60 mM for both rhG-CSF variants. The purity of protein after this chromatography step, as judged by RP-HPLC analysis, reached 78% and 73% for rhG-CSF (C17S, H43A, H170A) and (C17S, H43A, H52A, H156A), respectively.

As in the case of other rhG-CSF mutants [23], cation-exchange chromatography on a SP-Sepharose Fast Flow at pH 5.4 was used for the further purification. However, rhG-CSF (C17S, H43A, H52A, H156A) was not retained on SP-Sepharose column at this pH value. Such chromatographic behaviour of this rhG-CSF mutant may be explained by the fact that mutations of surface histidine residues result in a lowering of protein pI value. Therefore, rhG-CSF (C17S, H43A, H52A, H156A) and (C17S, H43A, H170A) were chromatographed on SP-Sepharose column at lower pH values – 4.8 and 5.0, respectively. The RP-HPLC purity of rhG-CSF mutants after the chromatography on SP-Sepharose and dialysis against storage buffer of pH

4.0 reached 92% and 99% for (C17S, H43A, H52A, H156A) and (C17S, H43A, H170A), respectively.

SDS-PAGE analysis indicated that each purified rhG-CSF mutant yielded a single broad band under both reducing and non-reducing conditions (Fig. 1). The high purity of rhG-CSF mutants was also demonstrated with isoelectric focusing gel electrophoresis. The values of their isoelectric points are presented in Table 1. All purified rhG-CSF histidine mutants were able to bind to monoclonal anti-rhG-CSF antibodies, as demonstrated by immunoblotting (Fig. 2). Purified rhG-CSF mutants exhibited biological activity with the magnitude comparable to that of intact rhG-CSF (Table 1). Differences in biological activity between intact rhG-CSF and its mutants were not found to be statistically significant (in all cases, $p > 0.05$). These facts suggest that mutations of rhG-CSF histidine residues to alanine have no significant effect for the specific interaction between rhG-CSF and its receptor, thus it seems that the structure of rhG-CSF histidine mutants is similar to that of intact rhG-CSF. Based on these results, it is possible to conclude that described procedures of refolding and purification allowed to obtain correctly folded and highly purified rhG-CSF (C17S) histidine mutants.

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

The binding strength of rhG-CSF (C17S) histidine mutants to chelated metal ions was estimated by means of IMAF (for review

Table 1
Properties of purified rhG-CSF (C17S) histidine mutants

Mutations	Biological activity ^a ($\times 10^8$ IU/mg)	Relative activity ^b (%)	pI
Without mutations	1.45 \pm 0.12	100	6.15
C17S, H43A	1.56 \pm 0.25	108	5.83
C17S, H43A, H52A	1.37 \pm 0.08	94	5.60
C17S, H43A, H52A, H156A	1.18 \pm 0.19	81	5.24
C17S, H43A, H170A	1.93 \pm 0.32	133	5.52

^a Each value represents the mean \pm SD for at least three independent experiments.

^b Relative activity is the ratio of mean biological activity for the mutant protein over biological activity for intact protein, expressed in percents. The mean activity value of 1.45×10^8 IU/mg, determined for intact rhG-CSF, corresponds to 100%.

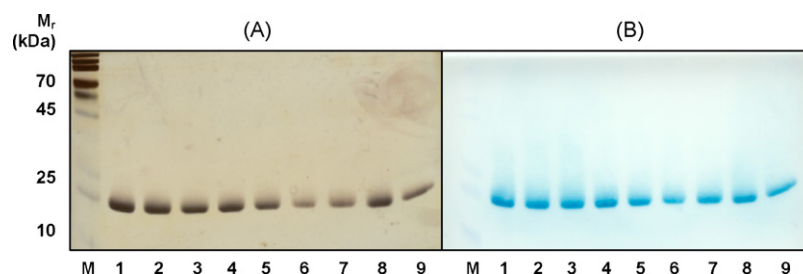


Fig. 2. Silver staining (A) and immunoblot (B) of SDS-PAGE of purified rhG-CSF (C17S) histidine mutants. M: Molecular mass markers. Lanes: (1) rhG-CSF standard; (2,3) rhG-CSF (C17S, H43A); (4,5) rhG-CSF (C17S, H43A, H52A); (6,7) rhG-CSF (C17S, H43A, H52A, H156A); (8,9) rhG-CSF (C17S, H43A, H170A).

