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Comparative studies of recombinant human granulocyte-colony stimulating factor, its Ser-17 and $(His)_6$-tagged forms interaction with metal ions by means of immobilized metal ion affinity partitioning
Effect of chelated nickel and mercuric ions on extraction and refolding of proteins from inclusion bodies

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Abstract

The chelation capability of the reactive dye Light Resistant Yellow 2KT towards metal ions, particularly mercury(II) was evaluated in the pH range 5.0–7.0, and it was shown that the dye–Hg(II) complex has a free site for the interaction with human recombinant granulocyte-colony stimulating factor (rhG-CSF) from *Escherichia coli*. Affinity partitioning of three rhG-CSF forms – native, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF was studied in aqueous two-phase systems, which contained metal ions – Cu(II), Ni(II) and Hg(II) – chelated by dye–poly(ethylene glycol) at pH 5.0 and 7.0, in the presence or absence of many selected agents. It was determined, that chelated Ni(II) ions exhibited stronger interaction with the hexahistidine-tagged protein form, while the extraction power of Cu(II) ions was found to be of comparable order of magnitude for all three protein forms at pH 7.0. A comparative study of rhG-CSF and both its forms partitioning in the presence of chelated Hg(II) ions at pH 7.0 and 5.0 revealed possible direct interaction between Hg(II) ions and unpaired Cys-17 of rhG-CSF. The partitioning of three rhG-CSF forms inclusion body extract was studied in the presence of chelated Ni(II) and Hg(II) ions thus explaining the efficiency of targeted proteins renaturation gained upon their inclusion body forms interactions with chelated metal ions.

Keywords: Immobilized metal affinity chromatography; Affinity adsorbents; Immobilized metal affinity partitioning; Partition coefficients; Proteins; Metal chelates; Poly(ethylene glycol); Dextran

1. Introduction

Expression of recombinant protein in *Escherichia coli* often leads to the formation of inactive aggregates (inclusion bodies) instead of the native protein

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[1,2]. These inclusion bodies can usually be solubilized with chaotropic agents, which unfold the protein macromolecule. Therefore, unfolded protein has to be refolded under strictly controlled conditions to the correct three-dimensional conformation of native and biologically active protein. Currently, various steps and methods have been developed for the recovery and in vitro folding (renaturation) of inclusion body proteins [2–9].

The efficiency of the renaturation process is thought to result from the kinetic competition between the correct folding pathway of partially folded intermediates that leads to an active native protein, and unproductive misfolding as well as aggregation [6,7,10]. To disentangle a correctly folded protein from its misfolded or aggregated forms, and to increase the yield of renaturation several chromatographic techniques, e.g., ion-exchange [11,12], high-performance liquid chromatography (HPLC) operating in the hydrophobic interaction mode [13] and size-exclusion chromatography [14–16] have been proposed. Procedures of proteins renaturation in reversed micelles [5,6,8], or in aqueous two-phase systems [17] have also been explored.

Recently, the strategy of fusion technique has been exploited for the iterative refolding of the hexahistidine tail containing eukaryotic proteins or protein domains upon reversible immobilization of denatured proteins on an Ni(II)–NTA (nitrilotriacetic acid) column with their subsequent renaturation by using a pair of reduced/oxidised glutathione [18]. The Ni(II)–NTA column was successfully used for renaturation of human pulmonary surfactant protein B [19], recombinant human proMMP-7 [20], and human prion proteins [21] expressed in *E. coli* as hexahistidine tail fusion proteins. Proteins were bound onto the column via a His tag under denaturing conditions and after the removal of denaturant were recovered in a refolded native state. It was also shown [7,22] that the fusion protein, which consisted of α-glucosidase and N- or C-terminal hexaarginine peptide, could interact in denatured state with heparin-Sepharose and refold after the removal of denaturant.

Studies on the interaction of proteins having engineered into them dihistidine metal-chelating motifs with immobilized metal ions performed by the group of Arnold [23–25] enabled one to show that metal ions can shift the thermodynamic folding–unfolding equilibrium for a peptide or protein by binding preferentially to the folded form. Thus metal ions can stabilise the existing or incipient structures. In accordance with this we have recently investigated the refolding possibilities of several recombinant proteins, that possessed either the naturally existing metal binding sites or were rich in histidine residues in the polypeptide chain by the procedure of immobilized metal ion affinity chromatography (IMAC). We showed [26,27], that human recombinant proteins such as interleukin-3 (IL-3, Zn-binding site), granulocyte colony (G-CSF, five His residues and unpaired Cys^{17}), and granulocyte macrophage colony (GM-CSF, three His residues) stimulating factors, all expressed in *E. coli* as inclusion body proteins, could be retained in denatured state by Sepharose-iminodiacetate (IDA) gels charged with Zn(II) or Ni(II) ions at an intermediate guanidine hydrochloride concentration in a loading buffer, and recovered by elution with a buffer containing imidazole in the correctly folded and native state with a protein-dependent yield. In addition, we recently demonstrated [28], that mercuric ions chelated by IDA gel are also able to extract rhG-CSF, if its solubilized inclusion bodies are being chromatographied. The target protein recovered from a column in the presence of β-mercaptoethanol or imidazole was presumed to be correctly folded, where free-reduced SH groups would be expected to be involved in the interaction with chelated mercury(II); though the studies of chromatographic behaviour of rhG-CSF and its chemically-modified forms on Hg(II)-loaded IDA gel did not establish, whether the unpaired cysteine of rhG-CSF was directly involved in the interaction with chelated Hg(II) ions.

More thorough studies on the interactions of rhG-CSF, its Ser-17 and $(His)_6$-tagged forms with chelated Hg(II), Cu(II) and Ni(II) ions, and studies of the refolding efficiency of this protein as a function of metal ion type were therefore performed by means of immobilized metal ion affinity partitioning (IMAP) and are now described. We were motivated in such studies for the following reasons. It was thought that comparison of the interactions of rhG-CSF and chelated metal ions, particularly, Hg(II) ions with that of its [$Cys^{17} \rightarrow Ser^{17}$] mutated form

might explain the role of unpaired Cys^{17}. The hexahistidine-tagged form might help to explore differences obtained in refolding efficiency when rhG-CSF internal His residues or the N-terminal $(His)_6$ tag were involved in the interaction with chelated metal ions, particularly, Ni(II) ions. We thought the chosen technique of IMAP might be appropriate for such studies since it has been previously used [23,29–31] as a sensitive tool to probe metal-binding sites and distinct surface differences of closely related proteins [23], cells [29] or enzymes [30,31]. In addition, the partitioning procedures are less time consuming, and the ability to add agents to the phase systems in order to alter the partitioning of the target biomolecule, etc., make the study of the binding features of rhG-CSF forms with metal ions easier. IMAP also provides an alternative tool for the study of refolding events of partitioned inclusion body protein forms because of the ability to analyse a protein in both phases and also at the interface between the phases, in contrast to IMAC, which just provides information on formed misfolded and/or aggregated species of the target protein.

2. Experimental

2.1. Materials

All cloning, DNA preparation, transformation and expression procedures were performed according to Ref. [32]. All enzymes, kits and DNA markers were from MBI Fermentas (Lithuania). Initial human G-CSF gene [33] was modified by polymerase chain reaction (PCR) by the introduction of NdeI and BamHI sites into the 5′- and 3′-ends of this gene, respectively, using synthetic primers. The amplified fragment was ligated into the same sites of the expression vector pET21b for production of the hG-CSF protein with natural amino acid sequence, and pET15b for the hG-CSF protein with the additional N-terminal $(His)_6$ tag. The gene for hG-CSF-Ser^{17} protein production was obtained with the help of PCR mutagenesis [34] on the plasmid pET21-hG-CSF. The expression of hG-CSF and its modified variants was achieved in the host strain *E. coli* BL-21 (DE3) after induction of middle log-phase cells with IPTG (isopropyl-β-thiogalactopyranoside) [35].

All forms of highly purified protein, e.g., rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and His_6–rhG-CSF were obtained from Biotechna UAB (Vilnius, Lithuania) as bulk solutions formulated in sodium acetate, pH 4.0 with protein concentration of about 1.0 mg/ml.

MES (2-morpholinoethanesulfonic acid) and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA). Poly(ethylene glycol) PEG 6000 was obtained from Fluka (Basle, Switzerland) and dextran 60 000 from the factory of Clinical Preparations (Saransk, Russian Federation). Reactive dye Light Resistant Yellow 2KT-Cu(II) (LR Yellow 2KT) was obtained from a local textile factory and was purified as described in Ref. [36].

Dialysis tubing (types 8/32 and 20/32) was purchased from Serva (Heidelberg, Germany). All other chemicals were commercially available and of analytical-reagent or puriss grade.

2.2. Demetallization of reactive dye Light Resistant Yellow 2KT

A 100-ml volume of stock solution (0.1%) of purified reactive dye in water was prepared. Dye concentration was calculated using molar extinction coefficient $\epsilon_{450\ nm}=24\ 100\ M^{-1}\ cm^{-1}$ and molecular mass $M_r=686.09$ [36]. 0.5 *M* H_2SO_4 was added into stock solution to obtain pH 2.0 and Cu^{2+} ions were removed by extraction of 10 ml of dye solution (pH 2.0) with 0.025% solution of dithizone in chloroform (5×50 ml). To remove the remainder of the dithizone the dye solution was washed with chloroform (3–4×10 ml), then 0.2 *M* solution of NaOH was added to obtain pH 6.0.

2.3. Difference spectroscopy

Difference spectral titration was performed in a double-beam spectrophotometer (Hitachi-330, Japan) in cells with a 10 mm optical path length. A 2.0-ml volume of either dye or dye–metal complex solution were poured into the spectrophotometer cells.

2.3.1. Dye titration with metal ions

Stock solutions of demetallized dye (25 μ*M*) and Cu^{2+}, Ni^{2+}, Zn^{2+} and Hg^{2+} ions (0.5–20 m*M*) were prepared freshly before use in the following buffers: (a) 50 m*M* MES–NaOH, 0.25 *M* Na_2SO_4 (pH 5.0); (b) 50 m*M* MES–NaOH (pH 6.0); (c) 50 m*M* HEPES–NaOH (pH 7.0). Dye was titrated by the addition of metal salt solution (5–100 μl) and difference spectra were recorded after each addition against a reference solution to which an equal volume of buffer was added. Difference spectra were recorded in the spectral region of 340–550 nm at pH 5.0 or 350–600 nm at pH 6.0 and 7.0, until the maximal value of optical density was reached. The dissociation constant (K_d) for the dye–metal ion complex was calculated from the double-reciprocal plot of ΔA_{max} versus metal ion concentration by using weighed regression analysis as described in Ref. [37].

2.3.2. Titration of dye–metal complexes with rhG-CSF

A stock solution of dye–metal complex (25 μ*M*) in a respective buffer was prepared by mixing demetallized dye solution with an excess of respective metal salt. The concentration ratio metal ion/dye was selected from the point of the difference spectrum maximum at which demetallized dye was saturated with the respective metal ion. The sample of hG-CSF was dialysed before each experiment against a buffer solution necessary for titration. Dye–metal complexes were titrated by 50 μ*M* (pH 5.0 and 6.0) and 60 μ*M* (pH 7.0) solution of native rhG-CSF or by 37 μ*M* solution of diethyl pyrocarbonate (DEP)-modified rhG-CSF (pH 7.0). A 10–100-μl volume of protein solution was added to the sample cell and the difference spectra were recorded after each addition against a reference solution into which an equal volume (10–100 μl) of buffer was added. Difference spectra were recorded in the region 350–600 nm, until maximal value of differential optical density was reached. The dissociation constant (K_d) of the ternary complex dye–metal ion–protein was calculated from the double-reciprocal plot of ΔA_{max} versus protein concentration and regression analysis [37].

