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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

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Abstract

Site directed mutagenesis of Cys17→Ser17 form of recombinant human granulocyte colony stimulating factor (rhG-CSF C17S) for sequential replacing of surface His^{43} and His^{52} with alanine was used to identify residues critical for the protein interaction with metal ions, in particular Ni^{2+} chelated by dye Light Resistant Yellow 2 KT (LR Yellow 2KT)–polyethyleneglycol (PEG), and refolding after partitioning of inclusion bodies in aqueous two-phase systems. Strong binding of rhG-CSF (C17S) to PEG–LR Yellow 2KT–Cu(II) complex allowed for the adoption of affinity chromatography on Sepharose–LR Yellow 2KT–Cu(II) that appeared to be essential for the rapid isolation of mutated forms of rhG-CSF. Efficiency of that purification stage is exemplified by isolation of rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) mutants in correctly folded and highly purified state. Affinity partitioning of rhG-CSF histidine mutants was studied in aqueous two-phase systems containing Cu(II), Ni(II) and Hg(II) chelated by LR Yellow 2KT–PEG at pH 7.0 and Cu(II)—at pH 5.0. It was determined, that affinity of rhG-CSF mutants for metal ions decreased in the order of C17S>C17S, H43A>C17S, H43A, H52A for Cu(II), and C17S=C17S, H43A>C17S, H43A, H52A for Ni(II) ions, while affinity of all rhG-CSF mutants for Hg(II) ions was of the same order of magnitude. Influence of His^{43} and His^{52} mutation on protein refolding was studied by partitioning of the respective inclusion body extract in aqueous two-phase systems containing Ni(II) and Hg(II) ions. Data on rhG-CSF histidine mutant partitioning and refolding indicated, that His^{52} mutation is crucial for the strength of protein interaction with chelated Ni(II) ions and refolding efficiency.

Keywords: Aqueous two-phase systems; Partition coefficients; Affinity adsorbents; Proteins; Metal chelates

1. Introduction

Aqueous two-phase systems are widely used in biochemistry and biotechnology as a simple and mild separation technique for purification of biological materials [1], for a recent review see Ref. [2]. During recent years a lot of attention has been paid to the use of aqueous two-phase systems for recovery and renaturation of inclusion bodies protein. Aqueous PEG–salt systems containing moderate concentration

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of guanidinium chloride were studied for oxidative refolding of lysozyme [3], while PEG–Na_2SO_4 and PEG–dextran systems, containing a high urea concentration were evaluated in respect to the phase behavior and influence of phase components on stabilization of protein structure [4,5]. A set of new polymers, that have thermoseparating properties have been recently introduced as a phase forming components. Such novel thermoseparating polymer-containing aqueous two-phase systems were shown to be promising for use in biotechnical extraction [6,7] as they ensured high recovery yield of proteins and operated at moderate temperatures and salt concentrations with a possible recovery of phase system forming polymers. Approaches on the use of thermoseparating polymer two-phase systems for the refolding of inclusion bodies proteins seem very attractive. Their ability to enhance protein renaturation and to improve the yield of refolding was shown by studying renaturation process of β-lactamase when inclusion bodies solubilized in GdmCl were partitioned [8], and by modeling the unfolding-refolding process of chymotrypsin inhibitor 2 [9]. The use of those polymers offers possibilities to develop the protein refolding process in aqueous two-phase systems, that may mimic the refolding function of natural chaperones [10].

In a recent work [11] we have shown the potential use of aqueous two-phase systems, containing PEG–dextran for the studies of refolding efficiency of rhG-CSF forms enhanced by the presence of chelated metal ions. For this purpose the top-phase of the system was modified by introducing PEG–LRY 2KT–metal ion (II) complex. The mode of partition technique, which is well known as immobilized metal ion affinity partitioning (IMAP) (for review see Refs. [12,13]) was chosen for refolding studies since it is a sensitive and convenient tool both to probe metal-binding sites and differentiate surface dissimilarities of closely related proteins [12], and to detect changes at the protein surface due to mutations of the residues responsible for metal ion binding.

The three-dimensional structure of recombinant hG-CSF was determined by X-ray crystallography in 1993 [14] and later confirmed by NMR spectroscopy [15]. If to display NMR resolved structure of rhG-CSF (Brookhaven code 1GNC) by RasMol (version 2.6, R. Sayle, Glaxo Research and Development, Greenford, Middlesex, UK), it is evident, that at least four histidines—His^{43}, His^{52}, His^{156} and His^{170}—are surface exposed to such degree, that may be accessible for the interaction with metal ions. Two of them, His^{52} and His^{156} appear to be of special interest, because of their position in close proximity (see Fig. 1). It is well established that two histidine residues brought into close proximity due to protein folding, commonly favour the stronger binding to the chelated metal ions [16,17]. Since the retention of a protein molecule by chelated Ni(II) ions requires the presence of at least two histidines [17], the distances and relative orientations of both His^{52} and His^{156} may fulfil the requirement for chelation of the same Ni(II) atom in case of rhG-CSF. The interaction between rhG-CSF and chelated Ni(II) ions may have a substantial effect on stabilization of a nascent correctly folded conformation of the protein during its refolding.

The main objective of this work is to study the interaction of rhG-CSF (C17S) histidine mutants primarily with chelated Ni(II) ions as well as with

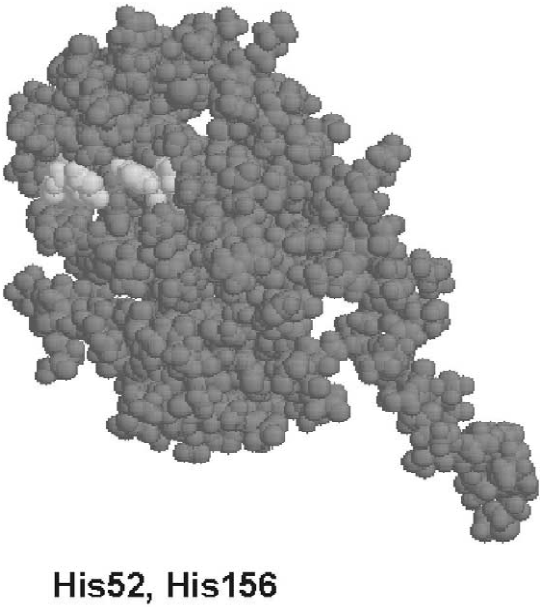

Fig. 1. Space-filling model of rhG-CSF with exhibited positions of surface-exposed His^{52} and His^{156} (grey).

Cu(II) and Hg(II) for comparison, and evaluate the effect of those mutations for refolding of protein after partitioning of solubilized inclusion bodies in aqueous two-phase systems PEG–dextran, containing Ni(II) ions. The site-directed mutagenesis of more stable [Cys17→Ser17] variant of rhG-CSF for sequential replacing of His residues, starting from His^{43} mutation was chosen. Correctly folded and highly purified mutated forms were obtained in quantities large enough for partitioning studies on the basis of developed semi-preparative purification scheme, that is described below.

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 (morpholinoethanesulphonic acid) and imidazole (buffer substance) were purchased from Merck (Darmstadt, Germany). HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulphonic acid), Trizma Base (Tris[hydroxymethyl]aminomethane) and isopropyl β-D-thiogalactopyranoside were purchased from Sigma (St. Louis, MO, USA). Poly(ethylene glycol) PEG 6000 was obtained from Fluka (Buchs, Switzerland) and dextran 60000 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. [18].

Triton X-100 and Tween 80 were purchased from Ferak (Berlin, Germany). Phenylmethylsulfonylfluoride (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. Synthesis of Sepharose CL-6B-LR Yellow 2KT–Cu(II)

Immobilization of reactive dye LR Yellow 2KT–Cu(II) on Sepharose CL-6B was performed according to the method for coupling Cibacron Blue F3GA to Sephadex G-200 [19].

