Journal of Chromatography, 627 (1992) 163–172
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 610

Purification and determination of the binding site of lactate dehydrogenase from chicken breast muscle on immobilized ferric ions

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(First received May 14th, 1992; revised manuscript received August 31st, 1992)

ABSTRACT

Lactate dehydrogenase from chicken breast muscle was purified to homogeneity in one step by immobilized metal ion affinity chromatography. The purified enzyme was used to localize the binding site to immobilized Fe(III) ions. After cyanogen bromide degradation and digestion with trypsin, small enzyme fragments capable of binding to immobilized Fe(III) ions were obtained. It is proposed that several histidyl groups are involved in the binding.

INTRODUCTION

Several years of investigation of the mechanism of adsorption on immobilized transition metals, such as Cu(II), Ni(II) and Zn(II), indicate that histidyl groups are the dominant electron donors. The number and sometimes the distribution of histidyl groups dictate almost exclusively their retention behaviour on these metals when immobilized on iminodiacetate-agarose (IDA-agarose) [1].

Protein binding to immobilized hard metals such as Fe(III), Al(III) or Ca(II) clearly involves oxygen atoms as electron donors. Therefore, phosphate [2,3] or carboxylate groups [4] are proposed to be primarily responsible for the adsorption on these metals.

Recently, we showed that lactate dehydrogenase (LDH) from chicken muscle, which lacks phosphate, binds very strongly to Fe(III) [5]. With respect to immobilized metal ion affinity chromatography (IMAC), the M form of this enzyme has an intriguing amino acid sequence: residues Nos. 5, 8, 11, 15, 17 and 19 are histidyl residues [6]. These observations stimulated us to examine the possibility to purify the enzyme on Cu(II)–, Ni(II)– or Zn(II)–IDA-agarose and to localize the binding site for immobilized Fe(III) ions.

EXPERIMENTAL

Materials

Frozen chicken breast muscle was purchased from food stores. Iron(III) chloride was obtained from Merck–Schuchardt (No. 803945; Munich, Germany), nickel nitrate hexahydrate from J. T. Baker (Lot No. 12944; Phillipsburg, NJ, USA), cyanogen bromide and 2,4,6-trinitrobenzenesulphonic acid (TNBS) from Fluka (Buchs, Switzerland), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Type XIII) from Sigma (St. Louis, MO, USA), chelating Sepharose FF and Pep RPC HR 5/5 from Pharmacia–LKB (Uppsala, Sweden) and trifluoroacetic acid (TFA) and acetonitrile from Merck (Darmstadt, Germany).

Synthetic peptides were generously supplied by

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0021-9673/92/$05.00

Dr. G. Lindeberg, Institute of Medical Immunology, Biomedical Centre, Uppsala, Sweden. The peptide preparations were not subjected to a final purification.

Chromatography

Chelating Sepharose FF packed into a column (5.4 × 2 cm I.D.) was charged with Ni(II) ions by applying four column volumes of 20 m*M* $Ni(NO_3)_2 \cdot 6H_2O$, followed by washing with four column volumes of deionized water and equilibration with 50 m*M* sodium phosphate–60 m*M* imidazole (1.0 *M* NaCl) (pH 7.1).

Columns of Fe(III)–IDA-agarose (2 ml) were prepared by applying sterile-filtered 20 m*M* iron (III) chloride. Excess of metal ions were washed out with deionized water. The columns were then washed with 5 ml of 0.1 *M* sodium phosphate (pH 7.1), followed by the equilibrating buffer. Reversed-phase chromatography was performed using the fast protein liquid chromatography system of Pharmacia–LKB on a Pep RPC HR 5/5 column with a linear gradient of acetonitrile.

Analysis of chromatographic fractions

Protein concentrations were calculated from absorbance measurements at 280 nm. Peptide peaks in eluates from reversed-phase chromatography were detected at 214 nm. Peptide concentrations were determined after reaction with TNBS [7]: 0.5 ml of 0.1 *M* borax, 0.5 ml of sample and 0.5 ml of 0.1% TNBS were mixed in glass tubes (75 × 12 mm I.D.), the tubes were left for 1 h at 40°C and the absorbance was measured at 420 nm against a blank. A calibration graph was prepared with glycine. Muscle extracts, purified enzyme and large peptides were analysed by sodium dodecyl sulphate (SDS) gel electrophoresis on Pharmacia Phast System using Pharmacia Phast Gel Gradient slabs 8–25 and SDS buffer strips. For peptide fractions, Pharmacia High Density Phast Gel was used. Peptide components in gel slabs were revealed by silver staining. Peptides were identified by determination of amino acid compositions after hydrolysis for 24 h with 6 *M* hydrochloric acid.

Protein and peptide cleavage procedures

Cyanogen bromide (CNBr) cleavage was carried out by a standard procedure [8] in 70% formic acid using a 50-fold excess of CNBr. The reaction mixture was freeze-dried twice after dilution with deionized water. Tryptic hydrolysis of peptides was done by addition of 30 μg of TPCK-treated trypsin to 1 μmol of peptide dissolved in 2 ml of 25 m*M* piperazine-N,N′-bis(2-ethanesulphonic acid) disodium salt (PIPES)–20 m*M* $CaCl_2$ (pH 7.1). The reaction was allowed to proceed for 3 h at 37°C.