see [22,35]) since it is a sensitive and convenient tool both to probe metal binding sites and differentiate surface dissimilarities of closely related proteins [22,36]. Aqueous two-phase systems composed of 5% (w/w) PEG and 8% (w/w) dextran were used for all partitioning experiments. In the systems containing demetallized LR Yellow 2KT-PEG at pH 7.0 (Table 2), $\log K$ values for rhG-CSF (C17S, H43A, H170A) and (C17S, H43A, H52A, H156A) mutants were similar to those for rhG-CSF (C17S, H43A) and (C17S, H43A, H52A), respectively. These data indicate only a negligible effect of His156 and His170 mutations on protein partitioning in the presence of demetallized LR Yellow 2KT-PEG.

Partitioning data of rhG-CSF (C17S) histidine mutants in two-phase systems containing Cu(II), Ni(II) and Hg(II) ions chelated by LR Yellow 2KT-PEG at pH 7.0 are summarized in Table 2. It is seen that in two-phase systems containing chelated Cu(II) ions the $\Delta \log K$ value for (C17S, H43A, H170A) mutant (1.34) was considerably lower compared to that for (C17S, H43A) – 2.06. The mutation of His156 also led to a decrease in the strength of protein interaction with chelated Cu(II) ions, $\Delta \log K$ values for (C17S, H43A, H52A) and (C17S, H43A, H52A, H156A) were 1.49 and 1.15, respectively. Thus, both His156 and His170 residues are surface-exposed and contribute to rhG-CSF interaction with chelated Cu(II) ions at pH 7.0.

Our previous studies on rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) partitioning in two-phase systems containing Cu(II)-LR Yellow 2KT-PEG [23] showed that His43 and His52 residues are involved in rhG-CSF interaction with chelated Cu(II) ions at pH 7.0. $\Delta \log K$ values for rhG-CSF (C17S), (C17S, H43A) and (C17S, H43A, H52A) mutants in two-phase system containing Cu(II) ions at pH 7.0 were 2.38,

2.06 and 1.49, respectively [23]. Thus, four histidine residues of rhG-CSF – His43, His52, His156 and His170 – contribute to the protein interaction with chelated Cu(II) ions at pH 7.0.

However, individual histidine residues contributed to a different extent to the interaction of rhG-CSF with chelated Cu(II) ions. Accordingly, His52 and His170 side chains dominate in this interaction, while the contributions of His43 and His156 are lower. Variations in the affinity of individual histidine residues to chelated Cu(II) ions may result from differences in histidyl 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 [37]. The protein affinity for chelated metal ion is also dependent on steric accessibility of the histidine imidazole nitrogen [22].

Among selected rhG-CSF (C17S) histidine mutants, (C17S, H43A) displayed the highest affinity for chelated Cu(II) ions in two-phase systems at pH 7.0 (Table 2). Partitioning behaviour of rhG-CSF histidine mutants in the systems containing Cu(II) ions at pH 7.0 is in agreement with their chromatographic behaviour on Sepharose–LR Yellow 2KT–Cu(II) column at pH 6.5. RhG-CSF (C17S, H43A) was recovered from this column at 75 mM imidazole [23], while other three rhG-CSF histidine mutants were eluted at approximately 60 mM imidazole.

In aqueous two-phase systems containing chelated Ni(II) ions, $\Delta \log K$ value of rhG-CSF (C17S, H43A, H170A) was only 0.50, compared to 1.01 for (C17S, H43A) variant (Table 2). Such a substantial decrease in $\Delta \log K$ shows very important contribution of His170 residue in the interaction of rhG-CSF with chelated Ni(II) ions. Mutation at His156 had no effect on protein partitioning in the presence of chelated Ni(II) ions (Table 2),

Table 2

Partitioning of purified rhG-CSF (C17S) histidine mutants in two-phase systems containing metal ions chelated by LR Yellow 2KT-PEG at pH 7.0^a

rhG-CSF mutant	M(II)							
	–		Cu(II)		Ni(II)		Hg(II)	
	$\log K$		$\log K$	$\Delta \log K$	$\log K$	$\Delta \log K$	$\log K$	$\Delta \log K$
C17S, H43A ^b	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 ^b	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 Na₂SO₄, 50 mM HEPES–NaOH buffer, pH 7.0. The amount of demetallized dye or chelated metal ion was 800 μ mol/kg of the two-phase system.