2.3. Cloning and cell growth

All cloning, DNA preparation, transformation and expression procedures were performed according to Ref. [20]. All enzymes, kits and DNA markers were obtained from MBI Fermentas (Vilnius, Lithuania). Initial human G-CSF gene [21] was modified by the introduction of NdeI and BamHI sites into the 5′- and 3′-ends of this gene, respectively, using synthetic primers. The genes for hG-CSF–Ser^{17}–Ala^{43} and hG-CSF–Ser^{17}–Ala^{43}–Ala^{52} protein production were obtained with the help of PCR mutagenesis [22] on the plasmid pET21-hG-CSF. The amplified fragments were ligated into the same sites of the expression vector pET21b for production of the hG-CSF (C17S, H43A) and hG-CSF (C17S, H43A, H52A) proteins. The expression of modified hG-CSF variants was achieved in the host strain *E. coli* BL-21 (DE3) after induction of middle log-phase cells with isopropyl-β-thiogalactopyranoside (IPTG) [23]. Cells were grown in 37 °C until 0.6–1.0 OD_{550} and induced with 1 m*M* of IPTG for 2–3 h.

2.4. Isolation of inclusion bodies from E. coli

Harvested *E. coli* cells (10 g) were homogenized in 100 ml 0.1 *M* Tris–HCl buffer, pH 7.0, containing 5 m*M* EDTA, 0.1 g lysozyme, 0.1 ml Triton X-100, 1.0 ml 100 m*M* PMSF (phenylmethyl sulfonyl fluoride) and 0.7 ml 2-mercaptoethanol, stirred for 30 min and disrupted by sonication on ice. The homogenizate was centrifuged at 24 500 *g* for 25 min. The supernatant was removed, the insoluble fraction was homogenized twice with 100 ml of 1 *M* NaCl, 0.1% Tween 80 solution and once with 100 ml of distilled water. The suspension obtained after each homogenization was centrifuged at 24 500 *g* for 25 min.

2.5. Refolding of rhG-CSF (C17S) histidine mutants from inclusion bodies

Refolding of rhG-CSF (C17S) histidine mutants was performed by the method of Lu et al. [24] with some modification. rhG-CSF (C17S, H43A) (7.7 g) or 4.6 g rhG-CSF (C17S, H43A, H52A) inclusion bodies were solubilized in 100 ml, 10 m*M* Tris–HCl buffer, pH 7.0, containing 7 *M* GdmHCl by stirring overnight at 4 °C. The protein solution was centrifuged at 40 000 *g* for 25 min. The solubilized fraction was mixed with 10 m*M* Tris–HCl buffer, pH 7.0, 7 *M* GdmHCl in 10 m*M* Tris–HCl buffer, pH 7.0, and 40 m*M* $CuSO_4 \cdot 5H_2O$ solution in water to adjust total protein concentration to 1 mg/ml (measured by the method of Bradford), GdmHCl to 6 *M* and Cu^{2+} ions to a final concentration of 20 μ*M*. The mixture was stirred for 1 h at room temperature, 100 m*M* EDTA solution was added to the final 10 m*M* concentration, pH was adjusted to 5.4 with 1 m*M* HCl and centrifuged at 40 000 *g* for 25 min.

For the removal of GdmHCl, 500 ml of Sephadex G-25 medium was packed into a glass column (2.6× 100 cm, Pharmacia, Uppsala, Sweden) and the column was equilibrated with 25 m*M* Tris–maleic acid–NaOH buffer, pH 6.5, 0.25 *M* Na_2SO_4. Protein solution after refolding (170 ml) was applied on to the column and elution was performed at a linear velocity 20 cm/h. Eluted protein fractions were pooled and centrifuged at 40 000 *g* for 25 min.

Identity and purity of the protein was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) [25] and by reversed-phase HPLC analysis (see Section 2.13).

2.6. Purification of rhG-CSF (C17S) histidine mutants

For the initial purification stage, 50 ml of Sepharose CL-6B containing covalently attached dye LR Yellow 2KT–Cu(II) (4.2 μmol/ml) was packed into a glass column (2.6×24 cm, Pharmacia, Uppsala, Sweden) which was equilibrated with 25 m*M* Tris–maleic acid–NaOH buffer, pH 6.5, 0.25 *M* Na_2SO_4. A 156–170-ml pool of the protein solution after gel permeation chromatography through Sephadex G-25 (0.57–1.06 mg/ml) was loaded on the column at the velocity 15 cm/h, not retained material was washed with five bed volumes of buffer and the elution of adsorbed protein was achieved by the linear 0–300 m*M* imidazole gradient at a linear velocity 25 cm/h. Protein fractions from imidazole gradient were combined and dialysed against 25 m*M* Na–acetate buffer, pH 5.4 or 5.0 for rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) at 4 °C, respectively, and centrifuged at 40 000 *g* for 25 min.

For the next purification stage, 40 ml of SP-Sepharose was packed into a glass column (2.6×24 cm). The column was equilibrated with 25 m*M* Na–acetate buffer, pH 5.4 or pH 5.0, for rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A), respectively. rhG-CSF (C17S, H43A) solution (88 ml) with a protein concentration 0.45 mg/ml, or 84 ml of rhG-CSF (C17S, H43A, H52A) (protein concentration 0.86 mg/ml) was loaded onto the column at a velocity of 20 cm/h, not retained material was washed out with five bed volumes of buffer and the elution of adsorbed protein was achieved by the linear 0–500 m*M* NaCl gradient at a linear velocity of 25 cm/h. Desorbed protein fractions were pooled, dialysed against 10 m*M* 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 [26].

2.7. Protein characterization and purity control

Identity and purity of the protein after each stage of purification was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) [25] and by RP-HPLC analysis (see Section 2.13). Finally purified proteins were also analysed by isoelectric focusing gel electrophoresis according to [27] and by mass-spectrometry.

2.8. Synthesis of the PEG–dye derivatives

The dye–liganded PEG 6000 derivative was synthesized as described previously [28].

For PEG–LR Yellow 2KT–Ni(II) and PEG–LR Yellow 2KT–Hg(II) synthesis 1 g of relevant metal salt was dissolved in 10 ml Na–acetate buffer (pH

4.0), 1 g of demetallized PEG–dye was added and the mixture was stirred for 3 h. PEG–dye–metal complex was extracted with chloroform (3×20 ml). Chloroform extracts were pooled, kept over anhydrous Na_2SO_4, and dried by rotary evaporation.

The amount of dye and dye–Cu(II) in dye–PEG derivatives was calculated from their absorption spectra using an extinction coefficient of 20 300 M^{-1} cm^{-1} at 405 nm for demetallized dye and 24 100 M^{-1} cm^{-1} at 450 nm for the Cu(II) complex, respectively [18]. The amount of Ni^{2+} ions was determined by atomic absorption spectrometry. The amount of Hg^{2+} ions was determined as described in Ref. [29].

2.9. 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 60000. 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 Results and discussion section). The IMAP experiments were performed by replacing part of PEG for PEG–dye or for PEG–dye–metal ion complex. The amount of dye–PEG derivative is expressed as metal ion concentration (μM/kg) per kg of two-phase system.

2.10. Partitioning of rhG-CSF (C17S) histidine mutants

The solution of respective protein in 10 m*M* Na–acetate buffer (pH 4.0) was dialysed against either 20 m*M* MES–NaOH buffer, pH 5.0 or 20 m*M* HEPES–NaOH buffer, pH 7.0. Subsequently, the sample containing 0.8 mg of the respective protein 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 *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 [26]. Both demetallized dye and dye–metal ion complexes absorb in the visible region and give residual absorbance in a protein assay at 595 nm. To exclude the side effects similar two-phase systems, but without a protein were prepared for each partitioning experiment. The samples withdrawn from such systems were equal in volume to that 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 and lower phase. The protein affinity for immobilized metal ion was expressed in terms of Δ log *K*, defined as the difference between the logarithmic partition coefficient of protein in the presence of metal–dye–PEG (K_{Me}) and the logarithmic partition coefficient in the presence of demetallized dye–PEG (K_{dye}) ($\Delta \log K = \log K_{Me} - \log K_{dye}$). The alteration of the protein partition coefficient (Δ log *K*), when selected agents were introduced into the two-phase systems, was expressed as a percentage of the initial value of Δ 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 Δ log *K* is given as the mean of two separate determinations.

