Journal of Chromatography, 539 (1991) 267–277
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 876

Immobilized metal ion affinity partitioning, a method combining metal–protein interaction and partitioning of proteins in aqueous two-phase systems[a]

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ABSTRACT

Immobilized metal ions were used for the affinity extraction of proteins in aqueous two-phase systems composed of polyethylene glycol (PEG) and dextran or PEG and salt. Soluble chelating polymers were prepared by covalent attachment of metal-chelating groups to PEG. The effect on the partitioning of proteins of such chelating PEG derivatives coordinated with different metal ions is demonstrated. The proteins studied were α_2-macroglobulin, tissue plasminogen activator, superoxide dismutase and monoclonal antibodies. The results indicate that immobilized metal ion affinity partitioning provides excellent potential for the extraction of proteins.

INTRODUCTION

Immobilized metal ion affinity chromatography (IMAC), which makes use of the selective retention of proteins on transition metal ions chelated to an insoluble matrix such as agarose, was introduced by Porath *et al.* [1]. The important contribution of Sulkowski [2] to the understanding of the underlying mechanism of this selective recognition has made this concept one of the best suited in separation technology, as evidenced by the increasing number of papers appearing in this field. While the concept of protein affinity for metal chelates has been extensively studied by the chromatography of proteins [3], peptides [4] and, more recently, of whole cells [5], very few attempts have been made to introduce this affinity principle to the other analytical and preparative separation methods [6]. The utilization of aqueous

[a] This paper is dedicated to Professor P.-Å. Albertsson on the occasion of his 60th birthday.

0021-9673/91/$03.50

two-phase systems for the affinity purification of many molecules, using pseudo-specific ligands such as triazine dyes, is now well established [7]. Hence, it is of considerable interest to exploit the affinity of proteins for immobilized (chelated) metals in such partitioning systems. Both of these aspects are the subject of this paper.

However, certain requirements have to be met in order to exploit successfully the IMA principle in affinity partitioning. First, an appropriate chemistry must be developed for the preparation of soluble polymers with covalently coupled chelating groups. Second, the impact of the protein surface topography on their recognition by metal chelates has to be considered. Moreover, the microenvironment of the electron donor grouping [5] on the protein surface may result in a variable mechanism of protein recognition by a metal ligand.

EXPERIMENTAL

Polyethylene glycol (PEG) 6000 and 1540 were obtained from Serva (Heidelberg, Germany). Dextran T-70 was obtained from Pharmacia (Uppsala, Sweden) and bromoacetic acid from Aldrich (Steinheim, Germany).

α_2-Macroglobulin (α_2-M) was purified from fresh plasma [8]. Copper–zinc superoxide dismutase (SOD) was a gift from Symbicom (Umeå, Sweden). Single-chain tissue plasminogen activator (t-PA) was kindly provided by Professor P. Wallen, University of Umeå, Sweden.

Preparation of metal chelate PEG

Iminodiacetate-PEG (IDA-PEG) was synthesized by reaction of bromoacetic acid with aminomonomethoxy-PEG (amino-M-PEG) 5000. Amino-M-PEG was prepared according to Cordes [9]. Briefly, 60 g of M-PEG 5000 (Sigma) were melted at 65°C and water was removed under vacuum. After addition of 3 ml of distilled thionyl chloride, the sample was rotated for 6 h at 65°C under a nitrogen atmosphere to exclude moisture. After removing excess of thionyl chloride by evaporation, the residue was dissolved in 3 l of absolute ethanol and precipitated at 4°C. The dried Cl-M-PEG was dissolved in 150 ml of water and 150 ml of ammonia solution (25%) were added. The solution was placed in a sealed plastic tube and left for 100 h at 55°C in a dry oven. After evaporation of the solvent, 53 g of amino-M-PEG were obtained.

