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THE ROLE OF LIPID PHASE STRUCTURE IN THE INTERACTION OF LACTATE DEHYDROGENASE WITH PHOSPHATIDYLSERINE. ACTIVITY STUDIES

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Abstract: Lactate dehydrogenase is one of the enzymes of the glycolytic path. It has been shown to be able to bind *in vitro* to cellular membranes. The presence of anionic phospholipids induces changes in the catalytic properties of the enzyme similar to those found when the enzyme is bound to natural membranes. In this study, a nonionic detergent (Tween 20), at concentrations not affecting the catalytic activity of LDH, was used to study the role of the lipid supra-molecular structure in the interaction between pig skeletal muscle lactate dehydrogenase and phosphatidylserine. Tween 20 changes the equilibrium of concentrations between the lipid supra-molecular forms. The detergent at the used concentration values did not alter the activity of the enzyme when it was used on its own, but did diminish the level of inhibition induced by the studied phospholipid. The obtained results showed that the interaction is reversible and that the bilayer structure of the lipid is essential for the inhibition.

Key Words: Lactate Dehydrogenase, Phosphatidylserine, Enzyme-Lipid Interaction, Liposomes, Tween 20.

INTRODUCTION

Glycolytic enzymes are soluble cytoplasmic proteins. However, some of them have been shown to be able to reversibly bind *in vitro* to intracellular membranes and to some components of the cytoskeleton. This binding is suggested to be controlled by electrostatic interactions, since it is very sensitive to pH and ionic strength changes [1-7]. However, the occurrence of other much weaker

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interactions (e.g. hydrogen bonds) cannot be excluded. If this binding occurred *in vivo* it could be a very important post-translational regulatory factor responsible for the regulation of the kinetic properties of the enzymes. Lactate dehydrogenases from various sources are adequate representatives for this group of enzymes [5, 8-9]. Lactate dehydrogenase (LDH) isoforms are the enzymes of the glycolytic pathway that catalyze the following reaction:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{NAD}^+$$

Our previous studies on this enzyme as well as on some other glycolytic enzymes showed that the presence of anionic phospholipids has effects on it similar to those seen in the presence of membranous structures [10-13]. Since phospholipids in water can exist in various supra-molecular forms, one of the questions arising from such studies is whether or not the supra-molecular structure of lipids is crucial for this interaction and binding. In aqueous environments, phospholipids at concentrations higher than CMC form at least three different structures existing in equilibrium: single molecules, micelles and vesicles (liposomes) [14, 15, 16]. Theoretically, the observed effects may result from the interaction of the enzyme with one particular form of those mentioned above, or from a combination of interactions with two or more forms. In our research, we attempted to discover which mechanism is responsible for the observed inhibition of the enzyme in the presence of phosphatidylserine, used as a representative anionic phospholipid: adsorption of the enzyme onto the lipid vesicle surface, or the formation of specific molecular enzyme-phospholipid complex(es). A nonionic detergent (Tween 20) was used in the experiments. The presence of such detergents changes the equilibrium of concentrations between the lipid supra-molecular forms [17, 18]. Since anionic phospholipids, e.g. phosphatidylserine (PS), phosphatidylinositol (PI), and cardiolipin (CL), have a much stronger influence on the activity of the muscle form (M_4) than the heart form (H_4) of the enzyme, therefore the muscle form was chosen for the experiments (LDH-M_4 from pig skeletal muscle).
The aim of the study was to attempt to determine whether a lipid bilayer is necessary for functional interactions between the enzyme and phospholipids.

MATERIALS AND METHODS

Chemicals

The substrates of LDH: pyruvate, the NADH and the Tween 20 used were purchased from Roche Molecular Bochemicals (Germany). All the other chemicals used were from Sigma-Aldrich and were of analytical grade.
A lactate dehydrogenase muscle isoenzyme (LDH-M_4) preparation from pig muscle was obtained commercially from Roche Molecular Biochemicals as a suspension in a 3.2 M ammonium sulphate solution. To prepare the enzyme for the experiments, the suspension was centrifuged down, and the pellet was dissolved in 100 mM Tris/HCl buffer (pH 7.5) and dialysed exhaustively against

the buffer at a temperature of 5ºC. This procedure produced the apo-form of the enzyme; the A_{280}/A_{260} ratio value was about 1.8. Only those preparations of the dialyzed enzyme with a specific activity not lower than 450 U/mg protein under standard conditions (pH=7.5) were used. One unit of LDH activity (U) represents the amount of the enzyme which converts 1 μmole of coenzyme per minute under test conditions.