^b For comparison, partitioning data of rhG-CSF (C17S, H43A) and (C17S, H43A, H52A) from the previous work are presented [23].

therefore, His156 does not contribute to protein interaction with Ni(II).

Our previous studies on rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) partitioning in two-phase systems containing Ni(II) ions chelated by LR Yellow 2KT-PEG at pH 7.0 [23] demonstrated that the contribution of His52 to the protein interaction with chelated Ni(II) ions is significant, while His43 is not important in this interaction. Thus, two histidine residues – His52 and His170 dominate rhG-CSF binding to chelated Ni(II) ions at pH 7.0.

According to the results on metal affinity partitioning of rhG-CSF histidine mutants, the same histidine residues – His52 and His170 – play a major role in rhG-CSF interaction with Cu(II) and Ni(II) ions at pH 7.0. The contribution of His43 and His156 in the interaction between rhG-CSF and chelated Cu(II) ions is lower, and these histidine residues do not contribute to the protein interaction with chelated Ni(II) ions. Consequently, chelated Ni(II) ions display higher selectivity to the surface-exposed His residues of rhG-CSF than Cu(II) ions. This finding may be attributed to the lower affinity of single His residues for chelated Ni(II) ions [22,37].

Partitioning of rhG-CSF (C17S) histidine mutants was also studied in the presence of Hg(II) ions chelated by LR Yellow 2KT-PEG at pH 7.0 (Table 2). The $\Delta\log K$ values for rhG-CSF (C17S, H43A, H170A) and (C17S, H43A, H52A, H156A) in two-phase systems containing chelated Hg(II) are of comparable magnitude and do not differ substantially from $\Delta\log K$ values of rhG-CSF variants without His170 and His156 mutations, i.e. (C17S, H43A) and (C17S, H43A, H52A). According to this, His156 and His170 residues are not important in rhG-CSF interaction with chelated Hg(II) ions. As it was shown recently [23], His43 and His52 residues also have no effect on rhG-CSF interaction with chelated Hg(II) ions in two-phase systems at pH 7.0. Consequently, surface-exposed histidine residues of rhG-CSF – His43, His52, His156 and His170 – are not involved in protein interaction with chelated Hg(II) ions or their contribution to this interaction is negligible.

Partitioning studies of rhG-CSF histidine mutants in aqueous two-phase systems containing chelated metal ions at pH 7.0 demonstrated that surface histidine residues dominate rhG-CSF interaction with chelated Cu(II) and Ni(II) ions, however, His residues do not play any important role in protein interaction with chelated Hg(II) ions. According to this, the mechanism of rhG-CSF interaction with chelated Hg(II) ions is different from that with Cu(II) and Ni(II) ions.

RhG-CSF and rhG-CSF (C17S) display relatively strong binding to Cu(II) ions chelated by LR Yellow 2KT-PEG in two-phase systems at pH 5.0, $\Delta\log K$ values of protein variants were 1.73–1.78 [18]. The assumption of possible involvement of histidine residues in this interaction was made since the implication of His residue of D-xylose isomerase in the interaction with Cu(II)-IDA-Sepharose under slightly acidic conditions was demonstrated by Mrabet [38]. Recently, the effect of His43 and His52 mutations on rhG-CSF (C17S) partitioning in the presence of Cu(II) ions chelated by LR Yellow 2KT at pH 5.0 was evaluated [23] and it was shown that His52 may be involved in the interaction of protein with chelated Cu(II) ions at this pH

Table 3

Partitioning of purified rhG-CSF (C17S) histidine mutants in two-phase systems containing Cu(II) ions chelated by LR Yellow 2KT-PEG at pH 5.0^a

rhG-CSF mutant	M(II)		$\Delta\log K$
	–	Cu(II)	
	$\log K$	$\log K$	
C17S, H43A ^b	0.27	1.88	1.61
C17S, H43A, H170A	0.33	1.71	1.38
C17S, H43A, H52A ^b	0.44	1.27	0.83
C17S, H43A, H52A, H156A	0.48	1.42	0.94

^a Composition of the two-phase system was identical to that in Table 2, except for the buffer 50 mM MES–NaOH, pH 5.0.