For the preparation of IDA-PEG, 15 g of amino-M-PEG dissolved in 100 ml of water were reacted with 15 g of bromoacetic acid at pH 8.5 for 12 h and room temperature. Then, 100 ml of water were added and the IDA-M-PEG was extracted three times with 300 ml of chloroform. Phase separation was accelerated by centrifugation. The combined chloroform phases were dried over anhydrous sodium sulphate and the solvent was removed by distillation. After two crystallizations in absolute ethanol, 12–13 g of IDA-PEG were obtained.

Metal charging of IDA-PEG was done by dissolving 2 g of IDA-PEG in 10 ml of 50 m*M* sodium acetate (pH 4.0) containing the respective metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+}, Fe^{3+}) in a 10–20-molar excess over the IDA-PEG. The solution was stirred for 1 h and then extracted with chloroform. The chloroform was dried and evaporated, yielding 1.9 g of metal–IDA-PEG.

The metal content in solutions was determined by flame photometry using appropriate standards.

Preparation of two-phase systems

The two-phase systems were prepared from stock solutions of polymers in water–40% (w/w) PEG and 20% (w/w) dextran. The polymer solutions were mixed with buffer, water and protein sample to give the final concentration as indicated in the legends to the figures. Affinity partitioning was performed by replacing part of the total PEG with metal–IDA-PEG. The systems were equilibrated by 40 inversions at 22°C. Phase separation was accelerated by centrifugation at 2000 *g* for 2 min. Aliquots were withdrawn from the top and bottom phases and analysed for the metal or protein content. Similarly, two-phase systems composed of PEG and salt were prepared from stock solutions and treated as described above.

Determination of the partition coefficient

The partition coefficient, K, was calculated as the ratio of the concentration or radioactivity of a species in the upper and lower phase. The effect of the affinity of a protein for the metal–IDA-PEG was expressed in terms of $\Delta \log K$, given by $\Delta \log K = \log K_{aff} - \log K_0$, where K_{aff} and K_0 are the partition coefficients of the protein in the presence and absence of metal–IDA-PEG in the system, respectively, other conditions being identical. The concentrations of α_2-M and monoclonal antibodies were determined by enzyme-linked immunoadsorbent assay (ELISA).

Iodination

SOD and t-PA were iodinated with 0.5 mC of ^{125}I (Amersham, U.K.), applying the Iodobeads technique [10].

RESULTS

Preparation of metal–IDA-PEG

The soluble chelating polymer was synthesized by three steps starting from monomethoxy-PEG as outlined in Fig. 1. Under the conditions used, mostly monosubstituted chelating PEG was formed. The nitrogen content of IDA-PEG as determined by elemental analysis was 0.32%, which is higher than expected (theoretical value 0.28%). Partial dimethylation during the reaction may account for this discrepancy.

The tridentate IDA-PEG may occupy a maximum of three coordination sites in the metal coordination sphere, leaving three coordination sites free for interaction withe water or proteins at least in the case of a hexacoordinate central metal ion. The amounts of the metal ions such as Cu^{2+}, Zn^{2+}, Ni^{2+} and Fe^{3+} chelated by the IDA-PEG are given in Table I. With Cu^{2+} and Zn^{2+}, about 1 mol of metal ion is chelated per mol of the polymer; Ni^{2+} and Fe^{3+} gave lower binding ratios.

Partitioning of metal–IDA-PEG in two-phase systems

The partition coefficients of metal–IDA-PEG complexes in PEG–dextran and PEG–salt sysems are compared in Table II. In PEG–dextran systems, the metal ions partition in favour of the PEG-rich top phase owing to chelation with IDA-PEG. Even in PEG–salt systems, a high partition coefficient was observed at least for Cu^{2+}. On the other hand, Zn^{2+}, Ni^{2+} and Fe^{3+} are probably released from the chelating polymer, as deduced from their low K values in systems containing high concentrations of phosphate.

