ELSEVIER

Journal of Chromatography A, 678 (1994) 25–34

JOURNAL OF CHROMATOGRAPHY A

Immobilized metal-ion affinity partitioning of NAD$^+$-dependent dehydrogenases in poly(ethylene glycol)–dextran two-phase systems ☆

Henrikas Pesliakas*, Vilius Žutautas, Birutė Baškevičiūtė

Department of Research, Institute of Biotechnology "Fermentas", Graičiūno 8, Vilnius 2028, Lithuania

(First received December 16th, 1993; revised manuscript received March 26th, 1994)

Abstract

Affinity partitioning of yeast alcohol dehydrogenase (YADH), lactate dehydrogenase from rabbit muscle (MLDH) and lactate and malate dehydrogenases from pig heart (HLDH and HMDH, respectively) were studied in aqueous two-phase systems containing metal ions (Cu^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+}) chelated by iminodiacetate–poly(ethylene glycol) (IDA–PEG). The partitioning behaviour of the enzymes in the presence of Cu(II)–IDA–PEG was studied as a function of the concentration of NaCl, the pH of the medium and the concentration of added selected agents. It was demonstrated that the partition effect (Δ log K) of dehydrogenases in the presence of Cu(II)–IDA–PEG and the affinity of enzymes for immobilized Cu^{2+} ions increases in the order MLDH > YADH > HMDH ≥ HLDH. It was shown that the determined variations in the enzyme affinities for Cu(II)–IDA–PEG might be related to the differences in the content of histidine residues accessible to the solvent.

1. Introduction

Immobilized metal ion affinity chromatography (IMAC) of proteins was introduced by Porath et al. [1]. The basic principle of this method is the coordination between transition metal ions chelated by iminodiacetic acid (IDA) and electron-donor groups on the protein surface. Surface-exposed histidine, cysteine and tryptophan residues have attracted attention as the primary sites responsible for protein interactions with immobilized metal ions [1–4]. More recent studies have demonstrated that of the amino acids, the available histidyl residues in terms of their topography on the macromolecule surface seem to be the critical factor dictating the selective retention on metal-affinity columns [4–10].

Immobilized metal ion affinity partitioning has been developed recently to enhance the selective partitioning of proteins in aqueous two-phase systems [11]. The method is based on the interactions between accessible amino acid residues on the protein surface and transition metal ions charged on IDA–poly(ethylene glycol) (PEG). The partitioning behaviour of native [12–16], genetically engineered proteins carrying high-

* Corresponding author.

☆ Presented at the *8th International Conference on Partitioning in Aqueous Two-Phase Systems, Leipzig, August 22–27, 1993.*

0021-9673/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(94)00420-E

affinity His–X_3–His sites engineered into their surface [16–19], erythrocytes [20] and isoenzymes of lactate dehydrogenase [21] has been studied in aqueous PEG–salt and PEG–dextran two-phase systems. Interactions between Cu(II)–IDA–PEG and surface-exposed histidines in aqueous two-phase systems have also been explored theoretically [13].

Recently [22,23], affinity partitioning has been used to study the interaction of many NAD(H)-dependent dehydrogenases with immobilized Cu(II) complexes of dye ligands. It was found that the Cu^{2+} ions had a discriminating ability to affect the specificity of dye–enzyme complex formation between the yeast and horse liver ADH on the one hand, and LDH from rabbit muscle on the other. In this respect, it was of interest to evaluate the binding properties of these enzymes towards Cu^{2+} and other metal ions charged on chelating ligands such as IDA, excluding the dye ligand effect.

2. Experimental

2.1. Materials

Yeast alcohol dehydrogenase (EC 1.1.1.1, specific activity ca. 400 U/mg) was obtained from Boehringer (Mannheim, Germany). Lactate dehydrogenase from rabbit muscle (EC 1.1.1.27, specific activity ca. 650 U/mg) was purchased from Serva (Heidelberg, Germany). Lactate dehydrogenase from pig heart (EC 1.1.1.27, specific activity ca. 300 U/mg) and malate dehydrogenase from pig heart (EC 1.1.1.37, specific activity ca. 1100 U/mg) were kindly provided by T. Bodneva, Institute of Biotechnology. Substrates and substances for Good's buffers —MES (morpholinoethanesulfonic acid), HEPES (N - 2 - hydroxyethylpiperazine - N′ - 2 - ethanesulfonic acid) and TAPS [N- tris(hydroxymethyl)methyl - 3 - aminopropanesulfonic acid]— were obtained from Serva or Sigma (St. Louis, MO, USA). Poly(ethylene glycol) PEG 6000 was obtained from Serva or Fluka (Basle, Switzerland) and dextran 60 000 from the Factory of Clinical Preparations (Krasnojarsk, Russian Federation). Iminodiacetic acid (IDA) and epichlorohydrin were obtained from Fluka. All other chemicals were commercially available and of analytical-reagent or puriss grade.

