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Interaction of bovine skeletal muscle lactate dehydrogenase with liposomes. Comparison with the data for the heart enzyme

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The effects of pH, salt concentration and the presence of oxidized and reduced forms of coenzyme on the interaction of skeletal muscle lactate dehydrogenase with the liposomes derived from the total fraction of bovine erythrocyte lipids were investigated by ultracentrifugation and were compared with those results obtained using the heart-rate isoenzyme which we have previously studied. Liposomes are good adsorptive systems for both types of isoenzyme. In the presence of erythrocyte lipid liposomes, bovine muscle and heart lactate dehydrogenases form two kinds of complex: lactate dehydrogenase adsorbed to liposomes and soluble lactate dehydrogenase-phospholipid complexes. Soluble protein-phospholipid complexes reveal different dependences of their stabilities on pH values and it seems that the nature of the binding site in either isozyme is different. In addition, absorption of the isoenzymes on the liposomes also reveals in difference in the effects of NAD and NADH. While the presence of NAD dissociates LDH-H_4 from the liposomes and NADH does not influence its adsorption, NAD promotes the binding of LDH-M_4, and NADH favors the dissociation.

Introduction

It is now well-documented that at least some glycolytic enzymes can associate with membranes in cells [1–5]. Some role of the association in regulatory processes of glycolysis is suggested by the finding that the association is affected by some metabolites such as substrates, products, coenzymes and the like [5–8]. Since the association with the membranes is also affected by such factors as pH, ionic strength and ionic metabolites, it is generally believed that the binding is controlled by electrostatic interactions. The biological implications of the association raise questions such as: what is the influence of the association on the catalytic properties of the enzyme and, on the other hand, whether or not the properties of membranes are affected by this association. Some recent studies have strongly suggested that LDH isozymes may be included in the group of membrane-associated enzymes and that the binding to membranes occurs through the proteins [9,10].

Using the method of ultracentrifugation of lipid suspensions in the presence of bovine heart LDH, it has previously been found that the surface of phospholipid liposomes can reversibly adsorb the enzyme [11].

In the present work we have investigated the association of bovine muscle LDH to liposomes from the total fraction of bovine erythrocyte lipids in order to compare it with those data for the heart-type isozyme which were described elsewhere [11].

Materials and Methods

LDH

Bovine muscle LDH was prepared from bovine skeletal muscle (M gluteus maximus) according to Pesce et al. [12]. It was purified by CM-Sephadex column chromatography and by subsequent repeated recrystallization from ammonium sulfate. Its specific activity was 300 U/mg and the preparation gave one band during polyacrylamide gel electrophoresis (LDH-5).

Before the experiments the LDH suspension in ammonium sulfate solution was centrifuged down and the pellet was dissolved in 10 mM Tris-HCl/1 mM EDTA (pH 7.5) and subsequently dialyzed for 48 h against this buffer at a temperature of 5°C. The final protein concentration was adjusted to 1 mg/ml. This procedure produced the apo-form of the enzyme, since the A_{280}/A_{260} value was about 1.8.

Enzyme assay

The amount of LDH in supernatant was determined by the enzyme-activity assay according to the method of

Abbreviations: LDH, lactate dehydrogenase (EC 1.1.1.27).

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Bergmeyer et al. [13]. The assay sample (3 ml) contained 0.2 mM NADH and 10 mM sodium pyruvate in 100 mM phosphate buffer (pH 7.5). One unit of the enzyme activity (U) is defined as the amount of the enzyme which converts 1 μmol of the coenzyme per 1 min at room temperature. For the calculation of concentration a value of 6200 $M^{-1} \cdot cm^{-1}$ was used as a molar absorption coefficient.

Determination of the protein concentration

In centrifugation studies the concentration of the protein was determined by theeh biuret method [14] in the supernatant. LDH of known concentration (determined by measurement of absorbance at 280 nm) was used as a standard.