^b For comparison, partitioning data of rhG-CSF (C17S, H43A) and (C17S, H43A, H52A) from the previous work are presented [23].

value. In order to evaluate the contribution of other two histidine residues of rhG-CSF – His156 and His170 – to this interaction, partitioning of respective rhG-CSF histidine mutants was studied in two-phase systems containing Cu(II) ions chelated by LR Yellow 2KT-PEG at pH 5.0 (Table 3).

As seen in Table 3, $\Delta\log K$ value for rhG-CSF (C17S, H43A, H170A) mutant in two-phase systems containing chelated Cu(II) ions was only slightly lower, compared to that for (C17S, H43A). The $\Delta\log K$ values for rhG-CSF (C17S, H43A, H52A, H156A) and (C17S, H43A, H52A) in these systems were of similar magnitude. Accordingly, His170 residue could play only a minor role in rhG-CSF interaction with chelated Cu(II) ions at pH 5.0, whereas His156 does not contribute to this interaction.

3.3. Effect of imidazole on the partitioning of rhG-CSF (C17S) histidine mutants in the presence of chelated metal ions

Partitioning of rhG-CSF (C17S) histidine mutants in two-phase systems containing Cu(II), Ni(II) and Hg(II) ions, chelated by LR Yellow 2KT-PEG at pH 7.0 was also studied in the presence of imidazole, which is capable of coordinating metal ions (Table 4). Imidazole at a 10 mM concentration resulted in a substantial decrease in $\Delta\log K$ values for rhG-CSF (C17S, H43A, H170A) and (C17S, H43A, H52A, H156A) mutants in

Table 4

Effect of imidazole on the partitioning of purified rhG-CSF (C17S) histidine mutants in two-phase systems containing metal ions chelated by LR Yellow 2KT-PEG at pH 7.0^a

rhG-CSF mutant	$\Delta\log K$ reduction (%)		
	Cu(II)	Ni(II)	Hg(II)
C17S, H43A ^b	90	58	81
C17S, H43A, H170A	83	68	86
C17S, H43A, H52A ^b	90	66	95
C17S, H43A, H52A, H156A	84	73	96

^a The concentration of imidazole was 10 mM. Other components of two-phase systems and their concentrations were identical to those in Table 2.

^b For comparison, partitioning data of rhG-CSF (C17S, H43A) and (C17S, H43A, H52A) from the previous work are presented [23].

the presence of all selected metal ions. The same effect of 10 mM imidazole on the partitioning of rhG-CSF (C17S, H43A) and (C17S, H43A, H52A) in two-phase systems containing chelated metal ions was observed (Table 4). Thus, imidazole strongly diminished the binding of rhG-CSF (C17S) histidine mutants to chelated metal ions at pH 7.0. Since two-phase systems contained 0.25 M Na₂SO₄ for the suppression of ionic interactions between protein and chelated metal ion, the ability of imidazole at low 10 mM concentration to substantially diminish the binding of rhG-CSF mutants to chelated metal ions suggests that this binding is governed by the coordination between metal ion and electron donor group (groups) from the protein surface.

3.4. Partitioning and refolding of rhG-CSF (C17S) histidine mutants from inclusion bodies in two-phase systems containing chelated metal ions

The partitioning data for rhG-CSF (C17S) histidine mutants from solubilized inclusion bodies in the presence of chelated Ni(II) and Hg(II) ions at pH 7.0 are presented in Table 5. The $\Delta \log K$ values for inclusion body proteins in two-phase systems containing chelated Ni(II) ions were not calculated because the large amount of protein formed a film-like layer at the interface between PEG and dextran phases and protein partitioning yields were only 14–50% (Table 5). In the presence of chelated Hg(II) ions, inclusion body proteins partitioned only in PEG and dextran phases of two-phase system. The dependence of the partitioning behaviour of rhG-CSF (C17S) histidine mutants from solubilized inclusion bodies on the type of metal ion may be related to the different mechanisms of protein interaction with chelated Ni(II) and Hg(II) ions.

