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Immobilized metal affinity chromatography for the separation of photosystems I and II from the thermophilic cyanobacterium *Synechococcus elongatus*

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ABSTRACT

Immobilized metal affinity chromatography (IMAC) of solubilized, photosystem II (PS II) enriched particles from the thermophilic cyanobacterium *Synechococcus elongatus* was studied. A chelating Sepharose Fast Flow column was charged with various metal ions (Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+}, Zn^{2+} and Cu^{2+}) and their affinity to photosystem I (PS I) and PS II was examined. Among all the metal ions tested, only copper was able to bind the two protein complexes. For elution of the column, a pH gradient, a pH step gradient and gradients of imidazole, amino acids, organic acids and various other eluents were tested; only the pH step gradient, which selectively eluted PS II at a pH between 6 and 5, was useful for the separation of PS I and PS II. All other gradients proved to be inappropriate for the separation of these two photosystems. Mechanisms of protein elution by these compounds are discussed. Alternatively, a separation of PS I and PS II at pH 7.5 could be achieved when an IMAC column was used on which the free coordination positions of the bound copper ions were occupied by imidazole. When solubilized photosystems were loaded on to this column, PS I replaced imidazole and remained bound on the column, whereas PS II was highly enriched in the effluent.

INTRODUCTION

Cyanobacteria contain, similarly to higher plants, two photochemical reaction centres [1]. They are localized in the thylakoid membranes and enable the cyanobacteria to evolve oxygen by splitting water and to reduce $NADP^+$. Water is split in the so-called photosystem II (PS II) and $NADP^+$ is the terminal electron acceptor of photosystem I (PS I). Both photosystems are multi-protein complexes that consist of various amounts of proteins and pigments depending on their state of purification.

In thylakoid membranes (TM), PS II of cyanobacteria consists of at least six subunits: the reaction centre protein D1 with an apparent molecular mass (M_r) of 31 000, the reaction centre protein D2 with an apparent M_r of 32 000, the M_r 47 000 chlorophyll (Chl) protein (CP 47), the M_r 43 000 chlorophyll protein (CP 43), the M_r 33 000 water splitting protein and cytochrome *b* 559 with M_r *ca.* 13 000. These proteins are encoded by the psbA, psbD, psbB, psbC, nuclear psb1, psbE and psbF, respectively [2].

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The primary charge separation takes place in the reaction centre proteins D1 and D2, which contain the primary electron donor P 680, two pheophytin molecules, two molecules of chlorophyll, two quinones and one non-haeme iron [2].

Photosystem I consists of two reaction centre proteins with M_r *ca.* 83 000 [3], containing the primary electron donor P 700, the primary acceptor A_0 (chlorophyll *a*), the electron acceptor A_1 (phylloquinone) and the iron–sulphur centre F_x. These proteins are encoded by the genes psaA and psaB. The reaction centre proteins also contain about 60 molecules of chlorophyll *a* and ten carotinoid molecules [4]. In addition to these two polypeptides, PS I consists of at least two other polypeptides: an M_r 10 000 protein (psaC gene product), containing the iron–sulphur centres F_a and F_b, and an M_r 15 000 polypeptide. Both are localized on the stromal side [5].

In the last few years, many different purification procedures for PS I and PS II complexes from thylakoid membranes of various organisms have been developed [6–8]. Most of these preparations include a selective extraction of the photosynthetic complexes from the membranes by the use of detergents. For the solubilization of the membranes the nature of the detergent (ionic or non-ionic), the ratio of detergent to chlorophyll (Chl) and other parameters such as pH and concentration of salts are of importance. Usually these preparations yield complexes which are pure for most purposes. Sometimes, however, it is necessary to eliminate even small amounts of impurities, especially when kinetic studies are done or the crystallization of the protein is attempted.

In 1984, Schatz and Witt [9] introduced a method for the selective extraction of PS II from the thermophilic cyanobacterium *Synechococcus* sp. by the use of the zwitterionic detergent SB 12. This purification was further improved by Rögner *et al.* [10] using density gradient centrifugation for the elimination of remaining impurities.

We applied a similar method to the thermophilic cyanobacterium *Synechococcus elongatus* and found a small amount of PS I still present even after density gradient centrifugation. A further purification by either anion-exchange chromatography (Q-Sepharose) or gel filtration (Superose 6 B) (data not shown) did not result in a satisfactory separation of PS I and PS II. We only succeeded in eliminating this contamination by immobilized metal affinity chromatography (IMAC) on a Chelating Sepharose column using Cu^{2+} as the immobilized metal ion.