Sample preparation

Chicken muscle extract was prepared according to Petell *et al.* [9]. Proteins and peptides were desalted or transferred into equilibration buffers by gel filtration on Sephadex columns. Small peptide sample (0.75 ml) were applied on a Sephadex G-10 column (5 × 1.8 cm I.D.), equilibrated with 20 m*M* acetic acid and the fraction between effluent volumes of 2.75 and 3.75 ml was recovered.

Enzyme assay

LDH was determined according to the method described by Kubowitz and Ott [10].

RESULTS

Purification of lactate dehydrogenase

A 100-ml volume of muscle extract was chromatographed on a Sephadex G-25 (medium) col-

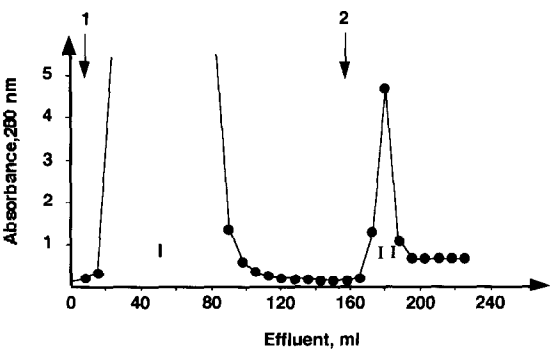


Fig. 1. Preparative purification of LDH on Ni(II)-chelating Sepharose FF. A 150-ml volume of chicken muscle extract in 50 m*M* sodium phosphate–1.0 *M* sodium chloride–60 m*M* imidazole (pH 7.1) was loaded on to an Ni(II)–Chelating Sepharose FF column (5.4 × 2 cm I.D.) equilibrated with the same buffer. The non-adsorbed material was washed out with the equilibrium buffer (arrow 1). The adsorbed material was eluted with 50 m*M* sodium phosphate–1.0 *M* sodium chloride–0.3 *M* imidazole (pH 7.1) (arrow 2). The flow-rate was 2.5 ml/min throughout.

umn (19 × 5 cm I.D.) in 50 m*M* sodium phosphate–1.0 *M* NaCl–60 m*M* imidazole (pH 7.1). Pooled fractions containing the enzyme (150 ml) were then applied, at a flow-rate of 2.5 ml/min, to a column of Ni(II)–IDA-agarose (5.4 × 2 cm I.D.) equilibrated in the same buffer. Fig. 1 illustrates the purification of the enzyme. Non-bound proteins were washed out with additional buffer. On increasing the imidazole concentration to 300 m*M* a second protein peak appeared. Gel electrophoresis showed that the breakthrough peak contained most of the applied proteins, whereas the second peak contained a single protein with a relative molecular mass of 40 000 (Fig. 2, lanes 2 and 3, respectively). This component represented 95% of the applied LDH activity. Fractions containing the purified LDH were pooled, desalted by dialysis and freeze-dried.