The $\Delta \log K$ value for rhG-CSF (C17S, H43A, H170A) mutant from solubilized inclusion bodies in two-phase system containing chelated Hg(II) ions at pH 7.0 was similar to that for (C17S, H43A) (Table 5). His43 and His52 mutations also resulted in only a slight effect on the partitioning of rhG-CSF (C17S) from solubilized inclusion bodies in this two-phase system [23]. However, the $\Delta \log K$ value for rhG-CSF (C17S, H43A,

H52A, H156A) from inclusion bodies in the presence of chelated Hg(II) ions was lower compared to those for other rhG-CSF histidine mutants (Table 5). In summary, mutations at three histidines – His43, His52 and His170 – have only a slight effect on the partitioning of rhG-CSF (C17S) from solubilized inclusion bodies in two-phase systems containing chelated Hg(II) ions at pH 7.0. This fact is not surprising since the contribution of histidine residues is not important in the interaction between rhG-CSF and chelated Hg(II) ions.

Hg(II)-LR Yellow 2KT-PEG was found to be more effective in extracting proteins of solubilized inclusion bodies (Table 5) than respective purified rhG-CSF mutants (Table 2). This fact may be explained assuming that a partially folded protein form which contains only one of two native disulphide bonds is predominant in the inclusion body extracts of rhG-CSF (C17S) histidine mutants at pH 7.0 analogously as in the case of rhG-CSF (C17S) [18]. The interaction of two free –SH groups with chelated Hg(II) ions may outweigh the effect of accompanying *E. coli* proteins since both SDS-PAGE and RP-HPLC analysis indicated that rhG-CSF mutant forms predominate in the inclusion body extract.

A scheme for the refolding and purification process of rhG-CSF and its mutants in aqueous two-phase systems containing chelated metal ions is represented in Fig. 3. In these systems, rhG-CSF or its mutant from solubilized inclusion bodies interact with chelated metal ions and is extracted into the upper PEG-rich phase, while the majority of *E. coli* proteins prefer the lower dextran-rich phase (Fig. 3,(1)). The PEG phase, containing chelated metal ions and extracted rhG-CSF, is then separated from the dextran phase, containing *E. coli* proteins and transferred on fresh dextran phase of the two-phase system without metal chelate and protein (Fig. 3,(2)). Finally, refolded

Table 5
Partitioning of solubilized inclusion bodies of rhG-CSF (C17S) histidine mutants in two-phase systems containing metal ions chelated by LR Yellow 2KT-PEG at pH 7.0^a

rhG-CSF mutant	Ni(II)		Hg(II)	
	$\Delta \log K$	Y^b (%)	$\Delta \log K$	Y (%)
C17S, H43A ^c	n.c.	28	2.13	101
C17S, H43A, H170A	n.c.	14	2.11	103
C17S, H43A, H52A ^c	n.c.	21	2.41	85
C17S, H43A, H52A, H156A	n.c.	50	1.72	96

n.c., not calculated.

^a Two-phase system (4 g) contained 5% (w/w) PEG 6000, 8% (w/w) dextran 60,000, 0.25 M Na₂SO₄, 50 mM HEPES–NaOH buffer (pH 7.0), 0.8 mg of total protein and 800 μ mol/kg of chelated metal ion.

^b Partitioning yield.

^c For comparison, partitioning data of solubilized inclusion bodies of rhG-CSF (C17S, H43A) and (C17S, H43A, H52A) from the previous work are presented [23].