2.11. Preparation of crude inclusion bodies extracts of rhG-CSF (C17S) histidine mutants

Inclusion bodies (0.25 g) was homogenized in 10 ml of 20 m*M* HEPES–NaOH buffer, pH 7.0. Three to four drops of 2 *M* NaOH solution were introduced into the homogenizate to obtain pH 12–13. After 2 min the homogenizate was titrated with 0.25 *M* H_2SO_4 up to pH 7.0. The protein extract was centrifuged at 40 000 *g* for 25 min.

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

Partitioning of rhG-CSF (C17S) histidine mutants from their inclusion-bodies extracts was investigated in phase systems composed of 5% (w/w) PEG 6000–8% (w/w) dextran 60000 in 50 m*M* HEPES–NaOH buffer, pH 7.0, containing 0.25 *M* Na_2SO_4,

and 800 μ*M*/kg of immobilized Ni^{2+} or Hg^{2+} ion. The amount of total protein was 0.8 mg.

For refolding of rhG-CSF (C17S) histidine mutants from inclusion bodies extract, three two-phase systems, which contained PEG–dye–metal ions were prepared. The extract of inclusion body containing approximately 1.5 mg of total protein in 20 m*M* HEPES–NaOH buffer, pH 7.0, was introduced into the two of these systems. Into the third one, the buffer instead of a protein was introduced. In parallel, three two-phase systems both free of the extract of inclusion bodies and PEG–dye–metal ion were prepared. After mixing and phase separation, the upper phase of the systems without extract of inclusion bodies was replaced by the upper phase, which contained the extracted protein. The selected desorption agents were introduced into the ''mixed'' two-phase systems. Both mixing and phase separation were repeated. Then, the 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* guanidine hydrochloride solution in 10 m*M* Tris–maleic acid–NaOH buffer, pH 6.5 and centrifuged at 24 500 *g* for 10 min. The samples of the initial extract of inclusion bodies and of dextran phases of the two-phase systems, which contained selected agents, and the sample of the interface layer were taken and analyzed by reversed-phase HPLC to detect the conformation changes of the target proteins in the two-phase systems. SDS–PAGE [25] of the samples was performed to assess purity of proteins.

2.13. RP-HPLC analysis

Recombinant hG-CSF (C17S) histidine mutants, their folding intermediates and the state of their conformation were analysed by RP-HPLC using a C_4 (Hi-Pore RP-304, 250×4.6 mm I.D., 30 nm, Bio-Rad, Hercules, CA, USA) reversed-phase column similar to that described in Ref. [24]. 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 to 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 individual purified rhG-CSF and its mutants were used as references.

3. Results and discussion

3.1. rhG-CSF (C17S) histidine mutant refolding and purification

As it was cited above (see Introduction), two residues of His^{52} and His^{156} are in close proximity on the surface of rhG-CSF molecule (Fig. 1). Therefore, they may be involved in the coordination with a single metal ion. His^{43} is in vicinity with Tyr^{39} and this site also could be coordinated by a single metal ion. Thus, we first decided to substitute His^{43} for Ala^{43} in the gene of hG-CSF-Ser^{17}. Furthermore, the second variant of the hG-CSF gene with an additional His^{52} mutation into alanine was obtained. Both histidine mutants were expressed in *E. coli* as inclusion body proteins, so the renaturation step was mandatory for their recovery in a native state.

Data on refolding and purification of both rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) mutants are summarized in Table 1. It can be seen, that after the step of oxidative refolding with Cu^{2+} ions in 6 *M* GdmHCl solution, the RP-HPLC purity of rhG-CSF (C17S, H43A) protein has reached 82%. After this stage RP-HPLC purity of the protein with two mutation was lower −46% (Table 1). Part of the protein aggregated after the removal of GdmHCl and this resulted in a loss of protein by 50 and 37% for rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) mutant, respectively. The electrophoretic patterns of rhG-CSF (C17S, H43A) samples after renaturation and desalting steps are shown in Fig. 2.

Our earlier investigations [11] showed relatively strong binding of rhG-CSF, rhG-CSF (C17S) and

Table 1
Refolding and purification of rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A)[a]

No.	Stage	Amount of protein (mg)		Protein recovery (%)	Protein concentration (mg/ml)	Buffer composition	RP-HPLC purity (%)
		Initial	After stage				
1	Oxidative refolding in solution	180 (295)	178.4 (281.4)	99 (95)	0.98 (1.56)	5.4 *M* GdmHCl, 20 μ*M* $CuSO_4$, 10 m*M* EDTA[b], pH 5.4	82 (46)
2	Desalting over Sephadex G-25	175.4 (279.2)	89.5 (184.8)	50 (63)	0.65 (1.56)	25 m*M* Tris–maleic acid–NaOH, pH 6.5, 0.25 *M* Na_2SO_4	95 (47)
3	Sepharose–LR Yellow 2KT–Cu(II)	88.9 (183.4)	70.4 (147.7)	39 (50)	0.92 (1.93)	25 m*M* Tris–maleic acid–NaOH, pH 6.5, 0.25 *M* Na_2SO_4	– (54)
4	Dialysis	70.4 (146.7)	39.8 (103.2)	22 (35)	0.51 (1.26)	25 m*M* Na–acetate, pH 5.4	92 (53)
5	SP-Sepharose FF	39.6 (73.1)	19.5 (20.2)	11 (7)	0.74 (0.64)	25 m*M* Na–acetate, pH 5.4	97 (91)
6	Dialysis	19.2 (19.8)	13.0 (19.2)	7 (7)	0.51 (0.65)	10 m*M* Na–acetate, pH 4.0	97 (94)

[a] Data for rhG-CSF (C17S, H43A, H52A) are in parentheses.
[b] Oxidation with Cu(II) was terminated with 10 m*M* EDTA.

His_6-rhG-CSF to PEG–LR Yellow 2KT–Cu(II) complex in aqueous two-phase systems (the Δ log *K* values within 2.20–2.38 were determined). Therefore, we supposed the adsorbent containing Cu(II) complex of dye LR Yellow 2KT might also be promising for an initial separation of rhG-CSF histidine mutant from contaminating *E. coli* proteins according to the methodology of IMAC [12]. Since chelated Cu(II) ions quite strongly coordinate protein macromolecules with even one available histidine [16,17] the use of this selected adsorbent might cover purification of all mutated rhG-CSF forms up to the form with at least one non-mutated histidine residue.

The chromatographic profile for rhG-CSF (C17S, H43A) on the Sepharose–LR Yellow 2KT–Cu(II) is shown in Fig. 3. As can be seen, the greater part of loaded protein was retained and rhG-CSF (C17S, H43A) recovered by a linear gradient elution with imidazole (its peak concentration was 75 m*M*). The electrophoretic pattern of the protein recovered after this purification stage is shown in Fig. 2.

The elution profile of rhG-CSF (C17S, H43A, H52A) from the Sepharose–LR Yellow 2KT–Cu(II) was very similar, too, but the peak imidazole concentration was lower (60 m*M*). This was evidence of a slightly weaker interaction of rhG-CSF (C17S, H43A, H52A) mutant with Cu(II)–dye complex. The purity of protein, as judged by RP-HPLC analysis after chromatography on a Sepharose–LR Yellow 2KT–Cu(II), reached 92 and 54%, for rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A), respectively.