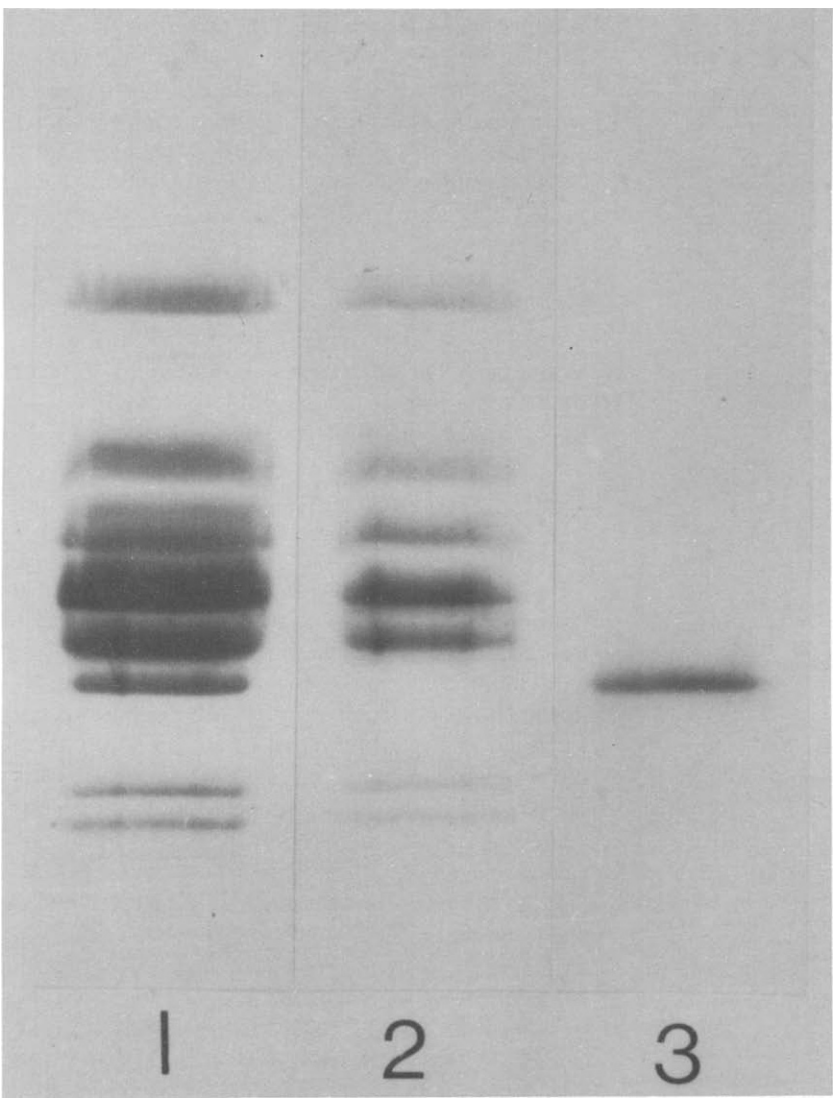


Fig. 2. SDS electrophoresis of the fractions obtained after the purification of LDH. Lanes: 1 = chicken muscle extract; 2 = peak I (Fig. 1), non-adsorbed material; 3 = peak II (Fig. 1), eluted with 0.3 *M* imidazole.

Chromatography of CNBr fragments on immobilized Fe(III) ions

The buffer systems used were (a) 25 m*M* acetate (pH 5.6) and (B) 25 m*M* PIPES (pH 7.1).

In the first experiment, 10 mg of CNBr fragments were dissolved in system A (4 ml). The sample was applied to Fe(III)–IDA-agarose equilibrated with the same buffer. Non-adsorbed material was washed out as peak I (Fig. 3a). Elution of the adsorbed peptides was accomplished by introducing 25 m*M* sodium phosphate (pH 7.0) (peaks II_1 and II_2) and further elution (peak III) by applying 25 m*M* sodium phosphate (1.0 *M* NaCl) (pH 7.0). Amino acid analyses (Table I) revealed that peak II_2 had a composition similar to the enzyme fragment 1 (residues 1–32) and peak III corresponded in composition to the enzyme fragment 8 (residues 264–279). Peak II_1 did not correspond to any of the expected nine CNBr fragments and was discarded.

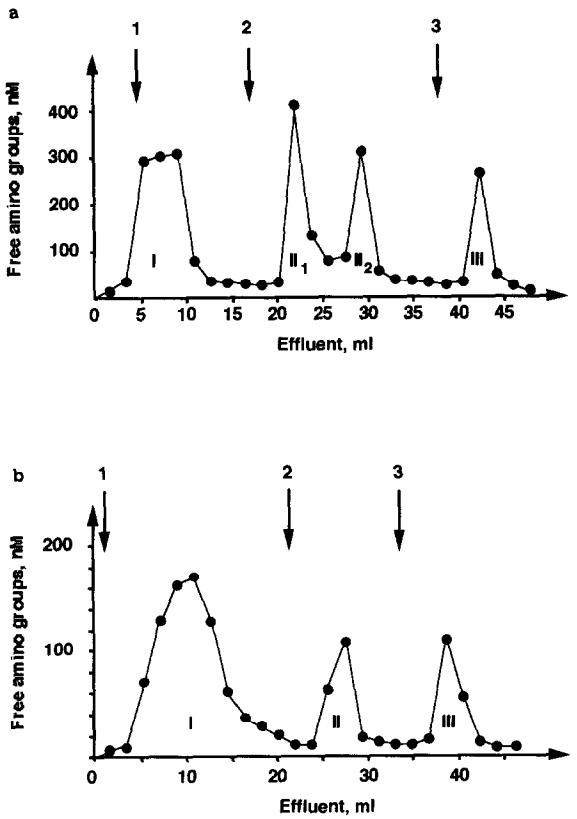


Fig. 3. Chromatography of CNBr fragments from LDH on Fe(III)–Chelating Sepharose FF. (a) 10 mg of CNBr fragments in 4 ml of 25 m*M* sodium acetate (pH 5.6) were loaded on to an Fe(III)–Chelating Sepharose FF column (2.55 × 1 cm I.D.) equilibrated with the same buffer. The non-adsorbed material was washed out by the equilibrium buffer (arrow 1). The adsorbed material was eluted by 25 m*M* sodium phosphate (pH 7.0) (arrow 2), followed by 25 m*M* sodium phosphate–1.0 m*M* chloride (pH 7.0) (arrow 3). The flow-rate was 30 ml/h throughout. (b) 10 mg of CNBr fragments in 4 ml of 25 m*M* PIPES (pH 7.1) was loaded on to an Fe(III)–Chelating Sepharose FF column (2.55 × 1 cm I.D.) equilibrated with the same buffer. The non-adsorbed material was washed out by the equilibrium buffer (arrow 1). The adsorbed material was eluted by 25 m*M* sodium phosphate (pH 7.0) (arrow 2), followed by 25 m*M* sodium phosphate–1.0 *M* sodium chloride (pH 7.0) (arrow 3). The flow-rate was 30 ml/h throughout.