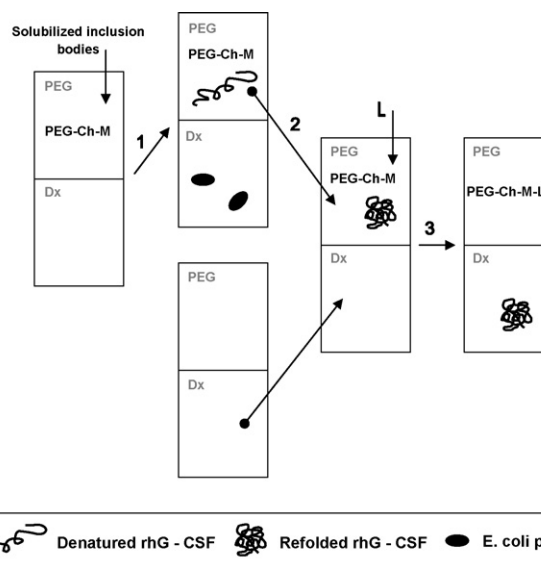


Fig. 3. A scheme for the refolding and purification of rhG-CSF in polyethylene glycol (PEG)–dextran (Dx) two-phase systems, containing Ni(II) or Hg(II) ions chelated by LR Yellow 2KT-PEG. Ch–M:LR Yellow 2KT–Ni(II) or–Hg(II) chelate; L: imidazole. (1) Partitioning of rhG-CSF inclusion body proteins; (2)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

rhG-CSF is desorbed to the lower dextran phase with imidazole or 2-mercaptoethanol (Fig. 3,(3)).

The effect of partitioning of rhG-CSF (C17S) histidine mutants from inclusion body extract on protein refolding was examined in the presence of chelated Ni(II) and Hg(II) ions at pH 7.0. After the partitioning of inclusion body extract and release of rhG-CSF mutant from chelated metal ions by imidazole, partial precipitation of the protein at the interface was evident in two-phase systems containing chelated Hg(II) ions. In the systems containing chelated Ni(II) ions, only very low amount of protein precipitated at the interface. The relative amounts of correctly folded rhG-CSF mutants in the samples of the solubilized interface layer and dextran-rich phase of two-phase systems were estimated using RP-HPLC analysis. RP-HPLC chromatograms of rhG-CSF (C17S, H43A, H170A) samples drawn from two-phase systems are shown in Fig. 4. In the initial inclusion body extract (Fig. 4A and B, chromatogram 2), rhG-CSF (C17S, H43A, H170A) conformation, which retention time is notably longer than that of correctly folded mutant, predominates, and the amount of the latter is negligible. It means that folding intermediates of rhG-CSF mutant with one reduced disulphide bond predominate in the inclusion body extract, analogously as it was described for rhG-CSF

[33]. After the partitioning of rhG-CSF (C17S, H43A, H170A) inclusion body extract in two-phase systems containing chelated Ni(II) ions, the relative amount of correctly folded protein in the sample from the dextran-rich phase was nearly equal to that of partially folded protein (Fig. 4A, chromatogram 3). In the sample from the dextran phase of two-phase systems containing chelated Hg(II) ions, the correctly folded rhG-CSF (C17S, H43A, H170A) form predominates (Fig. 4B, chromatogram 3).

In two-phase systems the amount of correctly folded protein was found to be higher in the dextran-rich phase than in the interface layer (not shown). The relative amounts of correctly folded rhG-CSF (C17S) histidine mutants in samples from the dextran-rich phase of two-phase systems, determined using RP-HPLC analysis, are presented in Table 6. In the dextran phase of two-phase systems containing chelated Ni(II) ions, the amount of correctly folded rhG-CSF (C17S, H43A, H170A) was 28%. The refolding of rhG-CSF variant with three histidine mutations (C17S, H43A, H52A, H156A) was inefficient in this system, the amount of correctly folded protein reached only 8% (Table 6). The amounts of correctly folded protein in the dextran phase of two-phase system containing chelated Ni(II) ions for rhG-CSF (C17S, H43A, H170A) and (C17S, H43A, H52A, H156A) were noticeably lower in comparison to those for rhG-CSF (C17S, H43A) and (C17S, H43A, H52A), respectively (Table 6). Thus, both His170 and His156 mutations resulted in a decrease of protein refolding efficiency in two-phase systems containing chelated Ni(II) ions.