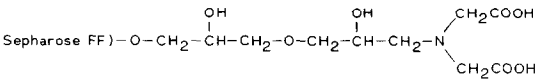

Fig. 1. Partial structure of Chelating Sepharose Fast Flow stationary phase. Assuming an octahedral coordination of the metal ion, iminodiacetic acid can only saturate three coordination positions, while the remaining three positions are free for interaction with proteins [12].

Chelating Sepharose (see Fig. 1) consists of iminodiacetic acid groups on spacers, coupled to a highly cross-linked agarose matrix by stable ether linkages [11].

Immobilized metal affinity chromatography [13] offers a new approach for the purification of PS I and PS II because the binding of metal ions is a specific property of a protein and differences in the binding of these metal ions may be used for the separation of these proteins. In addition, it is possible to alter parametes such as adsorption and desorption conditions and salt concentrations, which may determine the purity and stability of the sample.

EXPERIMENTAL

Prepartion of PS II particles

The thermophilic cyanobacterium *Synechococcus elongatus* strain KOVROV 1972/8 was grown in an inorganic medium according to Castenholz [14], supplemented with 10 m*M* $NaHCO_3$, at 57°C in a chemostat culture aerated with 2% (v/v) CO_2. The irradiation regime was chosen so as to favour a high ratio of PS II to PS I. For this purpose the cyanobacteria were grown in the tubular culture unit at a dilution rate of $D = 0.1\ h^{-1}$. The cells were irradiated at 50 W m^{-2} for several hours. Then a dark period of 12 h was inserted and the flow was stopped during that time. After this period, the cells were irradiated at 200 W m^{-2} for 6 h at the same dilution rate. This resulted in an increase in the PS II/PS I ratio from 1:2 at an irradiation of 50 W m^{-2} to *ca.* 2.5:1 when the cells were grown under the conditions mentioned above.

For the preparation of the thylakoid membranes (TM), the method of Schatz and Witt [9] was slightly modified. The cells were harvested at a density of 10^8 cm^{-3} by centrifugation, washed with buffer A [30 m*M* K_2HPO_4–5 m*M* $MgCl_2$–1 m*M* ε-aminocaproic acid (pH 7.5)] and treated with 0.3% lysozyme in buffer B [30 m*M* K_2HPO_4–500 m*M* mannitol–5 m*M* $MgCl_2$–35 m*M* NaCl–1 m*M* $CaCl_2$–1 m*M* ε-aminocaproic acid (pH 6.5)] at 47°C for 1 h in the dark with shaking. Lysozyme was removed by a centrifugation step at 6000 *g*. The spheroplasts in the pellet were disrupted by osmotic shock with buffer A and washed twice with the same buffer to remove phycobilisomes (PBS). Thylakoid membranes were collected by centrifugation at 24 000 *g* for 30 min at 2°C. They were resuspended in buffer B (pH 6.5), stirred for 15 min and centrifuged at 6000 *g* to remove whole cells. The supernatant was stored in the presence of 20% glycerol in liquid nitrogen. The activity of TM was determined to be 180 μmol O_2 (mg Chl)$^{-1}$ h^{-1} at 40°C with benzoquinone (BQ) as an electron acceptor.

For the preparation of the PS II particles, the TM were diluted with buffer B (pH 6.5) to a concentration of 1 mg Chl ml^{-1} and stirred with the detergent sulphobetaine 12 (SB 12; N-dodecyl-N,N-dimethyl-3-amino-1-propanesulphonate) at a ratio of detergent to chlorophyll of 3.3:1 (w/w) at room temperature for 30 min in the dark. Then the sample was centrifuged at 160 000 *g* for 2 h at 2°C. PS II particles were solubilized in the superatant, while the sedimented membrane fragments were enriched in PS I. This supernatant (further designated as PS II particles) was stored in the presence of 20% glycerol in liquid nitrogen. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that this PS II preparation still contained a large amount of low-molecular-mass proteins, free PBS and a small amount of PS I.