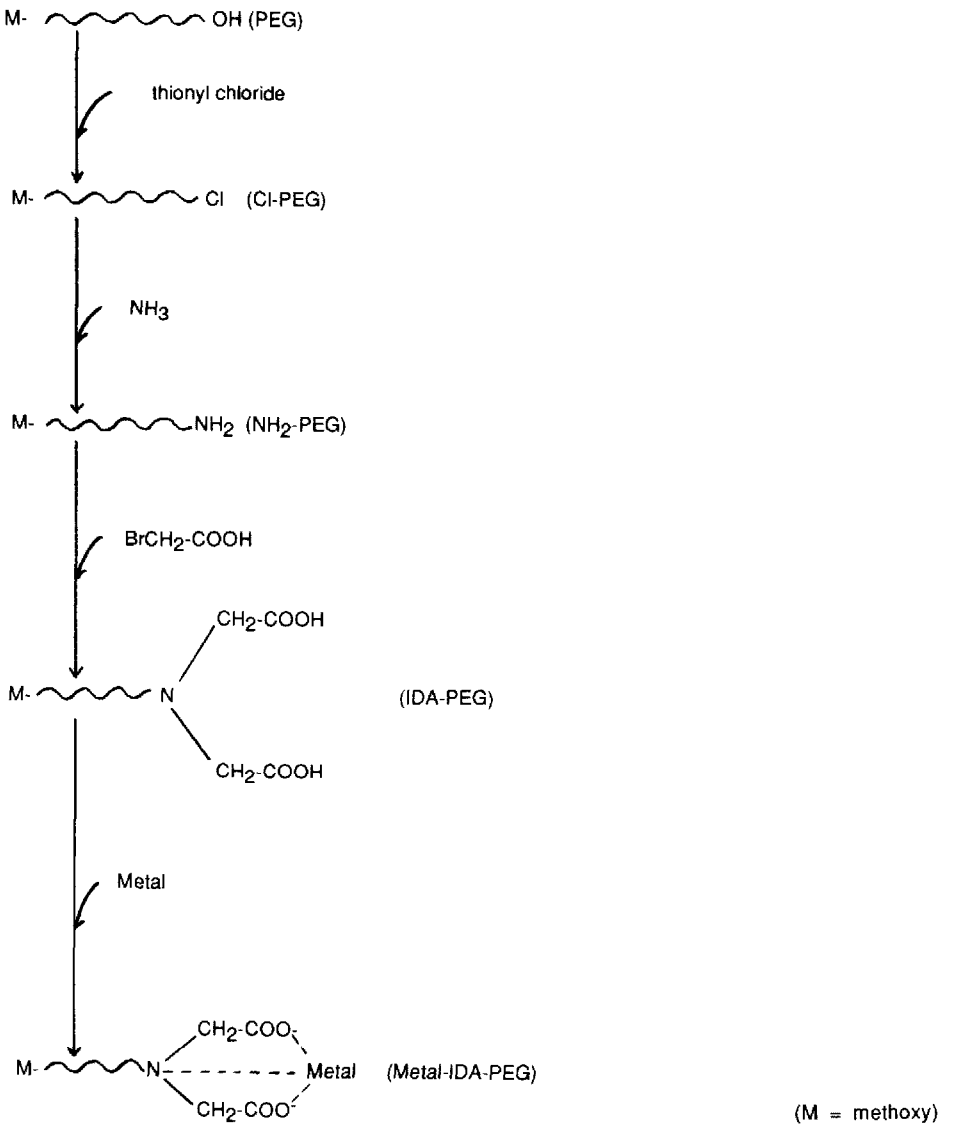

Fig. 1. Synthesis of metal–IDA-PEG.

Effect of metal–IDA-PEG on partitioning of α_2*-M*

The proteinase inhibitor α_2-M was used as a model protein to study the principles of metal chelate affinity partitioning. When α_2-M was added to a system containing increasing concentrations of metal–IDA-PEG, different extraction curves were obtained (Fig. 2). Cu^{2+} IDA-PEG causes a change in the partition coefficient of more than 1000-fold ($K_0 = 0.085$; $K_{aff} = 85$) yielding a maximum $\Delta \log K$ of *ca.* 3.0. The complexed metals Zn^{2+} and Ni^{2+} give rise to lower efficacy; Fe^{3+} seems to have a negligible affinity to the protein.

