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Interaction of bovine heart lactate dehydrogenase with erythrocyte lipids

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The interaction between bovine heart lactate dehydrogenase and erythrocyte lipid suspension as a function of pH, NAD, NADH, lipid and salt concentration was studied by ultracentrifugation. In the presence of erythrocyte lipid liposomes the enzyme forms two kinds of complex: lactate dehydrogenase adsorbed to liposomes and soluble lactate dehydrogenase-phospholipid complexes. The two complexes reveal different dependence of their stability on pH values. Lactate dehydrogenase decreases its specific activity when it binds to the phospholipid molecules. Efficient adsorption of lactate dehydrogenase to liposomes occurs in their pH range 6.0–8.0 and at low ionic strength. The adsorption is diminished in the presence of NAD^+ but it is not influenced by NADH. Possible mechanisms of the interaction and implications for the function in vivo are discussed.

Introduction

It is now well known that glycolytic enzymes can reversibly associate with intracellular membrane structures. The association is usually modulated by pH value, ionic strength and/or the concentration of specific metabolites. In this way the metabolic state of the cell determines the subcellular localization of the enzymes. The phenomenon is probably very important for the regulation of glycolysis, as the adsorbed enzymes mostly have kinetic properties different from those of the unadsorbed ones (for reviews see Refs. 1–3).

Lactate dehydrogenase EC 1.1.1.27) belongs to this class of enzyme. Its capacity for adsorption on erythrocyte and muscle cell subfraction membranes has been postulated several times [4–7]. Tetrameric isoenzymes of lactate dehydrogenase are composed of two types of subunits produced by two different genes: muscle type (M) and heart type (H). The two types of subunits may form five lactate dehydrogenase isoenzymes [8]. The question arising from this is whether the different isoenzymes have different capacities for binding to membranes and what is the distribution of lactate dehydrogenase localization in cell. Hultin et al. [7] showed that in chicken breast muscle cells only the M subunits bind to membranes. They also demonstrated the differentiation of the affinities to membranes between various lactate dehydrogenase isoenzymes; the lactate dehydrogenase 5 had the most affinity and lactate dehydrogenase 1 did not associate at all.

Baba and Sharma [9] and Skilleter had Kun [10] found that a small fraction of heart lactate dehydrogenase (predominantly isoenzyme 1) was associated with the inner membrane of heart and pectoralis muscle mitochondria. A similar result was obtained by Sjödin [11] in studies of the association of heart lactate dehydrogenase with human muscle mitochondria. It is of interest to investigate the association of the enzyme with artificial well-defined adsorptive systems. Such investigations can provide results from which it may be easier to draw conclusions about type of the interaction and its molecular mechanism.

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In this work we have investigated the association of bovine heart lactate dehydrogenase to vesicles made of total erythrocyte lipids in order to determine whether the lipid fraction of the membrane may be responsible for the association and what the conditions of the association are. We used the method of ultracentrifugation of the lipid suspensions in the presence of the enzyme.

Materials and Methods

Bovine heart lactate dehydrogenase (EC 1.1.1.27)

The enzyme in ammonium sulfate solution was obtained from POCH (Gliwice, Poland). The producer assesses the purity according to Boehringer's criteria. The preparation had a specific activity of 300 U/mg and gave one band during polyacrylamide gel electrophoresis. To prepare the enzyme for experiments the lactate dehydrogenase suspension was centrifuged down and the pellet was dissolved in 10 mM Tris-HCl/1 mM EDTA buffer (pH 7.5) and dialysed for 48 h against the buffer at a temperature of 5°C. The final protein concentration was adjusted to 3 mg/ml. This procedure produced the apo-form of the enzyme, since the A_{280}/A_{260} ratio value was about 1.8.

Preparation of erythrocyte lipids and liposomes

Lipids were extracted from bovine erythrocytes with *n*-butanol according to the method described by Zahler et al. [12]. The butanol solution of the lipids was evaporated to dryness under a nitrogen stream. To the resulting thin lipid film the appropriate amount of the buffer solution was added and the liposome suspension was obtained by mechanically shaking with glass beads for 30 min at room temperature. The concentration of lipids was calculated from the weight of dried matter or indirectly by phosphorus determination according to Bartlett [13]. Thin-layer chromatography was performed on silica gel-coated aluminium plates produced by Merck. Chromatograms were evaluated with a chloroform/methanol/water mixture.

Determination of the protein concentration

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

Enzyme assay

Lactate dehydrogenase activity was assayed by the method of Bergmeyer et al. [15] using sodium pyruvate as substrate. The assay sample (3 ml) contained 0.2 mM NADH and 10 mM sodium pyruvate in 100 mM phosphate buffer of pH 7.5. One unit of lactate dehydrogenase activity (U) represents the amount of enzyme which converts 1 μmol of the coenzyme per min under the test conditions. For the calculation of concentration a value of 6200 $M^{-1} \cdot cm^{-1}$ was used as a molar extinction coefficient.