Preparation of erythrocyte lipids and liposomes

Lipids were extracted from bovine erythrocyte with *n*-butanol according to the method described by Zahler et al. [15]. The butanol of the lipids was evaporated to dryness under a nitrogen stream. To the thin lipid film obtained the appropriate amount of the buffer solution was added and the liposome suspension was produced by mechanical shaking with glass beads for 30 min at room temperature. The concentration of lipids was calculated indirectly by phosphorus determination according to Bartlett [16]. Preparations of liposomes were not contaminated by the protein which was determined by the biuret method.

Adsorption of the enzyme of liposomes

The same experimental procedure as previously in the case of the heart isoenzyme was applied [11] for determination of the adsorption of the enzyme to liposomes.

The enzyme and liposome mixtures were incubated at room temperature for 60 min and then centrifuged for 60 min at 100000 × *g* using the MSE-50 ultracentrifuge. After centrifugation, the lipid protein concentrations were determined in the supernatant. The lipid and protein content of the pellets were calculated from the difference between the initial concentration and that in the supernatant. Control centrifugation of the enzyme alone did not give any pellet.

Results

Association of the skeletal muscle LDH with liposomes is pH-dependent. The curve shown in Fig. 1 reveals that the optimum of binding occurred at pH 6.8. At that pH, and at saturating lipid concentration, liposomes were adsorbing 40% of the total amount of the enzyme. As was previously shown for the heart LDH isoenzyme, LDH-M_4–liposome binding was sensitive to ionic strength, decreasing rapidly with the increase of salt concentration. No adsorption was observed at KCl

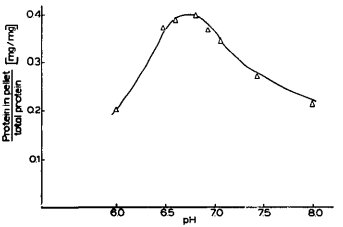

Fig. 1. Adsorption of bovine muscle LDH to liposomes. Protein and lipid concentrations were 0.4 and 1.8 mg/ml, respectively; sample volume: 3.5 ml.

and Na_3PO_4 concentrations above 0.1 M (data not shown).

As can be seen in Figs. 2 and 3, the enzyme-liposome binding was influenced by the presence of coenzyme. Its oxidized and reduced forms revealed opposite effects: while the presence of NAD^+ promoted LDH-M_4 adsorption to liposomes increasing the bound amount to 60%, the reduced form (NADH) favored the dissociation of the complex.

A solubilizing effect of the muscle LDH on liposomes was observed. While a suspension of lipid alone pelleted completely during centrifugation at the pH range from 6.0 to 8.0, in the presence of LDH-M_4 some amount of phospholipids remained in solution forming soluble lipid-LDH complexes (see Fig. 4). This phenomenon was pH-dependent and alkalization of the medium promoted formation of soluble complexes. These were

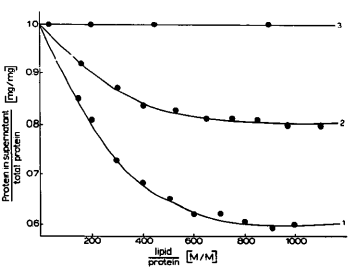

Fig. 2. Effect of NADH on the adsorption of the bovine muscle LDH to liposomes as a function of lipid concentration. Protein concentration in all samples was 0.4 mg/ml, sample volume: 3.5 ml, pH = 6.8, NADH concentrations: (1) 0, (2) $3.5 \cdot 10^{-7}$ and (3) $1.8 \cdot 10^{-5}$ mol/l.

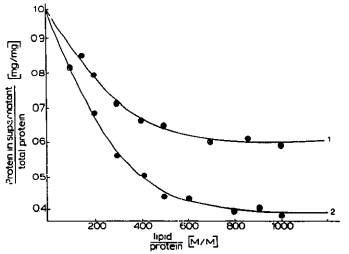

Fig. 3. Effect of NAD on the adsorption of the bovine muscle LDH to liposome as a function of lipid concentration. NAD concentrations: (1) 0 and (2) $1.8 \cdot 10^{-4}$ mol/l. Other conditions as in Fig. 2.

extracted from supernatant with chloroform and lipids were separated by thin-layer chromatography. Phosphatidylethanolamine was found as a main constituent of soluble complexes, while acid phospholipids (phosphatidylserine and phosphatidylinositol) were present in small amounts. Formation of soluble complexes with phospholipid inactivated the enzyme, as shown in Fig. 5. The decrease of activity correlated with the amount of lipid in supernatant.

