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DEVELOPMENT OF IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

II. INTERACTION OF AMINO ACIDS WITH IMMOBILIZED NICKEL IMINODIACETATE

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SUMMARY

The retention of natural amino acids on immobilized nickel iminodiacetate was studied at neutral pH. Three amino acids, L-cystine, L-histidine and L-tryptophan, were retained most strongly, L-glutamic and L-aspartic acids were not retained at all and L-arginine and L-lysine displayed higher retentions than other amino acids. The retention of the aromatic amino acids and glycine increased with pH between 7.5 and 9.0. An increase in ionic strength further enhanced that retention. Sodium chloride enhanced the retention of amino acids more than did other salts.

INTRODUCTION

Since the introduction of immobilized metal affinity chromatography (IMAC) of proteins[1], several successful applications of this separation principle have been reported[2]. In order to gain an insight into the physico-chemical basis of IMAC it is imperative to study the behaviour of model substances with immobilized metals. The information obtained from such studies should help us to proceed from purely empirical attempts at the purification of proteins towards deliberately selected chromatographic protocols.

In IMAC the nucleophilicity of the atoms (N, O, S) participating in coordination to metals is of primary importance. However, there are other contributory factors: steric fit or hindrance, entropic gain resulting from disorganization of some hydration water and coulombic interactions.

The behaviour of amino acids on hydrophobic gels (Chelex-100, etc.) has been studied by "ligand-exchange chromatography"[3]. These studies, however, offer little guidance in the elucidation of the parameters involved in the interaction of proteins with metal ions immobilized on hydrophilic gels such as agarose or cross-linked dextran (Sephadex).

The aim of this contribution is to commence a fundamental evaluation of the adsorption phenomena in IMAC. To this end we have undertaken a study of the

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interaction of amino acids with an immobilized metal, nickel iminodiacetate (IDA–Ni^{2+}). Selection of the experimental conditions has been of necessity somewhat arbitrary given the present state of our knowledge.

EXPERIMENTAL

Materials

The amino acids, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-cystine, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine and L-serine, were purchased from Merck (Darmstadt, F.R.G.). L-Glutamic acid, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine and L-valine were obtained from Fluka (Buchs, Switzerland). L-methionine was from Koch-Light (Colnbrook, U.K.). N-Ethylmorpholine was purchased from Fluka, nickel sulphate was from Merck and Sephadex G-25 was from Pharmacia (Uppsala, Sweden). All other chemicals were reagent grade.

The chelating gel, IDA-Sephadex G-25, was prepared by coupling iminodiacetate to an epoxy-activated matrix according to an established procedure[4–6]. The gel was saturated with an aqueous solution of $NiSO_4$, and washed with water. The retained Ni^{2+} was measured as described previously[6,7] and amounted to 80 μmol per ml gel.

Chromatographic procedure

A column (15 × 2 cm) of IDA-Sephadex G-25 was washed with water and charged with a 20 m*M* solution of nickel sulphate in water until saturated. The column was then rinsed thoroughly with water and equilibrated with the buffer of choice. All amino acids except L-tyrosine were dissolved in an equilibration buffer to a final concentration of 2 m*M*; L-tyrosine was dissolved in an equilibration buffer to saturation. Samples of 0.5 ml amino acid were employed. The column was developed at a flow-rate of 16 ml/cm^2/h at ambient temperature. Fractions of 1 ml were collected. The elution of the amino acids was monitored with the trinitrobenzene–sulphonic acid test[8], ninhydrin reagent[9,10] or by direct spectrophotometry at 280 nm. The retention of amino acids is expressed as V_e/V_t, where V_e is the volume of the eluate at the peak of a chromatographic zone and V_t is the volume of the column gel bed.