Adsorption of the enzyme to liposomes

The enzyme and liposome mixtures were incubated at room temperature for 60 min, then centrifuged for 60 min at 100 000 $\times$ *g* in an MSE-50 ultracentrifuge. After centrifugation the lipid and protein concentrations were determined in the supernatant. Lipid and protein contents 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. The dependence of the adsorption on lipid concentration, pH, NAD and NADH concentration was studied. To obtain proper pH values 10 mM Tris-HCl/1 mM EDTA buffer adjusted with 1 M HCl was used in all experiments. Each point in the presented plots represents the mean value of the determinations of three independent samples.

Results

In this study we have used liposomes made of total lipid fraction from bovine erythrocytes. Since there is a small amount of lipids in the erythrocyte plasma [16], it is assumed that the composition of the fraction is close to that of the lipid fraction of the plasma membranes. Ultracentrifugation of the liposome suspension in the presence of bovine heart lactate dehydrogenase showed that the enzyme pelleted together with liposomes at pH 7.0 (Fig. 1, curve 1). The abscissa in Fig. 1 represents the lipid concentration in the suspension before the ultracentrifugation. In saturating concentra-

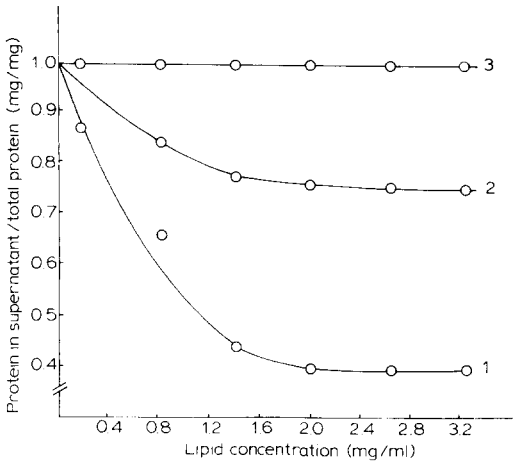

Fig. 1. Effect of NAD^+ concentration on the adsorption of lactate dehydrogenase to liposomes as a function of lipid concentration. Protein concentration in all samples, 0.35 mg/ml ($2.5 \cdot 10^{-6}$ M); volume, 3.2 ml; pH 7.0; NAD^+ concentrations: 1, zero; 2, $5 \cdot 10^{-6}$ M; 3, $5 \cdot 10^{-5}$ M.

tions the liposomes adsorb 60% of the total amount of the enzyme. As can be also seen in Fig. 1 (curves 2 and 3), the adsorption of the enzyme onto liposomes is very sensitive to the presence of NAD^+. Addition of the oxidized form of the coenzyme decreased the binding and at a concentration of $5 \cdot 10^{-5}$ M we observed no adsorption. In contrast with NAD^+, NADH does not influence the adsorption: concentrations up to 1.5

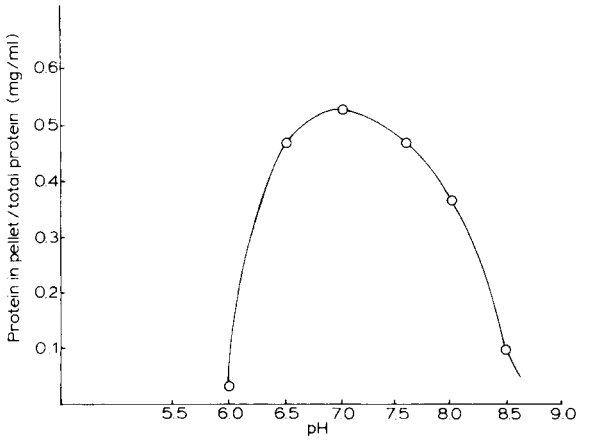

Fig. 2. Dependence of the lactate dehydrogenase adsorption to liposomes on pH values. Protein and lipid concentrations were 0.35 mg/ml and 1.6 mg/ml, respectively; sample volume, 3.2 ml.

$\cdot 10^{-3}$ M had a small effect, less than 10% (not shown). To test the specificity of the effect, the adsorption of the enzyme onto the liposomes in the presence of several NAD^+ analogues, nicotinamide-adenine dinucleotide phosphate (NADPH), adenosine phosphates (ATP, ADP, AMP) and cytidine triphosphate (CTP), was investigated. At pH 7.5 and at a concentration of $5 \cdot 10^{-5}$ M only $NADP^+$ caused a marked decrease in the adsorption (40%) but the effect of the other analogues was less than 5%. Thus, the adsorption is specifically sensitive to the interaction of the oxidized form of the coenzyme and its phosphate analogue.