According to the recommendations for the optimized analytical separation of charged isomers of rhG-CSF at pH 5.4 described in Ref. [27], strong cation-exchange chromatography was selected for the final purification of both mutated forms of rhG-CSF. Therefore, SP-Sepharose Fast Flow (FF) was selected for further semi-preparative purification of both proteins. The chromatographic profile for rhG-CSF (C17S, H43A, H52A) mutant passed through SP-Sepharose FF is exemplified in Fig. 4. Data in Fig. 2 and Table 1 proved the high efficiency of SP-Sepharose FF for final purification of rhG-CSF histidine mutant forms—the RP-HPLC purity of protein after this stage has reached 97 and 91% for H43A and for H43A, H52A mutants, respectively. Purified mutants were finally dialysed against storage buffer of pH 4.0. The electrophoretic purity of both

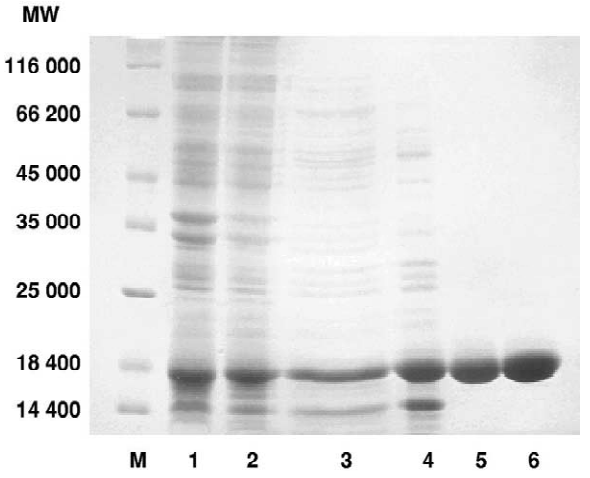

Fig. 2. SDS–PAGE analysis of rhG-CSF (C17S, H43A) samples (12% polyacrylamide gel, stained by Coomassie Brilliant Blue R-250). M, molecular mass markers. Lanes: 1, inclusion-bodies solubilized in 10 m*M* Tris–HCl, pH 7.0, 7 *M* GdmHCl; 2, after renaturation with 20 μ*M* $CuSO_4$ in 5.4 *M* GdmHCl, pH 5.4; 3, after desalting over Sephadex G-25; 4, pool of fractions recovered from Sepharose–LR Yellow 2KT–Cu(II) and dialysed against 25 m*M* Na–acetate, pH 5.4; 5, pool of fractions recovered from SP-Sepharose FF with a linear 0–500 m*M* NaCl gradient (0.13 *M* NaCl); 6, dialysis against 10 m*M* Na–acetate, pH 4.0. For lanes 1–2, 3–4 and 5–6, the amounts of total protein were 20, 13 and 10 μg, respectively.

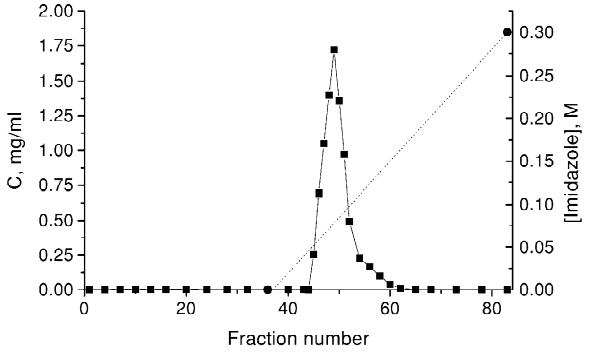

Fig. 3. Elution of rhG-CSF (C17S, H43A) from Sepharose–LR Yellow 2KT–Cu(II). Forty millilitres (bed volume) of LR Yellow 2KT gel was charged with Cu(II) (4.2 μmol/ml). Refolded rhG-CSF (C17S, H43A) [88.9 mg (0.57 mg/ml, 156 ml)] in 25 m*M* Tris–maleic acid–NaOH, pH 6.5, 0.25 *M* Na_2SO_4. Linear velocities at loading, washing and elution were 15, 40 and 25 cm/h, respectively. Fraction volume at elution was 8.5 ml. Adsorbed protein was developed with a linear gradient to 300 m*M* of imidazole (fraction 36–82). ■, protein concentration (mg/ml); ●, imidazole concentration (*M*).

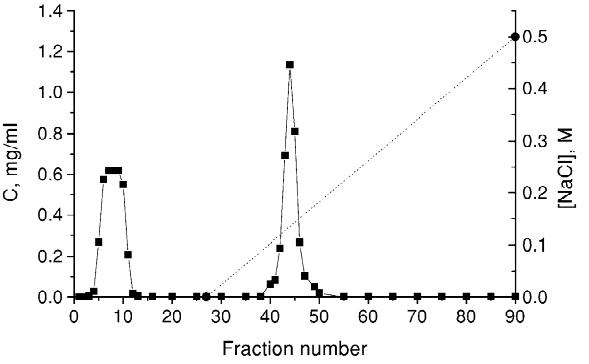

Fig. 4. Elution of rhG-CSF (C17S, H43A, H52A) from SP-Sepharose FF. rhG-CSF (C17S, H43A, H52A) [73.1 mg (0.87 mg/ml, 84 ml)] was recovered from Sepharose–LR Yellow 2KT–Cu(II) and dialysed against 25 m*M* Na–acetate, pH 5.0 was loaded onto 40 ml (bed volume) of SP-Sepharose FF in 25 m*M* Na–acetate buffer, pH 5.0. Linear velocities at loading, washing and elution were 20, 40 and 25 cm/h, respectively; fraction volume at elution was 6.25 ml. Adsorbed protein was developed with a linear gradient to 500 m*M* of NaCl (fraction 27–90). ■, protein concentration, (mg/ml); ●, NaCl concentration (*M*).

rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A mutant at this formulation is shown in Fig. 5. Only one visible band for purified proteins and reference sample is observed in a silver-stained gel

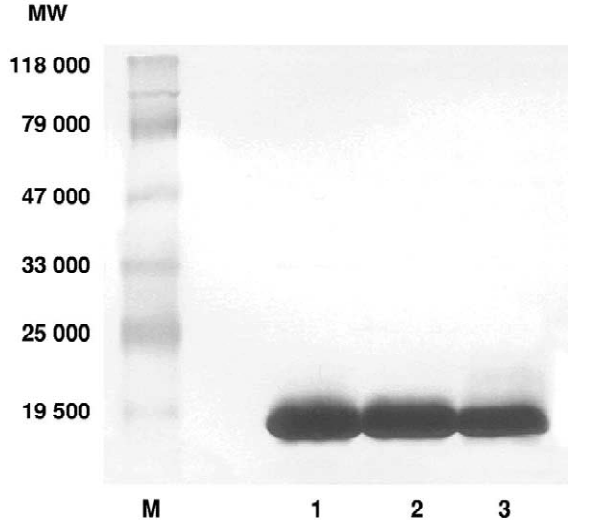

Fig. 5. SDS–PAGE analysis of purified rhG-CSF (C17S) histidine mutants (12% polyacrylamide gel, stained by Ag). M: pre-stained molecular mass markers. Lanes: 1, rhG-CSF (C17S, H43A); 2, rhG-CSF (C17S, H43A, H52A); 3, rhG-CSF standard. For each lane the amount of protein was 2 μg.

(Fig. 5, lanes 1–3). In addition to RP-HPLC and SDS–PAGE analysis, the purity of rhG-CSF histidine mutants was estimated by isoelectric focusing gel electrophoresis. It is seen from Fig. 6, that purified rhG-CSF mutants are homogenous—one band only for each lane containing samples of H43A (lanes 3–5) and H43A, H52A (lanes 6–8) mutant is visible as well as for a reference sample (lane 2). However, the pattern in Fig. 6 clearly indicated, that histidine mutations resulted in a lowering of the value of isoelectric points of rhG-CSF—p*I* values of 5.84 and 5.66 were determined for H43A and for H43A, H52A mutants, respectively.