2.2. Synthesis of IDA–PEG derivatives

IDA–PEG was synthesized in two steps by reaction of iminodiacetic acid with a monosubstituted derivative of epichlorohydrin-activated PEG. Epichlorohydrin-activated PEG was prepared according to the usual method for the synthesis of glycidyl ethers as described by Ulbrich et al. [24]. Briefly, 100 g of PEG 6000 (M_r 6000–7500) were dissolved in 500 ml of absolute benzene, then 5 ml of boron trifluoride ethyl etherate and 1.32 ml of epichlorohydrin (0.5 mol/mol PEG) were added dropwise and the reaction mixture was stored at room temperature for 48 h. Thereafter the mixture was gently stirred and 1.2 ml of a 45% solution of NaOH was slowly added dropwise and the solution was again stored at room temperature for 2 h. The reaction mixture was decanted, treated with 500 ml of diethyl ether and the precipitate was filtered off and dried. The product was dissolved in 200–300 ml of absolute benzene, precipitated repeatedly with 500 ml of diethyl ether and dried.

A 50-g amount of the product obtained was dissolved in 250 ml of 2 M Na_2CO_3 solution containing 25 g of iminodiacetic acid and the mixture was stirred at 65°C for 24 h. After the mixture had been cooled, the product was extracted with 750–1000 ml of chloroform. The chloroform phases were pooled, dried over anhydrous Na_2SO_4 and the solvent was removed by rotary evaporation. After two crystallizations in absolute ethanol, the IDA–PEG derivative was obtained (the yield was 25–30% of total PEG).

The Cu(II) complex of IDA–PEG was obtained by dissolving 25 g of IDA–PEG in 25 ml of 50 mM sodium acetate buffer (pH 4.0) containing 12.5 g of Cu_2SO_4. The solution was stirred at room temperature for 1 h and then

extracted twice with 100–150 ml of chloroform. The combined chloroform phases were dried and the solvent was evaporated. The yield of Cu(II)–IDA–PEG was 15–20 g. Other metal ion (Ni^{2+}, Zn^{2+} and Cd^{2+}) IDA–PEG complexes were obtained in a similar manner using a 10 molar excess of appropriate metal salts over IDA–PEG.

The metal ion concentration in the metal chelate–PEG derivatives was measured by atomic absorption spectrometry.

2.3. Enzyme assays

The enzyme activities were determined spectrophotometrically at 340 nm and 30°C. Alcohol dehydrogenase and both lactate dehydrogenases were determined as described previously [22,23]. The activity of malate dehydrogenase was measured as described [25].

2.4. Two-phase systems

Two-phase systems (4 g) were prepared by weighing from stock solutions of polymers in water, viz., 20–50% (w/w) PEG and 20–30% (w/w) dextran. The final concentrations of PEG and dextran in the aqueous two-phase systems were 6.5% (w/w) PEG 6000–10% (w/w) dextran 60 000. All necessary ingredients, buffer, water, enzyme samples and selected agents, were mixed with polymer solutions to give the desired final concentrations as indicated in the tables. IMA partitioning experiments were performed by replacing part of PEG with IDA–PEG or metal ion complexes with IDA–PEG. The amount of IDA–PEG derivative is given as a percentage of the total mass of PEG present in the system or expressed as metal ion concentration (m*M*/kg) per kg of two-phase system.

2.5. Partitioning of enzymes

After 17–24 units of YADH, 4–12 units of MLDH, 30–43 units of HLDH or 20–47 units of HMDH has been introduced into the two-phase systems, the mixture obtained was shaken gently for about 15 s, kept for 5 min and then centrifuged for about 2 min at 2000 *g* to complete the phase separation. Samples of known volume were withdrawn from each phase and the enzyme activity was determined. The partition coefficient of the enzyme, K, was defined as the ratio of the enzyme concentration in the upper and lower phases. The affinities of enzymes for metal ions were expressed in terms of $\Delta \log K$, defined as the difference between the logarithmic partition coefficients of enzymes in the presence (K) and in the absence (K_0) of metal–IDA–PEG ($\Delta \log K = \log K - \log K_0$). The change in the enzyme partition coefficients when selected agents were introduced into the two-phase systems was expressed as a percentage of the initial value of $\Delta \log K$ in the presence of metal–IDA–PEG and the absence of the agent.

All partitioning experiments were carried out in duplicate at room temperature and the value of $\Delta \log K$ is given as the mean of two separate determinations.

3. Results

3.1. Synthesis of IDA–PEG derivatives

The commonly used method for coupling IDA to PEG includes a three-step synthesis starting from monomethoxy-PEG [14]. The intermediate aminomonomethoxy-PEG derivative was treated with bromoacetic acid, yielding IDA–PEG. In an attempt to obtain a water-soluble chelating polymer carrying iminodiacetate groups coupled to the polymer in a similar manner to the synthesis of the well known Porath's adsorbent on Sepharose [2], another route to IDA–PEG synthesis was chosen. PEG was activated with a stoichiometric amount of epichlorohydrin to obtain the monosubstituted derivative, which was subsequently treated with the IDA according to Porath and Olin [2].

The metal ion contents combined with IDA–PEG are given in Table 1.