RESULTS

The retention data for amino acids on IDA–Ni^{2+}-Sephadex G-25 are collated in Table I and illustrated in Fig. 1. The retention was studied in 0.2 *M* N-ethylmorpholine buffer, pH 7.0, and in the same buffer containing 0.5 *M* potassium sulphate so that the influence of the ionic strength could be measured. A series of control experiments established that the interaction of all amino acids with deactivated epoxy-Sephadex G-25 and with IDA-Sephadex G-25 (not charged with Ni^{2+}) was minimal ($V_e/V_t \approx 1$), independent of the ionic strength and uniform for all amino acids. Therefore, the retention of the amino acids on IDA–Ni^{2+}, as reported in Table I and Fig. 1, is the result of their specific interaction with the metal chelate of IDA.

Three amino acids, L-cystine, L-histidine and L-tryptophan, were retained ex-

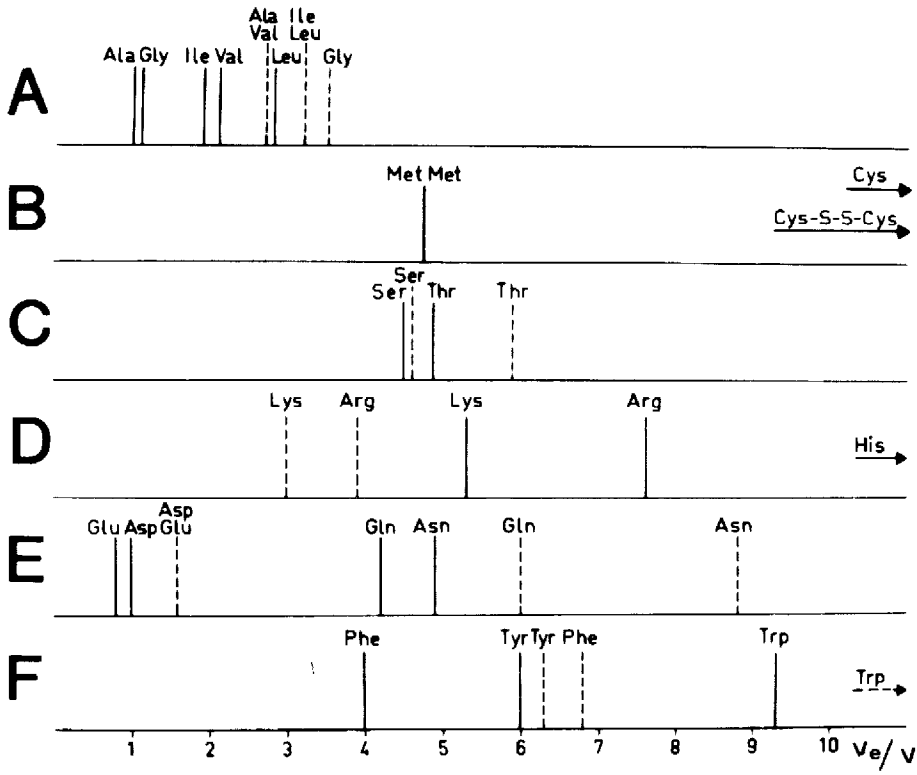


Fig. 1. Retention of L-amino acids on IDA Ni^{2+} in 0.2 *M* ethylmorpholine–acetate, pH 7.0 (solid bars) and in the same buffer containing 0.5 *M* potassium sulphate (dashed bars). Side-chain type: A, "pure" aliphatic; B, sulphur-containing; C, hydroxylic; D, basic; E, acidic or amidic; F, aromatic.

tremely strongly, having retention times well outside the range observed for all other amino acids. Therefore, no numerical values are presented for these amino acids. The extent of the interaction of other amino acids with IDA–Ni^{2+} can best be appreciated from Fig. 1.

Group A

Glycine and L-alanine were not retained in buffer alone. However, the introduction of 0.5 *M* K_2SO_4 caused significant retention. This effect of salt, perhaps, ought to be evaluated primarily in terms of an electrostatic interaction.

The retention of L-valine, L-leucine and L-isoleucine may to a large measure, be due to an hydrophobic effect of their side chains, although it does not correlate directly with their relative hydrophobicities.

Group B

L-Methionine was retained more strongly than all the hydrophobic (aliphatic) amino acids listed in group A. Clearly, some other forces must be invoked, presumably involving metal–sulphur affinity. Although salt had no observable effect on the retention, this might reflect a balancing of opposing forces such that the net effect was null.