Purification of PS II particles by sucrose density gradient centrifugation according to Rögner et al. [10]

PS II particles prepared as described above were layered on to a six-step sucrose density gradient (20–45%) and centrifuged at 160 000 *g* for 16 h at 2°C. We obtained six bands which differed in absorption spectra, photochemical activity (Hill reaction) and content of protein. The highest oxygen evolution of 230 μmol O_2 (mg Chl)$^{-1}$ h^{-1}, measured polarographically using a Clark-type electrode in the presence of 0.5 m*M* *p*-benzoquinone and 1 m*M* hexacyanoferrate(III) was observed in fraction 5 (designated as purified PS II particles), which corresponds to a sucrose concentration of 35%. The absorption maximum of this fraction in the red region was at 673 nm, and SDS-PAGE

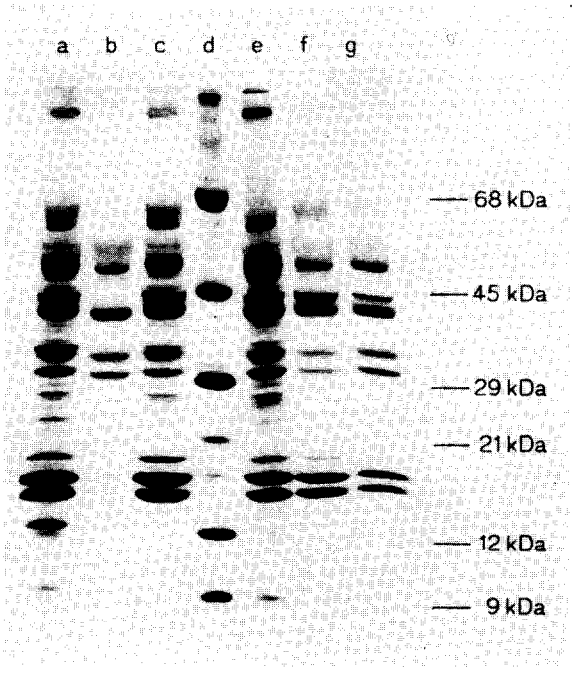

Fig. 2. SDS-PAGE for the purification of PS I and PS II from purified PS II particles (see Experimental). The bands at about M_r 110 000 and at M_r 80 000 belong to the PS I complex, the bands at M_r 47 000, 43 000, 33 000, 31 000 and 9000 belong to the PS II complex. Phycobilisomes have an apparent M_r of 15 000–18 000. No other bands will be considered here. Lanes a–c show the purification of the two photosystems with the pH step gradient. Lane a shows the purified PS II particles (see Experimental), lane b shows the fraction that was obtained by washing the column with buffer of pH 5, lane c shows the proteins which remained bound after the pH 5 washing and could be eluted with a buffer at pH 4, lane d contains marker proteins at M_r 68 000, 45 000, 29 000, 21 000, 12 000 and 9000 and lanes e–g show the separation of PS I and PS II by means of an imidazole-pre-charged column. In lane e the fraction is shown which bound to the first imidazole-pre-charged column (fraction G_1). It is obvious that both PS I and PS II were bound. Lane f shows the fraction which was bound to the second imidazole-pre-charged column (fraction G_2) and lane g shows the effluent of the second imidazole-pre-charged column (fraction E_2). It is clearly visible that the content of PS I in the effluent is drastically decreased in comparison with the purified PS II particles.

showed, in addition to the proteins of the PS II complex, the presence of PBS proteins and some PS I (see Fig. 2, lane a).

Immobilized metal affinity chromatography

For IMAC, Chelating Sepharose Fast Flow was packed in an HR 10/10 column (Pharmacia–LKB, Uppsala, Sweden). The bed height was 8 cm and the diameter was 10 mm. Then the Chelating Sepharose was loaded with a solution of a metal salt in distilled water at a concentration of 1 mg/ml and washed with three column volumes of distilled water [13] and three column volumes of buffer C [25 m*M* 2-(N-morpholino)ethanesulphonic acid (MES) (pH 7.5)–500 m*M* NaCl–0.02% β-D-dodecyl maltoside (DM)] at a flow-rate of 0.5 ml min^{-1}.

Then 8 ml of a solution of PS II particles or purified PS II particles (prepared as described above) at a concentration of 0.5 mg ml^{-1} in buffer B were applied to the column at 4°C and a flow-rate of 0.5 ml min^{-1}. We found that both PS I and PS II were bound to the column under these conditions.

For elution of the protein complexes, either a pH gradient, a pH step gradient or a gradient of eluents (imidazole, amino acids, organic acids or various other substances) were used.