When a similar run was performed using system B (Fig. 3b), the amount of adsorbed material became lower and only a single peak was obtained when 25 m*M* phosphate was applied. This peak (II) contained the N-terminal CNBr fragment (residues 1–32) as evidenced by amino acid analysis (Table I). Peak III again corresponded to CNBr fragment 8 (residues 264–279). Control experiments showed that the material present in peak II was unretarded on a metal-free column under the conditions described in the latter experiment (not shown), whereas the peak III material was bound. This confirms that the binding of the material in peak II was due to the presence of Fe(III) on the adsorbent.

Preparation of purified binding fragment 1

Material from peak II (Fig. 3b) was applied to a Pep RPC HR 5/5 column equilibrated with 0.1% TFA. A linear gradient of acetonitrile was used to elute the adsorbed material shown in Fig. 4a. The desired fragment appeared at 28% acetonitrile. This experiment was used as a guide for obtaining the desired material from crude CNBr peptides as illustrated in Fig. 4b. Several runs were made to recover sufficient material for subsequent experiments. The purity of the collected material was ascertained from SDS gel electrophoresis (Fig. 5). Its relative molecular mass was estimated to be 3300 (Fig. 5, lane 3). The material could be identified as the predicted CNBr fragment (residues 1–32).

Preparation of subfragments with binding properties

The purified and freeze-dried fragment (residues 1–32) was dissolved and subjected to trypsinolysis. The peptide mixture obtained was immediately chromatographed on Fe(III)–IDA-agarose equilibrated in 25 m*M* PIPES (pH 7.1). The result is

TABLE I

AMINO ACID COMPOSITION OF THE PEPTIDE FRACTIONS NUMBERED IN FIGS. 3a AND 3b

Amino acid residue	Fig. 3a, peak II_2		Fig. 3a, peak III		Fig. 3b, peak II	
	Detected (nmol per nmol peptide)	Expected (nmol per nmol peptide)	Detected (nmol per nmol peptide)	Expected (nmol per nmol peptide)	Detected (nmol per nmol peptide)	Expected (nmol per nmol peptide)
Aspartic acid	2.9	3	1.0	1	3.2	3
Threonine	0.2	0	1.0	1	0.2	0
Serine	2.2	2	1.0	1	2.3	2
Proline	0	0	1.0	1	0.2	0
Glutamic acid	2.4	2	0	0	2.1	2
Glycine	3.3	3	1.0	1	3.4	3
Alanine	2.9	3	1.0	1	3.1	3
Valine	4.5	5	2.0	2	4.5	5
Isoleucine	1.9	2	1.0	1	2.0	2
Leucine	2.1	2	1.0	1	2.1	2
Histidine	5.1	6	0.9	1	5.1	6
Lysine	2.7	3	2.0	2	3.2	3
Arginine	0.1	0	1.8	2	0.1	0
Homoserine	0.8	1	0.5	1	0.9	1