Table 1
Contents of metal ions combined with IDA–PEG

Metal ion	Metal ion content (mol/mol PEG)[a]
Cu^{2+}	0.43–0.58
Ni^{2+}	0.19–0.32
Zn^{2+}	0.09–0.13
Cd^{2+}	0.20–0.30

[a] The metal ion content in IDA–PEG is expressed as moles of the respective metal ion per mole of PEG.

3.2. Partitioning of dehydrogenases as a function of salt concentration and metal ions

In the two-phase system containing 6.5% (w/w) PEG and 10% (w/w) dextran, in the absence of the IDA–PEG derivative all the dehydrogenases studied were partitioned in favour of the dextran-rich bottom phase. The partitioning behaviour of any enzyme introduced into a system containing an increasing concentration of metal ions, e.g., Cu^{2+} ions chelated by IDA–PEG, was greatly changed. This may be exemplified by the YADH partitioning data. As can be seen from Table 2 and Fig. 1, the presence of Cu^{2+} ions in two-phase system dramatically enhances the extraction of enzyme into the metal–IDA–PEG-containing upper phase. Less extraction of YADH occurs in the presence of Ni^{2+} ions and PEG–IDA with no metal ion seems to have a negligible effect on the partitioning of the enzyme even when 5% of a portion of PEG was replaced with PEG–IDA.

Table 2
Effect of liganded PEG on the partitioning of yeast ADH

Liganded PEG	Δ log *K*
IDA–PEG	0.41
Ni(II)–IDA–PEG	0.72
Cu(II)–IDA–PEG	2.30

Two-phase system (4 g) contained 6.5% (w/w) PEG 6000, 10% (w/w) dextran 60 000, 17–24 units of enzyme, 5% liganded PEG and 10 m*M* MES buffer (pH 6.5). The amount of liganded PEG is expressed as a percentage of the total mass of PEG present in the system.

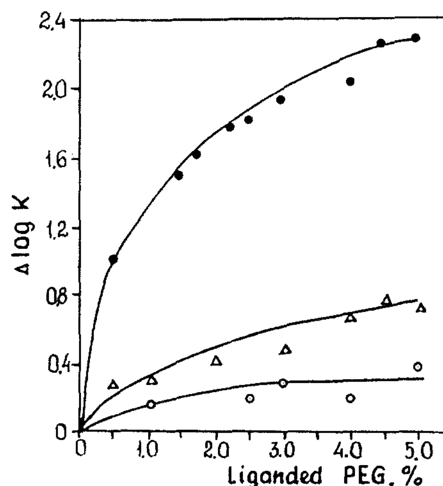

Fig. 1. Partitioning of yeast ADH in PEG–dextran systems containing increasing amounts of liganded PEG. Two-phase system (4 g) contained 6.5% (w/w) PEG 6000, 10% (w/w) dextran 60 000, 17–24 units of enzyme and 10 m*M* MES buffer (pH 6.5). The amount of liganded PEG is given as a percentage of the total mass of PEG in the system. ● = Cu(II)–IDA–PEG; △ = Ni(II)–IDA–PEG; ○ = IDA–PEG.

Addition of NaCl and an increase in its concentration in the two-phase systems to 0.6–1.0 *M* had different effects on the partitioning of the dehydrogenases studied. As can be seen from Table 3 and Fig. 2, NaCl greatly affects the partitioning of MDH and LDH from pig heart. An increase in NaCl concentration in the system to 0.6 *M* in the presence of Cu(II)–IDA–PEG caused an increase in the Δ log *K* value of HMDH from 0.89 (in the absence of NaCl) to 1.32 (at 0.3 *M* NaCl). However, with HLDH an increase in NaCl concentration to 0.6 *M* gave a decrease in Δ log *K* of the enzyme from 1.30 (in the absence of salt) to 0.41–0.56 (with 0.2–0.4 *M* NaCl), suppressing its binding to Cu(II)–IDA–PEG. This means that in addition to the coordination, another type of bonding, probably electrostatic, is involved in the interaction of HLDH with Cu(II)–IDA–PEG. Only the part of the Δ log *K* value equal to 0.41–0.56 may be attributed to the contribution of coordination bonds. On the basis of this effect of NaCl, all further experiments on HMDH partitioning were carried out in the presence of 0.25 *M* NaCl, and with HLDH NaCl was omitted. Introduction of

Table 3
Effect of NaCl concentration on the partitioning of MDH and LDH from pig heart in the presence of Cu(II)–IDA–PEG

NaCl (*M*)	Δ log *K*	
	HMDH	HLDH
–	0.89	1.30
0.1	1.15	0.50
0.2	1.15	0.41
0.3	1.32	0.50
0.4	1.05	0.56
0.5	1.09	0.54
0.6	1.08	0.44

Two-phase systems (4 g) were composed of PEG and dextran as in Table 2, 10 m*M* HEPES buffer (pH 7.0), 22–34 units of enzyme and increasing concentration of NaCl. The Cu(II)–IDA–PEG concentration in the system is expressed as the concentration of Cu^{2+} ions per kg of two-phase system and was equal to 0.21 and 0.42 m*M*/kg for HMDH and HLDH, respectively.