TABLE I

RETENTION OF L-AMINO ACIDS ON IDA–Ni^{2+}

All amino acid samples were applied on columns equilibrated with 0.2 *M* ethylmorpholine–acetate buffer, pH 7.0, or with the same buffer containing either 0.5 *M* potassium sulphate or 4 *M* NaCl. All other details are given in Chromatographic procedure.

Amino acid	V_e/V_t		
	Buffer	*Buffer (0.5 M K_2SO_4)*	*Buffer (4 M NaCl)*
L-Alanine	1	2.7	2
L-Arginine	7.6	3.9	11
L-Asparagine	4.9	8.8	9
L-Aspartic acid	1	1.6	2
L-Cysteine	>20	>20	>20
L-Cystine	>20	>20	>20
L-Glutamine	4.2	6	7.5
L-Glutamic acid	0.8	1.6	2.2
Glycine	1.1	3.5	2
L-Histidine	>20	>20	>20
L-Isoleucine	1.9	3.2	3.3
L-Leucine	2.8	3.2	3.3
L-Lysine	5.2	3	3
L-Methionine	4.7	4.7	5
L-Phenylalanine	4	6.8	>20
L-Serine	4.5	4.6	5
L-Threonine	4.9	5.9	6
L-Tryptophan	9.3	>10	>40
L-Tyrosine	6	6.3	>20
L-Valine	2.1	2.7	2.8

Group C

The interaction of L-serine and L-threonine with IDA–Ni^{2+} was stronger than that of the amino acids of group A and comparable to that of L-methionine. The influence of salt on the extent of their interaction could be taken as resulting from the presence of the methyl group of the side chain of L-threonine. However, the retention of both amino acids *vis à vis* that of some amino acids of group A (hydrophobic) clearly requires another interpretation. We postulate, therefore, that the strong retention of L-serine and L-threonine is likely to reflect the nucleophilic character of the hydroxylic oxygen atom, resulting in the tridentate coordination.

Group D

The interaction of L-lysine and L-arginine with IDA–Ni^{2+} was strong and complex. The most conspicuous feature of this interaction is the reversal of the salt effect: both amino acids were less retained in the presence of salt (0.5 *M* K_2SO_4) than in its absence. This may be rationalized by the assumption that the IDA–Ni^{2+} complex, due to its association with the acetate ion of the buffer, carries an overall negative charge. The electrostatic attraction of the positively charged L-lysine and L-ar-

ginine might then be attenuated in the presence of the counter-ions provided by salt. The extent of the retention itself, high by comparison to other amino acids, must arise from other forces including coordination of the ε-amino group of L-lysine and the guanidino group of L-arginine.

Group E

L-Aspartic and L-glutamic acids were not retained in buffer alone and were weakly retained in the presence of salt. Both observations are readily explainable in terms of coulombic repulsion. By contrast, their amides are strongly retained on IDA–Ni^{2+} and the retention is further enhanced by the addition of salt. This may be due to an hydrophobic effect and, in particular, to the nucleophilic character of the carboxamide nitrogen atom, resulting in tridentate coordination of the L-asparagine and L-glutamine.

Group F

The retention of aromatic amino acids on IDA–Ni^{2+} was strong and is probably due predominantly to the lyotropic character of their side chains and to π-complex formation with the metal.

The retention of most amino acids (Table I) in 4 *M* NaCl on IDA–Ni^{2+} is not significantly higher than that in 0.5 *M* K_2SO_4, with the notable exceptions of aromatic amino acids and arginine. The retention of the three aromatic amino acids increases considerably upon increasing the NaCl concentration, due to the contribution of hydrophobic interactions. The reason for the very high retention of L-arginine in 4 *M* NaCl as compared to L-lysine is not obvious.