2.4. rhG-CSF modification with diethyl pyrocarbonate

Modification of rhG-CSF was generally performed as described in Ref. [28], except for the protein sample, which was prepared by dialysis against 0.1 *M* sodium phosphate buffer at pH 5.85 instead of pH adjustment by the potassium phosphate solution. Briefly, 60 μ*M* solution of rhG-CSF in 10 m*M* sodium acetate buffer, pH 4.0 was dialysed against 0.1 *M* sodium phosphate buffer, pH 5.85. After dialysis optical density of protein solution at 280 nm and 240 nm was measured against buffer, pH 5.85. The theoretical value of differential optical density at 240 nm after modification reaction was calculated using molar extinction coefficient of *N*-carbethoxyhistidine, 3200 M^{-1} cm^{-1}. rhG-CSF was modified with the tenfold excess of DEP (3 m*M* concentration in a reaction mixture). For this, 2.5 ml of protein solution in 0.1 *M* sodium phosphate buffer, pH 5.85 was added to both spectrophotometer cells and the reaction was started by adding 20 μl of stock DEP solution and 20 μl of absolute ethanol into the sample and reference cells, respectively. During modification, difference spectra were recorded in 2–5 min intervals. The modification reaction was performed at room temperature until the differential absorbance at 240 nm reached theoretically calculated value 0.960 (20–25 min), which corresponded to carbethoxylation of five histidine residues of rhG-CSF. The reaction was stopped by adding 200 μl of 0.2 *M* solution of imidazole (pH 5.85) into the sample cell (15 m*M* imidazole in the cell). Modified protein solution was desalted over a Sephadex G-25 PD-10 column equilibrated with 50 m*M* HEPES buffer, pH 7.0.

2.5. Synthesis of the PEG–dye derivatives

The dye–liganded PEG 6000 derivative was synthesised as described previously [38].

For PEG–LRY 2KT–Ni(II) and PEG–LRY 2KT–Hg(II) synthesis 1 g of relevant metal salt was dissolved in 10 ml of sodium acetate buffer (pH 4.0), 1 g of demetallized PEG–dye was added and the mixture was stirred for 3 h. PEG–dye–metal complexes were 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 extinction coefficient 20 300 M^{-1} cm^{-1} at 405 nm for demetallized dye and 24 100 M^{-1} cm^{-1} at 450 nm for Cu(II) complex, respectively [36]. The amount of Ni^{2+} ions was determined by atomic absorption spectrometry. The amount of Hg^{2+} ions was determined spectrophotometrically as described in Ref. [28].

2.6. 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. Partitioning of pure rhG-CSF was performed in two-phase systems, whose composition varied from 4 to 10% (w/w) of PEG and from 6.5 to 10.5% (w/w) of dextran. Protein partitioning experiments in the presence of either PEG–dye or PEG–dye–M(II) derivative were carried out in aqueous two-phase systems composed of 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 the desired final concentration as indicated in tables (see Results section). Immobilized metal ion affinity partitioning experiments were performed by a partial replacement of PEG with PEG–dye–metal ion complex. The amount of dye–PEG derivative is given as a percentage of the total mass of PEG present in the system or expressed as metal ion concentration (μM/kg) per kg of two-phase system.

2.7. Partitioning of pure rhG-CSF and its mutant forms

Before the experiment the solution of rhG-CSF or its mutant form in 10 m*M* sodium 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 0.8–1.2 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. Samples of known volume were withdrawn from each phase and protein concentration was determined by the method of Bradford [39]. Both demetallized dye and dye–metal ions complexes absorb in the visible region and give residual absorbance in a protein assay at 595 nm by this method. To exclude this, analogously composed 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 as in case of two-phase systems with a protein and were used to prepare the reference solutions for a protein assay.

The partition coefficient of the protein, *K*, was defined as the ratio of protein concentration in the upper and lower phase. The protein affinity for immobilized metal ion was expressed in terms of $\Delta\log K$, 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}) having subtracted the logarithmic partition coefficient of the protein in an empty two-phase system in the absence of dye (K_0) [$\Delta\log K = \log K_M - (\log K_{dye} - \log K_0)$]. The alteration of the protein partition coefficient ($\Delta\log K$), when selected agents were introduced into 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.

2.8. Isolation of inclusion bodies from E. coli cell culture

A 5-g amount of harvested *E. coli* cells was homogenized in 50 ml 0.1 *M* Tris–HCl buffer, pH 7.0, containing 5 m*M* EDTA, 0.05 g lysozyme, 0.05 ml Triton X-100, 0.5 ml 100 m*M* PMSF (phenylmethyl sulfuonyl fluoride) and 0.35 ml 2-mercaptoethanol, stirred for 30 min and lysed by sonication on ice. The homogenate was centrifuged

at 14 000 *g* for 25 min. Supernatant was removed, insoluble fraction was homogenized twice with 50 ml of 1 *M* NaCl, 0.1% Tween 80 solution and once with 50 ml of distilled water. After each homogenization the suspension obtained was centrifuged at 14 000 *g* for 25 min.

2.9. Preparation of crude inclusion body extracts of rhG-CSF, rhG-CSF[Cys^{17}→Ser^{17}] and (His)$_6$–rhG-CSF

A 0.25-g amount of the inclusion bodies was homogenized in 10 ml of 20 m*M* HEPES–NaOH buffer, pH 7.0. One to two drops of 8 *M* NaOH solution were introduced into the homogenizate to obtain pH 12–13. After 2–3 min the homogenizate was titrated with diluted H_2SO_4 (1:10) up to pH 8.0, and then to pH 7.0 with more diluted H_2SO_4 (1:50). The protein extract was centrifuged at 18 000 *g* in the Beckman centrifuge for 25 min.

2.10. Partitioning of rhG-CSF and its mutant forms inclusion bodies extracts

Partitioning of rhG-CSF, $(His)_6$–rhG-CSF and rhG-CSF[Cys^{17}→Ser^{17}] from their inclusion body extracts was investigated in phase systems composed of 5% (w/w) PEG 6000–8% (w/w) dextran 60 000 in 50 m*M* HEPES–NaOH buffer, pH 7.0, containing 0.25 *M* Na_2SO_4. These two-phase systems contained 800 μ*M*/kg of immobilized Ni^{2+} ions or 825 μ*M*/kg of Hg^{2+} ions.

Three two-phase systems, which contained PEG–dye–metal ion, were prepared. A 1.0–1.2-ml volume of rhG-CSF or its mutant forms extract (concentration of protein varied from 0.5 to 1 mg/ml) in 20 m*M* HEPES–NaOH buffer, pH 7.0, was introduced into two of these systems. Into the third one, the buffer without a protein was introduced. In parallel, three two-phase systems free of both the extract of inclusion bodies and PEG–dye–metal ion were prepared. Both mixing and phase separation were performed as in two-phase systems, which contained pure protein. After that, the upper phase of the systems without extract of inclusion bodies was replaced by the upper phase, which contained the extracted protein. Selected desorption agents were introduced into the ''mixed'' two-phase systems. Both mixing and phase separation were performed. After that, the samples of known volume were withdrawn from each phase for a protein assay. The film-like layer between the phases was collected and dissolved in 1.0 ml of 6 *M* guanidine hydrochloride solution in 10 m*M* Tris–maleic acid–NaOH buffer, pH 6.5. The solution obtained was centrifuged in an Eppendorf centrifuge for 10 min. The samples of the initial extract of inclusion bodies, and of dextran phases of two-phase systems, which contained selected agents, and the sample of the interface layer solution were taken and analysed by reversed-phase (RP) HPLC [40] to detect the conformation changes of the target proteins in the two-phase systems. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [41] of the above mentioned samples was performed to assess purity of proteins. rhG-CSF samples withdrawn from dextran phases of two-phase systems, which contained selected agents were also tested for rhG-CSF activity.

2.11. RP-HPLC analysis

Recombinant hG-CSF, its folding intermediates, the denatured, and the reduced denatured protein as well as its both mutated and $(His)_6$ forms and the state of their conformation were analysed by RP-HPLC using a C_4 (Bio-Rad, Hi-Pore RP-304, 250× 4.6 mm I.D., 30 nm) reversed-phase column similarly as described in Ref. [40]. Solvent A: 0.1% trifluoroacetic acid (TFA) in water, solvent B: TFA–water–acetonitrile (0.1:9.9:90) were used for gradient elution. The column was initially equilibrated with solvent A at a flow-rate of 1 ml/min. The chromatographic run was performed with a 5 min linear gradient to 55% B followed by a 50 min linear gradient to 80% B on an HP 1100 liquid chromatographic system (Hewlett-Packard) 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 recombinant rhG-CSF, its mutated or $(His)_6$-tagged proteins were used as references.

3. Results and discussion

3.1. Preliminary investigation of rhG-CSF interaction with chelated metal ions within pH 5.0–7.0 by difference spectroscopy

The reactive dye LR Yellow 2KT possesses an azobond and two adjacent hydroxyl groups, which are involved in coordination with d group metal ions (Fig. 1). Some of previously [42] tested metal ions, e.g., Cu^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+} and Mg^{2+} showed the highest relative affinity for this dye among the reactive dyes investigated by difference spectroscopy in 10 m*M* Tris–HCl buffer, pH 6.5. Therefore, complexation of this dye with Cu^{2+}, Ni^{2+}, Zn^{2+} and Hg^{2+} ions over the range of pH 5.0–7.0 was examined by difference spectroscopy. The dye is a Cu(II) complex, thus it was demetallized before titration.

The introduction of metal ions into the dye solution induced the red shift and difference spectra formation. The dye formed complexes with metal ions including Hg^{2+} over the pH range investigated. According to data (Table 1) affinity of all investigated metal ions to the dye increased in the range of pH 5.0–7.0. Fig. 2A exemplifies a difference spectral titration of LR Yellow 2KT with Hg^{2+} ions.

The interaction of dye–metal ion complex with a protein leads to a red shift in the dye spectrum. Therefore, difference spectroscopy is a convenient

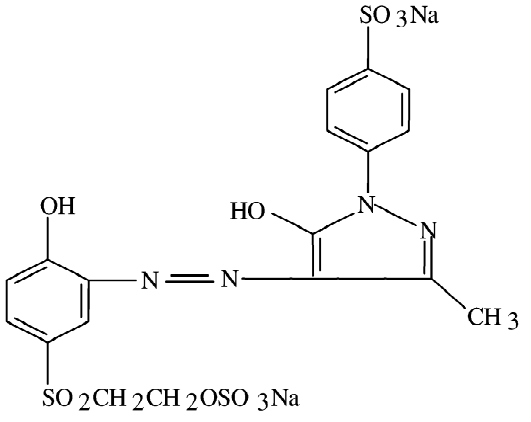

Fig. 1. Structural formula of reactive dye Light Resistant Yellow 2KT. The azobond and two adjacent hydroxyl moieties are involved in complexation with d group metal ions.

Table 1
Dissociation constants (K_d, m*M*) of LR Yellow 2KT–M^{2+} complex[a]

pH	Cu^{2+}	Ni^{2+}	Zn^{2+}	Hg^{2+}
5.0	0.055	0.339	0.190	0.544
6.0	0.046	0.029	0.047	0.104
7.0	0.037	0.021	0.031	0.027

[a] Difference spectral titration was performed at room temperature in buffer solutions: 50 m*M* MES–NaOH (pH 5.0), containing 0.25 *M* Na_2SO_4, 50 m*M* MES–NaOH (pH 6.0), and 50 m*M* HEPES–NaOH (pH 7.0).

method to investigate the interaction of proteins with chelated metal ions. The net charge of the dye over pH range 5.0–7.0 is negative. The isoelectric point (p*I*) of rhG-CSF is 5.9–6.1 [43]. Therefore, the electrostatic interaction between the protein and the dye may occur at pH 5.0. To exclude such interaction, the experiments were carried out in a buffer containing 0.25 *M* Na_2SO_4. No protein interaction with the demetallized dye was registered by difference spectroscopy over the pH range investigated, but difference spectra were recorded for some dye–metal complexes during titration. The example of the dye–Hg(II) complex titration with rhG-CSF and the subsequent determination of the dye–metal–protein dissociation constant (K_d) from the double-reciprocal plot of $1/\Delta A$ versus $1/[\text{rhG-CSF}]$, μM^{-1} are shown in Fig. 2B and C, respectively. The determined values of dissociation constants (K_d) for the four ternary dye–metal ion–rhG-CSF complexes are noted in Table 2. It can be seen, that the dye–Ni(II) and –Zn(II) complexes did not interact with rhG-CSF at pH 5.0 and 6.0 probably due to protonation of most His residues in proteins. Turbidity of the solution occurred during titration at pH 6.0 in the presence of dye–Cu(II) and –Hg(II) complexes. Probably, their binding to rhG-CSF reduced protein solubility near its isoelectric point. Only the dye–Cu(II) formed a complex with the protein with its K_d value of 22.5 μM at pH 5.0. Three of four dye–metal ion complexes interacted with the protein and their affinity for rhG-CSF increased at pH 7.0 (Table 2) in the range Hg(II)<Ni(II)<Cu(II). Difference spectrum for dye–Ni(II) complex was not generated. This may evidence the absence of its interaction with the protein. We also previously observed such behaviour of this dye–Ni(II) complex [42] in studies of

