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Short Communication

Evaluation of the interaction of protein α-amino groups with M(II) by immobilized metal ion affinity chromatography

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ABSTRACT

The adsorption properties of various peptides and proteins, lacking histidyl groups, on immobilized Cu(II), Ni(II), Zn(II) and Co(II) ions are described; at pH 6 and below they were little retarded. At higher pH the retention became pronounced for iminodiacetate (IDA)–Cu(II) gel. This effect seems to be related to the presence of a terminal α-amino group; in the absence of this group the retention of the protein was largely eliminated. At pH 8.5 a terminal α-amino group is adsorbed as strongly as a histidyl group. IDA–Ni(II), IDA–Zn(II) and IDA–Co(II) gels display little or no attraction for the terminal α-amino group of a protein.

INTRODUCTION

Immobilized metal ion affinity chromatography (IMAC) is a type of chromatography designed for the separation of biomolecules such as proteins and nucleic acids. It was introduced by Porath *et al.* in 1975 [1] and has since gained worldwide acceptance, as documented by many review articles.

Investigation into the interaction between immobilized iminodiacetate (IDA)–Cu(II) and IDA–Ni (II) ions and proteins has shown that, at neutral pH, accessible histidines are the primary adsorption sites [2]. One of the imidazole nitrogens is believed to form a coordination bond with the metal ion, resulting in increased retention. This model is supported by the observation that the retention is highly dependent on the pH of the surrounding medium and on the pK_a of the imidazole [3].

The practical applications of IMAC are numerous. To a large extent this is because of the possibility of designing an advanced protocol for the purification if the structures of a peptide or protein are known. It is, for example, known that one single exposed histidine will be recognized by immobilized IDA–Cu(II). As suggested by Sulkowski [3], immobilized Ni(II) requires two exposed histidines for an appreciable retention of a protein, whereas immobilized IDA–Zn(II) or IDA–Co(II) ions seem to recognize such groups if they are in a vicinal position.

Much effort has been devoted to the identification of the nature of the other protein functional groups which may contribute to retention on an IMA column. From the beginning the cysteinyl thiol and tryptophyl groups were considered [1]. Some other groups, such as tyrosyl, carboxylate

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and α-amino groups, were also proposed. However, none of these has been systematically studied, and the few data now available rather indicate that they are less important. *A priori*, the only group, besides imidazole, on a protein that is likely to be an electron donor for coordination to metals at neutral or near-neutral pH is the α-amino group. Hemdan and Porath [4] and Belew and Porath [5] chromatographed amino acids and short peptides on immobilized IDA–Ni(II) and IDA–Cu(II) ions at or near neutrality and observed the interaction of α-amino groups with these metals. It is reasonable to expect that such an interaction should be pH-dependent because it requires that the amino group occurs in its basic form.

In the present report we have investigated the affinity of the α-amino group for immobilized and chelated Cu(II), Ni(II), Zn(II) and Co(II) ions and its possible role in IMAC. To facilitate the interpretation of data it is necessary to avoid any contribution to binding by an imidazole group. Therefore we chose as models proteins lacking histidine, *i.e.* duck lysozyme, thaumatin and aprotinin. The pH range used was also restricted to ensure that neither lysyl amino groups nor tyrosyl groups coordinated to a metal ion.

EXPERIMENTAL

Chelating Superose was obtained from Pharmacia-LKB (Stockholm, Sweden). The gel was packed in a glass column (21 × 10 mm I.D.), bed volume 1.65 ml. The capacity for Cu(II) ions was almost 30 μmol/ml.

Barbary duck lysozyme was a gift from Drs. F. Hemman and A. Paraf of the Institut National de la Recherche Agronomique (Nouzilly, France). Its purity was ascertained by sodium dodecyl sulphate (SDS) gel electrophoresis and reversed-phase chromatography and its authenticity was judged from the amino acid analysis. The analysis showed that histidine was not a part of its composition.

Bovine insulin α-chain was prepared by performic acid oxidation and isolated by ion-exchange chromatography on DEAE–Sepharose at pH 9.5. Its purity was checked by amino acid analysis.

The dodecapeptide NH_2-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Lys-COOH was synthesized and purified by Mr. P. Hansen at this institute. Its size was verified by mass spectrometry.

Thaumatin, from *Thaumatococcus danielli* (mol. wt. 21 000), lot No. 108F0299, was from Sigma (St. Louis, MO, USA). Aprotinin (mol.wt. 6500), lot No. 119F8095, was also from Sigma. Those preparations of proteins were found to contain some minor impurities which were readily separated by IMAC. The major components were analysed and found to contain no histidine.