The samples of purified rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) were also analyzed by mass spectrometry (ESI MS). The determined molecular mass (M_r) values of 18 715.00 and 18 648.03 were in good correlation with the calculated M_r values—18 716.76 and 18 650.70 for rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A), respectively.

The proposed purification scheme for histidine mutant of rhG-CSF (C17S) consisting of oxidative refolding of the solubilized inclusion bodies with Cu^{2+} ions with the further two-step chromatographic purification of renatured proteins by immobilized metal ion affinity chromatography on Sepharose–LR Yellow 2KT–Cu(II) and SP-Sepharose FF ensured effective isolation of the target protein from the majority of contaminating E.coli proteins as well as from misfolded species and allowed to obtain highly purified mutated form of rhG-CSF.

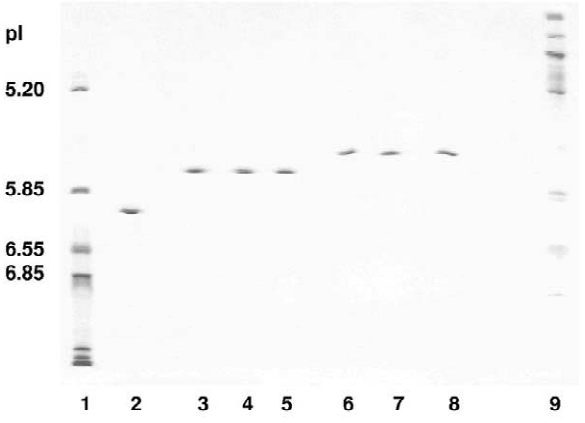

Fig. 6. Isoelectric focusing gel electrophoresis of purified rhG-CSF (C17S) histidine mutants (stained by Coomassie Brilliant Blue R-250). Lanes: 1, high p*I* markers (5.0–10.5) (Pharmacia); 2, rhG-CSF standard, 5 μg; 3–5, rhG-CSF (C17S, H43A), 5 μg; 6–8, rhG-CSF (C17S, H43A, H52A), 5 μg; 9, low p*I* markers (2.5–6.5), (Pharmacia).

3.2. Immobilized metal ion affinity partitioning of purified rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A)

The two-phase system composed of 5% (w/w) PEG and 8% (w/w) dextran was selected for all partitioning experiments because the lowest partition constant for rhG-CSF was determined in this system [11]. Data presented in Table 2 indicated, that the partition coefficients (log *K* value) for rhG-CSF mutants slightly increased in the ''empty'' system containing PEG–demetallized dye due to the increase of the number of His mutations from 0.11 for the protein form without histidine mutation to the values of 0.28 and 0.38 for H43A and for H43A, H52A mutants, respectively. The log *K* value for protein in the systems without demetallized dye were also found to follow the same tendency (data not shown here). This indicates, that His replacement for alanine may result in a slight increase of protein hydrophobicity.

Partitioning data of rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) in the two-phase systems containing Cu(II), Ni(II) and Hg(II) ions chelated by the LR Yellow 2KT–PEG at pH 7.0 are summarized in Table 2 and Fig. 7. It is seen that in the phase systems containing chelated Cu(II) ions

Table 2
Partitioning of purified rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) in the presence of metal ions chelated by LR Yellow 2KT–PEG at pH 7.0[a]

M^{2+}	C17S[b]		C17S, H43A		C17S, H43A, H52A	
	log *K*	Δ log *K*	log *K*	Δ log *K*	log *K*	Δ log *K*
–	0.11	–	0.28	–	0.38	–
Cu^{2+}	2.49	2.38	2.34	2.06	1.87	1.49
Ni^{2+}	1.10	0.99	1.29	1.01	0.79	0.41
Hg^{2+}	1.40	1.29	1.72	1.44	1.75	1.37

[a] Two-phase system (4 g) contained 5% (w/w) PEG 6000, 8% (w/w) dextran 60000, 0.8 mg of protein, 0.25 *M* Na_2SO_4 and 50 m*M* Hepes–NaOH buffer, pH 7.0. The amount of demetallized dye or metal ion was 800 μmol per kg of the two-phase system.
[b] For comparison, partitioning data of rhG-CSF (C17S) from the earlier work [11] are presented.

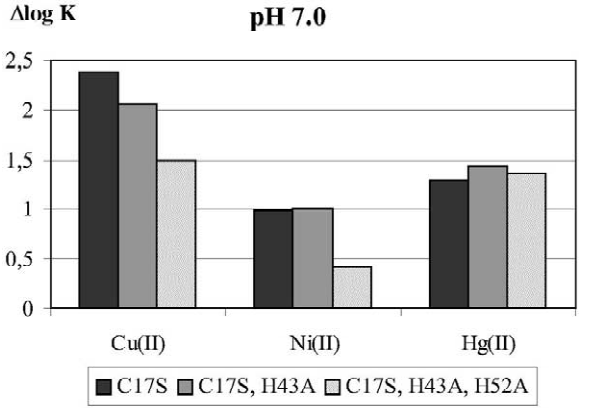

Fig. 7. Partitioning of purified rhG-CSF mutants—C17S, H43A and C17S, H43A, H52A—in the presence of metal ions chelated by LR Yellow 2KT–PEG at pH 7.0. Two-phase systems (4 g) contained 5% (w/w) PEG 6000, 8% (w/w) dextran 60000, 0.8 mg of protein, 0.25 *M* Na_2SO_4 and 50 m*M* HEPES–NaOH buffer, pH 7.0. Metal ion concentration was 800 μmol per kg of the two-phase system. For comparison, data from the earlier work [11] for purified rhG-CSF (C17S) partitioning are presented.

the Δ log *K* value for H43A mutant reached 2.06, which lower but comparable with a Δ log *K* value of 2.38 for the protein form without His replacement that has been recently determined [11]. In contrast to that, mutation of the second histidine—His^{52}—has led to the decrease in the Δ log *K* value to 1.49. Such alteration of the Δ log *K* value is obviously related to the number of histidine mutations in the protein molecule, and confirmed the evidence, that $His^{43}$and His^{52} are surface-exposed, but His^{52} is more important for interaction. Partitioning data of rhG-CSF histidine mutants in the systems containing Cu(II) ions at pH 7.0 are in agreement with the behaviour of these forms of protein on Sepharose–LR Yellow 2KT–Cu(II) column at pH 6.5, where the weaker retention of rhG-CSF (C17S, H43A, H52A) form was observed (see Section 3.1).

Partitioning of rhG-CSF histidine mutants in the presence of Ni(II)–LR Yellow 2KT–PEG at pH 7.0 showed (Table 2, Fig. 7), that the replacement of His^{43} had no effect on rhG-CSF (C17S, H43A) mutant interaction with Ni(II) ions because the Δ log *K* value of 0.99 is the same as that found for rhG-CSF (C17S) protein (Δ log K=1.01, Table 2) without His mutation. Once again, the Δ log *K* value for rhG-CSF (C17S, H43A, H52A), containing two mutated His was found to be only 0.41, i.e. approximately 2.4 times lower than that for the H43A variant. However, the comparison of the partitioning data depending on the number of mutated His residues in the presence of Ni(II) ions compared to Cu(II) indicated, that the strength of interaction with Ni(II) ions was weaker approximately 2 and 3.6 times for protein with one and two His mutations, respectively. This clearly shows the greater contribution of His^{52} for the interaction with Ni(II) than Cu(II) ions and let us to define it as a critical for metal binding.

Partitioning of rhG-CSF histidine mutants was also evaluated in the presence of Hg(II) ions chelated by LR Yellow 2KT–PEG at pH 7.0 and data was presented in Table 2 and Fig. 7. It is seen that the Δ log *K* values for H43A and for H43A, H52A mutants of 1.44 and 1.37, respectively, are equal and do not differ substantially from the Δ log *K* value of 1.29 found earlier [11] for the partitioning of protein without His mutation. Thus, His^{43} and His^{52} do not play any important role for the interaction of rhG-CSF (C17S) form with chelated Hg(II) ions.