NaCl at concentrations up to 1.0 *M* into the systems containing MLDH in the presence of Cu(II)–IDA–PEG had no appreciable effect on the partitioning of the enzyme, whereas with YADH there was a slow increase in Δ log *K* as the NaCl concentration was increased from 0.2 to 0.6 *M* (see Figs. 5 and 6).

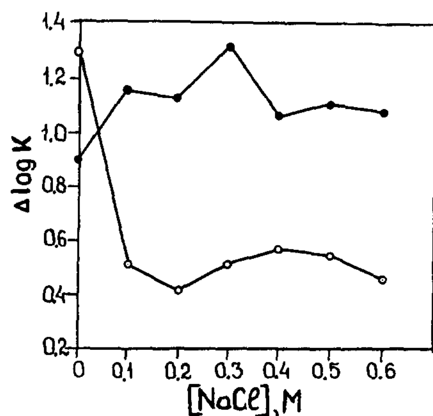

Fig. 2. Effect of NaCl concentration on the partitioning of MDH and LDH from pig heart in the presence of Cu(II)–IDA–PEG. Two-phase systems (4 g) were composed of PEG and dextran as in Fig. 1, 10 m*M* HEPES buffer (pH 7.0), 22–34 units of enzyme and increasing concentrations of NaCl. The Cu(II)–IDA–PEG concentration in the system is expressed as the concentration of Cu^{2+} ions in m*M* per kg of two-phase system and was equal to 0.21 and 0.42 m*M*/kg for HMDH and HLDH, respectively. ● = HMDH; ○ = HLDH.

Partitioning of dehydrogenases in the presence of PEG–IDA conjugates combined with various metal ions in summarized in Table 4. According to the results in Table 4, it is evident that Cu^{2+} ions, as would be expected, exhibited the strongest effect on the partitioning of the dehydrogenases studied. Of the other metal ions, only Ni^{2+} ions had an appreciable effect on the affinity partitioning efficiency (Δ log *K*) of YADH and MLDH. The extraction power into the upper phase of dehydrogenases in the presence of Cu(II)–IDA–PEG differed significantly and revealed differences in the surface properties of the enzymes. For example, a high binding of YADH to Cu(II)–IDA–PEG (Δ log $K = 2.62$) might be expected, based on the predominant contribution of the Cu^{2+} ion coordination bonds previously found [22] in the interaction of this enzyme with Cu(II) complexes of many dye ligands. The possibility of coordination of the Cu^{2+} ions with the histidine residue located at the coenzyme-binding site of YADH was proposed. With LDH from rabbit muscle we observed [23] a smaller contribution of Cu^{2+} ions to the dye–enzyme complex formation. However, as can be seen from Table 4, LDH from rabbit muscle displayed a much stronger binding to Cu(II)–IDA–PEG (Δ log $K = 3.72$) than YADH. This obviously indicated that MLDH possesses surface-exposed amino acid residues available for the interaction with Cu^{2+} ions. A much weaker interaction with Cu(II)–IDA–PEG was found for HMDL (Δ log $K = 1.45$). In contrast to LDH from rabbit muscle, the enzyme from pig heart interacts with Cu^{2+} ions weakly (Δ log $K = 1.33$). The partitioning behaviour of both LDH towards Cu(II) and Ni(II)–IDA–PEG determined in this work agrees very well with the partitioning results of these enzymes obtained recently by Otto and Birkenmeier [21]. Despite the differences in the composition of the aqueous two-phase systems used, the tendency of LDH partitioning in both instances was found to be the same: LDH from rabbit muscle displayed a much stronger binding to Cu(II) and

Table 4
Effect of metal ion chelated by IDA–PEG on the partitioning of different NAD^+-dependent dehydrogenases

Metal ion–IDA–PEG	Concentration of metal ion (mM/kg)	Δ log K			
		YADH (pH 6.5)	MLDH (pH 7.0)	HLDH (pH 7.0)	HMDH (pH 7.0 with 0.25 M NaCl)
–	–	K_0 = 0.0043 ± 0.0011	K_0 = 0.0089 ± 0.0004	K_0 = 0.0124 ± 0.0005	K_0 = 0.0898 ± 0.0090
Cu^{2+}	0.42	2.62 ± 0.04	3.72 ± 0.12	1.33 ± 0.08	1.45 ± 0.028
Ni^{2+}	0.25	1.10 ± 0.10	0.70 ± 0.03	0.32 ± 0.01	0.114 ± 0.035
Zn^{2+}	0.12	n.d.[a]	n.d	0.086 ± 0.026	0.03
Cd^{2+}	0.25	n.d.	n.d.	0.22 ± 0.078	0.10

System composition as in Table 2; 10 mM buffers: HEPES (pH 7.0) and MES (pH 6.5).
[a] n.d. = Not determined.