TABLE I

METAL ION CONTENT OF IDA-PEG AFTER CHARGING WITH Cu^{2+}, Zn^{2+}, Ni^{2+} AND Fe^{3+}

The metal content is expressed as μmol of the respective metal per μmol of IDA-PEG. The nitrogen content of IDA-PEG as determined by elemental analysis was 0.32%, giving rise to the assumption of monosubstituted PEG.

Metal ion	Metal ion content (μmol/μmol IDA-PEG)
Cu^{2+}	0.92
Zn^{2+}	0.80
Ni^{2+}	0.27
Fe^{3+}	0.40

Effect of pH and salt concentration on affinity partitioning of α_2-M

Coordinate binding of proteins to transition metals is expected to be sensitive to the pH of the medium. The magnitude of such pH effects can be exactly measured by titration experiments as shown in Fig. 3. The binding of α_2-M to Cu^{2+}–IDA-PEG is diminished as the pH of the phase system is decreased. The effect of salts such as NaCl and Na_2SO_4 on the partitioning of α_2-M was two-fold. At pH values above 5, the binding of α_2-M to Cu^{2+}–IDA-PEG was strengthened in the presence of salts, indicated by the higher $\Delta \log K$ values obtained. At lower pH values, salts seemed to suppress the binding, probably owing to quenching of non-coordination bonds.

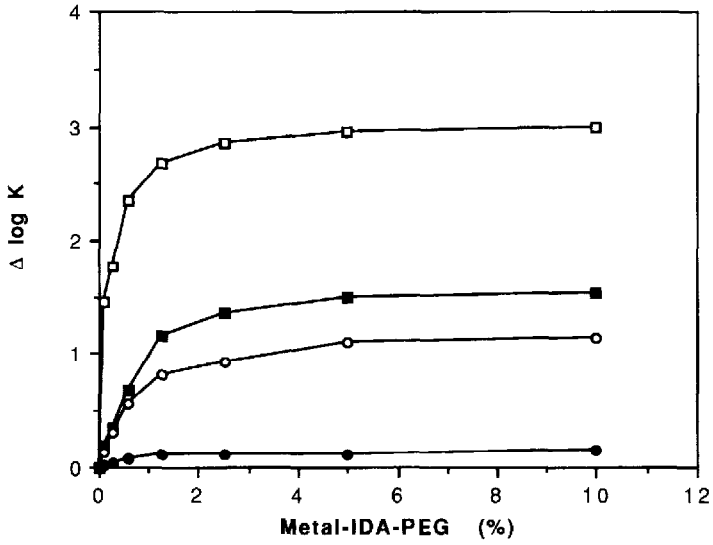

Fig. 2. Effect of metal–IDA-PEG on partitioning of α_2-M in PEG–dextran systems. Two-phase systems of 2 g were composed of 5% PEG 6000, 7.5% dextran T-70, 0.1 *M* Na_2SO_4, 0.01 *M* sodium phosphate buffer (pH 7.0), 20 μg of α_2-M and increasing amounts of metal–IDA-PEG. The concentration of the liganded polymer is expressed as a percentage of the total PEG in the system. □ = Cu^{2+}–IDA-PEG; ■ = Zn^{2+}–IDA-PEG; ○ = Ni^{2+}–IDA-PEG; ● = Fe^{3+}–IDA-PEG.

TABLE II

PARTITION COEFFICIENTS OF METAL IDA-PEG COMPLEXES IN PEG–DEXTRAN AND PEG–SALT SYSTEMS

PEG–dextran system: 5% PEG 6000, 7.5% dextran T-70, 0.1 *M* Na_2SO_4, 0.01 *M* sodium phosphate buffer (pH 7.0); 20% of the total PEG 6000 was replaced with different metal–IDA-PEG derivatives. PEG–salt system: 11% PEG 1540, 13.5% K_2HPO_4–KH_2PO_4 (pH 7.0), 2% PEG 6000; 20% of the total PEG 6000 was replaced with different metal–IDA-PEG derivatives. The partitioning was performed at 22°C. Aliquots were removed from the top and bottom phases, appropriately diluted and subjected to flame photometry. Blanks were systems without metal–IDA-PEG.