According to the value of Δ log *K* determined for both rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) mutants by their partitioning in two-phase systems containing chelated metal ions the following row for the strength of interaction (pH 7.0) was established: Ni(II) < Hg(II) < Cu(II).

Our earlier studies [11] on rhG-CSF and rhG-CSF (C17S) form partitioning in the two-phase systems containing Cu(II)–LR Yellow 2KT at pH 5.0 revealed the residual interaction of the proteins with Cu(II) ions (Δ log *K* 1.73–1.78). Therefore, the partitioning of rhG-CSF histidine mutants in the presence of demetallized dye and Cu(II)–dye complex at pH 5.0 was evaluated (Table 3). As can be seen from Table 3 the mutation of His resulted in a slight increase of Δ log *K* for hG-CSF forms in the presence of demetallized dye from 0.20 (for the form without His mutation) to 0.27 and 0.44 for protein with one and two His mutation, respectively. In the presence of immobilized Cu(II) ions the Δ log *K* values of 1.73, 1.61, and 0.83 were determined for the protein form without His mutation, with one and two His mutations, respectively. The lowest Δ log *K* value found for the partitioning of the protein with two His^{43} and His^{52} mutations agreed with the

Table 3
Partitioning of purified rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) in the presence of metal ions chelated by LR Yellow 2KT–PEG at pH 5.0[a]

M^{2+}	C17S[b]		C17S, H43A		C17S, H43A, H52A	
	log *K*	Δ log *K*	log *K*	Δ log *K*	log *K*	Δ log *K*
–	0.20	–	0.27	–	0.44	–
Cu^{2+}	1.93	1.73	1.88	1.61	1.27	0.83

[a] Composition of the two-phase system was similar to that in Table 2, except for the buffer—50 m*M* MES–NaOH, pH 5.0.
[b] For comparison, partitioning data of rhG-CSF (C17S) from the earlier work [11] are presented.

tendency of partition coefficient's alteration determined for interaction of this form with Cu(II) ions at pH 7.0 (Table 3) and lead to the assumption that His^{52} may be involved in the protein interaction with immobilized Cu(II) ions at pH 5.0, too. This assumption is supported by an analogous event recently described [30] for the interaction—exposed His of D-xylose isomerase–Cu(II)–IDA (iminodiacetate)–Sepharose at slightly acidic conditions.

3.3. Partitioning of rhG-CSF (C17S) histidine mutants in the presence of imidazole at pH 7.0

The interaction of rhG-CSF (C17S) histidine mutants with Cu(II), Ni(II) and Hg(II) ions, chelated by LR Yellow 2KT–PEG derivative was studied in the two-phase systems, containing imidazole at pH 7.0 (Table 4, Figs. 8–10).

Data in Table 4 indicate, that the introduction of imidazole, which competes with metal ions chelated by PEG–dye derivative caused a decrease in Δ log *K* for histidine mutants in the presence of all metal ions studied. The introduction into the phase systems of 1 m*M* concentration of imidazole resulted in a decrease of 53 and 43% of the Δ log *K* value for rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) in the presence of Cu(II) ions (Table 4, Fig. 8), and in a decrease of 11 and 20% for rhG-CSF (C17S) mutant with one and two mutated His in the presence of Ni(II) ions, respectively (Table 4, Fig. 9). The presence of 1 m*M* imidazole in the system with Hg(II) resulted in the same decrease in Δ log *K*

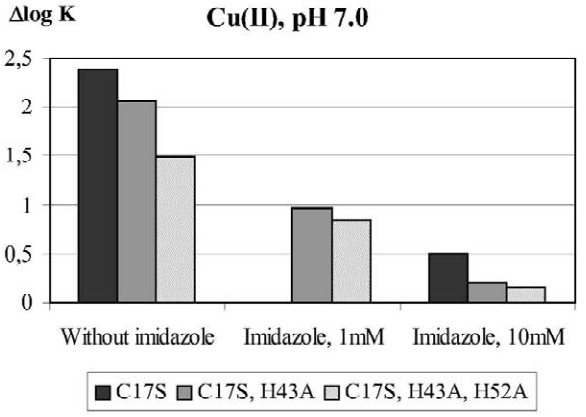

Fig. 8. Effect of imidazole on the partitioning of purified rhG-CSF mutants—C17S, H43A and C17S, H43A, H52A in the presence of Cu^{2+} ions—chelated by LR Yellow 2KT–PEG at pH 7.0. System composition and Cu^{2+} concentration were similar to those in Fig. 7. For comparison, data from the earlier work [11] for purified rhG-CSF (C17S) partitioning are presented.

Table 4
Partitioning of purified rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) in aqueous two-phase systems containing metal ions chelated by LR Yellow 2KT–PEG in the presence of imidazole at pH 7.0[a]

M^{2+}	Imidazole concentration (m*M*)	Δ log *K* reduction (%)		
		C17S[b]	C17S, H43A	C17S, H43A, H52A
Cu^{2+}	1	n.d.	53	43
	10	79	90	90
Ni^{2+}	1	n.d.	11	20
	10	65	58	66
Hg^{2+}	1	n.d	44	45
	10	81	81	95

[a] The system composition and metal ion concentration were similar to that in Table 2.
[b] For comparison, partitioning data of rhG-CSF (C17S) from the earlier work [11] are presented.

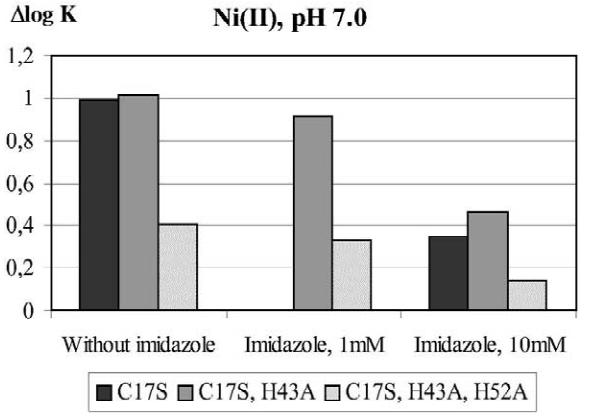

Fig. 9. Effect of the imidazole on the partitioning of purified rhG-CSF mutants—C17S, H43A and C17S, H43A, H52A in the presence of Ni^{2+} ions—chelated by LR Yellow 2KT–PEG at pH 7.0. System composition and Ni^{2+} concentration were similar to those in Fig. 7. For comparison, data from the earlier work [11] for purified rhG-CSF (C17S) partitioning are presented.

(44–45%) for both mutated forms of rhG-CSF (C17S) (Table 4, Fig. 10). The introduction of imidazole at a 10 m*M* concentration into phase systems caused the greater reduction in Δ log *K* for all rhG-CSF mutants, as shown in Table 4 and Figs. 8–10, and its Δ log *K*-reducing ability followed the same tendency as observed for 1 m*M* imidazole. Within the range of chelated Cu(II) and Ni(II) ions, the Δ log *K* values are reduced with 10 m*M* imidazole by 90% in the presence of Cu(II) and 58–66% in the presence of Ni(II) ions for rhG-CSF (C17S) mutant with one and two His mutations, respectively. The residual interactions of His mutant with Cu(II) ions is reduced with 10 m*M* imidazole to a greater extent than with Ni(II) ions. Thus, imidazole, irrespective of its concentration—1 or 10 m*M*—was the more effective Δ log *K*-reducing agent in the systems containing Cu(II) ions compared to that with Ni(II). The reason for this may be related to the larger coordination sphere of Ni^{2+} in comparison to Cu^{2+} ions. PEG–LR Yellow 2 KT derivative donated in our case three donor groups for metal ions, but in the chelated state Ni(II) has three while Cu(II) ions have only one residual coordination bond. Owing to this difference and the lack of two surface-exposed His^{43} and His^{52} Ni(II) ions may be involved in binding the larger number of other donor groups on the surface of rhG-CSF mutants than Cu(II) ions. Thus, even a 10-m*M* imidazole concentration in the phase system might be insufficient to completely reduce such interactions. In the presence of chelated Hg(II) ions, 10 m*M* imidazole resulted in decreases of Δ log *K* by 81 and 95% for the H43A and H43A, H52A mutants, respectively (Table 4, Fig. 10).