Selective blocking of the α-amino groups of several proteins was achieved by carbamoylation at pH 7.0 with potassium cyanate following the procedure and precautions previously outlined [6]. The extent of carbamoylation as estimated through reaction with trinitrobenzene-sulphonic acid [7] never exceeded 1 mol per mol of protein.

Chromatographic experiments were carried out using a high-performance liquid chromatographic (HPLC) system (LKB) comprising a high-precision pump and a variable-wavelength monitor. Proteins were monitored routinely at 280 nm; dodecapeptide was monitored at 215 nm. Elution profiles were obtained from a Model 2210 recorder. All runs were made at room temperature, 20–22°C.

The column was charged with metal after equilibration with 0.1 *M* sodium acetate (1 *M* sodium chloride), pH 4.0. A 0.70-ml aliquot of 0.050 *M* copper sulphate pentahydrate, nickel nitrate hexahydrate, zinc sulphate heptahydrate or cobalt chloride hexahydrate dissolved in the same buffer was injected followed by washing with 10 ml of the equilibrium buffer. Then the column was re-equilibrated with 15 ml of the starting buffer and the effluent pH-checked. By using this procedure a small portion of the gel remained uncharged with metal and a more stable baseline was obtained.

The buffer systems used were as follows:

(a) pH 4.0–5.5, 0.05 *M* acetic acid–sodium hydroxide (1 *M* sodium chloride).

(b) pH 6.0–6.8, 20 m*M* sodium dihydrogenphosphate–sodium hydroxide (1 *M* sodium chloride).

(c) pH 7.0–8.6, 20 m*M* sodium dihydrogen–phosphate–sodium hydroxide (1 *M* sodium chloride), pH 7.0 and 20 m*M* boric acid–hydrochloric acid (1 *M* sodium chloride), pH 8.6, were mixed in different proportions to give the desired pH.

(d) pH 9.0, 20 m*M* boric acid–sodium hydroxide (1 *M* sodium chloride).

Routinely, the amount of each protein used in a sample was 0.1–0.3 mg dissolved in the equilibrat-

ing buffer or transferred into the buffer by gel filtration on PD-10. The injected volume was 0.20 ml in all runs and the flow-rate was maintained throughout at 1 ml/min.

The chromatographic behaviour of the test substances was expressed as relative retentions $V_r = V_e/V_t$, where V_e and V_t are the elution volumes of the sample in the presence and in the absence of loaded metal, respectively. The recovery of each protein or peptide was estimated assuming that the recovery was 100% in the absence of bound metal.

RESULTS AND DISCUSSION

Chromatography on Cu(II)–IDA–Superose at different pH values

In order to appreciate a contribution of a single α-amino group to the retention on M(II)–IDA columns, some model proteins lacking histidine were selected; a single histidine residue might overwhelm the putative contribution of other groups at neutral pH. Because an influence from cysteine thiol groups cannot be ruled out model proteins should not contain such groups either.

In the first series of experiments aprotinin was chromatographed at many different pH values. Its interaction with the IDA–Cu(II) could be well demonstrated by isocratic elution. As can be seen in Fig. 1, it increased sharply with pH. The same type of

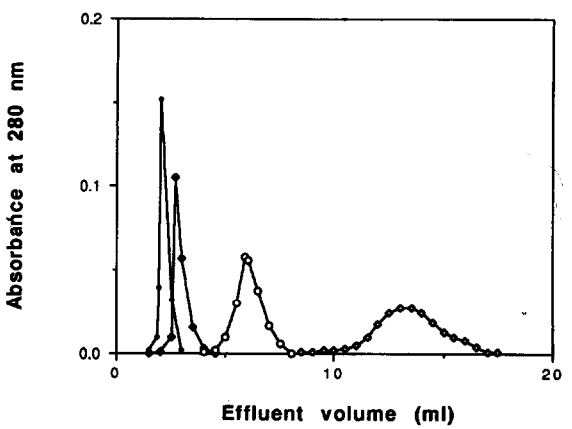

Fig. 1. Composite diagram showing the effect of pH on the retention of aprotinin on a column (21 × 10 mm I.D., 1.65 ml) of Cu(II)–IDA–Superose. Samples were run isocratically at a flow-rate of 1 ml/min. ● = pH 6.0; ◆ = pH 6.5; ○ = 7.0; ◇ = pH 7.5.