Metal–IDA-PEG	*K*	
	PEG–dextran	PEG–salt
Cu^{2+}–IDA-PEG	11.1	10.2
Zn^{2+}–IDA-PEG	5.5	0.55
Ni^{2+}–IDA-PEG	10.6	0.25
Fe^{3+}–IDA-PEG	11.3	0.44

Partitioning of α_2-M in PEG–salt systems in the presence of metal–IDA-PEG

Aqueous two-phase systems may also be formed by mixing PEG with high concentrations of salts such as sodium sulphate or sodium phosphate. In such systems the polymer is highly concentrated in the top phase, whereas the bottom phase is formed by the salt solution. Even there, Cu^{2+}–IDA-PEG was found to be very effective in extracting the protein from the salt-rich bottom phases (Fig. 4A and B). The highest $\Delta \log K$ values obtained are comparable to that yielded in PEG–dextran systems. The difference in the steepness of the partition curves indicates that the affinity of α_2-M for Cu^{2+}–IDA-PEG is obviously higher in PEG–sulphate than PEG–phosphate systems. This may reflect different effects of these salts on the water structure in the medium.

Dissociation of protein–metal–IDA complexes

Transition metal ions, particularly Cu^{2+}, Zn^{2+} and Ni^{2+}, bind with electron-donor atoms such as N, O and S in available amino acid residues in proteins. Hence, it would be expected that the dissociation of the metal chelate–protein complexes could be accomplished by competing electron donors, *e.g.*, free amino acids. As shown in Fig. 5, cysteine strongly diminished the binding of α_2-M to Cu^{2+}–IDA-PEG with increasing concentration in the two-phase system. In comparison, imidazole and tryptophan were less effective. No influence on the affinity partitioning of α_2-M was observed by the addition of ammonium ions at the pH of the experiment. As one would expect, the addition of EDTA at concentrations above 2 m*M* abolished the binding of α_2-M to Cu^{2+}–IDA-PEG.

Immobilized metal ion affnity partitioning of other proteins

The binding properties of different proteins towards selected chelated transition metals are compared in Table III. The partitioning experiments were conducted in PEG–dextran and PEG–salt systems. Apparently, uncharged IDA-PEG had only