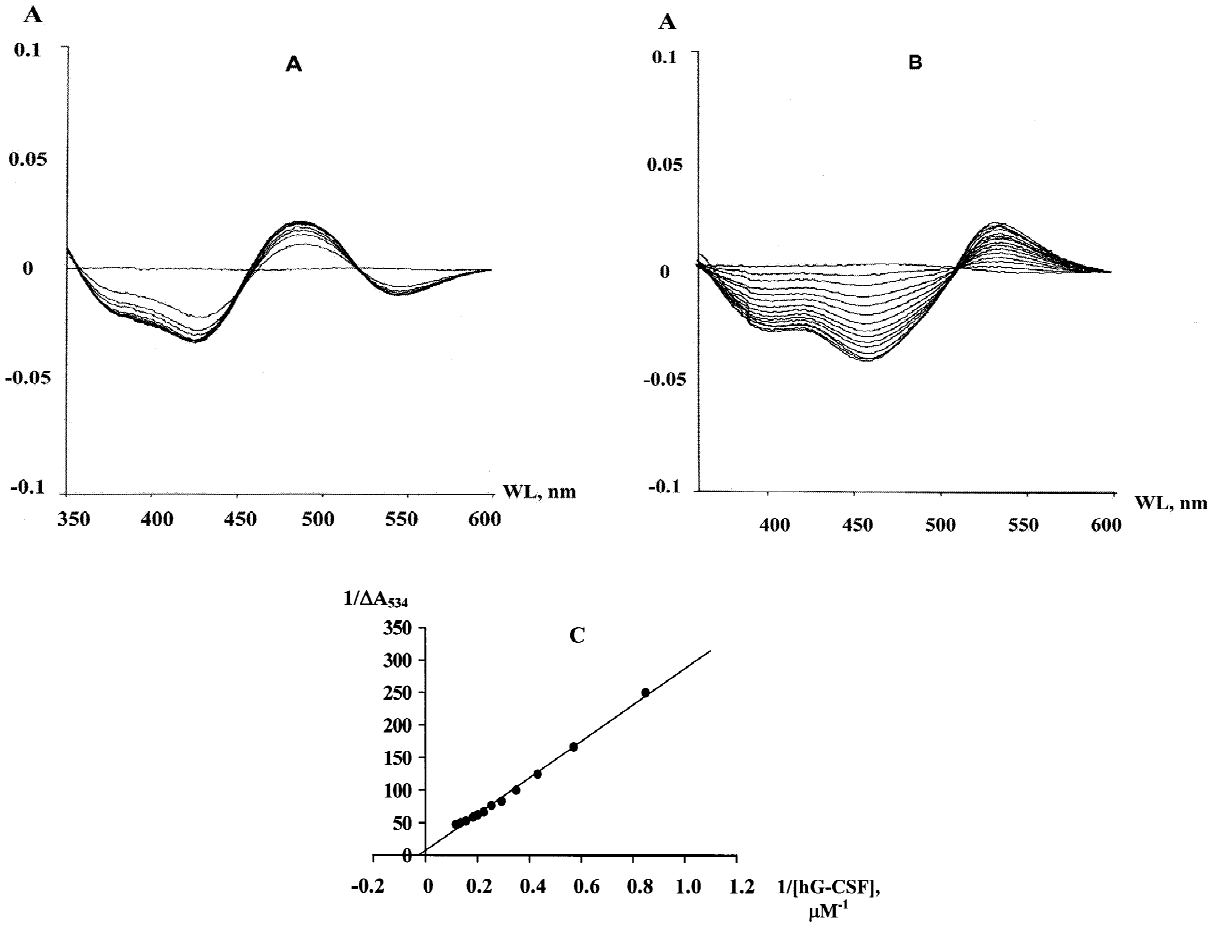

Fig. 2. Difference spectral titration for LR Yellow 2KT with Hg^{2+} ions (A) and for LR Yellow 2KT–Hg(II) with rhG-CSF (B) in 50 m*M* HEPES–NaOH buffer, pH 7.0. Differential optical density at 534 nm versus rhG-CSF concentration in double-reciprocal (C) plot. The initial dye–Hg(II) complex concentration, 25 μ*M*; rhG-CSF concentrations, 0–8.5 μ*M*.

Table 2
Dissociation constants (K_d, μ*M*) of the ternary complex rhG-CSF–M^{2+}–LR Yellow 2KT[a]

pH	Cu^{2+}	Ni^{2+}	Zn^{2+}	Hg^{2+}
5.0	22.5	n.i.	n.i.	[b]
6.0	[b]	n.i.	n.i.	[b]
7.0	8.8 (23.9)[c]	n.i.	17.1	38.7 (42.6)

[a] Difference spectral titration was performed at room temperature in the buffer solution as indicated in Table 1.

[b] Turbidity of solution occurred before the end of titration.

[c] K_d value for DEP modified rhG-CSF ternary complex is shown in the parentheses.

n.i. Interaction between protein and dye–metal complex was not detected.

yeast alcohol dehydrogenase interaction with dye metal ions (Cu^{2+}, Zn^{2+}, $Ni^{2+}$and Mn^{2+}) complexes by circular dichroism (CD) spectroscopy. In accordance with the results in Table 2 we can conclude that the ternary complex formation depends on both pH and the nature of metal ion.

rhG-CSF possesses five histidine residues and unpaired cysteine-17 in a polypeptide sequence [44], which can be involved in the interaction with metal ions. In a previous paper [28] we demonstrated, that the protein modified with diethyl pyrocarbonate lost, in contrast to the unmodified, its ability to interact with Ni(II)-charged Sepharose-IDA gel, but it was retained on the gel loaded with Hg(II) ions. This

suggested that histidine residues are not essential for the protein interaction with chelated mercury. To support this assumption, the interaction of DEP-modified protein with LR Yellow 2KT–Hg(II) and –Cu(II) complexes was evaluated. The dye–Ni(II) complex was not appropriate for such assessment since it did not interact with the unmodified protein (Table 2).

The kinetics of proteins modification with DEP has been already investigated over the range of pH 5.6–8.5 in 0.1 *M* potassium phosphate buffer [45]. The modification rate growth was apparent with the increase of pH, but selectivity for histidine residues modification was greater at lower pH (His residues were successfully modified even at pH 5.6). In accordance with this the rhG-CSF modification reaction with DEP was performed in 0.1 *M* sodium phosphate buffer at pH 5.85 (Fig. 3). The modified protein exhibited no interaction with demetallized dye LR Yellow 2KT at pH 7.0, but metal ions, e.g., Cu^{2+} or Hg^{2+} induced generation of difference spectra, and this led to the determination of K_d for dye–metal ions–DEP-modified rhG-CSF ternary complexes. For the mixed Cu(II) and Hg(II) complexes, the dissociation constants were calculated to be 23.9 μ*M* and 42.6 μ*M*, respectively. As can be seen from Table 2, the determined dissociation constants for dye–Hg(II) complexes with unmodified and modified protein were almost equal – 38.7 and 42.6 μ*M*, respectively, while the K_d value for dye–Cu(II) complex with DEP-modified protein increased approx. threefold. This indicates that modification of His residues reduced the protein interaction with the dye–Cu(II) complex but had no effect on the protein interaction with the dye–Hg(II) complex.

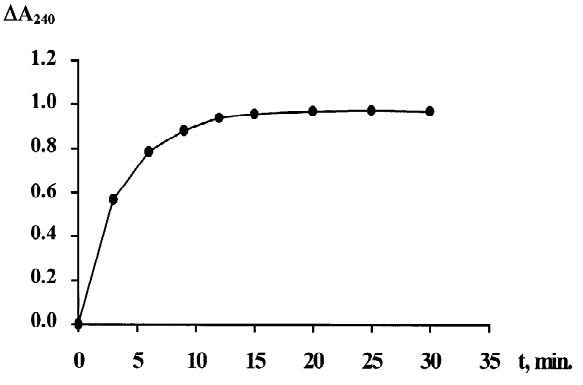

Fig. 3. The alteration of differential optical density at 240 nm during modification of 60 μ*M* rhG-CSF with 3 m*M* DEP in 0.1 *M* sodium phosphate buffer, pH 5.85.

3.2. Immobilized metal ion affinity partitioning of rhG-CSF, His_6–rhG-CSF and rhG-CSF[Cys^{17}→Ser^{17}]

The lowest partition constant of rhG-CSF (K_0 = 1.15) was found in the two-phase system composed of 5% (w/w) PEG and 8% (w/w) dextran. This composition therefore was used for all further rhG-CSF partitioning experiments. Partitioning of rhG-CSF at pH 7.0, when PEG was partially replaced with demetallized dye–PEG is summarised in Table 3. It can be seen, that the increase of dye concentration in the two-phase system caused a slight alteration in the protein logarithmic partition coefficient with respect to the empty two-phase system in the absence of the dye. Probably, a weak hydrophobic interaction between the protein and dye molecules occurred in the presence of 0.25 *M* Na_2SO_4. Therefore, for immobilized metal ion affinity partitioning experiments a moderate concentration of approximately 800 μ*M*/kg metal ion (in the form of PEG–dye–metal ion complex) was maintained.

rhG-CSF and its mutant forms were effectively extracted into the upper phase at pH 7.0 (Table 4 and Fig. 4) when metal ions from Cu(II) to Hg(II) chelated by the LR Yellow 2KT–PEG derivative were introduced into the two-phase system. The partition coefficients of the protein and the two forms

Table 3
Effect of demetallized dye LR Yellow 2KT on the partitioning of pure rhG-CSF[a]

No.	PEG–LR Yellow 2KT		*K*	Δlog *K*
	%	μ*M*/kg		
1	0	0	1.15	–
2	6	465	1.66	0.16
3	10	775	1.40	0.09
4	25	1938	1.22	0.03

[a] Two-phase system (4 g) contained 5% (w/w) PEG 6000, 8% (w/w) dextran 60 000, 0.8–1 mg of protein, 0.25 *M* Na_2SO_4 and 50 m*M* HEPES–NaOH buffer, pH 7.0. The amount of dye is expressed both as a percentage of the total mass of PEG present in the system and as μmol per kg of the two-phase system.

Table 4
Partitioning of pure rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF in the presence of metal ions chelated by LR Yellow 2KT–PEG at pH 7.0[a]

M^{2+}	C (M^{2+}), (μmol/kg)	rhG-CSF		rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$]		$(His)_6$–rhG-CSF	
		Log K	Δlog K	Log K	Δlog K	Log K	Δlog K
–	–	0.16	–	0.11	–	0.21	–
Cu^{2+}	816	2.38	2.22	2.49	2.38	2.41	2.20
Ni^{2+}	810	1.72	1.56	1.10	0.99	2.47	2.26
Hg^{2+}	836	2.35	2.19	1.40	1.29	2.31	2.10

[a] System composition was similar to that in Table 3. Metal ion concentration is expressed in μmol per kg of two-phase system.