The amounts of correctly folded rhG-CSF mutants in dextran-rich phase of two-phase systems containing chelated Ni(II) ions (Table 6) decreased in the order (C17S) \approx (C17S, H43A) > (C17S, H43A, H170A) \approx (C17S, H43A, H52A) > (C17S, H43A, H52A, H156A). Accordingly, mutations at His170, His52 and His156 led to the decrease in rhG-CSF (C17S) refolding efficiency in these systems. In addition, lower

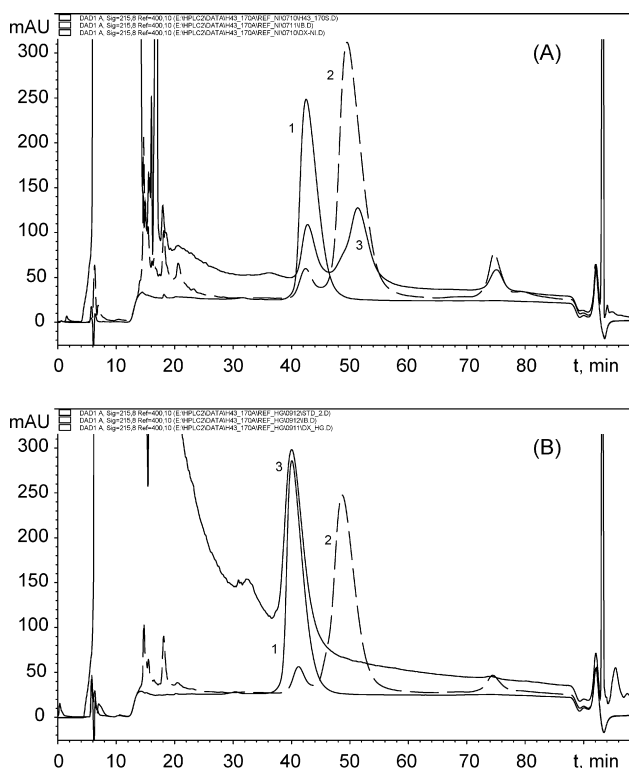


Fig. 4. RP-HPLC analysis of rhG-CSF (C17S, H43A, H170A) samples. Refolding of rhG-CSF mutant in aqueous two-phase systems containing chelated metal ions. (A1, B1) Purified rhG-CSF (C17S, H43A, H170A), 10 mM sodium acetate buffer, pH 4.0, 1.23 mg/ml, 40 μ l; (A2, B2) solubilized inclusion bodies, 20 mM HEPES–NaOH, pH 7.0, 2.21 mg/ml, 70 μ l (B2: 60 μ l); (A3) sample from the dextran phase of two-phase system containing chelated Ni(II) ions, 0.05 mg/ml, 1200 μ l; (B3) sample from the dextran phase of two-phase system containing chelated Hg(II) ions, 0.05 mg/ml, 1100 μ l. The composition of two-phase systems is indicated in Table 6.

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, their number and the nature of metal ion^a

rhG-CSF mutant	Relative amount of correctly folded protein in the dextran phase (%)	
	Ni(II)	Hg(II)
C17S ^b	56 (0.99) ^c	99
C17S, H43A ^b	67 (1.01)	94
C17S, H43A, H170A	28 (0.50)	92
C17S, H43A, H52A ^b	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 μ mol/kg M(II)–LR Yellow 2KT-PEG, 0.25 M Na₂SO₄ and 50 mM HEPES–NaOH buffer, pH 7.0. Imidazole concentration was 50 mM in the systems containing chelated Ni(II) ions, and 200 mM in those containing chelated Hg(II) ions.

^b For comparison, relative amounts of correctly folded rhG-CSF (C17S), (C17S, H43A) and (C17S, H43A, H52A) from the previous work are presented [23].

^c For comparison, Δ log *K* values of purified rhG-CSF mutants in two-phase systems containing chelated Ni(II) ions are presented.

refolding efficiency was obtained for those rhG-CSF mutants which contained less surface histidine residues. $\Delta \log K$ values of purified rhG-CSF mutants in two-phase systems containing chelated Ni(II) ions at pH 7.0 (Table 6) follow similar order: (C17S) \approx (C17S, H43A) > (C17S, H43A, H170A) \approx (C17S, H43A, H52A) = (C17S, H43A, H52A, H156A). Hence we may conclude that the direct correlation between the affinity of purified rhG-CSF mutant for chelated Ni(II) ions and its refolding efficiency in two-phase systems containing chelated Ni(II) ions exists (except for C17S, H43A, H52A, H156A).