The pH gradient was run by applying to the column a linear binary gradient (five column volumes) consisting of buffer C and a buffer of pH 4 containing 50 m*M* sodium acetate, 500 m*M* NaCl and 0.02% DM at a flow-rate of 0.7 ml min^{-1}. The pH gradient was controlled by measuring the pH of the fractions with a pH electrode (Ingold Messtechnik, Steinbach/Ts., Germany).

The pH step gradient was run by applying to the column buffers containing 50 m*M* sodium acetate, 500 m*M* NaCl and 0.02% DM, which were adjusted to pH 6, 5 and 4, respectively. The flow-rate was 0.7 ml min^{-1}. To perform the gradient of eluents, a linear gradient consisting of buffer C and this buffer containing the respective eluent was applied to the column. The gradient was run for five column volumes at a flow-rate of 0.7 ml min^{-1}. First gradients were run with a maximum concentration of eluent of 15 m*M*. If the protein did not elute at that concentration, this gradient was followed up by another gradient containing the concentration of eluent given in Tables II–IV.

Samples were concentrated with an ultrafiltration cell (Amicon, Witten, Germany) equipped with a Diaflo YM-100 membrane and run at a nitrogen pressure of 1.0 bar.

The spectra of the samples were measured with a Lambda 17 UV–VIS spectrophotometer (Perkin Elmer, Überlingen, Germany).

SDS-PAGE was performed according to Laemmli [15] using gels with a continuous polyacrylamide gradient of 12–24%, containing urea at a concentration of 7 *M*. The sample buffer consisted of 0.13 *M* tris(hydroxymethyl)aminomethane (Tris) (pH 9)–5.0% (w/v) SDS–2.5% (w/v) dithiothreitol–4 *M* urea. The electrophoresis was run at 7 mA constant current for 18 h at 18°C.

All chemicals were of analytical-reagent grade from Merck (Darmstadt, Germany).

RESULTS

Metals

Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Sr^{2+}, Ca^{2+}, Cu^{2+} ions were bound to the Chelating Sepharose. The column was equilibrated with buffer C and then PS II particles were applied. It was found that PS I and PS II only bound to copper whereas the other metal ions showed no binding capacity for these two protein complexes.

Buffers

Only buffers that have no strong interaction with metal ions can be used. The best buffers in this respect are 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), piperazine-N,N′-bis(ethanesulphonic acid) (PIPES) and MES whereas, for example, N-tris(hydroxymethyl)methylglycine (Tricine) elutes copper from the column at concentrations below 30 m*M*. Tris does not elute copper from the column at concentrations comparable to that of Tricine but it interacts in some way with copper and so reduces or modifies significantly the binding of proteins to the column. Buffers containing Tris should only be used for proteins that have a high affinity for metal ions.

TABLE I

ELUTION DATA FOR IMIDAZOLE AND IMIDAZOLE ANALOGUES

The top row gives the concentration of eluent that is necessary for protein elution and the bottom row the maximum concentration of eluent tested. Mg–ATP probably does not elute because of steric effects.

	Eluent			
	Imidazole	4-Methylimidazole	Adenine	Mg–ATP
Concentration for protein elution (m*M*)	<5	<5	<5	>15
Concentration for Cu^{2+} elution (m*M*)	>15	>15	>10	>15

Elution

pH gradient

When a pH gradient (pH 7.5–4) was applied to the column (see Experimental) we obtained an elution diagram (not shown) that showed two distinct but overlapping bands. PS II was eluted in fractions with pH between 6 and 5, whereas PS I was enriched in the fractions below pH 5.

pH step gradient

A satisfactory separation of PS I and PS II could only be obtained when a step gradient was applied to the column. When the column was washed with acetate buffer (pH 5), pure PS II eluted (see Fig. 2, lane b). PS I, PBS proteins and a residue of PS II remained bound on the column (see Fig. 2, lane c). These proteins could be eluted when the column was washed with acetate buffer of pH 4. Although this method resulted in a good separation of PS II from PS I, we observed partial inactivation of PS II photochemical activities (since light-induced kinetic measurements at 820 nm did not show the characteristic microsecond donation of an electron from the secondary donor Z (Tyr-161 of the reaction centre protein D1) to the primary donor P 680 [16] (data not shown). For this reason we tested eluents that eluted the protein complexes at higher pH.

Imidazole and imidazole analogues

Imidazole and imidazole analogues as eluents. Imidazole is commonly used for protein elution in IMAC. The elution properties of imidazole and some imidazole analogues are given in Table I.