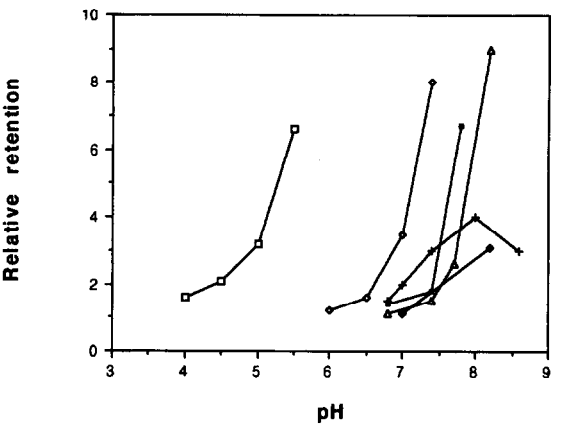

Fig. 2. Effect of pH on the relative retention of peptides and proteins on Cu(II)–IDA–Superose. Samples, 0.10–0.30 mg, were run isocratically at a flow-rate of 1 ml/min. □ = Chicken egg lysozyme; ◇ = aprotinin; ■ = Barbary duck lysozyme; △ = thaumatin; ◆ = cyanate-treated aprotinin; + = bovine insulin α-chain.

behaviour has previously been reported [3] for bovine serum albumin chromatographed isocratically on IDA–Ni(II) in the pH range 6.0–7.0, which was related to the titration of a histidyl group. Fig. 2 describes how the relative retention varies with the pH for native aprotinin and for α-carbamylaprotinin. There is a significant effect of blocking the amino group. These observations are consistent with the notion that the amino group coordinates to immobilized IDA–Cu(II). The pK_a of an α-amino group in a protein may vary, but judging from the pK_a data of peptides one can expect this value to extend down to 7.5 or even lower, especially if there are one or more basic residues nearby. At pH 8, there is a slight retention of the carbamoylated aprotinin which might be caused by unprotonated lysyl amino groups; this effect is presently under study.

The histidine- and thiol-lacking proteins, duck lysozyme and thaumatin, display largely the same behaviour: the relative retention seems to follow the deprotonation of the α-amino group. Blocking the terminal α-amino group in thaumatin clearly impedes the coordination to the metal. Much of the difference between these three proteins might be due to differences in the pK_a value of the terminal amino group. For the sake of comparison one protein which contains a single histidine, chicken egg lyso-

zyme, is included in Fig. 2. Clearly, the contribution from the histidine dominates the retention in the pH range 5–7.

Two peptides, oxidized bovine insulin α-chain and a dodecapeptide, were also examined. The retention of the α-chain increased with pH but to a much smaller extent than that of the proteins. In fact, the retention seemed to decrease at pH 8 and above. However, it was found that at this pH the retention was very dependent on the chemical nature of the buffer (not shown). The dodecapeptide displayed grossly a similar behaviour (not shown). These observations may reflect structural changes in the peptides leading to decreased accessibility for coordination or, alternatively, the formation of a high-affinity copper binding site which scavenges Cu^{2+} from IDA and does not contribute much to the retention [8]. The latter phenomenon has been reported for serum albumin [9] and for peptides [5]. The results now described amply illustrate the fact that retention data obtained from experiments with peptides with unblocked α-amino acids and carboxyl terminals cannot be applied to proteins [10].

Chromatography on Ni(II)-, Zn(II)- and Co(II)-chelating Superose

The corresponding chromatographic runs were made using nickel, IDA–Ni(II). The outcome is illustrated in Fig. 3. An obvious observation is that all proteins are much less strongly adsorbed on nickel. Thaumatin, for instance, was in the breakthrough at all pH values up to at least 9. Even chicken egg lysozyme, which has one histidine and one terminal α-amino group, was little retained at alkaline pH. Thus, it seems that the terminal amino group in proteins coordinates weakly or not at all with immobilized nickel ions. In clear contrast to this, both insulin α-chain and the dodecapeptide seem to have amino terminals that interact with the metal. There was no retention of insulin α-chain or any of the proteins on Zn(II)–IDA– or Co(II)–IDA–Superose.

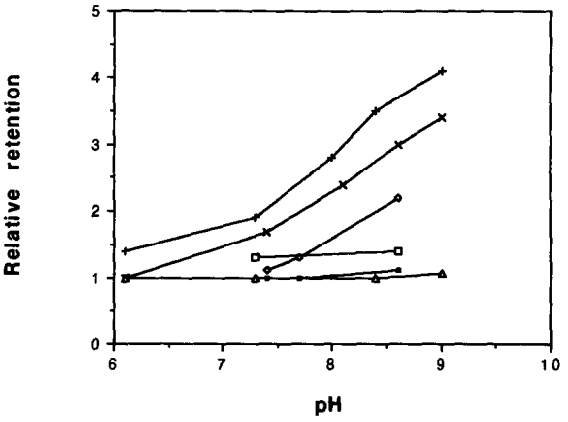

Fig. 3. Effect of pH on the relative retentions of peptides and proteins on Ni(II)–IDA–Superose. Samples, 0.10–0.30 mg, were run isocratically at a flow-rate of 1 ml/min. × = Dodecapeptide; + = bovine insulin α-chain; ◇ = aprotinin; ■ = Barbary duck lysozyme; □ = chicken egg lysozyme; △ = thaumatin.