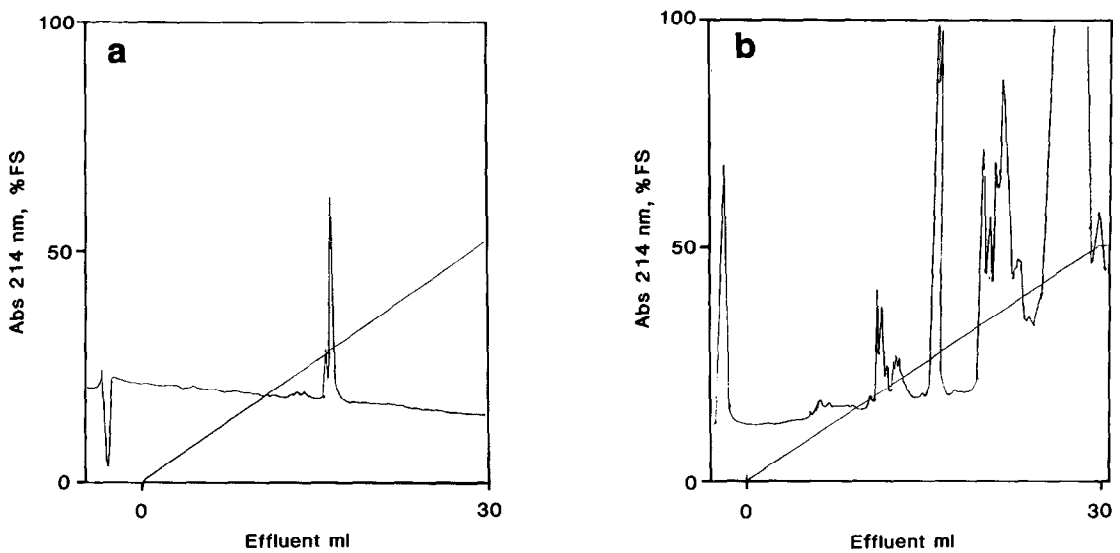


Fig. 4. RPC of CNBr fragments from LDH on Pep RPC HR 5/5. The samples dissolved in 0.1% TFA were loaded on to the reversed-phase chromatography column and eluted by a linear acetonitrile gradient from 0 to 50% in 0.1% TFA. The flow-rate was 0.7 ml/min throughout. The absorbance was monitored at 214 nm at a chart speed of 0.5 cm/ml. (a) 200 ml of peak II (Fig. 3b) were loaded on to the reversed-phase chromatography column. (b) 4.44 mg of CNBr fragments in 0.5 ml of 0.1% TFA were loaded on to the reversed-phase chromatography column. The same chromatography was performed nine times.

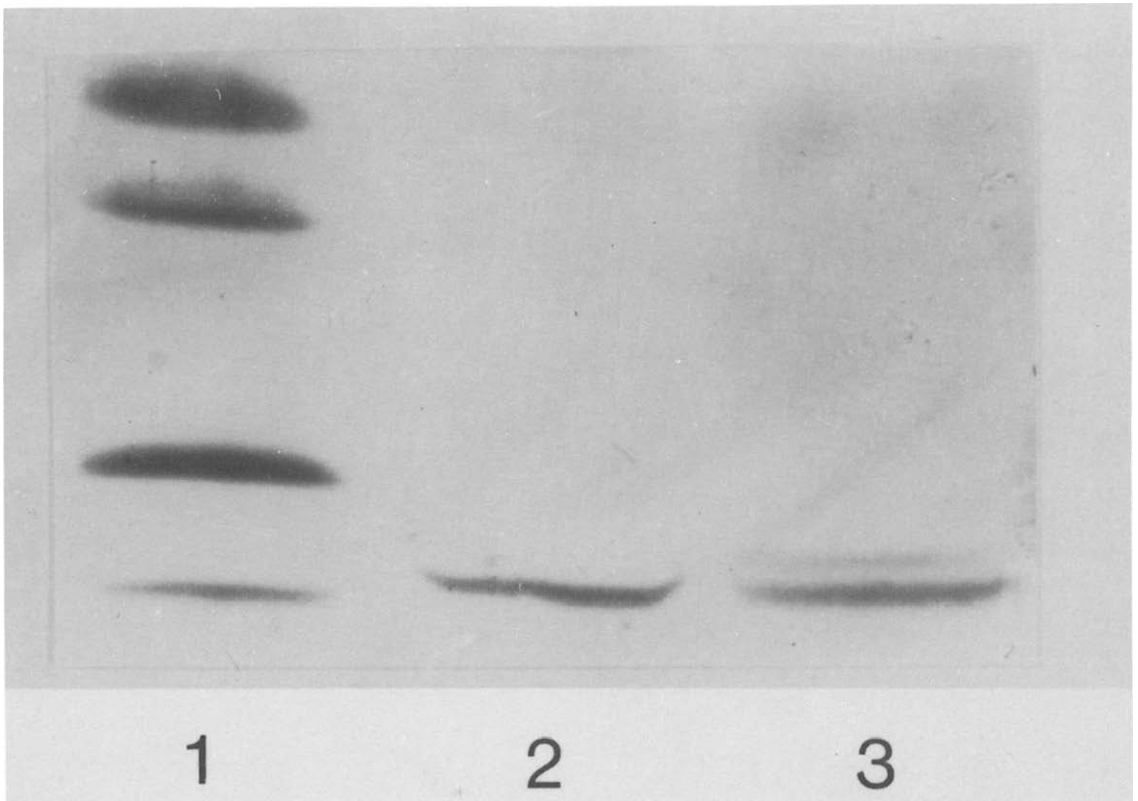


Fig. 5. SDS electrophoresis on High Density Phast Gel with Phast System. Lanes: 1 = Polypeptide molecular mass calibration kit (Pharmacia–LKB), M_r 10 804, 8266, 6413 and 2555 CNBr fragments from myoglobin; 2 = peak II (Fig. 3b); 3 = material containing CNBr fragments 1–32 obtained after reversed-phase chromatography represented in Fig. 4b.

shown in Fig. 6. Peak II, the material eluted with 25 m*M* phosphate, was analysed by SDS gel electrophoresis (Fig. 7). The relative molecular mass of the material in peak II was estimated to be 1000–1500.

The material was further chromatographed on a Superose 12 column equilibrated with 20 m*M* acetic acid. Essentially one peak was obtained (Fig. 8). Amino acid analysis of this material (Table II) showed the absence of the fragments 1–3 (SLK) and 22–32 (ISVVGVGAVGM), which obviously are trypsin degradation products that do not bind. The material corresponds to the peptide segment 4–21 (DHLIHNVHKEEHAHAHNK). Considering its low relative molecular mass it seemed that this material contained a roughly equimolar mixture of the two tryptic fragments (4–12) and (13–21). Apparently both subfragments, if present, became bound to Fe(III)–IDA-agarose.