According to this correlation, mutations of histidine residues, which contribute to rhG-CSF (C17S) interaction with chelated Ni(II) ions, led to the decrease in protein refolding efficiency in two-phase systems containing chelated Ni(II). Two reasons may be responsible for the effect of histidine mutations on protein refolding in these systems. Firstly, the partially folded form of rhG-CSF mutant, which contains more surface-exposed histidine residues, may bind a higher number of Ni(II)-LR Yellow 2KT-PEG molecules. Intermolecular interactions between such protein forms in the presence of Ni(II)-LR Yellow 2KT-PEG, leading to aggregation, can be less possible in comparison to protein forms which contain less surface-exposed histidine residues. The prevention of aggregate formation, in turn, may improve protein refolding efficiency. Secondly, 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 demonstrated that proteins which contain His-X₃-His site can be stabilized effectively by Cu(II)-IDA complex [39,40]. Likewise, rhG-CSF, which contains several histidine residues, could be stabilized by chelated Ni(II) ions to a certain extent. The stabilizing effect of chelated metal ions may be proportional to their binding strength to the protein and the number of metal-binding sites on the protein surface. Therefore, the decrease in a number of binding sites for chelated Ni(II) on the protein surface result in the lower stabilizing effect and refolding efficiency in two-phase systems containing chelated Ni(II) ions.

After the partitioning of solubilized inclusion bodies of rhG-CSF mutants in two-phase systems containing chelated Hg(II) ions and the addition of imidazole, the amounts of correctly folded rhG-CSF (C17S, H43A, H170A) and (C17S, H43A, H52A, H156A) in the dextran phase were 92% and 91%, respectively (Table 6). The similar amounts of correctly folded rhG-CSF variants without histidine mutations (C17S) and with one histidine mutation (C17S, H43A) were previously determined in these systems. Only the amount of correctly folded rhG-CSF (C17S, H43A, H52A) was slightly lower (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 the protein with chelated Hg(II) (see Section 3.2).

The refolding efficiency of rhG-CSF (C17S) and its histidine mutants was higher in two-phase systems containing chelated Hg(II) ions compared to that in the systems containing Ni(II)

ions (Table 6). The effect of metal ion type on the refolding of rhG-CSF mutants in two-phase systems may result from the different mechanisms of protein interaction with chelated Ni(II) and Hg(II) ions.

4. Conclusions

The role of His156 and His170 residues in the interaction of rhG-CSF (C17S) variant with metal ions, chelated by LR Yellow 2KT-PEG, was studied at pH 7.0 by means of metal affinity partitioning of respective rhG-CSF (C17S) mutants. Partitioning behaviour of rhG-CSF (C17S) histidine mutants in aqueous two-phase systems containing chelated metal ions revealed that His156 and His170 residues are involved in the interaction of protein with chelated Cu(II) ions. The contribution of His170 is very important in rhG-CSF (C17S) interaction with chelated Ni(II) ions. Histidine residues, however, are not involved in protein interaction with chelated Hg(II) ions or their contribution to this interaction is negligible.

The effect of His156 and His170 mutations on the refolding efficiency of rhG-CSF (C17S) in aqueous two-phase systems containing chelated metal ions was investigated. Both histidine mutations resulted in a significant decrease of the refolding efficiency of rhG-CSF (C17S) in two-phase systems containing chelated Ni(II) ions. In the presence of chelated Hg(II) ions, these mutations caused only the slight alterations in protein refolding efficiency.

Partitioning and refolding studies of rhG-CSF (C17S) mutants, containing different numbers of surface histidines revealed that the direct correlation exists between the affinity of purified rhG-CSF mutant for chelated Ni(II) ions and its refolding efficiency in two-phase systems containing chelated Ni(II) ions. Refolding efficiency of rhG-CSF mutants in these systems containing Ni(II) decreased according to the number of surface histidine residues, which may contribute to the protein interaction with chelated Ni(II) ions.

This study demonstrated that the number of surface histidines and metal ion type may influence protein refolding efficiency in two-phase systems PEG-dextran containing chelated metal ions. Aqueous two-phase systems with metal chelates could also be applicable for the refolding of other recombinant proteins such as interferons, granulocyte macrophage-colony stimulating factor, interleukins etc., which contain surface histidine or cysteine residues.

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