Ni(II)–IDA–PEG than the enzyme from pig heart. The observed orders of magnitude of the Δ log K values of the LDH obtained in this work and that reported by Otto and Birkenmeier [21] are similar: 3.72 and 0.41–0.56 (with 0.2–0.4 M NaCl) in the presence of Cu(II)–IDA–PEG for MLDH and HLDH, respectively, in this work and 4.5 and 0.4 determined by Otto and Birkenmeir [21]. In general, the partitioning behaviour of both types of LDH parallels also the chromatographic behaviour of the LDH isoenzymes from hog on an Ni(II)–nitrilotriacetic acid (NTA) column as determined by Hochuli et al. [26]. They observed the retention of the muscle-type isoenzyme on the Ni(II)–NTA column and a lack of retention by the heart-type enzyme.

3.3. *Effect of pH on affinity partitioning of dehydrogenases*

The affinities of all the dehydrogenases studied, except the LDH from pig heart, for Cu(II)–IDA–PEG, and also the MLDH affinity for Ni(II)–IDA–PEG, were found to be sensitive to pH. In the presence of Cu(II)–IDA–PEG the Δ log K values of YADH increase monotonically with increase in pH from 5.0 to 8.0, as indicated in Table 5 and Fig. 3. The alteration of

Table 5
Effect of pH on the partitioning of dehydrogenases in the presence of metal–IDA–PEG

pH	Δ log K			pH	Δ log K	
	YADH (Cu^{2+})	MLDH (Cu^{2+}; Ni^{2+})			HMDH (Cu^{2+})	HLDH (Cu^{2+})
5.0	1.68	1.60	n.d.[a]	6.0	1.04	1.37
5.5	2.04	2.00	1.24	7.0	1.45	1.33
6.5	2.20	2.79	1.86	8.0	1.74	1.46
7.0	n.d.	3.03	2.30	9.0	1.25	n.d.
7.5	2.30	n.d.	n.d.			
8.0	2.38	2.99	2.09			

Two-phase systems (4 g) were composed of PEG and dextran as in Table 2. Concentrations of metal ions: Cu^{2+}, 0.2234 mM/kg for YADH and MLDH and 0.42 mM/kg for HMDH and HLDH; Ni^{2+}, 7.03 mM/kg for MLDH. Two-phase systems contained 10 mM buffers for YADH, MLDH and HLDH and 10 mM buffers with 0.25 M NaCl for HMDH: MES (pH 5.0–6.5), HEPES (pH 7.0) and TAPS (pH 8.0–9.0)
[a] n.d. = Not determined.

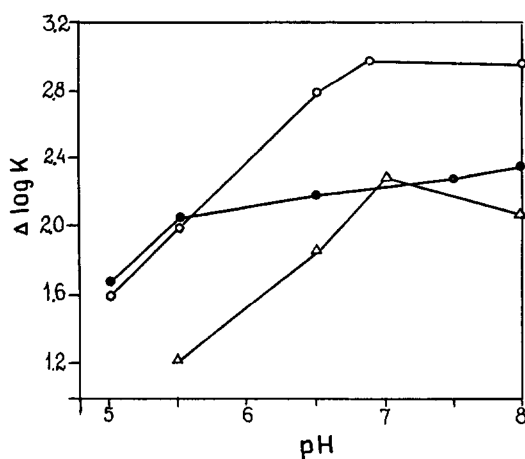

Fig. 3. Effect of pH on the partitioning of yeast ADH in the presence of Cu(II)–IDA–PEG and LDH from rabbit muscle in the presence of Cu(II) and NI(II)–IDA–PEG. Two-phase systems (4 g) were composed of PEG and dextran as in Fig. 1. Concentrations of metal ions: Cu^{2+}, 0.2234 m*M*/kg; Ni^{2+}, 7.03 m*M*/kg. 10 m*M* buffers: MES (pH 5.5–6.5), HEPES (pH 7.0) and TAPS (pH 8.0–9.0). ○ = MLDH (CU^{2+}); ● = YADH (Cu^{2+}); △ = MLDH (Ni^{2+}).

the partition coefficients of MLDH, Δ log *K*, in the presence of immobilized Cu^{2+} or Ni^{2+} ions is highly sensitive to pH, increasing in the pH range 5.0–7.0 and beginning to decrease above pH 7.0 (Table 5, Fig. 3). The dependence of the affinities of MDH and LDH from pig heart for Cu(II)–IDA–PEG on the pH of the medium differs greatly (Table 5, Fig. 4). With HMDH an

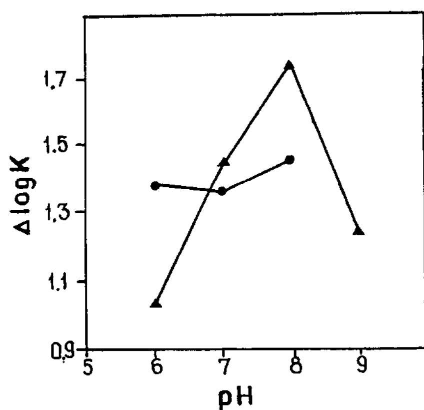

Fig. 4. Effect of pH on the partitioning of LDH and MDH from pig heart in the presence of Cu(II)–IDA–PEG. System composition as in Fig. 3. Concentration of Cu^{2+} ions, 0.42 m*M*/kg. ▲ = HMDH; ● = HLDH.

increase in pH in the systems from 6.0 to 8.0 caused an increase in Δ log *K*, followed by a decrease at higher pH. In contrast, the affinity partitioning effect (Δ log *K*) of HLDH is virtually insensitive to pH variations in the range 6.0–8.0 (Fig. 4).