The chromatographic behaviour of the synthesized peptides DHLIHNVHK (synthetic peptide 1) and EEHAHAHNK (synthetic peptide 2) was investigated. These peptides correspond to the two tryptic subfragments found to be bound to immobilized Fe(III). The peptides were run according to the same protocol in 25 m*M* PIPES (pH 7.1) on Fe(III)–IDA-agarose and separately on metal-free IDA agarose. On the former gel the peptides were retained in the starting buffer and eluted with 25 m*M* phosphate (Fig. 9a and b), but appeared in the breakthrough fraction from the metal-free gel (not shown). Amino acid analyses of the peaks eluted with phosphate were in agreement with the expected results. The material from unretained peak I (Fig. 9b) had a composition lacking one histidine compared with that of peptide (13–21) (Table III). Analysis by mass spectrometry confirmed that the

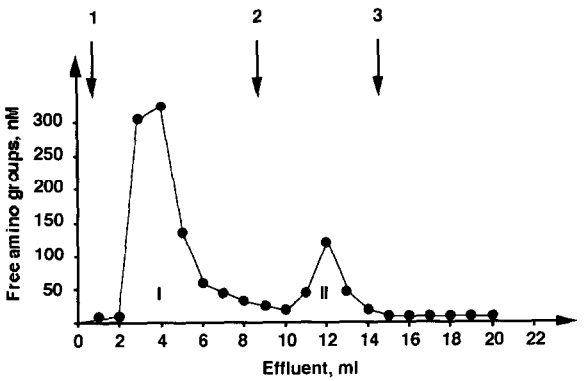


Fig. 6. Chromatography of the tryptic digest of fragment 1 on Fe(III)–Chelating Sepharose FF. A 2-ml volume of 25 m*M* PIPES (pH 7.1) containing the tryptic digest of fragment 1 was applied to an Fe(III)–Chelating Sepharose FF column (2.55 × 1 cm I.D.) equilibrated with the same buffer. The non-bound material was washed out with the equilibrium buffer (arrow 1). The adsorbed material was eluted by 25 m*M* sodium phosphate (pH 7.0) (arrow 2), followed by 25 m*M* sodium phosphate–1.0 *M* sodium chloride (pH 7.0) (arrow 3). A flow-rate of 30 ml/h was kept constant during the chromatography.

second synthetic preparation contained a substantial amount of a substance with a relative molecular mass lower than that of synthetic peptide 2 by one histidyl residue.

DISCUSSION

Work in this and other laboratories has shown that two exposed histidyl groups are a minimum requirement for adsorption of a protein molecule to immobilized Ni(II) ions [1]. Unusually tight binding of the M form of LDH subunit to immobilized Cu (II), Ni(II) and Zn(II) ions can be expected in view of the chemical structure of its N-terminal part, where six histidyl residues occur in a stretch of 20 residues [6]. In contrast, the H subunit does not contain a single residue in the same region. The isolation procedure thus selects the M subunit of the enzyme. Control experiments with the different forms of isoenzymes confirm this (not shown).