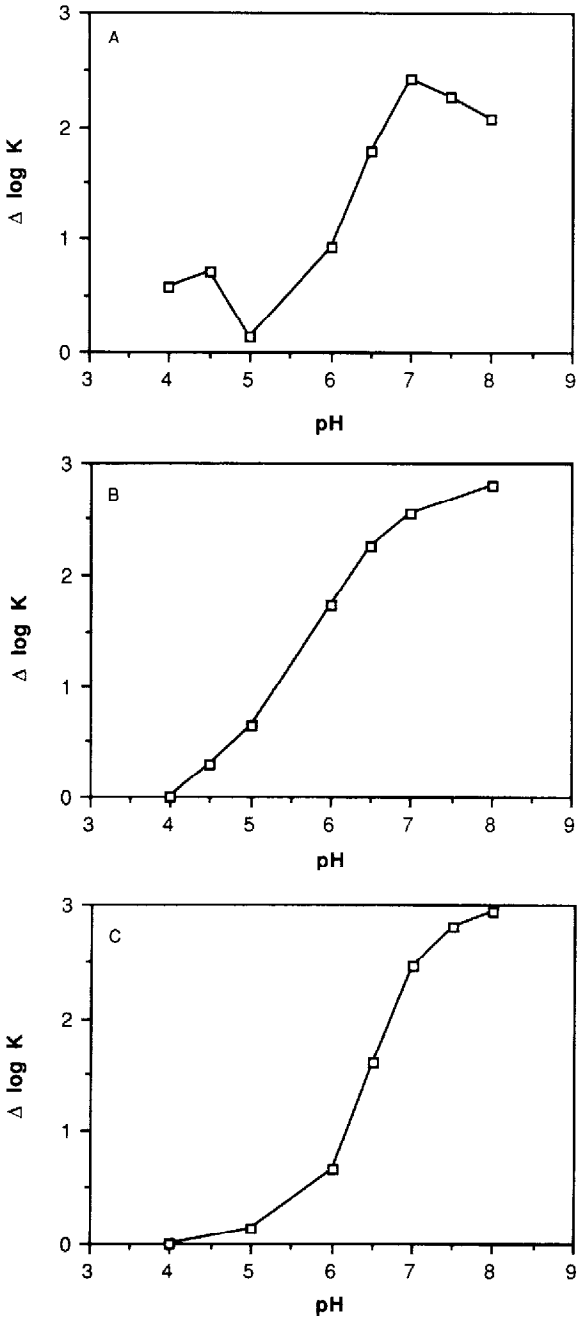

Fig. 3. Effect of pH and salt on affinity partitioning of α_2-M. Two-phase systems of 12 g, composed of 5% PEG 6000, 7.5% dextran T-70, 240 μg of α_2-M in the presence or absence of salt and Cu^{2+}–IDA-PEG, were adjusted to pH 8.0 with 0.1 *M* NaOH with continuous stirring at 22°C. After 5 min of equilibration, the pH was decreased stepwise by adding 0.1 or 0.01 *M* HCl, and aliquots of 1 ml were withdrawn, immediately centrifuged and analysed for the protein in the top and bottom phases. Composition of the systems: (A) 2.5% Cu^{2+}-IDA-PEG, no salt; (B) 2.5% Cu^{2+}–IDA-PEG, 1 *M* NaCl; (C) 2.5% Cu^{2+}–IDA-PEG, 0.1 *M* Na_2SO_4.