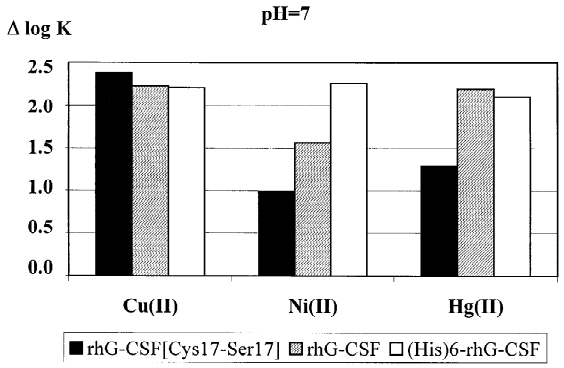

Fig. 4. Partitioning of pure rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF in the presence of metal ions chelated by LR Yellow 2KT–PEG at pH 7.0. Two-phase systems (4 g) contained 5% (w/w) PEG 6000, 8% (w/w) dextran 60 000, 0.8–1 mg of protein, 0.25 *M* Na_2SO_4 and 50 m*M* HEPES–NaOH buffer, pH 7.0. Concentrations of Cu^{2+}, Ni^{2+} and Hg^{2+} per kg of two-phase system were 816 μ*M*/kg, 810 μ*M*/kg and 836 μ*M*/kg, respectively.

studied markedly changed from a Δlog K value of 0.99 for the $Cys^{17}\rightarrow Ser^{17}$ mutant in the presence of Ni(II) ions to 2.19 for rhG-CSF in the presence of chelated mercury, and to a value equal or exceeding 2.20 for all three protein forms in the presence of Cu(II) ions. Comparison of Δlog K values for rhG-CSF in the presence of selected metal ions at pH 7.0, shows that the PEG–dye–Cu(II) and –Hg(II) complexes exhibited greater protein extraction capacities than Ni(II) complexes. But it should be noted that the dye–Ni(II) complex, being linked to PEG, is able, in contrast to its free form in solution (Table 2), to interact with rhG-CSF. This is consistent with the commonly accepted observation that immobilized metal ions fundamentally differ from freely soluble metal ions [46]. The extraction efficiency of rhG-CSF by the PEG–dye–Ni(II) complex was found to be sensitive to pH. The Δlog K value decreased from 1.56 (pH 7.0) to 0.28 (pH 5.0), as indicated in Table 4, Fig. 4 and Table 5, Fig. 5, respectively. Such a decrease in Δlog K value by lowering pH may be related to protonation of the imidazole group of the His residue and the lack of its ability to interact with Ni(II) ions at pH 5.0. This suggested that the surface exposed His residue of rhG-CSF is involved in the interaction with chelated Ni(II) ions, a suggestion supported by the good correlation between the partitioning behaviour of rhG-CSF in the presence of

Table 5
Partitioning of pure rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF in the presence of metal ions chelated by LR Yellow 2KT–PEG at pH 5.0[a]

M^{2+}	C (M^{2+}), (μmol/kg)	rhG-CSF		rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$]		$(His)_6$–rhG-CSF	
		Log K	Δlog K	Log K	Δlog K	Log K	Δlog K
–	–	0.20	–	0.20	–	0.10	–
Cu^{2+}	816	1.98	1.78	1.93	1.73	2.34	2.24
Ni^{2+}	810	0.48	0.28	n.d.	n.d.	n.d.	n.d.
Hg^{2+}	836	2.05	1.85	1.10	0.90	2.26	2.16

[a] Two-phase system composition was similar to that in Table 3, except for the buffer, 50 m*M* MES–NaOH, pH 5.0.
n.d., Not determined.

the PEG–dye–Ni(II) complex at both pH values and the chromatographic behaviour of its DEP-modified and unmodified form on Ni(II)-charged Sepharose-IDA columns. As recently reported [28], DEP-modified protein was no longer retained by the column at pH 7.0, while unmodified protein was not retained at pH 5.0 in the presence of salt. Comparison of Δlog *K* values determined for tagged (2.26) and non-tagged protein (1.56), respectively (Table 4), clearly shows that in the case of $(His)_6$–rhG-CSF, the tag may play an essential role in the interaction with Ni(II)–dye–PEG.

The partitioning of rhG-CSF and both the forms studied in the presence of Cu(II) complexes of LR Yellow 2KT–PEG at pH 7.0 demonstrated (Table 4, Fig. 4), that the extraction power of Cu(II) ions towards all three protein forms was of the same range, the Δlog *K* values being within 2.20–2.38. Data in Table 4 clearly indicated, that the strength of rhG-CSF and its [$Cys^{17}\rightarrow Ser^{17}$] mutant interactions with chelated Cu(II) ions were considerably higher than with the PEG–dye–Ni(II) complex, and were as order of magnitude comparable with that of the $(His)_6$-tagged protein interaction with Cu(II) or Ni(II) ions. The Δlog *K* value of rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] mutant (2.38) even slightly exceeded (pH 7.0) the Δlog *K* value of rhG-CSF (2.22). This raised doubts as to the involvement of cysteine-17 of rhG-CSF in the interaction with the PEG–dye–Cu(II) complex. Δlog *K* of rhG-CSF and its mutated [$Cys^{17}\rightarrow Ser^{17}$] form decreased to the same range of 1.73–1.78 when protein partitioning was performed at pH 5.0 (Table 5 and Fig. 5). The Δlog *K* of the $(His)_6$–rhG-CSF form did not decrease. It is possible that such pH-dependent decreases in Δlog *K* may be associated with the protonation of the same type of exposed His residue. On the other hand, this decrease in Δlog *K* values was not so pronounced in comparison to the decrease in Δlog *K* value for rhG-CSF, observed by protein partitioning at pH 5.0 in the presence of the PEG–dye–Ni(II) complex (Table 5). This may mean that the other amino acid residues of rhG-CSF and its mutant [$Cys^{17}\rightarrow Ser^{17}$] are involved in the interaction with Cu(II) ions at pH 5.0, which would be consistent with the data presented in Table 2. The strength of DEP-modified rhG-CSF (with all five His residues) interaction with the Cu(II)–dye complex in

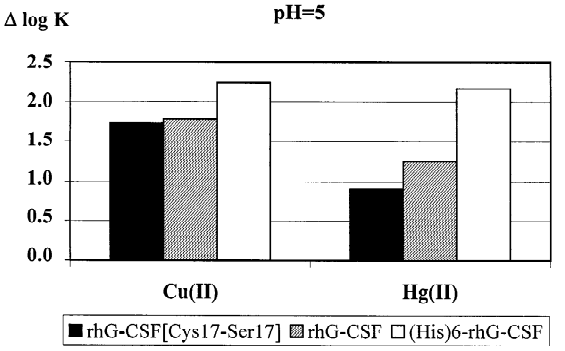

Fig. 5. Partitioning of pure rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF in the presence of metal ions chelated by LR Yellow 2KT–PEG at pH 5.0. System composition was similar to that in Fig. 4, except for the buffer, 50 m*M* MES–NaOH, pH 5.0.

solution at pH 7.0 was reduced approx. threefold, i.e., the K_d increased from 8.8 to 23.9 μ*M*, but despite this the residual interaction still occurred. The Δlog *K* values for the partitioning of $(His)_6$-tagged rhG-CSF in the presence of PEG–dye–Cu(II) complex were similar at pH 7.0 (2.20) and pH 5.0 (2.24). Therefore, the prevalence of $(His)_6$ tag in the interaction with the PEG–dye–Cu(II) complex may be proposed at pH 7.0, and might be also plausible at pH 5.0, assuming that among the stretch of His residues representatives with a lower pK_a exist. The possible involvement of the exposed His residue of D-xylose isomerase in the interaction with Cu(II)–IDA-Sepharose under slightly acidic conditions was previously demonstrated by Mrabet [47].

A comparative study of the partitioning of rhG-CSF and the two forms studied, in the presence of PEG–dye–Hg(II) complexes performed at pH 7.0 and pH 5.0 are summarised in Tables 4 and 5 and Figs. 4 and 5. It is worth noting that the extraction of rhG-CSF and $(His)_6$–rhG-CSF into the upper phase occurred at both pH values more efficiently than the extraction of rhG-CSF, which contains Ser-17 instead of Cys-17. The determined Δlog *K* values for rhG-CSF exceeds the Δlog *K* value of rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] approx. 1.7 (at pH 7.0) and 2.0 (at pH 5.0) fold. No doubt, this could reflect the contribution of the free SH group of cysteine-17 of rhG-CSF to the protein extraction power by the PEG–dye–Hg(II) complex, and clearly evidences, that the direct interaction between chelated mer-

cury(II) and unpaired Cys residue of rhG-CSF is possible. The same may be valid for $(His)_6$-tagged form of the protein because its Δlog K value (2.10, pH 7.0) is almost similar to that of rhG-CSF (Δlog K=2.19).

Despite the lack of a free SH group, rhG-CSF[Cys^{17}→Ser^{17}] is still able to interact with chelated Hg(II) ions at pH 7.0 and pH 5.0. According to the Δlog K values shown in Tables 4 and 5, it can be seen that the strength of rhG-CSF[Cys^{17}→Ser^{17}] interaction with all three studied PEG–dye–metal ion complex derivatives increases in the order Ni(II)<Hg(II)<Cu(II), similarly as for rhG-CSF, with the substantial exception of the latter, which interacts more strongly with Ni(II) and Hg(II) complexes. This indicates, that in the absence of an available SH group in a polypeptide chain of the protein, Hg(II) ions complexed by PEG–dye may also coordinate other appropriate amino acid side chains of rhG-CSF[Cys^{17}→Ser^{17}] at pH 7.0 and pH 5.0. In this context, the high value of Δlog K for $(His)_6$–rhG-CSF at 5.0 (Table 5) does not reject the possibility of the involvement of any histidine residue with a lower pK_a, donated by $(His)_6$ stretch in the interaction with PEG–dye–Hg(II) complex, similarly, as it was assumed for this protein interaction with the dye–Cu(II) complex.

3.3. Partitioning of rhG-CSF and its mutant forms in the presence of selected agents

The interaction of rhG-CSF with Cu(II), Ni(II) and Hg(II) ions immobilized on the LR Yellow 2KT–PEG derivative was studied when selected agents such as imidazole, β-mercaptoethanol, sodium chloride, L-aspartic acid, oxidised glutathione, L-tyrosine and sodium acetate were present in the two-phase systems. For comparison, the partitioning of rhG-CSF[Cys^{17}→Ser^{17}] and $(His)_6$–rhG-CSF was studied after the addition of appropriate concentrations of selected agents.

The data in Table 6 indicate, that the introduction

Table 6
Partitioning of pure rhG-CSF, rhG-CSF[Cys^{17}→Ser^{17}] and $(His)_6$–rhG-CSF in two phase systems containing metal ions chelated by LR Yellow 2KT–PEG in the presence of the selected agents at pH 7.0[a]

M^{2+}	Agent	Concentration	Δlog K reduction (%)		
			rhG-CSF	rhG-CSF[Cys^{17}→Ser^{17}]	$(His)_6$–rhG-CSF
Cu^{2+}	Immidazole	0.1 mM	1	n.d.	n.d.
		1 mM	3	n.d.	n.d.
		10 mM	91	79	38
Ni^{2+}	Immidazole	0.1 mM	63	n.d.	n.d.
		1 mM	71	n.d.	n.d.
		10 mM	84	65	69
	β-Mercaptoethanol	1 mM	58	n.d.	n.d.
		10 mM	73	1	46
Hg^{2+}	Imidazole	10 mM	81	60	22
	β-Mercaptoethanol	1 mM	66	n.d.	n.d.
		10 mM	98	77	98
	Oxidised glutathione	1 mM	0	n.d.	n.d.
		10 mM	45	n.d.	n.d.
	NaCl	0.25 M	54	n.d.	n.d.
		0.5 M	66	n.d.	n.d.
		0.75 M	69	n.d.	n.d.
	L-Asp–NaOH, pH 7.0	3 mM	3	n.d.	n.d.

[a] Both the system composition and metal ion concentration were similar to those in Table 4.
n.d., Not determined.

of imidazole, which competes with metal ions, e.g., Cu(II) or Ni(II) complexed with PEG–dye derivatives, at concentrations of up to 10 m*M* in the phase systems at pH 7.0 caused an effective decrease in the Δlog *K* value of rhG-CSF by 91–84%. Its effect was concentration dependent (Table 6). For example, a low concentration (0.1 m*M*) resulted in a decrease in the Δlog *K* value of rhG-CSF by 63% in the presence of Ni(II) ions and had no effect in the presence of Cu(II) ions. This indicated a stronger interaction of rhG-CSF with Cu(II) than Ni(II) ions, an observation that correlates well with data in Table 4, which indicated that the rhG-CSF extraction power of the PEG–dye–Cu(II) complex was substantially higher than that of the PEG–dye–Ni(II) complex. The effect of β-mercaptoethanol on the Δlog *K* value of rhG-CSF in a system, which contained the PEG–dye–Ni(II) complex was lesser than that of imidazole. Mercaptoethanol at concentrations of 1.0–10 m*M* caused a decrease in Δlog *K* values by 58 and 73% respectively, while similar concentrations of imidazole decreased Δlog *K* values by 71% (1 m*M*) and 84% (10 m*M*), respectively (Table 6).