We found that imidazole eluted both PS I and PS II at any concentration below 5 m*M*. We obtained an elution diagram (see Fig. 3) that showed that PS I and PS II were eluted closely together. The separation could not be improved by applying a less steep gradient. Therefore, imidazole was not useful

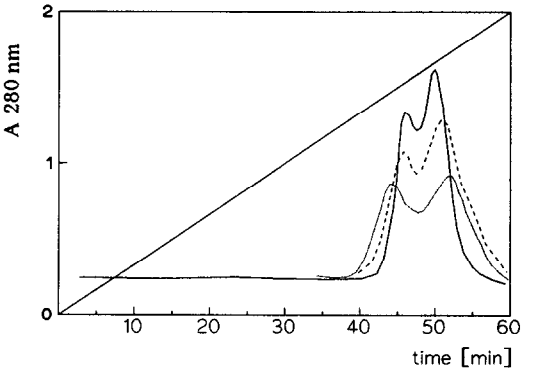

Fig. 3. Schematic elution diagrams of the elution of purified PS II particles (see Experimental) with gradients of imidazole (solid line) and amino acids (dashed line, group A; dotted line, group B). In order to be able to compare the elution profiles of the different eluents, the eluents and their concentrations are not given. The elution data are presented in Table I for the elution with imidazole and in Table III for the elution with amino acids. The amino acids listed in group A elute the protein complexes at concentrations below 15 m*M* and those amino acids listed in group B elute the protein complexes at concentrations below 60 m*M*. These elution profiles demonstrate that the two peaks cannot be separated, so that both peaks contain PS I and PS II. PS II is enriched in the peak eluting earlier, whereas PS I is enriched in the peak eluting later in the gradient. It is obvious that the resolution is best in the gradients with the amino acids eluting at higher concentrations.

for the separation of these two protein complexes. Similar results were obtained with its analogues 4-methylimidazole and adenine, while the strongly reduced elution capability of Mg–ATP is probably due to steric effects.

Pre-charging the column with imidazole. Alternatively, we did not use imidazole as an eluent, but pre-charged the column with imidazole. In this experiment we washed the Cu^{2+}-loaded column with 15 m*M* imidazole in buffer C until the colour of the column changed from light to dark blue, indicating that all free binding sites of copper were occupied with imidazole. The column was then washed with three column volumes of buffer C in order to remove surplus imidazole. After sample application we washed the column again with three column volumes of buffer C. In contrast to the experiments with the non-pre-charged column, only a minor part of the protein complexes was bound to the column, while the major part passed through (further mentioned as fraction E_1). Together with fraction E_1 there also eluted some imidazole, which was released from the column when protein was bound. The bound material was eluted in a step gradient (three column volumes, 0.7 ml min^{-1}) with 15 m*M* imidazole in buffer C (further mentioned as fraction G_1). Then the column was equilibrated with three column volumes of buffer C.

SDS-PAGE (see Fig. 2, lanes e–g) showed that fraction G_1 consisted mainly of PS I and a small amount of PS II whereas fraction E_1 contained mainly PS II complexes. For further purification, fraction E_1 was concentrated in an Amicon cell equipped with a YM 100 membrane. The concentrated sample was diluted tenfold with buffer C and concentrated once again in order to remove imidazole. This is necessary because imidazole at higher concentrations prevents the protein complexes from binding to the column. (The more PS I is bound, the more imidazole is released. As a consequence, imidazole concentrates along the column until a concentration is reached where it acts as an eluent. Then the remainder of the protein that did not yet bind to the column is prevented from binding.) The concentrated fraction E_1 was then applied once more to the same column. This time almost all the PS I that did not bind to the column in the first run and some PS II were bound.

The bound material was eluted with 15 m*M* imidazole as described above (further mentioned as fraction G_2). Again most of the PS II did not bind and was found in the effluent (further mentioned as fraction E_2). The UV–VIS spectrum of the purified PS II particles (see Experimental) is given in Fig. 4a and the spectra of fractions G_1 and E_2 are shown in Fig. 4b.

This method removes PS I satisfactorily but it cannot remove PBS proteins from the sample (see Fig. 2, lane g).