Discussion

Results presented in this paper show similarities and differences in the interaction of skeletal muscle LDH

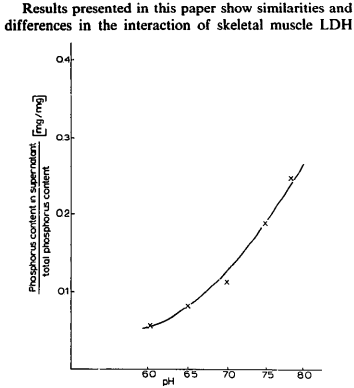

Fig. 4. Dependence of the amount of phospholipid retained by the enzyme in the supernatant on pH. Experimental conditions: see Fig. 1.

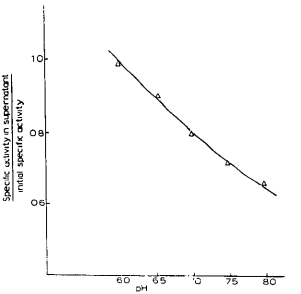

Fig. 5. Effect of the phospholipid on the specific activity of the enzyme as a function of pH. Experimental conditions as in Fig. 1.

isoenzyme with liposomes, as compared with the interaction of the heart isoenzyme. In both cases, two kinds of enzyme-lipid complex are formed: insoluble, precipitating in ultracentrifugation, where the enzyme is adsorbed to liposomes, and soluble LDH-phospholipid complexes, present in supernatant.

As for the precipitating complexes, it was shown that the pH-dependence of their formation with LDH-M_4 was similar to that of LDH-H_4, revealing an optimum at pH 6.8. In the case of both isoenzymes, association with liposomes diminished with increasing salt concentration. This dependence seems to strongly suggest the electrostatic nature of binding forces. However, a simple explanation of the adsorption in terms of multielectrostatic interactions between the net charge of the protein molecule and the liposome surface seem to be insufficient. Muscle isoenzyme of LDH shows a positive net electric charge at pH 6.5 [17], and the surface of liposomes, derived from total lipid fraction of erythrocytes, bears a negative charge over a wide pH range. Simple overall electrostatic attraction between the two systems should cause a monotonic increase of the isoenzyme absorption with decreasing pH. Occurrence of the optimum in the pH-dependence curve, and the differentiated effects of the oxidized and reduced forms of the coenzyme on the lipid-LDH adsorption indicate rather complex mechanisms of binding. Our observation that the presence of NAD^+ promoted binding, while its reduced form favored dissociation, seems to support the supposition that some factors other than electrostatic attraction contribute in lipid binding. In our opinion, the effect of coenzymes may be to identify the local conformation near the binding site(s). Obviously, this hypothesis needs further support by means which would enable-monitoring the conformational changes.

Comparison of the effects of coenzymes on liposome–LDH-M_4 binding with those for the heart isoenzyme revealed some interesting differences. While the presence of NAD^+ dissociates LDH-H_4 from liposomes, and NADH does not influence the adsorption, in the case of LDH-M_4, NAD^+ promoted the binding, and NADH, the dissociation, of the enzyme. The data obtained in ultracentrifugation experiments are not sufficient to give an exact interpretation of these differences, but they evidently indicate that LDH isoenzymes show different adsorptive properties.

It was shown that, in the presence of LDH-M_4, some amount of phospholipids, mainly phosphatidylethanolamine, was present in the supernatant, forming soluble complexes with the enzyme. Complexed LDH-M_4 showed lower activity than the uncomplexed isoenzyme. Soluble complex formation and enzyme inactivation was observed also with the heart LDH isoenzyme [11]. However, the pH-dependence of these phenomena differed in the two isoenzymes. With LDH-M_4, alkalization, and with LDH-H_4, the acidification of the environment, favored soluble complex formation and enzyme inactivation.

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