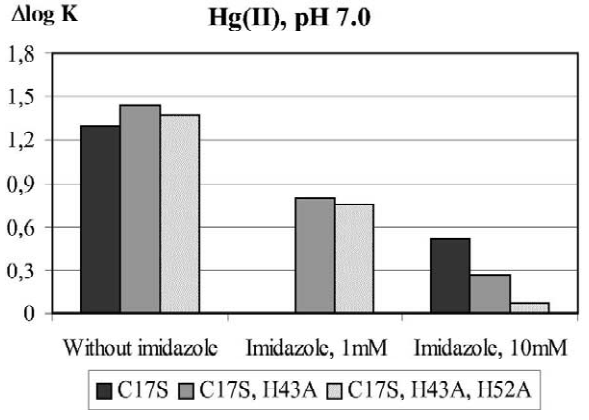

Fig. 10. Effect of the imidazole on the partitioning of purified rhG-CSF mutants—C17S, H43A and C17S, H43A, H52A in the presence of Hg^{2+} ions, chelated by LR Yellow 2KT–PEG at pH 7.0. System composition and Hg^{2+} concentration were similar to those in Fig. 7. For comparison, data from the earlier work [11] for purified rhG-CSF (C17S) partitioning are presented.

3.4. Partitioning of rhG-CSF (C17S) histidine mutants from inclusion bodies extracts in the presence of PEG–LR Yellow 2KT–Ni(II) and Hg(II) complex

The partitioning of rhG-CSF (C17S) histidine mutants from solubilized inclusion bodies was studied in the presence of chelated Ni(II) and Hg(II) ions. Studies with chelated Hg(II) ions were performed for comparison, because the rhG-CSF (C17S) form contains, and both His mutant can contain in inclusion body solubilizate species with free –SH groups, that might interact, as we showed earlier [11] with chelated Hg(II) ions. Data on partitioning of solubilized inclusion bodies of rhG-CSF mutants in the two-phase systems at pH 7.0 are summarized in Table 5. It can be seen from Table 5, that the yields of protein partitioning were within 21–28% in the systems with Ni(II) and within 85–

Table 5
Partitioning of inclusion bodies extract of rhG-CSF (C17S, H43A) and rhG-CSF (C17S, H43A, H52A) in the presence of metal ions chelated by LR Yellow 2KT at pH 7.0[a]

M^{2+}	C17S[b]		C17S, H43A		C17S, H43A, H52A	
	$\Delta \log K$	Y[c] (%)	$\Delta \log K$	Y (%)	$\Delta \log K$	Y (%)
Ni^{2+}	n.c.	26	n.c.	28	n.c.	21
Hg^{2+}	2.26	98	2.13	101	2.41	85

n.c., not calculated.
[a] Two-phase system (4 g) contained 5% (w/w) PEG 6000, 8% (w/w) dextran 60000, 0.8 mg of total protein, 800 μmol/kg of chelated metal ion, 0.25 *M* Na_2SO_4 and 50 m*M* HEPES–NaOH buffer, pH 7.0.
[b] For comparison, partitioning data of rhG-CSF (C17S) inclusion bodies extract from the earlier work [11] are presented.
[c] Partitioning yield.

101% in the systems with Hg(II) ions. In the phase systems, containing Ni(II) ions the major part of introduced inclusion body protein formed a film-like layer at the interface of PEG and dextran-rich phases of the two-phase system. Therefore, in such cases the protein partition constant K was not calculated. In the systems containing Hg(II) ions the proteins from introduced inclusion bodies partitioned between PEG and Dx phases. This difference reflected a different mode of protein interaction with PEG–dye–Ni(II) and PEG–dye–Hg(II) complexes when inclusion bodies extract of rhG-CSF (C17S) and its histidine mutant partitioned. The $\Delta \log K$ value for rhG-CSF (C17S) histidine mutants inclusion bodies extract partitioning in the systems containing Hg(II) ions were determined to be 2.13 and 2.41 for the protein with one and two histidine mutations, respectively, and are close to the $\Delta \log K$ value of 2.26 that has been found for the rhG-CSF (C17S) form. Thus, the magnitude of $\Delta \log K$ was not related to the number of His mutations in the rhG-CSF (C17S) molecule. The comparison of $\Delta \log K$ value after partitioning of inclusion-bodies extract (Table 5) and purified rhG-CSF (C17S) mutants (Table 2) in the systems containing Hg(II) ions showed about a 2-fold increase of $\Delta \log K$ values in favour of the protein forms from their respective inclusion bodies. This fact may be explained assuming that a protein form with one disulphide bridge may be present in the inclusion-bodies extract at pH 7.0 similarly, as we proposed in Ref. [11]. The correctly folded form of native rhG-CSF (C17S) has no free –SH group. Thus, the effect of two free –SH groups may outweigh the effect of accompanying *E. coli* proteins since both SDS–PAGE and RP-HPLC analysis indicated a relative amount of the targeted protein forms in the inclusion-bodies extract of 75–91%.

We have also examined the effect of refolding of rhG-CSF (C17S) histidine mutant forms in the inclusion body extracts partitioning experiments in the presence of chelated Ni(II) and Hg(II) ions at pH 7.0. The relative amount of correctly folded protein conformation was evaluated using RP-HPLC analysis of protein samples taken from both the interface and the dextran-rich phase of the two-phase systems. RP-HPLC analysis data are exemplified in Fig. 11 for rhG-CSF (C17S, H43A, H52A) inclusion body extract partitioning in the system containing Ni(II) ions. Fig. 11 shows, that in the initial inclusion body

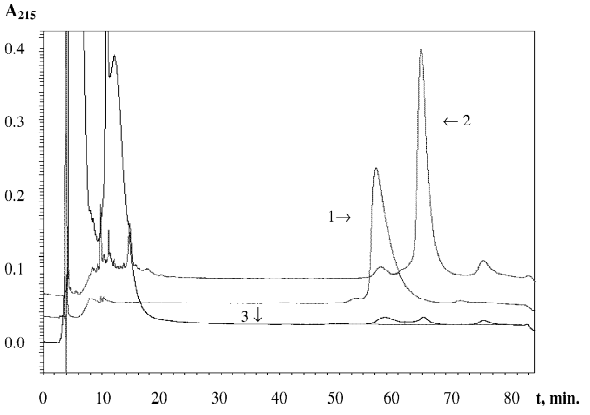

Fig. 11. RP-HPLC analysis of rhG-CSF (C17S, H43A, H52A) samples. 1, purified rhG-CSF (C17S, H43A, H52A) in 10 m*M* Na–acetate, pH 4.0, 0.96 mg/ml, 50 μl; 2, inclusion bodies extract, 20 m*M* HEPES–NaOH buffer, pH 7.0, 2.27 mg/ml, 100 μl; 3, sample from the dextran phase of the two-phase system (5% PEG, 8% dextran, 800 μmol/kg Ni^{2+} chelated by LR Yellow 2KT–PEG, 50 m*M* imidazole, 0.25 *M* Na_2SO_4, 50 m*M* HEPES–NaOH, pH 7.0), 0.05 mg/ml, 900 μl.