The Δlog *K*-decreasing capacity of 10 m*M* imidazole introduced into the phase system, which contained PEG–dye–Cu(II) complex when rhG-CSF and its two forms were partitioned at pH 7.0, differed markedly and decreased (Fig. 6) in the order rhG-CSF (91%)>rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] (79%)> $(His)_6$–rhG-CSF (38%), clearly indicating that such

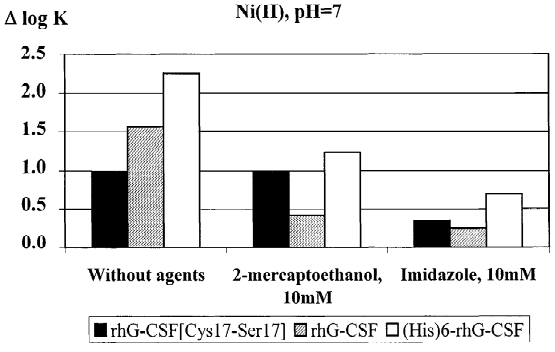

Fig. 6. Effect of the selected agents on the partitioning of pure rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF in the presence of Ni^{2+} ions chelated by LR Yellow 2KT–PEG at pH 7.0. System composition and Ni^{2+} concentration were similar to those in Fig. 4.

concentrations of imidazole are insufficient to reduce His-tagged protein binding to Cu(II) ions. Together with the data in Table 4 this supports the notion, that the $(His)_6$ tag may dominate in the interaction with Cu(II) ions. Not pronounced, but detectable differences in the ability of 10 m*M* imidazole to decrease Δlog *K* of rhG-CSF and its [$Cys^{17}\rightarrow Ser^{17}$] mutant (by 91 and 79%, respectively), were consistent with the data on protein partitioning (Table 4). The extraction power of rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] by the PEG–dye–Cu(II) complex at pH 7.0 was slightly greater than that of rhG-CSF and, as a result, the Δlog *K*-decreasing capacity of 10 m*M* imidazole was lower in the case of the [$Cys^{17}\rightarrow Ser^{17}$] mutant interaction with Cu(II) ions. According to data in Table 6, 10 m*M* imidazole exhibited a more pronounced Δlog *K*-decreasing capacity in the case of rhG-CSF interaction with Ni(II) complex, while the Δlog *K* of [$Cys^{17}\rightarrow Ser^{17}$] mutant and $(His)_6$-tagged form were decreased by 65–69%. The lower Δlog *K* reduction of the $(His)_6$-tagged rhG-CSF in comparison to non-tagged protein is in agreement with data in Table 4, and may indicate the involvement of the tag's histidine residues in the interaction with the PEG–dye–Ni(II) complex. In the case of the [$Cys^{17}\rightarrow Ser^{17}$] mutant a Δlog *K* reduction similar to that for tagged proteins was observed. As indicated in Table 4, the extraction power of the [$Cys^{17}\rightarrow Ser^{17}$] mutant by the PEG–dye–Ni(II) complex was lower than that of rhG-CSF and even lower than that of the tagged protein form. Such a discrepancy in rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] mutant partitioning behaviour suggests that in addition to the histidine residue, the OH group of Ser-17, or another donor group might be involved in the interaction with Ni(II) ions.

The effects of various selected agents on the Δlog *K* values of rhG-CSF at pH 7.0 in the systems which contained PEG–dye–Hg(II) complex differed considerably, as indicated in Table 6. Oxidised glutathione (1 m*M*) and L-aspartic acid (3 m*M*) had no significant effect, while 1 m*M* β-mercaptoethanol caused a decrease in the Δlog *K* value by 66%. An increase in the concentration of oxidised glutathione, imidazole and β-mercaptoethanol of up to 10 m*M* generated a decrease in Δlog *K* of rhG-CSF by 45, 81 and 98%, respectively. Introduction of NaCl at increasing concentrations from 0.25 to 0.75 *M*

caused a decrease in Δlog *K* of rhG-CSF in the presence of PEG–dye–Hg(II) by 54–69%. The ability of 10 m*M* β-mercaptoethanol to reduce almost completely (by 98%), and the ability of NaCl to reduce substantially (by 69%) rhG-CSF interaction with chelated Hg(II) ions reflects their high Δlog *K*-decreasing capacity that parallels their capacity to elute proteins retained by the Hg(II)–charged Sepharose-IDA gel [28].

The effect of imidazole (10 m*M*) and β-mercaptoethanol (10 m*M*) on the Δlog *K* values was compared by partitioning rhG-CSF and its two forms in the presence of PEG–dye–Ni(II) (Fig. 6) and PEG–dye–Hg(II) (Fig. 7) complexes at pH 7.0. As indicated in Fig. 6, imidazole was a more effective competing agent than β-mercaptoethanol in the interaction of all three protein forms with the PEG–dye–Ni(II) complex. The introduction of imidazole to the phase system caused a decrease in Δlog *K* of rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF by 84, 65 and 69%, respectively, while Δlog *K* of rhG-CSF, its [$Cys^{17}\rightarrow Ser^{17}$] mutant and $(His)_6$-tagged form were decreased by 73, 1.0 and 46%, respectively, when the same concentration of β-mercaptoethanol was present (Table 6). In contrast, Fig. 7 shows that in the presence of the PEG–dye–Hg(II) complex the competing capacity of β-mercaptoethanol was more pronounced than that of imidazole. A 10-m*M* concentration of imidazole caused a decrease in Δlog *K* of rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF by 81, 60

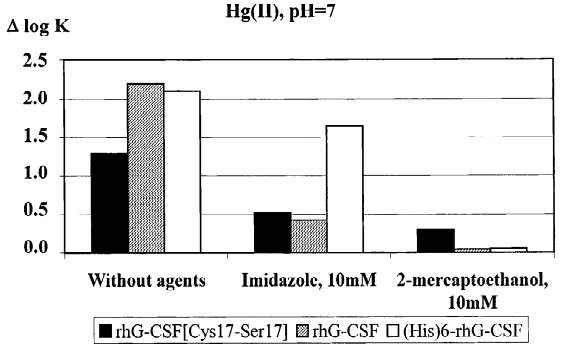

Fig. 7. Effect of the selected agents on the partitioning of pure rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF in the presence of Hg^{2+} ions chelated by LR Yellow 2KT–PEG at pH 7.0. System composition and Hg^{2+} concentration were similar to those in Fig. 4.

and 22%, respectively, while 10 m*M* β-mercaptoethanol caused a decrease in Δlog *K* of [$Cys^{17}\rightarrow Ser^{17}$] mutant of 77%, and in the Δlog *K* of rhG-CSF and its $(His)_6$-tagged form of 98% (Table 6). The reduction of the interaction of rhG-CSF and its $(His)_6$-tagged form with Hg(II) ions by the same extent is presumably related to the effect of Hg(II) ions on the partitioning of these protein forms (Δlog $K=2.19–2.10$, Table 4). This observation supports the suggestion that Cys-17 of the tagged protein form may also be responsible for interaction with chelated Hg(II) ions. The lower Δlog *K*-decreasing capacity of 10 m*M* β-mercaptoethanol found for the [$Cys^{17}\rightarrow Ser^{17}$] mutant (77%, Table 6) may be associated with and may reflect the involvement of donor groups differing from that donated by rhG-CSF or its $(His)_6$-tagged form in the interactions with chelated Hg(II) ions. SH group-containing agents also exhibited a relatively high ability to reduce the interaction between a protein non-containing free SH group and chelated Hg(II) ions. In general, Table 6 and Figs. 6 and 7 indicate, that the differences in Δlog *K*-decreasing capacity of imidazole and β-mercaptoethanol were primarily defined by the type of chelated metal ion that participates in the two-phase system, and by the suggested type (and possibly number) of donor groups donated by rhG-CSF and both the forms studied. Imidazole acts in our studied cases as a displacing agent causing a dissociation more pronounced than that of β-mercaptoethanol of rhG-CSF and both forms complex with chelated Ni(II) (pH 7.0) than Hg(II) ions. This tendency is in accordance with previously [31] observed effects of similar concentrations of imidazole and cysteine introduced to the two-phase system containing Cu(II)–IDA–PEG where the availability of His residues on the surface of NAD^+-dependent dehydrogenases was tested. In these experiments the effect of cysteine was smaller than that of imidazole. In addition, we showed recently [28] that the elution of rhG-CSF from Ni(II)–IDA-Sepharose was not effective using 5–10 m*M* β-mercaptoethanol or 1.0 *M* NaCl, but was using imidazole. Ultimately, the established order of amino acid interaction strength with Cu(II) and Ni(II)–IDA adsorbents at pH~7.0 proved the stronger retention of His than of reduced Cys [48,49]. Based on this finding it follows, that at the same concentration range of imidazole and β-

mercaptoethanol the Δlog *K*-decreasing capacity of imidazole should be greater when chelated Cu(II) or Ni(II) ions are present in the two-phase system. Indeed, Table 6 illustrates this tendency in the forms studied. Analogous considerations might be expanded for the systems containing chelated Hg(II) ions allowing for the exception that the affinity of agents containing free SH groups to mercury exceeds that of N-donor ligands, previously established by studying the binding of various low-molecular-mass compounds to an organomercurial copolymer of ethylene and maleic acid [50]. Low retention of amino acids, e.g., Lys, His or Arg, in contrast to mercaptoethanol, cysteine and other studied SH compounds was reported. Consequently the Δlog *K*-decreasing capacity of the same concentration of imidazole would be predicted to be lower than that of β-mercaptoethanol when rhG-CSF or the two forms studied were present in two-phase system containing chelated Hg(II) ions. This prediction is confirmed by data in Table 6 and Fig. 7, and is consistent with the recent observation that the elution of rhG-CSF retained on Hg(II)–IDA-Sepharose took place with eluents containing low concentrations (5–10 m*M*) of β-mercaptoethanol while the elution with imidazole required concentrations in the range of 100 mmol/l [28].

The PEG–dye–Ni(II) complex did not extract rhG-CSF into the upper phase at pH 5.0 (Table 5). The affinity partitioning of rhG-CSF and the forms studied in the presence of selected agents at pH 5.0 could therefore be evaluated in the phase system containing PEG–dye complexes with Cu(II) and Hg(II) ions. As indicated in Table 7, only two of the selected agents, e.g., β-mercaptoethanol (10 m*M*) or L-tyrosine (0.2 m*M*) decreased Δlog *K* of rhG-CSF to a considerable extent (by 77 and 46%, respectively) when chelated Cu(II) ions were added to the two-phase system. Other selected agents, e.g., 0.25 *M* sodium chloride, 50 m*M* sodium acetate (pH 5.0) or 3 m*M* L-aspartic acid–NaOH (pH 5.0) had only a negligible effect on rhG-CSF partitioning. In the two-phase system, which contained immobilized Hg(II) ions, two selected agents – 1 m*M* β-mercaptoethanol and 0.25 *M* sodium chloride – caused an effective decrease in Δlog *K* of rhG-CSF (by 98 and 94%, respectively), as indicated in Fig. 9 and Table 7. L-Aspartic acid (3 m*M*) also had an appreciable

Table 7
Partitioning of pure rhG-CSF, rhG-CSF[Cys^{17}→Ser^{17}] and $(His)_6$–rhG-CSF in two-phase systems containing metal ions chelated by LR Yellow 2KT in the presence of the selected agents at pH 5.0[a]

M^{2+}	Agent	Concentration	Δlog *K* reduction (%)		
			rhG-CSF	rhG-CSF[Cys^{17}→Ser^{17}]	$(His)_6$–rhG-CSF
Cu^{2+}	β-Mercaptoethanol	1 m*M*	7	n.d.	n.d.
		10 m*M*	77	61	6
	NaCl	0.25 *M*	9	n.d.	n.d.
	L-Tyr	0.2 m*M*	46	39	10
	Sodium acetate buffer, pH 5.0	2 m*M*	−3	n.d.	n.d.
		50 m*M*	−15	n.d.	n.d.
	L-Asp–NaOH, pH 5.0	3 m*M*	7	n.d.	n.d.
Hg^{2+}	β-Mercaptoethanol	1 m*M*	98	82	76
	NaCl	0.25 *M*	94	n.d.	n.d.
	L-Tyr	0.2 m*M*	8	n.d.	n.d.
	Sodium acetate buffer, pH 5.0	2 m*M*	1	n.d.	n.d.
		50 m*M*	1	n.d.	n.d.
	L-Asp–NaOH, pH 5.0	3 m*M*	74	11	13

[a] Both the system composition and metal ion concentration were similar to those in Table 5.
n.d., Not determined.

effect (Fig. 9), decreasing Δlog *K* of rhG-CSF by 74%, whereas the effect of L-Tyr (0.2 m*M*) or sodium acetate (2–50 m*M*), was negligible (Table 7). The agents, that exerted significant rhG-CSF Δlog *K*-decreasing capacity in the presence of PEG–dye–Cu(II) and –Hg(II) ions were then chosen for further comparative partitioning at pH 5.0. As indicated in Fig. 8, the introduction of 10 m*M* β-mercaptoethanol or 0.2 m*M* L-tyrosine caused a decrease in Δlog *K* values of rhG-CSF and its [$Cys^{17}\rightarrow Ser^{17}$] mutant in the presence of PEG–dye–Cu(II) complex, 77–61% with the introduction β-mercaptoethanol, and 46–39% with the addition of L-tyrosine (Table 7). However, these agents had a negligible effect on the Δlog *K* value of $(His)_6$-tagged rhG-CSF; only a 6–10% reduction was reached (Table 7). This, and data in Table 5 indicate therefore, that the interaction of $(His)_6$-tagged rhG-CSF with Cu(II) ions is stronger and may result from its binding through the His tag moiety. The Δlog *K*-decreasing capacity of low (0.2 m*M*) concentrations of L-Tyr in the case of rhG-CSF and its [$Cys^{17}\rightarrow Ser^{17}$] mutant partitioning in the presence of PEG–dye–Cu(II) complex at pH 5.0 can also be noted, as possible reflection of the involvement of the tyrosine residue in the interactions with Cu(II) ions.