It is difficult to find here any correlation between the chemical structure of the analogues (the presence of different chemical groups) and the difference in effects on the adsorption they reveal. Hence we think that the adsorption depends on the conformational state of the enzyme. It is likely that specific binding of the coenzyme or its phosphate analogue results in a conformational change in the binding site(s) region.

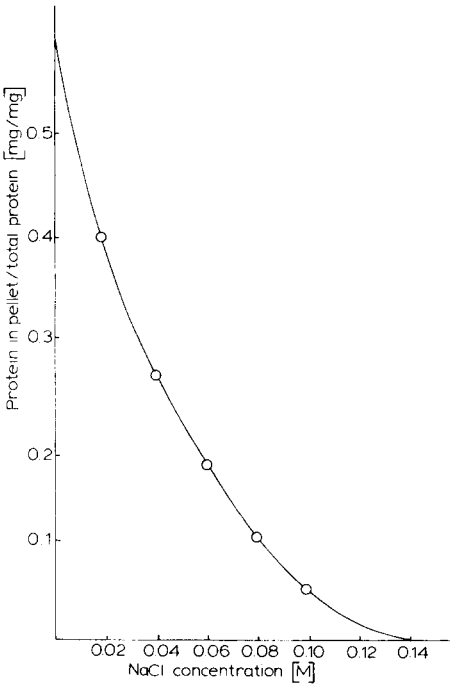

Fig. 3. Dependence of the lactate dehydrogenase adsorption to liposomes on NaCl concentration (pH 7.0). Other experimental conditions as in Fig. 2.

Next, a strong dependence of the binding on the pH value was observed (Fig. 2). Efficient adsorption occurs in a very narrow range of pH values: 6.5–8.0, with the optimum at pH 7.0. Practically no amount of the enzyme was found in the pellet at pH 6.0 and above pH 8.5.

Binding of the enzyme to liposomes decreased rapidly with increasing ionic strength (Fig. 3). An NaCl concentration of 0.14 M completely abolished the adsorption. A similar and even more pronounced decrease in the adsorption was induced by increasing concentrations of NaH_2PO_4. There was no binding of the enzyme by liposomes above a salt concentration of 0.1 M (not shown).

On the other hand, we have observed some solubilizing effect of lactate dehydrogenase on liposomes. A suspension of lipid pelletted completely during ultracentrifugation in the whole pH range studied (from 6.0 to 8.5), but after ultracentrifugation in the presence of lactate dehydrogenase some phospholipid (dependent on pH) was recovered in the supernatant (Fig. 4). Up to about 20% of total phospholipid at pH 6.0 could not be precipitated by ultracentrifugation in these conditions. The pH dependence of the solubilization phenomenon does not correlate with that for the enzyme coprecipitation with liposomes shown in Fig. 2. Solubilized fractions of lipids were extracted with chloroform from the supernatant and

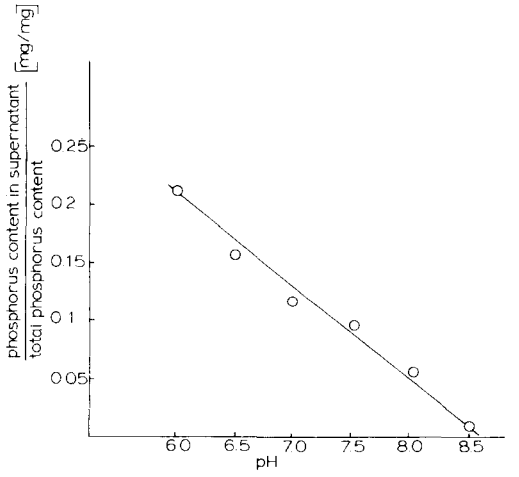

Fig. 4. Dependence on pH of the amount of phospholipid retained by the enzyme in the supernatant. Experimental conditions as in Fig. 2.

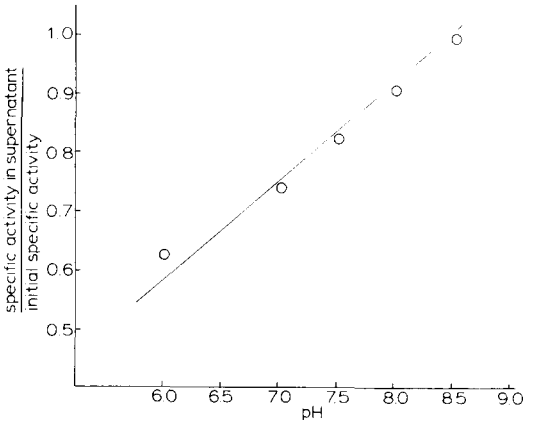

Fig. 5. Effect of the phospholipids on the specific activity of the non-adsorbed enzyme as a function of pH. Experimental conditions as in Fig. 2.

separated by thin-layer chromatography. In the chromatograms we found acidic phospholipids; mainly phosphatidylethanolamines, with fewer phosphatidylserines and phosphatidylinositols.