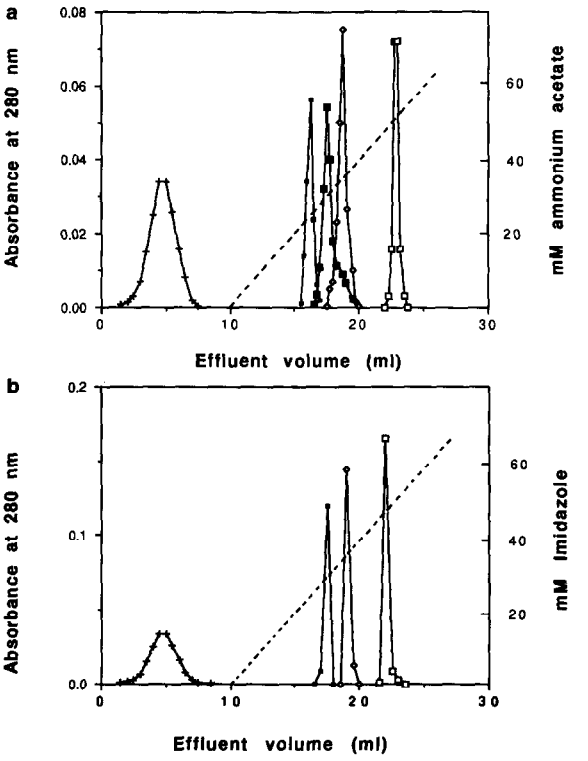

Fig. 4. Composite diagrams showing the elution profiles of some peptides and proteins on a column (21 × 10 mm I.D., 1.65 ml) of Cu(II)–IDA–Superose. Each protein was run separately at a flow-rate of 1 ml/min. Starting buffer was 20 m*M* boric acid–sodium hydroxide (1 *M* sodium chloride), pH 8.5. Samples: insulin α-chain, aprotinin and lysozymes. (a) Gradient was formed with 20 m*M* boric acid–0.10 *M* ammonia (1 *M* sodium chloride), pH 8.5. (b) Gradient was formed with 20 m*M* boric acid–0.10 *M* imidazole (1 *M* sodium chloride), pH 8.5. + = Bovine insulin α-chain; ◇ = aprotinin; ■ = Barbary duck lysozyme; □ = chicken egg lysozyme; ■ = cyanate-treated chicken egg lysozyme.

Comparing the binding of the α-amino group with imidazole group on Cu(II)–IDA–Superose

The following substances were chromatographed one by one on Cu(II)–IDA–Superose in boric acid buffer (1 *M* sodium chloride), pH 8.5: oxidized bovine insulin α-chain, chicken egg lysozyme, amino-terminal-blocked chicken egg lyzozyme, Barbary duck lysozyme and aprotinin. Shortly after the application the insulin α-chain appeared in the eluate. The other substances were eluted by using an ammonia gradient from 0 to 60 m*M*, as shown in Fig. 4a. This experiment allows a rough comparison between the binding of an α-amino terminal group and that of histidyl side-chain. A pair of these groups binds much more strongly than any one of them alone. Blocking the amino terminal has a very significant effect in decreasing the interaction. In fact, comparing duck lysozyme, which has one α-amino terminal but no histidine, and carbamoylated chicken egg lysozyme gives the impression that there is no great difference in binding between these two groups.

The outcome of a similar experiment using imidazole instead of ammonia is illustrated in Fig. 4b. Remarkably, imidazole seems to possess little eluting power at pH 8.5. At pH 7, for example, chicken egg lysozyme is readily eluted by 0.01 *M* imidazole (1 *M* sodium chloride) (not shown).

CONCLUSION

Taken together, the results of this preliminary study support some important notions. Firstly, our theory that an exposed histidyl group on a protein surface is the primary adsorption site at pH 5–7 is reinforced by the observation that none of the histidine-lacking proteins displays any significant retention. The amino terminal might, in theory at least, become of significance if the group is surrounded by positively charged groups, but this has to be yet experimentally established. Secondly, at a pH above 8, the α-amino terminal has an affinity for immobilized Cu(II) ions that is similar in strength to the affinity of histidine. Therefore, proteins lacking histidine [or with non-interacting histidine(s)] may be chromatographed as a result of the impact of the terminal amino group on binding: they can be separated from proteins with blocked N-terminals. Thirdly, assuming that the peptides in this study are representative of peptides in general, immobilized nickel ions might be useful for fractionation of peptides in slightly alkaline media.

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