Several attempts have been made in this labora-

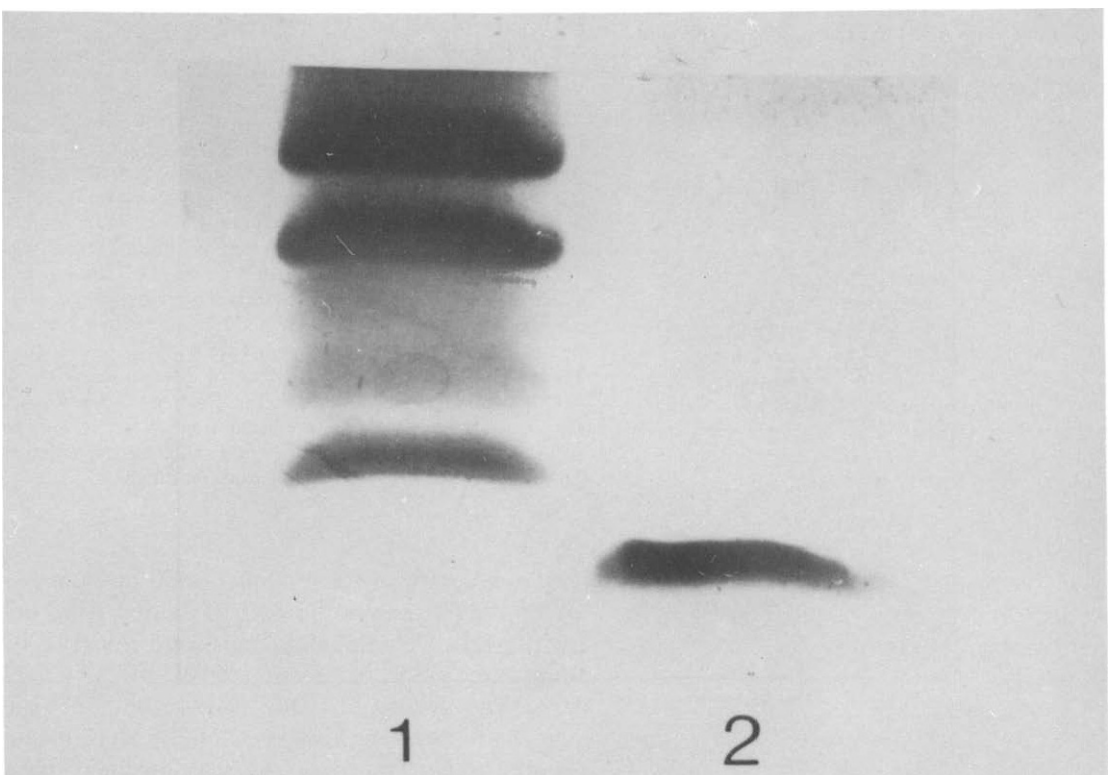


Fig. 7. SDS electrophoresis on High Density Phast Gel with Phast System. Lanes: 1 = Polypeptide molecular mass calibration kit (Pharmacia–LKB), M_r, 10 804, 8266, 6413 and 2555 CNBr fragments from myoglobin; 2 = peak II (Fig. 6).

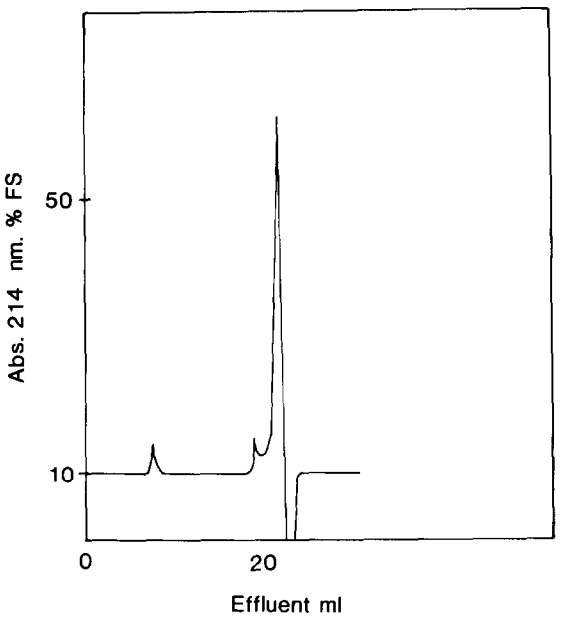


Fig. 8. Size exclusion chromatography of peak II, Fig. 6, on Superose 12. A 200-μl volume of peak II (Fig. 6) was applied to a Superose 12 HR 10/30 column equilibrated with 20 mM acetic acid at a flow-rate of 0.4 ml/min. The absorbance was followed at 214 nm at a monitor sensitivity of 0.1 a.u.f.s. and recorded at a chart speed of 0.25 cm/ml.

TABLE II

AMINO ACID COMPOSITION OF THE PEPTIDE FRACTION NUMBERED IN FIG. 6

Amino acid residue	Fig. 6, peak II	
	Detected (nmol per nmol peptide)	Expected (nmol per nmol peptide)
Aspartic acid	2.7	3
Threonine	0.2	0
Serine	0.2	0
Glutamic acid	2.0	2
Proline	0.3	0
Glycine	0.3	0
Alanine	2.0	2
Valine	1.3	1
Isoleucine	1.2	1
Leucine	1.2	1
Tyrosine	0.1	0
Phenylalanine	0.2	0
Histidine	5.1	6
Lysine	2.4	2
Arginine	0.4	0