We think that there are specific binding sites for some kinds of phospholipid on the enzyme molecules, and the formation of a soluble lipid-lactate dehydrogenase complex, besides the lactate dehydrogenase-liposomes complex, may be responsible for the phenomenon. The lack of correlation between the patterns of pH dependence of the coprecipitation with liposomes and the solubilizing effect indicates that the mechanisms responsible for the two phenomena are different.

The specific activity of the enzyme remaining in the supernatant was modified and the modification correlated with the phospholipid content of the supernatant (Fig. 5). The phospholipid lactate dehydrogenase complex becomes unstable when the pH increases, but it is less sensitive to salt concentration in comparison with the lactate dehydrogenase-liposome complex, because 0.1 M NaH_2PO_4 present in the activity-test solution did not dissociate the complex.

Discussion

The results which are presented above prove the existence of two types of reversible interaction between heart lactate dehydrogenase and erythrocyte lipid suspension. One type of interaction leads to adsorption of the enzyme to liposomes and the

second to formation of a soluble phospholipid-enzyme complex.

Usually, the adsorption of peripheral proteins to lipid bilayer or membranes is explained by multielectrostatic attraction. Also in this case, the diminishing effects of salt concentration and pH values above and below optimum on the adsorption to liposomes strongly suggest that interactions of ionic type are responsible for the adsorption. This interpretation is supported by the fact that a salt concentration completely dissociating the enzyme-liposomes complex can be achieved. The decrease in electric potential of the liposome surface brought about by increasing salt concentration results in a decrease in the coulombic attraction between the liposome surface and ionic groups of the protein. The heart lactate dehydrogenase has a negative net electric charge at pH 7.0 [17]. The same charge sign should be expected for the surface of the liposomes.

The main components of the erythrocyte lipid fraction are zwitterionic or neutral phosphatidylcholines, sphingomyelins and cholesterol [16]. A smaller amount of acidic phospholipids form the negative surface charge of liposomes which is stable over a wide pH range [16]. It is clear, therefore, that there must be specific domain(s) on the protein molecule acting as an adsorptive site. The adsorptive properties of the site are rather complex. It is clear that the decrease in positive charge with increasing pH may diminish the adsorption. We have previously observed [18] such a monotonic decrease in glyceraldehyde-3-phosphate dehydrogenase adsorption to phospholipid liposomes with increasing pH from 6.0 to 9.0. Here, unexpectedly, the adsorption is abolished at pH values below 6.5. It seems that, besides charge, other factors such as conformational changes can modify the adsorption properties. Changes in the protein net charge can induce, at least, local conformational rearrangement modifying the adsorptive sites. Such conformational changes have been postulated in the case of fructose-diphosphate aldolase adsorbed on phospholipid liposomes [19].

The adsorptive sites in proteins can be blocked by competitive binding of other substances or by their indirect modification mediated by conformational changes of the protein molecule [20]. Since a relatively low concentration of NAD^+ causes desorption of the enzyme from liposomes it is the latter mechanism which is believed to be responsible for the effect. Binding of NAD^+ to its specific binding sites probably modifies allosterically the adsorptive site, but it is not clear why the reduced form of the coenzyme does not influence the adsorption. It has been shown several times [6,7,21] that the adsorption of the muscle type of lactate dehydrogenase to various adsorptive systems such as homogenized tissues, subcellar structures or dextran sulfate is very sensitive to the presence of NADH. The differences provide evidence of the complex nature of the adsorptive sites in the heart-type enzyme. For an exact interpretation of the results further comparable investigations are necessary.

In many cases structural proteins have been found to be specific binding sites for enzymes. However, taking into account the domain-like nature of the membrane surface and the alteration of local concentrations of cofactors, we think that certain specifically arranged lipid domains can exist as the binding sites for the enzyme on membranes in vivo. The interactions with randomly distributed lipid in the bilayer surface are non specific but in the case of lipid domains specifity can be induced by its composition, local concentration of charge etc. Since the kinetics of most enzymes are altered on binding to membranes it is generally accepted that reversible adsorption is one of the important factors of metabolic regulation. As seen from experiments shown in Figs. 4 and 5, in the case of the studied enzyme another possibility for the change in specific activity appears. That is by formation of a soluble complex with single phospholipid molecules.

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