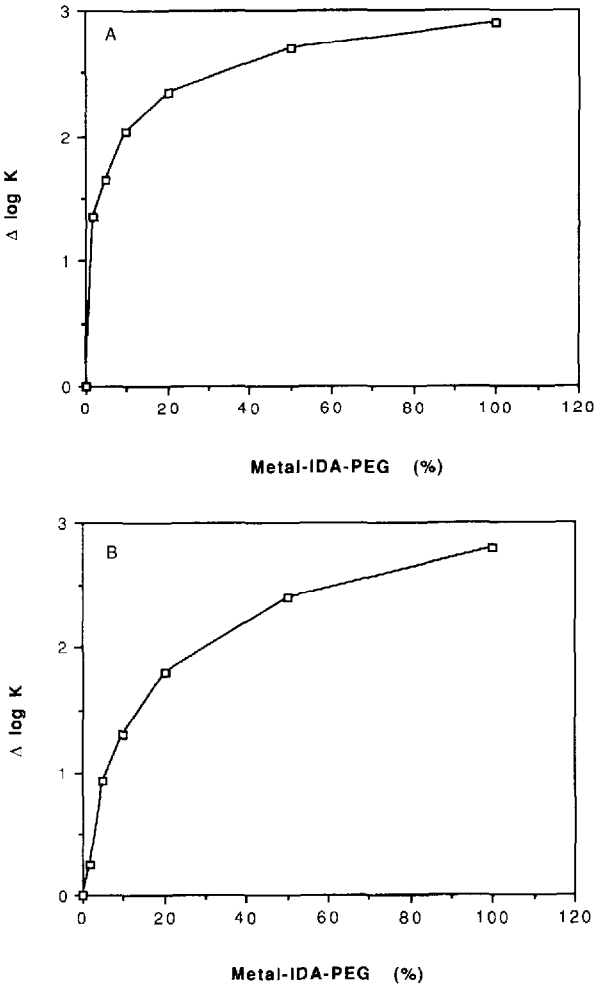

Fig. 4. Effect of metal–IDA-PEG on partitioning of α_2-M in PEG–salt systems. Two-phase systems of 2 g were composed of either (A) 10% PEG 1540, 10% Na_2SO_4, 2% PEG 6000, 10 m*M* sodium phosphate buffer (pH 7.0) and 20 μg of α_2-M or (B) 11% PEG 1540, 13.5% K_2HPO_4–KH_2PO_4, 2% PEG 6000 and 20 μg of α_2-M. The given percentage of liganded PEG expresses that part of the total PEG 6000 which was replaced with Cu^{2+}–IDA-PEG. Log *K* for α_2-M was −1.17 and −1.1 for the PEG–sulphate and PEG–phosphate systems, respectively.

marginal effects on the partitioning of the proteins in both kinds of two-phase systems. As expected, the binding properties of the proteins differed significantly owing to their different surface properties. In general, Cu^{2+}–IDA-PEG was found to be the most effective ligand with both PEG–dextran and PEG–salt systems. Zn^{2+}–, Ni^{2+}– and Fe^{3+}–IDA-PEG were less effective, especially when used in conjunction with PEG–phosphate systems. This is probably due to the formation of metal phosphates because Zn^{2+} and Ni^{2+} are known to be bound less strongly than Cu^{2+} by

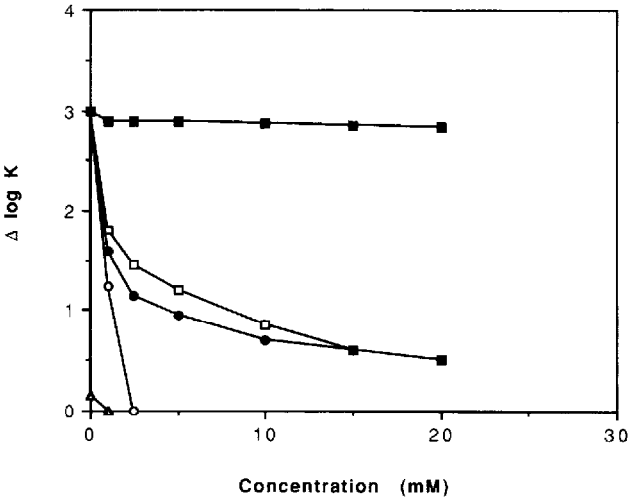

Fig. 5. Dissociation of the protein–metal–IDA-PEG complexes. The two-phase systems of 2 g were composed of 5% PEG 6000 including 2.5% Cu^{2+}–IDA-PEG, 7.5% dextran T-70, 0.1 *M* Na_2SO_4, 0.01 *M* sodium phosphate buffer (pH 7.0), 20 μg of α_2-M and different additives in the concentrations given on the abscissa. □ – Imidazole; ● – tryptophan; ○ – cysteine; △ = EDTA; ■ = ammonium ion. The $\Delta\log K$ values were calculated in comparison with systems of the same composition but without Cu^{2+}–IDA-PEG ($\log K_0$).

iminodiacetate chelator. Among the proteins tested, α_2-M displayed the strongest binding to metal–IDA-PEG. t-PA interacted preferentially with chelated Cu^{2+}. Its affinity to chelated Ni^{2+} was higher than that to chelated Zn^{2+}. SOD bound fairly selectively to Cu^{2+}–IDA-PEG whereas no interaction was found with chelated Zn^{2+}

TABLE III

EFFECT OF METAL–IDA-PEG ON PARTITIONING OF DIFFERENT PROTEINS IN PEG–DEXTRAN AND PEG–SALT SYSTEMS

PEG–dextran system: 5% PEG 6000, 7.5% dextran T-70, 0.1 *M* Na_2SO_4, 0.01 *M* sodium phosphate buffer (pH 7.0); for the calculation of $\Delta\log K$, 20% of the total PEG was replaced with uncharged IDA-PEG or metal–IDA-PEG. PEG–salt system: 11% PEG 1540, 2% PEG 6000, 13.5% K_2HPO_4-KH_2PO_4 (pH 7.0); for the calculation of $\Delta\log K$, 50% of the total PEG 6000 was replaced with uncharged IDA-PEG or metal–IDA-PEG.