3.4. Partitioning of dehydrogenases in the presence of selected agents

The interaction of dehydrogenases with chelated Cu^{2+} ions was studied when selected agents such as nucleotide ligands, chelating agents or amino acids were present in the two-phase systems. As can be seen from Table 6 and Figs. 5 and 6, the introduction into the two-phase systems of the chelating agent EDTA at concentrations up to 1 m*M* was sufficient to abolish the binding of all the dehydrogenases to Cu(II)–IDA–PEG, the Δ log *K* values decreasing to zero. Imidazole also had a strong ability, but discriminating among the enzymes studied, to reduce their interaction with immobilized Cu^{2+} ions. Table 6 shows that the addition of 1 m*M* imidazole to the two-phase systems decreased Δ log *K* to zero with MLDH and HMDH, but was less effective with YADH and HLDH. In the latter two cases, a further increase in imidazole concentration to 5 m*M* caused a 92% decrease in Δ log *K* of HLDH, whereas the Δ log *K* of YADH was decreased only to 48%. Table 6 shows that among the amino acids, tryptophan was the most effective for the dissociation of the HMDH–Cu(II)–IDA complex. A 1 m*M* concentration of tryptophan could reduce the value of Δ log *K* by 88%. Low concentrations of arginine (1 m*M*) resulted in decreases in Δ log *K* by 96, 74 and 53% for HMDH, HLDH and MLDH, respectively. The observed Δ log *K*-decreasing capacity of ammonium ion was the same as that of arginine for LMDH and lower for HMDH.

As can be seen from Table 6, cysteine at increasing concentrations in the two-phase systems diminished the binding of HLDH and HMDH to Cu(II)–IDA–PEG but to a smaller extent than imidazole. Nucleotide ligands such as adenine and NAD were able, as was shown

Table 6
Dependence of the affinity partitioning effect (Δ log K) of dehydrogenases on the concentration of selected agents

Agent	Concentration (mM)	Residual Δ log K (%)			
		YADH (pH 6.5)	MLDH (pH 7.0)	HLDH (pH 7.0)	HMDH (pH 8.0 with 0.25 M NaCl)
EDTA	1	0	0 (at 0.5 mM)	0	0
Imidazole	1	71	0	33	0
	5	48	0	8	0
NAD	1	49	n.d.[a]	n.d.	n.d.
	5	34	n.d.	n.d.	n.d.
NADH	1	n.d.	82	80	100[b]
	5	n.d.	71	55	100[b]
Nicotinamide	1	85	n.d.	n.d.	n.d.
	5	72	n.d.	n.d.	n.d.
Adenine	1	86	n.d.	n.d.	n.d.
	5	75	n.d.	n.d.	n.d.
Tryptophan	1	99	n.d.	61	12
	5	95	n.d.	23	0
Ammonium ion	1	n.d.	41	n.d.	59
	5	n.d.	19	n.d.	0
Arginine	1	n.d.	47	26	4
	5	n.d.	14	−12	−5
Cysteine	1	n.d.	n.d.	34	27
	5	n.d.	n.d.	21	16

System composition as in Table 2; 10 mM buffers: HEPES (pH 7.0), MES (pH 6.5) and TAPS (pH 8.0). Concentration of Cu^{2+} ions in the systems: 0.2234 mM/kg for YADH and MLDH, 0.21 mM/kg for HMDH and 0.42 mM/kg for HLDH.
[a] n.d. = Not determined.
[b] Two-phase system without NaCl.

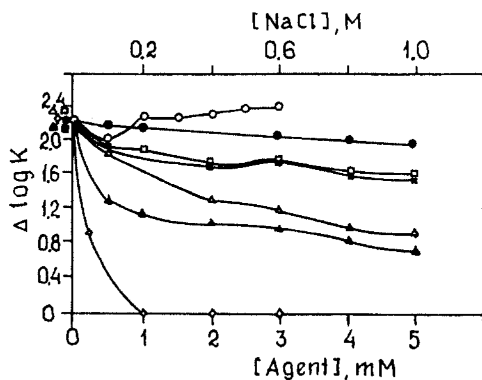

Fig. 5. Partitioning of yeast ADH in the systems containing increasing concentrations of selected agents in the presence of Cu(II)–IDA–PEG. System composition as in Fig. 1. Concentration of Cu^{2+} ions, 0.2234 mM/kg; 10 mM MES buffer (pH 6.5). ○ = NaCl; ● = tryptophan; □ = adenine; ■ = nicotinamide; △ = imidazole; ▲ = NAD; ◇ = EDTA.