In Fig. 2 (lanes e–g) an SDS gel is shown where the successive extraction of PS I from purified PS II particles is monitored. It can be seen that in the first run almost all PS I is bound. Later PS I and PS II are bound. This is probably due to the changing PS I/PS II ratio in the sample during the procedure. With regard to the results obtained by Porath and Olin [12], we assume that there is a competitive

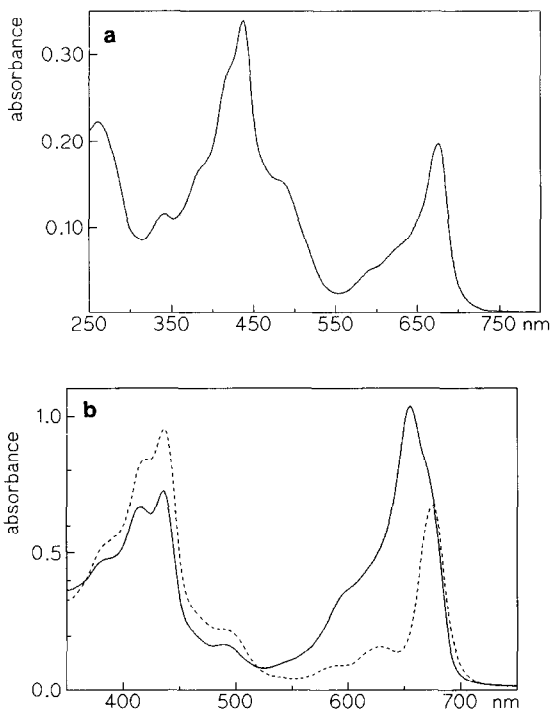

Fig. 4. Spectra from different states of purification. (a) Spectrum of the purified PS II particles derived from the sucrose density gradient centrifugation (see also Fig. 2, lane a). (b) Spectrum of the fraction that was bound to the first imidazole-pre-charged column, fraction G_1 (dashed line; see also Fig. 2, lane e), and that of the effluent, fraction E_2 (solid line; see also Fig. 2, lane g). The strong absorption of the effluent at about 654 nm is due to the high concentration of allophycocyanine in the sample.

TABLE II

ELUTION DATA FOR VARIOUS ORGANIC ACIDS THAT HAVE DIFFERENT CHELATING PROPERTIES

The concentrations listed are the maximum concentrations of eluent checked for the purpose of elution. The top row gives the concentration of eluent that is necessary for protein elution. The values in the bottom row indicate that no copper is eluted at the concentration of eluents tested.

	Eluent			
	Ascorbate	Acetate	Malonate	Oxalate
Concentration for protein elution (m*M*)	> 50	> 500	⩽ 60	< 30
Concentration for Cu^{2+} elution (m*M*)	> 50	> 500	> 60	> 30

binding of PS I and PS II to the copper where PS I shows a greater affinity for copper than PS II.

Finally all PS I could be removed from the sample.

Eluents

We also tested other compounds as possible selective eluents of the protein complexes (carboxylic acids, amino acids and others), but no separation of PS I and PS II comparable to the results just described could be obtained. We shall nevertheless briefly describe the results because they provide valuable data about the mechanism of protein elution in IMAC.

Carboxylic acids. Carboxylic acids present an ideal system for the investigation of the elution mechanism of proteins from the metal chelate column because they are available in all chain lengths. In addition, many oligofunctional carboxylic acids are available. Their elution characteristics are shown in Table II.

These data indicate that acetate, which is a monodentate ligand, did not elute protein at concentrations below 500 m*M*, whereas the bidentate ligands malonate and oxalate eluted protein at much lower concentrations. The elution diagrams of these carboxylic acids are comparable to those of the amino acids group B (see Fig. 3, and also the next sub-section. All these acids did not elute copper at the concentrations tested.

Amino acids. With amino acids as eluents, again no good separation of PS I and PS II could be obtained, similarly to the carboxylic acids. According to the data in Table III the amino acids can be divided into two groups: (A) amino acids that elute the protein complexes below a concentration of 15

TABLE III

ELUTION DATA FOR PROTEIN AND COPPER OF SOME AMINO ACIDS

The top row gives the concentrations of the individual amino acids that were necessary for protein elution. The bottom row gives the maximum concentration for the use of these amino acids because above these concentrations Cu^{2+} is released. N-Acetylhistidine is an exception; in contrast to all the other amino acids tested, no copper was eluted at the end of the gradient.