As can be seen from Table 7 and Fig. 9, β-mercaptoethanol was more effective at pH 5.0 in reducing the interactions of all three protein forms

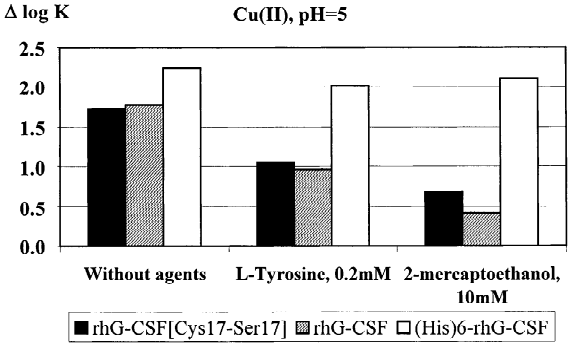

Fig. 8. Effect of the selected agents on the partitioning of pure rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF in the presence of Cu^{2+} ions chelated by LR Yellow 2KT–PEG at pH 5.0. System composition and Cu^{2+} concentration were similar to those in Fig. 5.

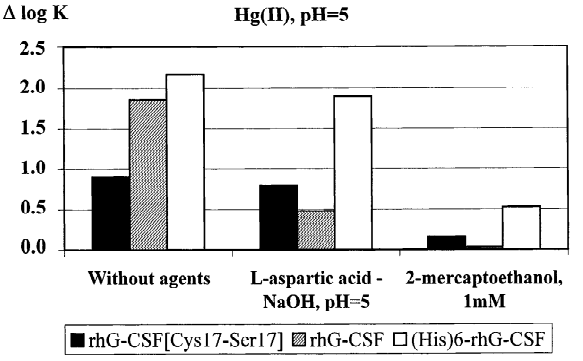

Fig. 9. Effect of the selected agents on the partitioning of pure rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF in the presence of Hg^{2+} ions chelated by LR Yellow 2KT–PEG at pH 5.0. System composition and Hg^{2+} concentration were similar to those in Fig. 5.

with the PEG–dye–Hg(II) complex than with the PEG–dye–Cu(II). At a concentration of 1 m*M* in the presence of chelated Hg(II) ions it decreased Δlog *K* of rhG-CSF and its [$Cys^{17}\rightarrow Ser^{17}$] mutant by 98–82%. Even the $(His)_6$-tagged protein Δlog *K* value decreased by 76%. Thus, β-mercaptoethanol at pH 5.0 maintained in general a Δlog *K*-decreasing tendency in the presence of PEG–dye–Hg(II) and PEG–dye–Cu(II) complexes similar to that observed at pH 7.0 (Table 6). Its higher affinity to chelated Hg(II) than Cu(II) or Ni(II) ions predetermines its effective concentration range. For example, at 10 m*M* in the phase system containing Cu(II) ions the Δlog *K* value of rhG-CSF and its [$Cys^{17}\rightarrow Ser^{17}$] form decreased by 77–61%, while at a tenfold lower concentration it decreased Δlog *K* values by 98–82% when partitioning of these proteins was performed in the presence of chelated Hg(II) ions. The latter observation also indicates the weaker interaction of rhG-CSF forms with chelated Hg(II) ions at pH 5.0 than at pH 7.0. The effect of L-aspartic acid on the Δlog *K* values of all three protein forms in the presence of the PEG–dye–Hg(II) complex differed greatly, as indicated in Fig. 9 and Table 7. At a concentration of 3 m*M* aspartic acid caused a considerable decrease in Δlog *K* value of rhG-CSF (74%), while it had only a negligible effect on the corresponding value for the $Cys^{17}\rightarrow Ser^{17}$ and $(His)_6$-tagged protein forms (11–13%).

3.4. Partitioning of rhG-CSF and its forms from inclusion body extracts in the presence of PEG–dye–Ni(II) and –Hg(II) complexes

The partitioning of rhG-CSF and its mutant forms extracted from inclusion bodies using an ''alkaline shock'' [51] procedure, in the presence of chelated Ni(II) or Hg(II) ions in the phase system is summarised in Table 8. Comparison of these results with the partitioning data on purified proteins, as given in Table 4, revealed some differences between the behaviour of purified proteins and extracted from inclusion bodies in the presence of the Hg(II)–dye–PEG. $\Delta\log K$ values of proteins at pH 7.0 were 1.5–4-times lower (except for rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$]) than those of the purified protein analogues. This could be caused by contaminating *E. coli* proteins, which are preferentially concentrated in the lower phase of two-phase systems. The increased PEG–dye–Hg(II) ions extraction power with respect to proteins from the rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] inclusion body extract ($\Delta\log K=2.26$, Table 8) compared to that of the purified protein ($\Delta\log K=1.29$, Table 4) may be explained by the possibility that a protein form with one reduced disulfide bridge may be present in the partitioned extract at pH 7.0. Lu et al. [40] recently described a longer-living partially oxidised species (folding intermediates) containing a single correct disulfide bond and two non-disulfide bonded Cys moieties in the folding process of rhG-CSF followed by RP-HPLC. Accordingly, the presence of such a species at pH 7.0 in rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] protein inclusion body extract may also be proposed. In the native, correctly folded state of the [$Cys^{17}\rightarrow Ser^{17}$] form of rhG-CSF there is no free –SH group, and its interaction with Hg(II) at pH 7.0 is half that for the other two protein forms (Table 4). Thus, the effect of two non-disulfide bonded SH groups could outweigh the effect of accompanying *E. coli* proteins since SDS–PAGE and RP-HPLC analysis both indicated a relative amount of the targeted protein forms in the inclusion body extract of 75–91%. The higher protein extraction ability of Hg(II)–dye–PEG as compared with the Ni(II)–dye–PEG-containing upper phase was also observed when the $(His)_6$–rhG-CSF inclusion body extract was partitioned (Table 8) – with $\Delta\log K$ values of 1.00 and 0.52, respectively. Analysis of the number of available SH groups using the Ellman reaction in the $(His)_6$–rhG-CSF inclusion body extract demonstrated approximately 3.07 mol of –SH groups/mol of the protein form. The correctly folded $(His)_6$–rhG-CSF has only one unpaired –SH group. Therefore, the emergence of additional free –SH group function in the inclusion body extract and the higher affinity of –SH groups to Hg^{2+} ions compared to Ni^{2+} ions, explain the stronger interaction with chelated Hg(II) ions when the inclusion body extract was partitioned at pH 7.0. The $\Delta\log K$ values of inclusion body extracts of rhG-CSF and its [$Cys^{17}\rightarrow Ser^{17}$] mutated form partitioning in the presence of Ni(II)–dye–PEG were not calculated because part of the protein formed a film-like layer at the interface. This fact may reflect that inclusion body extract of rhG-CSF and the [$Cys^{17}\rightarrow Ser^{17}$] mutant interacted with PEG–dye–Ni(II) complex in a mode that differed from their interactions with the dye–Hg(II) complex or the

Table 8
Partitioning of rhG-CSF, rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF inclusion bodies extract in the presence of metal ions chelated by LR Yellow 2KT at pH 7.0[a]

M^{2+}	C (M^{2+}), (μmol/kg)	rhG-CSF		rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$]		$(His)_6$–rhG-CSF	
		$\Delta\log K$	Y[b] (%)	$\Delta\log K$	Y (%)	$\Delta\log K$	Y (%)
Ni^{2+}	810	n.c.	28	n.c.	26	0.52	93
Hg^{2+}	802	1.42	99	2.26	98	1.00	97

[a] Two-phase system (4 g) contained 5% (w/w) PEG 6000, 8% (w/w) dextran 60 000, 0.8–1 mg of protein, 0.25 *M* Na_2SO_4 and 50 m*M* HEPES–NaOH buffer, pH 7.0.

[b] Partitioning yield.

n.c., Not calculated.

mode of $(His)_6$-tagged interaction with the dye–Ni(II) complex.

We have also examined the effect of refolding of rhG-CSF and its mutant forms in the inclusion body extracts partitioning experiments in the presence of chelated Hg(II) or Ni(II) ions. The relative amount of correctly folded protein conformation was estimated using RP-HPLC analysis data of protein samples taken from both the interface and the dextran-rich phase. In the case of inclusion body rhG-CSF extract partitioning the protein samples from the dextran phases were also tested for in vitro biological activity. The amount of correctly folded protein conformation, calculated from RP-HPLC chromatograms and rhG-CSF activity data are summarised in Table 9. Typical RP-HPLC analysis of protein samples drawn from the phase systems is illustrated in Fig. 10. In addition, protein samples were analysed by SDS–PAGE. Fig. 11 shows a typical SDS–PAGE pattern of inclusion body rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] extract samples from the interface and from the dextran phase of the phase systems. In the initial inclusion body rhG-CSF extract the folding intermediates with the reduced Cys^{36}–Cys^{42} or Cys^{64}–Cys^{74} disulfide bond, as previously described [52], clearly predominate and the relative amount of incipient correctly folded protein conformation is negligible (Fig. 10B). After inclusion body protein partitioning in the presence of chelated metal ions the relative amount of correctly folded rhG-CSF protein markedly increased and the amount in the dextran-rich bottom phase was higher than that in the interface layer. This tendency remained true for all three rhG-CSF forms irrespective of both metal ion and desorption agent used. However, some differences in the relative refolding efficiency of individual protein forms were noted. The largest amount of correctly folded protein conformation, occurred (Table 9) with the His-tagged protein (60%). Recovery of the correctly folded rhG-CSF [$Cys^{17}\rightarrow Ser^{17}$] was 56% and only 46% for rhG-CSF without mutations if partitioning of inclusion body extracts was performed in the presence of Ni(II) ions with subsequent imidazole-induced transition of target proteins to the dextran phase. The most reasonable explanation for this difference in refolding efficiency is the different binding strength of these rhG-CSF forms with Ni(II) ions, which in turn may be due to sequences of different metal-binding sites donated by the different protein forms. Indeed, albeit indirectly, the differences in the extraction power of pure rhG-CSF, its [$Cys^{17}\rightarrow Ser^{17}$] mutant and the His-tagged form by Ni(II) ions (Table 4), and the different effect of 10 m*M* imida-

Table 9
Dependence of relative amount of correctly folded protein on the nature of metal ion, protein form and desorption agent

No.	Protein form	Metal ion; desorption agent (m*M*)	Relative amount of correctly folded protein (%)	
			Interface	Dextran phase
1	rhG-CSF		12	46
		Ni^{2+};		$(6.45\cdot10^7)$[c]
2	$(His)_6$–rhG-CSF	50 m*M* imidazole	[a]	60
3	rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$]		27	56
4	rhG-CSF	Hg^{2+};	31	43
		10 m*M* 2-mercaptoethanol		$(5.40\cdot10^7)$
5	$(His)_6$–rhG-CSF		[a]	n.d.
6	rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$]		65	91
7	rhG-CSF	Hg^{2+};	26	17[b]
8	$(His)_6$–rhG-CSF	200 m*M* imidazole	[a]	n.d.
9	rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$]		90	99

[a] Protein concentration was less than detection limit.
[b] In the RP-HPLC analysis profile there is a peak with less retention time than of native conformation (relative amount, 82%).
[c] hG-CSF biological activity (U/mg) is shown in parentheses; pure native hG-CSF exhibit biological activity $1\cdot10^8$ U/mg.
n.d., Not determined.