Fig. 2 illustrates the pH dependence of the retention of aromatic amino acids, and glycine (used as a reference). Part A gives the retention *vs.* pH of the buffer and part B shows the same in the presence of salt (1 *M* NaCl). It is seen that differential retention of aromatic amino acids (A) becomes accentuated with increasing pH above 6.0. The retention of glycine is insensitive to pH up to 7.0 and then rises steeply within the range tested (up to pH 9.0). This behaviour can be explained in terms of an increasing proportion of non-protonated glycine species which can coordinate with IDA–Ni^{2+}. Above pH 6.0, the retention of aromatic amino acids was strongly enhanced by the addition of salt. In the case of glycine the salt only slightly enhanced the effect of increasing pH alone.

The data of Fig. 2 indicate clearly that the differential effect of pH on the retention of these amino acids (Phe, Tyr and Trp) can be modulated by the inclusion of salt. The retention of all aromatic amino acids was lowest in the presence of tetramethylammonium chloride (Table II); in fact, it was even lower than that in the buffer alone (Table I). This is as anticipated in view of the nature of the tetraalkylammonium ions as an hydrophobic electrolyte which can interfere with the hydration water around the adsorbent site.

The salt effects may be exploited for the optimization of the resolution of these amino acids and, presumably, of proteins which carry them on their surfaces.

Tables II and III summarize the retention data for aromatic amino acids in the presence of the chlorides of Li^+, Na^+, K^+, NH_4^+ and $(CH_3)_4N^+$ and in the presence of potassium salts of F^-, Cl^-, Br^-, I^- and SCN^-. Comparison of the values leads to the conclusion that Na^+ and Cl^- are the most efficient ions in pro-

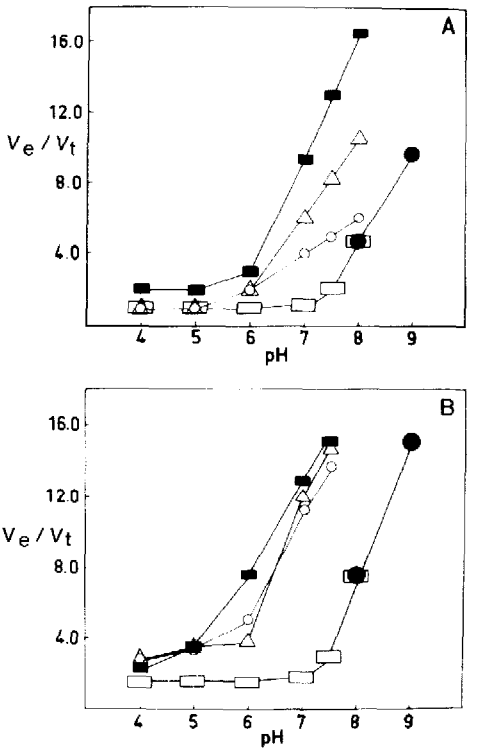


Fig. 2. The pH dependence of the retention of aromatic L-amino acids and glycine (a reference amino acid) on IDA–Ni^{2+}. A, In 0.2 *M* sodium acetate at pH 4 and 5, in 0.2 *M* ethylmorpholine–acetate at pH 6.0, 7.0, 7.5 and 8.0 and in 0.2 *M* potassium carbonate/bicarbonate at pH 8.0 and 9.0. B, As in A except for the inclusion of 1 *M* sodium chloride in all buffers. ■-■, L-tryptophan; △-△, L-tyrosine; ○-○, L-phenylalanine; □-□, glycine; ●-●, glycine in 0.2 *M* potassium carbonate/bicarbonate buffer.

moting the retention of all aromatic amino acids. Fig. 3 summarizes the experiments on the ionic strength dependence of the retention of aromatic amino acids on IDA–Ni^{2+}. Clearly, there is a positive correlation between V_e/V_t and the concentration of sodium chloride for all amino acids except glycine. Therefore, sodium chloride

TABLE II

INFLUENCE OF CATIONS ON THE RETENTION OF L-AROMATIC AMINO ACIDS ON IDA–Ni^{2+}

All amino acids were chromatographed in 0.2 *M* ethylmorpholine–acetate, pH 7.0, containing an appropriate salt (1 *M*).