	Amino acids group A						Amino acids group B			
	Arg	Lys	Asn	Pro	Trp	Cys	NAc-His	His	Met	Leu
Concentration for protein elution (m*M*)	< 15	< 15	< 15	< 15	< 15	–	< 50	< 50	⩽ 50	< 60
Concentration for Cu^{2+} elution (m*M*)	⩾ 15	⩾ 15	⩾ 15	⩾ 15	> 10	> 0	> 100	⩾ 50	⩾ 50	⩾ 100

TABLE IV

ELUTION DATA OF SUBSTANCES ASSUMED TO HAVE A HIGH AFFINITY TO COPPER AND THEREFORE BE GOOD ELUENTS

The concentrations given are the maximum concentrations that were checked for the purpose of elution. With thiourea and cystamine, copper was released from the column at the end of the gradient. In all other instances no copper was eluted.

	Eluent								
	Urea	Thiourea	Thiamine	Thiophene-2-carbonic acid	Cystamine	IPTG	Piperazine	Sodium azide	Biotin
Concentration for protein elution (m*M*)	> 2000	> 500	> 500	> 100	< 100	> 50	> 50	> 20	> 15
Concentration for Cu^{2+} elution (m*M*)	> 2000	⩾ 500	> 500	> 100	⩾ 100	> 50	> 50	> 20	> 15

m*M* (arginine, lysine, asparagine, proline and tryptophan) and (B) those which elute proteins below 60 m*M* (N-acetylhistidine, histidine, methionine and leucine). The elution profiles are shown in Fig. 3. Cysteine is an exception; it does not elute protein, but elutes copper immediately from the column.

The elution patterns of both classes of amino acids (see Fig. 3) are very similar. In all instances two distinct but overlapping bands were observed. This indicates that the elution mechanism should be similar. The only difference is that the elution peaks of the amino acids eluting at higher concentrations are much broader than those of the amino acids eluting at lower concentrations. Therefore, a slightly better separation of the two protein complexes can be obtained when amino acids are used for elution which elute at higher concentrations. Again it was not possible to improve the separation even with flatter gradients.

A comparison of the elution properties of imidazole and histidine (both containing an imidazole ring) indicates the different elution mechanisms for the two compounds. Whereas imidazole did not release copper from the column, copper was released by histidine at concentrations that were slightly above the concentrations at which protein was eluted.

Eluents containing nitrogen or/and sulphur atoms. We also tested various other compounds that we thought might be good competitors with the protein for the coordination positions of the metal ions. As copper has a great affinity to sulphur and nitrogen [17], substances were tried that contain either one or both of these atoms. The results are given in Table IV. Again a separation of PS I and PS II could not be obtained.

From the results, it is striking that with the exception of cystamine (which contains a sulphur–sulphur bond), none of the eluents eluted the protein at the concentrations tested. The results obtained with cystamine as an eluent are not very good, however. Thus it is not possible to separate PS I and PS II and it is a disadvantage that copper is eluted from the column at or even below a concentration of 100 m*M* cystamine in the elution buffer.

DISCUSSION

Separation of PS I and PS II

This work has shown that IMAC is a useful method for the separation of PS I and PS II. This can be achieved either by elution of the protein complexes at low pH or by using their different binding properties to the imidazole-pre-charged column.

The elution mechanism at low pH is probably based on the protonation of the amino acid residues responsible for the binding of the proteins to the metal ions. The protonation finally results in a release of protein from the column. Elution with buffer at low pH results in extensive purification of the PS II complex and can be used as a one-step procedure omitting the density gradient centrifugation.

Pre-charging the column with imidazole differs from conventional elution procedures because here the protein competes with the eluent for the coordination positions of the metal ions and not the reverse. When imidazole was used as an eluent on a non-pre-charged column, an excess of imidazole was necessary to elute the protein complexes and therefore only a mixture of PS I and PS II could be eluted. With an imidazole-pre-charged column however, imidazole only saturates the coordination positions of the metal ions and therefore no surplus imidazole is present. This technique allows PS I and PS II to be separate on the basis of their different abilities to replace imidazole from the metal ion binding sites, which is a consequence of their different affinities to Cu^{2+}.

Elution with various eluents

Selective elution of PS I and PS II was not achieved with various substances as eluents. The probable elution mechanisms of each group of eluents are discussed.

Imidazole and imidazole analogues

Imidazole and adenine cannot form a ring complex with copper and therefore they should act as a monodentate ligand, which coordinates the metal ion with its basic nitrogen. The other nitrogen is not available as an electron donor because its free electron pair is part of the aromatic system of the imi-

dazole ring (we assume that the delocalization of the electrons in the aromatic system is disturbed in the complexation state, so that the two nitrogen atoms can be distinguished). Kinetic studies also show that imidazole behaves like NH_3 in complex formation reactions [18]. The fact that Mg–ATP does not elute protein at concentrations comparable to that of adenine is probably due to steric hindrance [19,20].