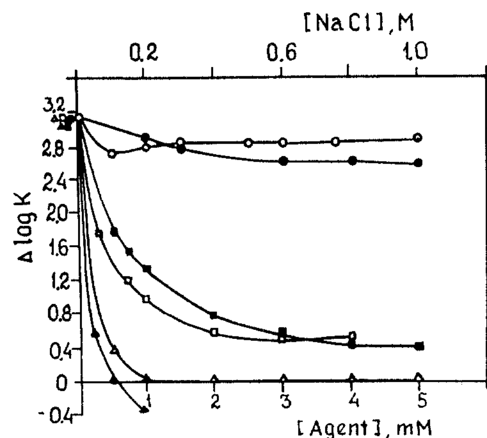

Fig. 6. Partitioning of LDH from rabbit muscle in systems containing increasing concentrations of agents in the presence of Cu(II)–IDA–PEG. System composition as in Fig. 1. Concentration of Cu^{2+} ions, 0.2234 mM/kg; 10 mM HEPES buffer (pH 7.0). ○ = NaCl; ● = NADH; □ = ammonium ions; ■ = arginine; △ = imidazole; ▲ = EDTA.

previously [27], to elute the enzyme retained on the adsorbent with the immobilized dye–Cu(II) complex. Therefore, they might act as displacing agents causing also the dissociation of the formed Cu(II)–IDA–PEG–enzyme complexes. However, in contrast to the high eluting capacity of adenine observed earlier [27] and its relatively high Δ log K-decreasing capacity, observed previously [22,23] by studying the partitioning of dehydrogenases in the presence of dye–Cu(II) complexes, in this study we found a much smaller effect of adenine on the Δ log K values. Table 6 and Fig. 5 show that even a 5 mM concentration of adenine caused a decrease in Δ log K of YADH in the presence of Cu(II)–IDA–PEG of only 25%. However, unexpectedly, we found a relatively high capability of NAD to reduce YADH binding to Cu^{2+} ions. A 5 mM concentration of NAD decreased Δ log K of YADH by 66% and gave a larger decrease in Δ log K when the same concentration of imidazole was presence in the two-phase system. This indicates that the coenzyme-binding site of YADH might be involved in the interaction with Cu(II)–IDA–PEG. Addition of NADH and an increase in its concentration to 5 mM had an appreciable effect, decreasing Δ log K of HLDH to 55%, whereas Δ log K of MLDH was decreased to 71% and no change in Δ log K was observed with HMDH. Nicotinamide, as can be seen from Table 6, affected the YADH partitioning with respect to the Cu(II)–IDA–PEG in a similar manner to adenine.

4. Discussion

As a continuation of our attempts to explore the role of metal ions on the specific recognition of ligands, e.g., biomimetic dyes by various NAD^+-dependent dehydrogenases, in this study the binding properties of four enzymes towards metal ions chelated by IDA–PEG were evaluated in an aqueous two-phase system composed of PEG and dextran.

The determined variations in the partition coefficients (Δ log K) of dehydrogenases (YADH, MLDH, HLDH and HMDH) studied in the presence of metal ions, primarily Cu^{2+}, immobilized on IDA–PEG clearly indicated (Table 4) the presence of and differences in the accessibility of metal-binding sites on the surface of the enzymes. Further, it was found that the interactions of many dehydrogenases with Cu(II)–IDA–PEG show a pronounced dependence on the pH of the medium. The determined increase in the Δ log K values of enzymes with increase in pH (Table 5) may be related, as has been indicated previously [18], to deprotonation of the imidazole group. Therefore, the differences in the partitioning behaviour of the dehydrogenases studied in the presence of Cu(II)–IDA–PEG could be due to the possible involvement in the interaction with metal ions of histidyl residues of enzymes exposed to the solvent. The dependence of metal affinity on histidine content, with the existence of a linear proportionality between the increase in partition coefficients and the protein surface histidine content, was clearly shown recently [16,18] by studying the partitioning of native and genetically engineered histidine-containing proteins in a PEG–dextran two-phase system containing Cu(II)–IDA–PEG. Despite the lack of complete information concerning the location and number of surface-exposed histidine residues in the dehydrogenases studied, some discussion is possible.

It is well known that the dehydrogenases studied differ in the number of histidine residues available for chemical modification by diethyl pyrocarbonate. As the latter reacts with all accessible histidines in proteins, the maximum number of histidine residues modified in dehydrogenases can be regarded, to a first approximation, as a relative measure defining the partitioning efficiency (Δ log K) of an enzyme in the presence of Cu(II)–IDA–PEG. The numbers of histidine residues per subunit of enzymes studied were found to be ten for YADH [28], eleven for MLDH [21], seven for HLDH [26] and thirteen per molecule of HMDH [29]. According to the literature, the maximum numbers of histidine residues modified per enzyme subunit were three (pH 6.0) for LDH from rabbit muscle [30], 2.5 (pH 7.0) for yeast ADH [31] and one (pH 6.0) for LDH from pig heart [32]. The number of

essential histidine residues for these three enzymes was established as one per enzyme subunit [31]. For MDH from pig heart, one essential histidine residue chemically alkylated with iodoacetamide per active site was found [29].