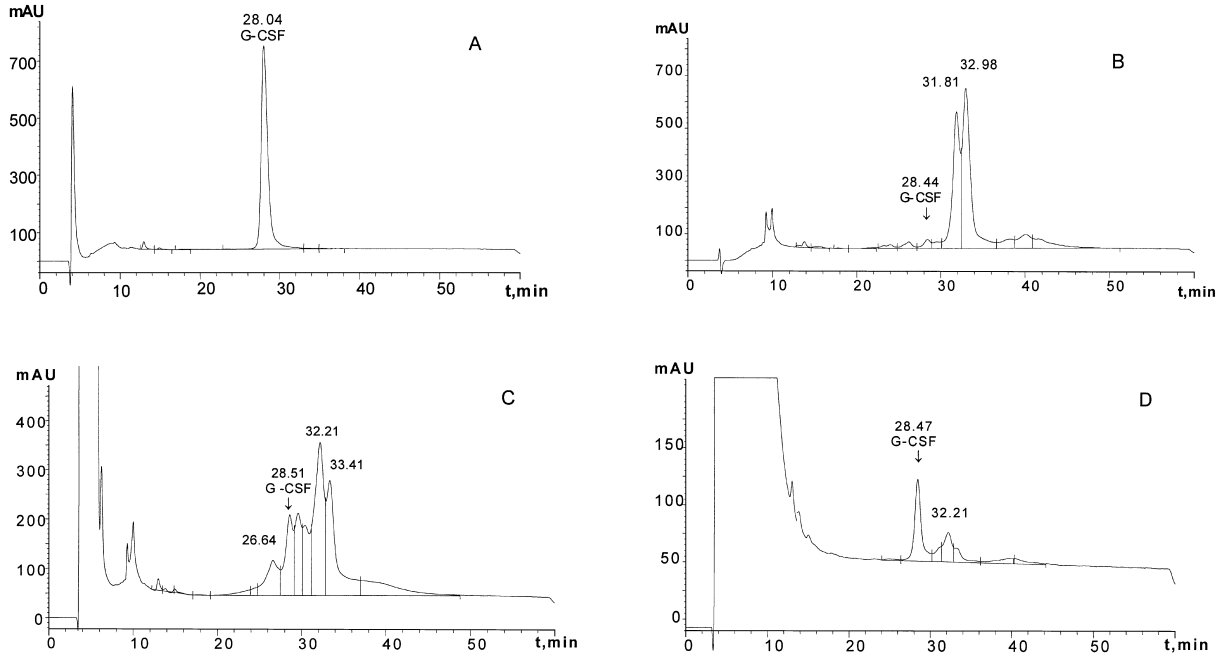

Fig. 10. RP-HPLC analysis. (A) rhG-CSF standard solution (1.042 mg/ml), injection volume 40 μl; (B) rhG-CSF inclusion bodies extract (2.25 mg/ml) in 20 m*M* HEPES–NaOH buffer, pH 7.0, 40 μl; (C) rhG-CSF interface layer solution in 6 *M* GdmHCl (0.29 mg/ml), 300 μl (two-phase system composed of 5% PEG, 8% dextran, 810 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); (D) rhG-CSF sample from the dextran phase of the above-mentioned system (0.06 mg/ml), 500 μl.

zole on Δlog *K* value reduction (Table 6) strongly support this explanation. It seems possible, that in the course of inclusion body extract partitioning, the involvement of the $(His)_6$ tag in the interaction with Ni(II) results in stronger binding and stabilization of the conformation state of the ternary complex of $(His)_6$–rhG-CSF and Ni(II)–dye–PEG compared to the case when similar complex formation occurs via involvement of internal amino acid residues, seem as the His residue, which is likely to be donated by the other two protein forms. In addition differences in environment close to the metal-binding site may also influence the amount of correctly folded protein conformation. Indeed, the increase in the relative amount of correctly folded $Cys^{17}\rightarrow Ser^{17}$ rhG-CSF protein conformation of up to 56% (Table 9), compared to the increase of 46% for rhG-CSF may be due to the replacement of Cys^{17} to Ser^{17}. This mutation eliminates the possibility of incorrect S–S bridge formation in the protein molecule itself, thus protein intermediates with two free –SH groups (instead of three in the case of rhG-CSF or its His-tagged form) may predominate in the initial inclusion body extract. Consequently, the relative amount of correctly folded protein in both the interface layer and the dextran-rich phase would increase. But in both cases, part of the protein released from Ni(II) ions with the aid of imidazole, would be collected at the interface layer. In the case of rhG-CSF, RP-HPLC analysis of the interface layer solubilized in 6.0 *M* guanidine hydrochloride (GdmCl) demonstrated (Fig. 10C), that in addition to 12% of the protein form being correctly folded, other forms of target proteins were also present, which would suggest, that after the addition of imidazole, the misfolded and/or aggregated proteins tend to assemble at the interface layer together with some properly folded proteins.

In the phase systems, which contained chelated Hg(II) ions, the relative amount of correctly folded rhG-CSF conformation was dependent on the type of desorption agent selected; 43% of the rhG-CSF

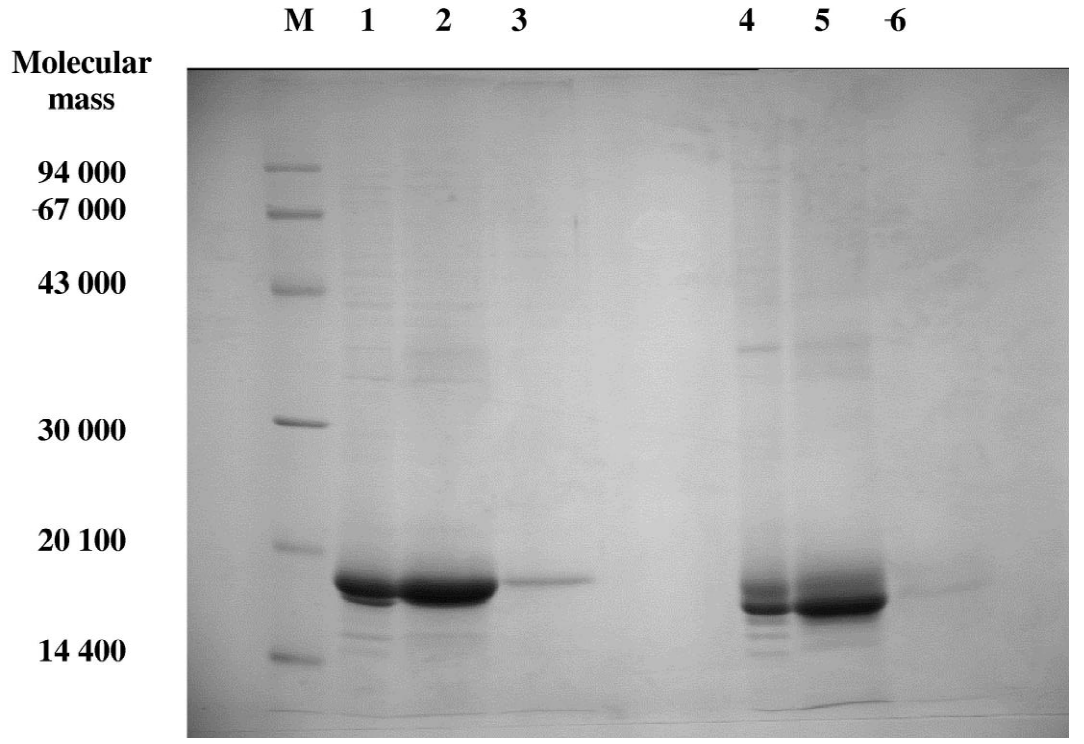

Fig. 11. SDS–PAGE analysis of rhG-CSF[Cys^{17}→Ser^{17}] inclusion bodies extract in 12% SDS–polyacrylamide gel, stained by Coomassie Brilliant Blue R 250. Lanes: 1–3: reducing conditions, 4–6: non-reducing conditions. M: Molecular mass markers; lanes: 2, 5: inclusion bodies extract in 20 m*M* HEPES–NaOH buffer, pH 7.0, 20 μg total protein; 3, 6: 6 *M* GdmHCl solution of protein from the interface of the two-phase system composed of 5% PEG–8% dextran, 0.25 *M* Na_2SO_4, 50 m*M* imidazole in 50 m*M* HEPES–NaOH, pH 7.0 containing 810 μ*M* of Ni^{2+} chelated by LR Yellow 2KT–PEG, 20 μg total protein; 4, 7: protein sample of the dextran phase of the above-mentioned two-phase system, 4.5 μg total protein.

protein was properly folded when the concentration of 2-mercaptoethanol in the phase system was increased to 10 m*M*, as compared to a value of 17%, when 200 m*M* of imidazole was used (Table 9). When imidazole was added a peak (whose area is about 82%) with a retention time shorter than that for the correctly folded protein conformation however was also detected, a peak which may be attributed to the misfolded form of rhG-CSF. Our data allowed us to conclude therefore that during rhG-CSF inclusion body extract partitioning in the presence of the Hg(II)–dye–PEG derivative, the formation of incorrect disulfide bridges may occur. The introduction of 2-mercaptoethanol, probably, allows partial disulfide bridges reshuffling, which in turn leads to the higher amount of correctly folded conformation noted. In contrast, the relative amount of correctly folded rhG-CSF was higher in the presence of Ni(II) (46%) than in the presence of Hg(II) ions (17%), when the protein was released from chelated metal ions with imidazole (Table 9). It seems likely, that if the rhG-CSF form interacts with Ni(II) ions via the histidine residues its conformation could be stabilised in a mode in which the possibility of incorrect disulfide bridge formation is reduced to favour correct folding. The interaction of a protein with Hg(II) ions chelated by dye–PEG usually occurs via the SH group of the unpaired cysteine. If in the rhG-CSF inclusion body extract the intermediate species with three unpaired Cys residues dominates, Hg(II) ions may interact not only with Cys^{17}, but also with the two other Cys residues donated by a productive folding intermediate. This seems possible in accordance with the free –SH groups analysis, as demonstrated for the $(His)_6$-tagged protein form in the inclusion body extract. When

a protein form, enriched with unpaired Cys residues is bonded to chelated Hg(II) ions, the participation of free Cys^{17} in incorrect disulfide bridge formation with other free –SH groups might be possible. Such bridges could be broken in the presence of low-molecular-mass compounds, which possess free –SH groups, e.g., 2-mercaptoethanol. In contrast to rhG-CSF, the influence of the agent on the relative amount of renatured $Cys^{17}\rightarrow Ser^{17}$ rhG-CSF protein was negligible. RP-HPLC analysis of protein samples drawn from the dextran-rich phases indicated that 91% and 99% of protein was correctly folded (Table 9) when 2-mercaptoethanol and imidazole were present, respectively. Thus, in parallel to the reasoning used with rhG-CSF, it is possible to assume, that folding intermediates, which possess only two unpaired Cys residues (Cys^{17} is replaced to Ser^{17}), may predominate in the initial $Cys^{17}\rightarrow Ser^{17}$ rhG-CSF inclusion body extract. Therefore, the process of incorrect disulfide bridge formation is less pronounced for this protein molecule, which may diminish its propensity for unproductive aggregation. In the interface layer, the differences in relative amount of properly folded protein, as determined by RP-HPLC analysis, reached 65% for the protein form released from chelated Hg(II) ions by 2-mercaptoethanol and 90% for that released by imidazole (Table 9). The lower amount found in the interface layer after its release from Hg(II) ions with 10 m*M* 2-mercaptoethanol, may be related to the ability of the latter to reduce disulfide bridges. On the other hand, this concentration of 2-mercaptoethanol may hinder disulfide bridge formation in a number of folding intermediates. Hence a number tend to assemble at the interface. The retention time of trace at the interface in RP-HPLC column was determined to be similar to that for the properly folded moieties from the dextran-rich phase.