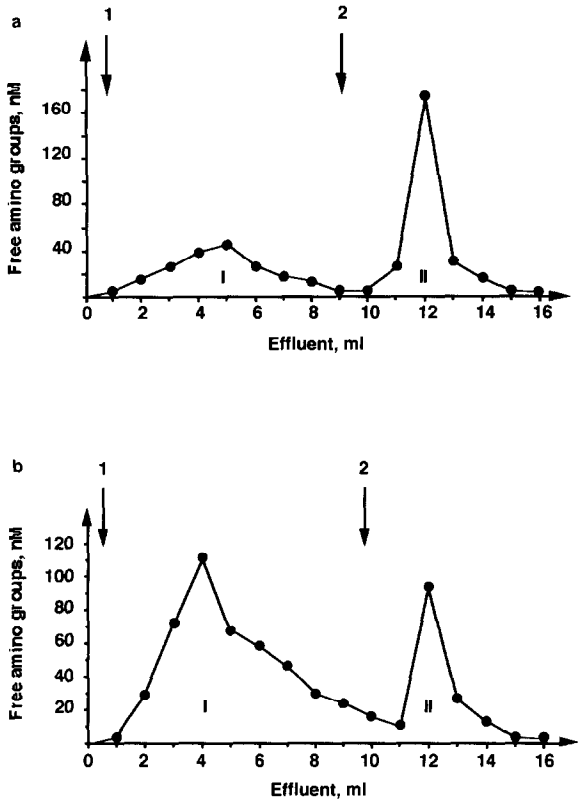


Fig. 9. Chromatography of synthetic peptides 1 and 2 on Fe(III)–Chelating Sepharose FF. (a) 0.42 mg of synthetic peptide 1 in 1 ml of 25 mM sodium acetate (pH 5.6) was loaded on to an Fe(III)–Chelating Sepharose FF column (2.5 × 1 cm I.D.) equilibrated with the same buffer. The non-adsorbed material was washed out by the equilibrium buffer (arrow 1). The adsorbed material was eluted by 25 mM sodium phosphate (pH 7.0) (arrow 2). The flow-rate was 30 ml/h throughout. (b) 0.5 mg of synthetic peptide 2 in 1 ml of 25 mM PIPES (pH 7.1) was loaded on to an Fe(III)–Chelating Sepharose FF column (2.5 × 1 cm I.D.) equilibrated with the same buffer. The non-adsorbed material was washed out by the equilibrium buffer (arrow 1). The adsorbed material was eluted by 25 mM sodium phosphate (pH 7.0) (arrow 2). The flow-rate was 30 ml/h throughout.

tory to account for the binding of non-phosphorylated proteins to immobilized Fe(III) ions. Affinities for the natural free amino acids were found to be small as judged from retention data [2,4]. Individual carboxylic groups [3] and clusters of carboxylic side-chains or combinations of tyrosyl phenol groups and carboxylate side-chain groups have been suggested as binding sites [11]. These models for binding are clearly not applicable to explain the

TABLE III

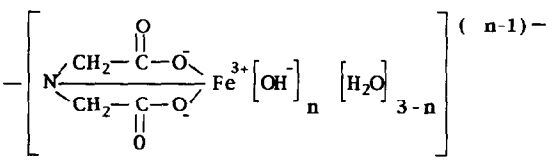
AMINO ACID COMPOSITION OF THE PEPTIDE FRACTIONS NUMBERED IN FIGS. 9 AND 9b

Amino acid residue	Fig. 9a, peak II		Fig. 9b, peak I		Fig. 9b, peak II	
	Detected (nmol per nmol peptide)	Expected (nmol per nmol peptide)	Detected (nmol per nmol peptide)	Expected (nmol per nmol peptide)	Detected (nmol per nmol peptide)	Expected (nmol per nmol peptide)
Aspartic acid	2.0	2	1.1	1	1.1	1
Glutamic acid	0	0	2.1	2	1.8	2
Alanine	0	0	2.0	2	1.8	2
Valine	1.0	1	0	0	0	0
Isoleucine	1.0	1	0	0	0	0
Leucine	1.0	1	0	0	0	0
Histidine	2.9	3	2.0	3	3.2	3
Lysine	1.1	1	1.1	1	1.1	1

properties of LDH. Our data rather suggest that the binding is confined to a restricted area of the protein molecule, namely segment (4–21), or rather to several side-chains within this segment. It seems safe to conclude that at least one and probably several histidyl side-chains participate in the binding. Possibly one or two carboxylate groups also interact with the metal, but this is less certain. One is tempted to propose that very likely at least three histidyl side-chains are necessary for sufficiently strong binding to immobilized Fe(III) ions. This proposal is supported by the behaviour of the synthetic peptides: the material from peak I, Fig. 9b, which contains only two histidyl groups is not retained on immobilized Fe(III) ions, whereas the material containing three histidyl groups (peak II, Fig. 9a and b) is adsorbed on the column in the starting buffer. Conclusive proof for this suggestion can only be obtained after more extensive studies with synthetic peptides of appropriate structures.

It should be noted that the putative binding peptide segment in the LDH molecule protrudes from the body of the protein molecule, forming a loosely structured part of the peptide chain. All side-chains are well exposed and accessible for binding [12]. LDH is built up of four identical subunits arranged in a symmetrical manner; this might be a feature that significantly enhances the binding to immobilized Fe(III) through multiple site interaction.

The enzyme fragment (residues 264–273) was adsorbed both on metal-loaded and metal-free gel. It contains five cationic side-chains, one of which is histidine. It is therefore likely that the adsorption is not due to the metal but to anionic groups on the agarose gel. Immobilized Fe(III) ions behave much as a cation exchanger under the conditions used in the experiments [13]. The interactive gel-immobilized ionic species may have a structure with a negative charge (*i.e.*, with $n = 2$ or 3):



In summary, the present results seem to indicate that the Fe(III)-binding site of each LDH subunit resides in a short segment at the N-terminus of the polypeptide chain. The binding peptide segment is coordinated to the metal by at least two side-groups, one of which must be a histidyl side-chain. This is a minimum requirement; probably there are more histidyl groups involved. That histidyl groups can participate in Fe(III) binding is well documented for several iron(III) metalloproteins [14–16]. More detailed information on the molecular interactions must await more sophisticated techniques than those available today.

ACKNOWLEDGEMENTS

We are grateful to Dr. David Eaker at the Institute of Biochemistry, Uppsala University, for linguistic revision of the manuscript and to Mrs. Inga Johansson for secretarial help.

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