Taking these considerations into account, one can observe that the dehydrogenase affinity to Cu(II)–IDA–PEG (Table 4) increases according to the number of histidine residues accessible for chemical modification with diethyl pyrocarbonate in the following order: MLDH > YADH > HMDH ≥ HLDH. Therefore, one of the possible ways to explore the location of the histidine residues that are involved at the interaction of the studied dehydrogenases with Cu^{2+} ions would be to carry out investigations with chemically modified enzymes. However, despite this, the data presented here demonstrated that immobilized metal ion affinity partitioning in aqueous two-phase systems can be used as a sensitive probe for metal-binding sites on the surface of NAD^+ dehydrogenases.

Acknowledgement

The authors thank Ms. Tamara Bodneva for providing the samples of purified MDH and LDH from pig heart.

References

[1] J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature*, 258 (1975) 598–599.
[2] J. Porath and B. Olin, *Biochemistry*, 22 (1983) 1621–1630.
[3] J. Porath, in H. Tschesche (Editor), *Modern Methods in Protein Chemistry*, Vol. 2, Walter de Gruyter, Berlin, 1985, p. 85.
[4] J. Porath, *Trends Anal. Chem.*, 7 (1988) 254–259.
[5] E. Sulkowski, *Trends Biotechnol.*, 3 (1985) 1–7.
[6] E.S. Hemdan, Y.J. Zhao, E. Sulkowski and J. Porath, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 1811–1815.
[7] T.T. Yip, Y. Nakagawa and J. Porath, *Anal. Biochem.*, 183 (1989) 159–171.
[8] M. Belew and J. Porath, *J. Chromatogr.*, 516 (1990) 333–354.
[9] Y.J. Zhao, E. Sulkowski and J. Porath, *Eur. J. Biochem.*, 202 (1991) 1115–1119.
[10] L. Andersson, E. Sulkowski and J. Porath, *Bioseparation*, 2 (1991) 15–22.
[11] G.E. Wuenschell, E. Naranjo and F.H. Arnold, *Bioprocess Eng.*, 5 (1990) 199–202.
[12] S.D. Plankett and F.H. Arnold, *Biotechnol. Tech.*, 4 (1990) 45–48.
[13] S.S. Suh and F.H. Arnold, *Biotechnol. Bioeng.*, 35 (1990) 682–690.
[14] G. Birkenmeier, M.A. Vijayalakshmi, T. Stigbrand and G. Kopperschläger, *J. Chromatogr.*, 539 (1991) 267–277.
[15] B.H. Chung and F.H. Arnold, *Biotechnol. Lett.*, 13 (1991) 615–620.
[16] F.H. Arnold, *Bio/Technology*, 9 (1991) 151–156.
[17] S.S. Suh, B.L. Haymore and F.H. Arnold, *Protein Eng.*, 4 (1991) 301–305.
[18] R.J. Todd, M.E. Van Dam, D. Casimiro, B.L. Haymore and F.H. Arnold, *Proteins Struct. Function Genet.*, 10 (1991) 156–161.
[19] F.H. Arnold and B.L. Haymore, *Science*, 252 (1991) 1796–1797.
[20] H.G. Botros, G. Birkenmeier, A. Otto, G. Kopperschläger and M.A. Vijayalakshmi, *Biochim. Biophys. Acta*, 1074 (1991) 69–73.
[21] A. Otto and G. Birkenmeier, *J. Chromatogr.*, 644 (1993) 25–33.
[22] J.-H.J. Pesliakas, V.D. Žutautas and A.A. Glemža, *Chromatographia*, 26 (1988) 85–90.
[23] V. Žutautas, B. Baškevičiūtė and H. Pesliakas, *J. Chromatogr.*, 606 (1992) 55–64.
[24] V. Ulbrich, J. Makeš and M. Mareček, *Collect. Czech. Chem. Commun.*, 29 (1964) 1466–1475.
[25] L.A. Decker (Editor), *Worthington Enzyme Manual*, Worthington Biochemical, Freehold, NJ, 1977, pp. 23–24.
[26] E. Hochuli, H. Döbeli and A. Schacher, *J. Chromatogr.*, 411 (1987) 177–184.
[27] S.S. Flaksaitė, O.F. Sūdžiuvienė, J.-H.J. Pesliakas and A.A. Glemža, *Biokhimiya*, 52 (1987) 73–81.
[28] H. Jörnvall, *Eur. J. Biochem.*, 72 (1977) 425–442.
[29] B.H. Anderton, *Eur. J. Biochem.*, 15 (1970) 562–567.
[30] C. Huc, A. Olomucki, Lê-Thi-Lan, D.B. Pho and N. van Thoai, *Eur. J. Biochem.*, 21 (1971) 161–169.
[31] M. Hennecke and B.V. Plapp, *Biochemistry*, 22 (1983) 3721–3728.
[32] J.J. Holbrook and V.A. Ingram, *Biochem. J.*, 131 (1973) 729–738.