Carboxylic acids

The results obtained with carboxylic acids indicate that those substances which form five-membered rings in the complexation state are most effective in eluting protein, whereas those which form larger or smaller rings are less useful.

Amino acids

The difference in the elution of protein between the two groups of amino acids may be explained by considering their mechanism of elution. In general, two ways of interaction of amino acids with metal ions are possible.

One is that they act as bidentate ligands which coordinate the free positions of the bound copper with the carboxylate group and the α-amino group [21]. In this instance the amino acids form five-membered ring complexes and the different elution concentrations among the various amino acids tested could be explained primarily by either steric effects or hydrogen bonding. For amino acids the elution capacity is not primarily due to the basicity of the nitrogen [20].

The second possibility is the interaction of the amino acid residues with the metal ion. In this instance one should observe various elution behaviours for the different amino acids. This was only true for cysteine, which complexed the copper ion immediately, probably with its sulphydryl group. As we did not observe significant differences in the elution properties of the other amino acids tested, our results confirm the data obtained by Makinen *et al.* [22] and thus show that amino acid residues are not primarily involved in the complexation of metal ions and therefore do not contribute much to the elution process.

Eluents containing nitrogen or/and sulphur atoms

Regarding the other eluents tested, it is surprising that piperazine does not elute protein at concentrations below 50 m*M*, though ethylenediamine is one of the best chelating agents for copper [23]. This ought to be due exclusively to steric effects.

Thiophene-2-carboxylic acid should also be able to form a five-membered ring complex and therefore elute protein from the column, but as discussed before, the sulphur atom is part of the aromatic system and so the lone electron pairs of the sulphur atom are not available for coordination of the copper ion.

Thiamine (vitamin B_1) was also tested as an eluent because of its substituted thiazole ring. We expected this structure to be an analogue of an N-substituted imidazole ring, which would help to clarify the role of the basic nitrogen in the elution mechanism. The fact that no elution of protein was observed at concentrations below 500 m*M* again supports the model that the free electron pair of the basic nitrogen is involved in the elution mechanism. Additionally, steric effects have to be taken into account when explaining the elution properties of this large system.

Among the compounds containing one sulphur atom which is not part of an aromatic system, only those with electron-rich sulphur atoms such as in sulphydryl groups (see *Amino acids*) are appropriate for copper complexation. The disadvantage is that these substances elute the copper. Less electron-rich compounds such as thiourea are not able to elute protein at comparatively low concentrations. Even at high concentrations thiourea did not elute protein, but eluted copper from the column. Isopropyl thiogalactoside (IPTG) may additionally be sterically hindered. Biotin was tested because the sulphur atom is part of a cyclic non-aromatic system so that the angles of the free electron pairs of the sulphur atom are different to those in linear systems. However, no elution of protein could be observed up to a concentration of 15 m*M*.

Sodium azide was used to investigate the elution-properties of a nitrogen ligand. As the azide anion is linear it can only act as a monodentate ligand. No elution of protein was observed up to a concentration of 20 m*M*.

Urea, which can provide two amino groups for the complexation of metal ions, can only form a four-membered ring with copper and is therefore a poor competitive ligand. This is the reason why the

column can be washed with urea at a concentration of at least 2 *M* without elution of either protein or copper. This may be advantagous for certain purification problems [13].

Although we were not successful in separating PS I and PS II, the results obtained with the various eluents tried showed that there are differences in their elution properties. It became clear that the elution properties of the eluents cannot be exactly predicted, either from the chemical nature or from the structure of the eluent, which gives room for further efforts to find new eluents.

Imidazole and adenine proved to be the "strongest" eluents for IMAC, although they are monodentate ligands. They eluted the protein early in the gradient, but did not elute copper from the column. Among the "weaker" eluents, those forming five-membered ring complexes with the metal ions elute protein much better than those which form larger cyclic systems [22]. In this respect it would be interesting to try β-amino acids. As they can form only six-membered ring complexes they should elute protein less effectively than α-amino acids, and this could result in a better separation of the proteins. Additionally, it would be worth trying N-substituted systems as they do not elute copper at concentrations where unsubstituted systems already do, but show the same elution patterns as unsubstituted systems.

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