Amino acid	V_e/V_t				
	LiCl	*NaCl*	*KCl*	NH_4Cl	$(CH_3)_4N^+Cl^-$
L-Phenylalanine	8	11.3	6	3	2
L-Tyrosine	10	12	7	4	3.2
L-Tryptophan	11	12.9	10.6	7	6.3

TABLE III

INFLUENCE OF ANIONS ON THE RETENTION OF L-AROMATIC AMINO ACIDS ON IDA–Ni^{2+}

All amino acids were chromatographed in 0.2 *M* ethylmorpholine–acetate, pH 7.0, containing an appropriate salt (1 *M*). In KI and KSCN the metal ion was displaced.

Amino acid	V_e/V_t		
	KF	*KCl*	*KBr*
L-Phenylalanine	3	6	4
L-Tyrosine	3	7	4
L-Tryoptophan	5	10.6	7

is the salt of choice for the chromatography of these amino acids, peptides containing them and proteins which bear them in exposed positions.

DISCUSSION

The binding of a protein molecule to an immobilized metal ion occurs via amino acid residues exposed on the protein surface and which are able to participate in a coordination bond. The innate potential of an individual amino acid residue to interact with immobilized metal ion will be modulated to a significant extent by its microenvironment, *i.e.*, vicinal amino acid side chains and the solvent in which the protein molecule is bathed. In order to analyze and interpret the relative contributions of a particular amino acid residue and the influence of its neighbours it is necessary to isolate that residue experimentally.

The simplest approach is to study the interaction of individual amino acids with immobilized metal ions. The information derived from such a study can then be extrapolated with due caution to the behaviour of amino acid residue present on

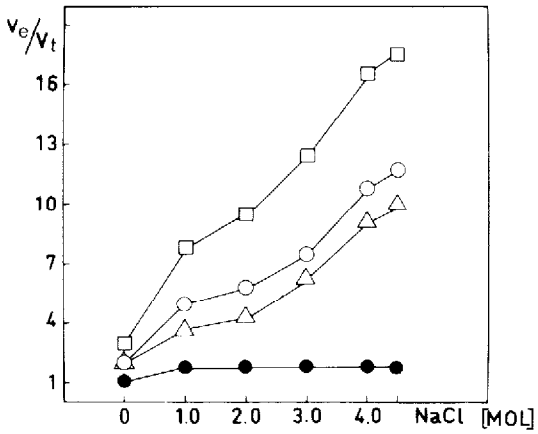
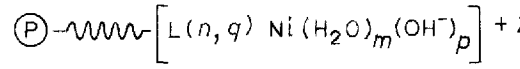


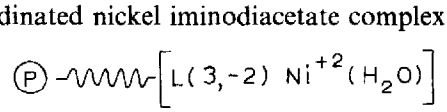
Fig. 3. The relationship between the concentration of NaCl and the retention of aromatic L-amino acids and glycine (a reference amino acid) on IDA–Ni^{2+}. Amino acids were applied in 0.2 *M* ethylmorpholine–acetate at pH 6.0 and in this same buffer supplemented with sodium chloride as indicated. □-□, L-Tryptophan; ○-○, L-phenylalanine; △-△, L-tyrosine; ●-●, glycine.

the protein surface. Sodium chloride and potassium sulphate often affect the adsorption behaviour of different proteins in very different ways, a fact that can be used to advantage in the fractionation of complex protein mixtures[11]. The results of the present investigation seem to afford a rational explanation for this differential nickel–protein affinity.

Divalent nickel exists in solution either in a tetra- or hexacoordinated form. When immobilized, some of the coordination sites must be occupied by the ligand atoms of the polymer-bound iminodiacetate and the remainder by water, hydroxyl groups or buffer ions. Generally the nickel ion complex can be represented as follows:



where (P) signifies the polymer matrix, L the ligand with dentation n and negative charge q; $m + n + p = 4$ or 6 for tetra- and hexacoordination, respectively. $|p + q| < 2$ will give a positively charged adsorbent (anion exchanger), $|p + q| = 2$ a neutral adsorbent and $2 < |p + q| < 4$ (or 6 in the case of hexacoordination) will give a negatively charged adsorbent (cation exchanger). For the case of the tetracoordinated nickel iminodiacetate complex we can write:



This means that in pure water, where this species will prevail, the residual ligand position will be occupied mainly by one water molecule and the complex will thus have zero net charge.