Protein	$\Delta\log K$							
	PEG–dextran: metal–IDA-PEG				PEG–salt: metal–IDA-PEG			
	Cu^{2+}	Zn^{2+}	Ni^{2+}	None	Cu^{2+}	Zn^{2+}	Ni^{2+}	None
α_2-Macroglobulin	3.00	1.50	1.10	0.17	2.4	0.06	0.18	0.02
Tissue plasminogen activator	1.59	0.78	1.20	0.14	1.23	0.46	0.31	0.03
Superoxide dismutase	1.30	0.05	0.18	0.08	0.83	0.10	0.20	0.05
Monoclonal antibody ($IgG_{1\kappa}$)	1.15	0.12	0.05	0.10	n.d.[a]	n.d.	n.d.	n.d.

[a] n.d. = Not determined.

and Ni^{2+}. When we tested a range of monoclonal antibodies, different partition coeffients were obtained. However, all displayed the strongest binding to chelated Cu^{2+}, as exemplified for a monoclonal antibody of the immunoglobulin (Ig) $G_{1\kappa}$ type. No correlation was found between the magnitude of binding ($\Delta \log K$) to chelated metal ions and a certain class or subclass specificity.

DISCUSSION

In this study the feasibility of complexation of proteins with chelated metal ions was exploited for affinity extraction in aqueous two-phase systems; so far the only published application is the extraction of haemoglobin [11].

Using α_2-M as a model protein, the effect of the transition metal ions Cu^{2+}, Zn^{2+}, Ni^{2+} and Fe^{3+} chelated to IDA-PEG on the partitioning of this protein was clearly shown. The results reveal that the type and concentration of the chelated metal ions are important parameters which influence the partition coefficient of the protein. The strong effect exerted by chelated Cu^{2+} on the partitioning of proteins in comparison with other metal ions parallels its strong tendency to coordinate predominantly with histidine residues on the protein surface. It is fairly clear that the accessibility of surface-exposed histidine residues is the dominant factor governing the binding strength of metal chelates to proteins [12]. Accordingly, the differences in the $\Delta \log K$ values obtained for α_2-M, t-PA, SOD and a monoclonal antibody may account for this fact.

To perform affinity partitioning, specific or pseudo-biospecific ligands such as inhibitors, antibodies or dyes were used [7]. However, the difficulty of reversing the ligand–protein interaction is often encountered with this technique. In the case of immobilized metal ion affinity partitioning (IMAP), several possibilities exist for dissociating the metal–protein complex under nondenaturing conditions. The addition of free electron-donating amino acids or EDTA was as effective as lowering the pH of the two-phase system (Figs. 3 and 5).

As shown in Fig. 4 and Table III, the binding of proteins to chelated metal ions occurs at high concentrations of salts in the phase system. In addition, a promoting effect of the water structure-forming salts such as sodium phosphate and sodium sulphate on binding of α_2-M to Cu^{2+}–IDA-PEG was observed. This offers interesting possibilities for performing IMAP also in PEG–salt systems, which can be handled and scaled up more easily than PEG–dextran systems.

Liquid–liquid partitioning in conjunction with metal affinity extraction should be an interesting approach for downstream processing of proteins including those of clinical and industrial interest. The growing use of recombinant DNA technology allows the modification of protein products in order to facilitate their purification. The introduction of "affinity tails" of desirable chromatographic properties, such as histidine-containing peptides, will make the use of IMAP a very attractive alternative for large-scale purifications [13].

ACKNOWLEDGEMENTS

Part of this work was carried out under the collaborative research programme between Karl-Marx-University of Leipzig, and the University of Technology of

Compiègne. G.B. gratefully acknowledges the hospitality offered to him at Umeå University. T.S. was supported by the Swedish Medical Research and Cancer Research Council. We acknowledge useful discussions with Dr. E. Sulkowski during the preparation of the manuscript.

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