Two protein samples drawn from the dextran-rich phase in rhG-CSF inclusion body extract partitioning experiments exhibited statistically reliable and similar biological activities of $5.40\cdot10^7$ IU/mg and $6.45\cdot10^7$ IU/mg, when the protein was released from Hg(II) ions with 10 m*M* 2-mercaptoethanol, or from Ni(II) ions with 50 m*M* of imidazole, respectively. These data correlate well with the relative amount of correctly folded protein determined by RP-HPLC; 43% [10 m*M* 2-mercaptoethanol, Hg(II) ions] and 46% [50 m*M* imidazole, Ni(II) ions], respectively (Table 9). Thus, biological activity data confirms that the interaction between the target protein extracted from inclusion bodies and chelated metal ion induces the generation of a correctly folded native protein possessing rhG-CSF activity.

4. General comments and conclusions

To date Hg(II) ions chelated by an appropriate ligand to probe the accessibility of the free –SH group of the Cys residue by the IMAC procedure have been little used, nor has the procedure of immobilized metal ion affinity partitioning been developed. We have chose the reactive dye LR Yellow 2KT as a chelator to allow us to monitor its interaction with Hg^{2+} ions by difference spectroscopy. Our earlier observations [42] on the ability of this dye to form relatively stable complexes with the first-row transition metal ions such as Cu(II), Ni(II) and Zn(II) at neutral pH were confirmed. And, what is more important, we found that Hg^{2+} ions also are able to form complexes with this dye within the studied pH range 5.0–7.0 (Fig. 2 and Table 1) and have free remaining sites, through which to interact with rhG-CSF in solution, as evidenced in Fig. 2B. The dissociation constants (K_d) for ternary dye–Hg(II) complexes with rhG-CSF (Table 2) and its DEP-modified form were shown to be almost equal at pH 7.0 to suggest, that His residues are not essential in the interaction with the dye–Hg(II) complex.

More precise information on the amino acid side chains of rhG-CSF that might be involved in binding with chelated metal ions was provided by the comparison of the interaction of hG-CSF, its mutated form rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] and $(His)_6$–rhG-CSF with metal ions, such as Cu(II), Ni(II) and Hg(II) by means of immobilized metal ion affinity partitioning. The decreases in $\Delta\log K$ values for rhG-CSF and rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] in the presence of Cu(II) ions in the two-phase system in the range of pH 7.0–5.0 (Figs. 4 and 5, Tables 4 and 5), and the observed threefold increase in K_d for the ternary complex dye–Cu(II)–DEP-modified rhG-CSF at pH 7.0, indicated the involvement of surface-exposed His residues in the interaction with Cu(II) ions.

Recently studies concerning alanine scanning mutagenesis of rhG-CSF [53,54], have identified many surface-exposed amino acid residues. Among them, surface-exposed His^{43}, His^{52} and His^{156} [54] are of primary interest since they may be involved in the interaction with Cu(II) ions. The strength of the interaction of rhG-CSF and its rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] mutant with Cu(II)–dye–PEG at pH 7.0 was found to be of the same order of magnitude as that for the $(His)_6$-tagged form (Table 4), to suggest the involvement of more than one His residue. Support for this is the fact that 1 m*M* imidazole added had negligible effect on the rhG-CSF $\Delta\log K$-decrease (Table 6), and a pronounced effect was observed only with 10 m*M* imidazole (Table 6). It was clearly demonstrated previously [55,56], that the multiplicity of available His residues on the protein surface defined the strength of its retention on the IDA–Cu(II) column, e.g., horse cytochrome *c* (one histidine) was not retained while yeast cytochrome *c* (two histidines) was retained in the presence of 1.0 m*M* imidazole and further eluted over a gradient of from 1.0 to 10 m*M* [56]. The linear dependence of apparent metal affinity on histidine content was also shown by the partitioning of native and recombinant forms of a protein in a PEG–dextran two-phase system, which contained Cu(II)–IDA–derivatized PEG [23,57–59]. The fact that the interaction of both rhG-CSF and its mutated form with Cu(II) ions at pH 5.0 (Table 5) was indistinguishable therefore may be attributed to the involvement of other residues, a suggestion possibly supported by the noticeable decrease in $\Delta\log K$ values of both rhG-CSF and its $Cys^{17}\rightarrow Ser^{17}$ mutant under the low concentration (0.2 m*M*) of L-tyrosine at pH 5.0 (Table 7). On the rhG-CSF surface Tyr^{39} was identified as an H-bonded exposed residue [53], a potential candidate to interact with Cu(II) ions. Such an idea is supported by recent fluorescence spectrometry studies of rhG-CSF [40]. The peak attributable to Tyr was detected in the fluorescence spectrum of the native protein at a slightly acidic pH (3.0), to suggest in our case that the Tyr residue might be able to interact with chelated Cu(II) ions at pH 5.0. The $(His)_6$–rhG-CSF, in contrast to the two other protein forms, equally strongly binds to Cu(II) at both pH values (Tables 4 and 5), demonstrating the importance of the His tag in the interaction with Cu(II) ions.

The interaction of rhG-CSF with Ni(II) was strongly reduced in the range of pH from 7.0 to 5.0 (Fig. 4, Tables 4 and 5), an observation associated with the protonation of the histidine imidazole ring, indicating the major role of surface-exposed His residues in the protein binding to Ni(II) ions. This interpretation was supported by the stronger decrease in $\Delta\log K$ caused by 10 m*M* imidazole compared to 10 m*M* 2-mercaptoethanol at pH 7.0 (Fig. 7, Table 6). The lower $\Delta\log K$ value for the $Cys^{17}\rightarrow Ser^{17}$ mutant compared to that of rhG-CSF in a system, which contained Ni(II) ions (pH 7.0, Table 4) suggested that in addition to His residues, the Cys^{17} of rhG-CSF might also be involved in complexation with Ni(II) ions. In favour of such proposal the slight increase in rhG-CSF $\Delta\log K$ observed in the presence of Ni(II) ions at pH 5.0 compared with that of the dye alone, as well the inability of 10 m*M* of 2-mercaptoethanol to reduce the $\Delta\log K$ of the rhG-CSF mutated form should be noted at pH 7.0 (Fig. 7). Moreover, the stronger interaction of $(His)_6$–rhG-CSF with Ni(II) ions compared to rhG-CSF confirms the involvement of more than one exposed His residue to ensure the effective interaction with chelated Ni(II) ions. It has been shown [56], that at least two available His residues are necessary to ensure the efficient protein retention on Ni(II)–IDA column. In this context, the Cys^{17} may participate in the rhG-CSF interaction with Ni(II) ions in addition to the exposed His residues.

As expected, the nature of the chelated Hg(II) ion interaction with rhG-CSF and its mutant forms was different to that of chelated Cu(II) and Ni(II) ions. The displacement of Cys^{17} to Ser^{17} in the rhG-CSF molecule caused a considerable decrease in $\Delta\log K$ at both pH values studied. 10 m*M* 2-mercaptoethanol at pH 7.0 (Table 6) or 1 m*M* at pH 5.0 (Table 7) were sufficient to abolish Hg(II) binding to all protein forms. In contrast to the phase system, which contained Cu(II) or Ni(II) ions, the introduction of 10 m*M* 2-mercaptoethanol in the system which contained Hg(II) ions caused a bigger decrease in $\Delta\log K$ compared to that of 10 m*M* imidazole (Table 6). Modification of rhG-CSF with DEP had almost no effect on the K_d of the ternary complex dye–Hg(II)–rhG-CSF. Our experimental findings suggest, that Cys^{17} plays a major role in the rhG-CSF interaction with Hg(II) ions. The decrease in $\Delta\log K$ for rhG-CSF caused by L-aspartic acid at pH 5.0

(Table 7) suggests that Asp or Glu residues also may be involved in the binding with Hg(II) ions. The presence of such residues exposed on the surface of rhG-CSF has been previously reported [53,54]. Nevertheless, additional studies are necessary for strict conclusions on the involvement of such residues in the interaction with Hg(II) ions at pH 5.0.

RP-HPLC analysis of the initial inclusion body extract revealed predominance of protein forms which possessed either one or two broken disulfide bridges. Approximately 3.07 mol of free-reduced –SH groups per mol of $(His)_6$–rhG-CSF in its inclusion body extract were measured, to suggest the dominance of protein intermediates with one reduced disulfide bond, either Cys^{36}–Cys^{42} or Cys^{64}–Cys^{74}. It seems possible that the participation of such species presumably, in interactions with chelated Hg(II) ions governed the extraction (Table 8) and the refolding efficiency (Table 9) of the target protein. The moderate amount of correctly folded rhG-CSF (43%) and high relative amount of correctly folded rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$] (91–99%) found when inclusion body extracts were partitioned in the presence of chelated Hg(II) ions, with subsequent protein release into the dextran-rich phase with 2-mercaptoethanol obviously indicates that such refolding events are possible. The reduction in correct folding of rhG-CSF seems to be associated with the participation of the unpaired Cys-17 of rhG-CSF in the misfolding events, so that the refolding efficiency become dependent on the type of agent selected for the release of the protein form from Hg(II) ions into the dextran phase. The replacement of Cys^{17} with Ser^{17} increased the relative amount of correctly folded protein. This was more noticeable in two-phase systems, which contained immobilized Hg(II) ions (Table 9), and might be related to the excluded possibility of the formation of incorrect disulfide bridges during the transition of the intermediate with one disulfide bond to the correctly folded protein. In the systems containing chelated Ni(II) ions the relative amount of correctly folded rhG-CSF and both its studied forms (Table 9) was found dependent on the type of metal-binding site participating in the interactions. The bigger refolding efficiency of the $(His)_6$-tagged rhG-CSF was obviously explained by the stronger binding of the $(His)_6$ tag with chelated Ni(II) ions which promoted protein refolding, as compared to the refolding efficiency when the internal His residues of rhG-CSF or its $Cys^{17}\rightarrow Ser^{17}$ form were involved in interactions with Ni(II) ions (Table 9). In general, the same tendency was also observed in studies of the refolding of these rhG-CSF forms when their respective inclusion body solutions in 3.0 *M* GdmCl were chromatographed on to the Ni(II) charged IDA-Sepharose column (unpublished data).

In conclusion, the comparative study of rhG-CSF and its $Cys^{17}\rightarrow Ser^{17}$ mutants partitioning behaviour in a two-phase system in the presence of the PEG–Light Resistant Yellow 2KT–Hg(II) derivative indicated that protein interactions with chelated Hg(II) ions may occur via the free –SH group of the unpaired Cys^{17}. The interactions of rhG-CSF forms studied with chelated Cu(II) ions was independent at pH 7.0, of either internal His residues or His tag involvement. However, the His tag introduction to rhG-CSF ensured strong protein interaction with chelated Ni(II) ions. Partitioning of inclusion body extracts of rhG-CSF forms revealed refolding effects which were dependent on the type of chelated metal ions and donor groups donated by the protein forms. In the phase systems containing Ni(II) ions the relative amount of correctly folded rhG-CSF forms found in dextran-rich phases increased in the order $(His)_6$–rhG-CSF > rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$]>rhG-CSF; in those containing chelated Hg(II) ions correctly folded rhG-CSF forms increased in the order rhG-CSF[$Cys^{17}\rightarrow Ser^{17}$]>rhG-CSF.

The data presented here indicates that the immobilized metal ion affinity partitioning technique may be used to probe the availability of unpaired Cys residues on the surface of native proteins using chelated mercuric ions, and may serve as an independent tool for the investigation of refolding events of recombinant inclusion body proteins which possess histidine, cysteine or other amino acids available for interaction with metal ions. To date, the larger application of this technique of studying recombinant inclusion body protein refolding is limited by the lack of two-phase systems, which might contain high concentrations of chaotropes, in particular GdmCl, commonly used to solubilize inclusion body proteins. But recent studies of the oxidative renaturation of hen-egg white lysozyme performed in an aqueous PEG–salt two-phase system containing 1 *M* of GdmHCl [60] indicate that these will be overcome.

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