The number of hydroxyl ions bound will depend upon the pH:

$$[L(3,-2)\ Ni^{2+}(H_2O)] \rightarrow [L(3,-2)\ Ni^{2+}(OH^-)]^- + H^+$$

This means that at alkaline pH and in the presence of negatively charged counter ions, the net charge on the complex will be negative.

Table I and Fig. 1 illustrate the retention of amino acids on IDA–Ni^{2+}. The most conspicuous finding is the observation that L-cystine (L-cysteine), L-histidine and L-tryptophan are retained much more strongly *vis-à-vis* other amino acids. This observation is in full agreement with the original supposition regarding the amino acid residues involved in the binding of proteins to immobilized metal chelates[1]. Quantitative evaluation of their affinities for immobilized metal ions will be the subject of a separate study. It is clear, however, that these amino acids (side chains) may be predominantly responsible for the adsorption of proteins in IMAC. Additional studies of dipeptides should buttress this view. Eventually, protein "models" have to be studied to confirm, or to dispel, the conclusions arrived at by the investigation of amino acids and peptides. Both of these approaches are at present being implemented in our laboratory.

The retention behaviour of other groups of amino acids on IDA–Ni^{2+} is quite complex, though it might be rationalized in terms of chelation, electrostatic attraction

or repulsion, hydrophobic effects and π-bonding. A detailed analysis of all these parameters may be premature in view of the somewhat arbitrarily chosen experimental conditions (buffer, pH, temperature, ionic strength). An additional survey of other buffers, higher and lower ionic strengths, temperature effects, etc. is required. It is clear, however, that the modes of retention of some amino acids are clearly distinct from those of others and that the retention of any particular amino acid is amenable to an experimental manipulation. Both of these conclusions are of importance in additional studies of the retention of model peptides and proteins.

The retention of aromatic amino acids, L-phenylalanine, L-tyrosine and L-tryptophan, on IDA–Ni^{2+} was studied as a function of pH (Fig. 2A) at low and high ionic strengths (Fig. 2B). Glycine was used as a reference amino acid. In the case of glycine a marked increase in the retention (pH 7.5) can be correlated with the extent of deprotonation of its amino group ($pK_2 \approx 9.6$) and implies the involvement of the latter in the chelate formation with IDA–Ni^{2+}. A similar pH dependence was also observed for aromatic amino acids, implying again the involvement of α-amino groups. However, the retention was significantly enhanced at pH just above 6, about three pH units away from the pK values (9.1–9.4) of their α-amino groups. This may indicate a contribution of aromatic side chains (π–π bonding) to the interaction of these amino acids with IDA–Ni^{2+}.

An increase in the ionic strength of the solvent enhanced the retention of glycine in the range pH 7.5–9.0. The retention of L-tryptophan was enhanced somewhat in 1.0 *M* NaCl. Both L-phenylalanine and L-tyrosine were retained significantly more strongly in the presence of 1.0 *M* NaCl. The data of Fig. 3 further support the observation that an increase in the ionic strength of the solvent enhances the interaction of all aromatic amino acids with IDA–Ni^{2+}.

An appropriate electrolyte for the enhancement of retention can be selected by use of the data of Tables II and III. It seems that sodium chloride is clearly the salt of choice among the alkali-metal halides. Coincidentally, this salt also tends to stabilize the tertiary structure of proteins.

All in all, the retention of aromatic amino acids is already considerable at pH 7 and can be further enhanced by salt. These observations are clearly pertinent to the selection of chromatographic parameters for the isolation of proteins.

In conclusion, the retention behaviour of individual amino acids on IDA–Ni^{2+}, selected for its intermediate chelation strength between IDA–Zn^{2+} and IDA–Cu^{2+}, revealed the propensity of some amino acids to interact with an immobilized metal and the influence of some factors (pH, ionic strength, nature of cation/anion) upon this interaction. The findings of this study will facilitate further refinement of experimental conditions for the interaction of small molecules (dipeptides, oligopeptides) and ultimately of proteins.

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