extract the folding intermediates with the reduced Cys^{36}–Cys^{42} or Cys^{64}–Cys^{74} disulfide bond predominate since the retention time is higher than that for the native protein. Fig. 11 shows that the relative amount of correctly folded protein conformation is negligible (curve 2). After partitioning of rhG-CSF (C17S, H43A, H52A) inclusion body extract in the phase systems containing Ni(II) ions, the relative amount of correctly folded conformation in the sample drawn from the lower dextran-rich phase increased and was higher compared to that as for folding intermediates (curve 3). In all two-phase systems studied where protein amount in the interface was sufficient for analysis, the amount of correctly folded protein was found to be higher in the dextran-rich bottom phase than in the interface layer (Table 6). In the systems containing Ni(II) ions, the relative amount of correctly folded rhG-CSF (C17S, H43A) was found to be 67% and it was approximately 1.7 times higher than the value of 39% determined for rhG-CSF (C17S, H43A, H52A). These values of the correctly folded form of rhG-CSF (C17S) histidine mutants found in dextran-rich phase of the systems with Ni(II) ions (Table 6) are closely related to the partitioning behavior of purified proteins (Table 2). As it was noted (Table 2), the Δ log K value of purified rhG-CSF (C17S) mutants in the systems containing Ni(II) ions were 0.99, 1.01 and 0.41 for the protein without His mutation, with one and two His mutations, respectively (Table 2). It seems, that the relative amount of correctly folded protein is approximately proportional to the strength of protein interaction with Ni(II) complex of LR Yellow 2KT–PEG. For example, His^{43} replacement has no effect on the magnitude of partition constant for purified protein in the presence of Ni(II) ions so the amount of correctly folded protein was close to that as for hG-CSF (C17S) form without His mutation. However, the additional His^{52} mutation resulted in an approximately 2.4-fold decrease in Δ log K value for purified rhG-CSF (C17S, H43A, H52A) compared to rhG-CSF (C17S) (Table 2), and this led in approximately 1.4–1.5-fold decrease in the amount of correctly folded protein (Table 6). An analogous correlation was observed in our earlier investigations [11] on the studies of the interaction and refolding of rhG-CSF and $(His)_6$-rhG-CSF in the two-phase systems, containing Ni(II) ions. The affinity of $(His)_6$-rhG-CSF for chelated Ni(II) ions at pH 7.0 was found to be more than two times higher compared to that as for rhG-CSF and the amount of correctly folded protein found after partitioning of inclusion body extracts was higher, i.e. 60% for His-tagged variant of rhG-CSF, compared to 46% found for rhG-CSF [11].

The strength of interaction of rhG-CSF forms with Ni(II) at pH 7.0 is mainly governed by His residues exposed to the surface, so for formation of a strong complex two neighboring His residues are necessary. The visualisation of rhG-CSF structure revealed, that two of the surface histidines—His^{52} and His^{156}—are in close vicinity and may be coordinated by a single Ni(II) ion. The marked decrease in the strength of rhG-CSF (C17S, H43A, H52A) mutant interaction with Ni(II) ions due to the replacement of His^{52} with alanine strongly supports this assumption. The decrease of binding strength of rhG-CSF (C17S, H43A, H52A) to Ni(II) chelate may lead to the less re-

Table 6
Dependence of relative amount of correctly folded rhG-CSF (C17S) histidine mutants on histidine mutations and metal ion

RhG-CSF mutant	Metal ion	Imidazole (mM)	Relative amount of correctly folded protein (%)	
			Interface	Dextran phase
C17S[a]	Ni^{2+}	50	27	56
C17S, H43A			b	67
C17S, H43A, H52A			b	39
C17S[a]	Hg^{2+}	200	90	99
C17S, H43A			48	94
C17S, H43A, H52A			40	76

[a] For comparison, relative amounts of correctly folded rhG-CSF (C17S) from the earlier work [11] are presented.
[b] Protein concentration was less than RP-HPLC detection limit.

stricted protein conformational freedom which in turn may result in a lower protein refolding yield compared to the form of protein with one His mutation.

When inclusion body extracts of rhG-CSF (C17S) mutated forms were partitioned in the systems containing Hg(II) ions, the significant part of the inclusion body proteins were partitioned after the addition of imidazole in favour of the interface of PEG–Dx system. RP-HPLC analysis of samples drawn from this series of experiments demonstrated, as shown in Table 6 the higher relative amount of correctly folded protein found in the lower dextran-rich phase, compared to that of the interface. For example, the relative amount of correctly folded protein in the dextran-rich phase of the system with Hg(II) was 94 and 76% for rhG-CSF (C17S) form with one and two histidine mutations, respectively. The amount of correctly folded protein for the sample of rhG-CSF (C17S) drawn from an analogous experiment was 99%, so this value is very close to that as for His^{43} mutation, and approximately 1.3 times higher than that for protein with two His mutation. The amounts of correctly folded protein at the interface of the two-phase system when partitioning of inclusion body extracts was performed in the presence of Hg(II) ions were 48 and 40% for rhG-CSF (C17S) with one and two histidine mutation, respectively. For comparison, the amount of correctly folded protein for the analogous sample of rhG-CSF (C17S) variant was found 90% [11], i.e. approximately two times higher compared to that as for hG-CSF (C17S) variants with one or two histidine mutations. During inclusion body extract partitioning, part of the proteins is prone to aggregate and precipitate at the interface of two-phase systems. These events might be caused by binding of non-polar and polar regions of the protein to a polymer and salt ions, respectively. It has been reported [31] that PEG may weakly bind to hydrophobic regions of unfolded and partially folded proteins. In the denatured state it is easier for salt ions to penetrate into the interior of the protein for binding to its charged residues. The overall effect of both interactions may destabilize the protein structure driving it to aggregation.

The replacement of His residue for alanine may change protein surface properties by increasing for example, surface hydrophobicity. The occurrence of such an event was assumed on the basis of a slight increase of the log K value for rhG-CSF (C17S) histidine mutants in an ''empty'' two-phase system without the presence of chelated metal ions. The increase of log K is in the order C17S<C17S, H43A<C17S, H43A, H52A was observed at both pH 7.0 (Table 2) and pH 5.0 (Table 3). If such alteration of surface hydrophobicity exists, this may result in a higher degree of aggregation of partially folded intermediates by lowering protein refolding efficiency and partitioning yield.

To sum up, the performed studies demonstrate the potential of aqueous two-phase systems containing chelated metal to detect changes on the surface of rhG-CSF molecule resulted from His mutations, and evaluate the influence of those mutations on the refolding efficiency of the protein.

4. Conclusions

The rapid and convenient scheme for the recovery and purification of rhG-CSF (C17S) histidine mutants, based on immobilized metal ion affinity and cation-exchange chromatography was developed. It allowed to prepare correctly folded and highly purified rhG-CSF mutants in quantities of tens of milligrams.

Partitioning behaviour of rhG-CSF (C17S) histidine mutants in aqueous two-phase systems modified with complexes of PEG–LR Yellow 2KT revealed that His^{43} and His^{52} residues are involved in the protein interaction with chelated Cu(II) ions. In the systems containing chelated Hg(II) ions, histidine mutations had no effect on protein partitioning and caused only a slight decrease in the amount of correctly folded protein when respective inclusion bodies were partitioned.

His^{52} was of crucial importance for interaction of the rhG-CSF (C17S) protein form with chelated Ni(II) ions and refolding by partitioning its inclusion body extract. Different mode of interaction of rhG-CSF histidine mutants with chelated Ni(II) and Hg(II) ions resulted in the difference of refolding efficiency when respective inclusion bodies extracts of rhG-CSF (C17S) histidine mutants were partitioned. The importance of His^{52} in the rhG-CSF

(C17S) interaction with Ni(II) ions and refolding allowed to propose a possible involvement of spatially adjacent His^{52} and His^{156} in coordination with chelated Ni(II) ions and the importance of such interactions for Ni(II)-assisted refolding.

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