Protein determination and enzyme assay

Protein concentrations were determined using the method of Bradford [19] using a Bio-Rad Protein Assay kit, Bio-Rad (Germany). Lactate dehydrogenase activity was assayed via the modified method of Bergmeyer *et al.* [20], using sodium pyruvate as a substrate and spectrophotometrically monitoring the decrease of absorbance at 340nm (changes in NADH concentration). The assay sample contained 0.2 mM NADH and 3 mM sodium pyruvate in a 100 mM MES/NaOH or 100 mM TRIS/HCl buffer of appriopriate pH. The reaction was started by the addition of a mixture of both substrates (NADH and pyruvate) to the sample after 5 minutes preincubation at 25ºC. All the assays were also performed at 25ºC. In all the assay samples, the concentration of the enzyme was 1.5 nM. All the reagents for the assay were purchased from Roche Molecular Biochemicals (Germany).

Each point in the presented plots represents the average of the results from assays of three independent samples.

Phospholipids and liposomes

All the phospholipids used were purchased from Avanti Polar Lipids, Inc. USA. Lipid suspensions were prepared by mechanically shaking a weighted amount of the lyophilized lipid with an appriopriate buffer at room temperature. The mixture was subsequently treated with ultrasound waves using a probe sonifier until the mixture became transparent. The concentration of the phopsholipid was calculated from the amount of dried lipid and the known volume of the buffer.

RESULTS AND DISCUSSION

It was previously found in our laboratory that the addition of a suspension of anionic phospholipids, such as phosphatidylserines, phosphatidylinositols or cardiolipin, to a solution of mammalian muscle LDH results in a decrease in enzyme activity. No effect was observed for phosphatidylcholine, which forms neutral lipid zwitterions [11, 12, 21]. For this study, we chose a preparation of natural phosphatidylserine (PS), a typical representative of the acidic phospholipids of membranes. Bearing three ionizable groups, PS molecules are negatively charged for pH values > 3.5 in an aqueous environment [22]. The effect of the phospholipid on the enzyme activity appeared to be strongly dependent on pH in the studied range of values – pH 5-8 (Fig. 1).

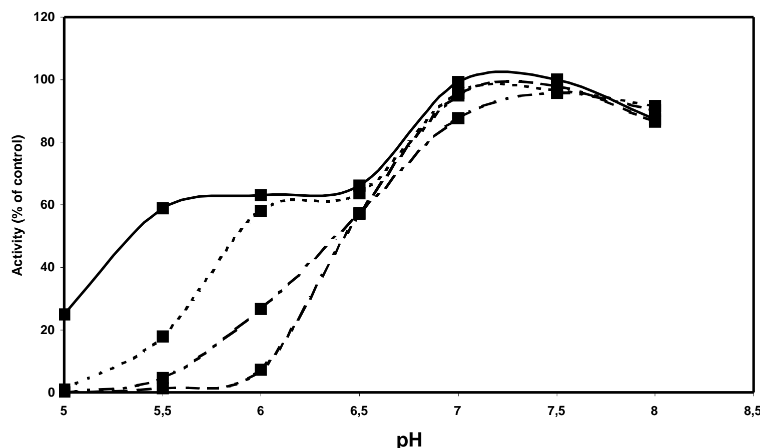

Fig. 1. Dependence of the inhibition of LDH activity by phosphatidylserine on pH. —— no PS, ······ 0.5 μM, –·–·– 5.0 μM, – – – 50 μM PS. A MES/NaOH buffer was used for pH 5-6.5 and Tris/HCl buffer for pH 7-8. The control is the activity of the sample without PS at pH 7.5.

Inhibition of the enzyme's activity in the presence of phosphatidylserines is diminished with rising pH. Above pH 6.5, the effect of the phospholipid on LDH activity is practically negligible. This result provides further evidence that the inhibition is controlled by ionic interactions. Moreover, it indicates that the sensitivity of the charge on the enzyme molecules to environment conditions is crucial for the changes in interaction. When pH is increased within this range, the negative charge of the phospholipid increases, while the protein passes through its isoelectric point. Increasing the phospholipid concentration results in a decrease in the enzyme specific activity, reaching full inhibition for values of the molar lipid/protein ratio around 1000 for bovine phosphatidylserine at the most effective pH value of 5.5 (Fig. 2 unbroken line). The observed relatively high saturating lipid/protein ratio value suggested that the interaction was an enzyme molecule-liposome type. The value of the fully inhibiting ratio depends the method of preparation of liposomes and does not reflect any specific lipid-protein stoichiometry. This conclusion was supported by the results of the experiment with a synthetic dipalmitoyl-PS preparation (Fig. 2, broken line). The curves are similar, differing mainly in their saturation value. Phospholipids in water form vesicles with bilayer walls, therefore, some molecules are not accessible for interaction. The suspensionsof liposomes of various lipids differ in terms of their parameters even when the same procedure is used. In our opinion, the difference in the curves is not due to the difference in fluidity between the lipid structures. Natural PS and synthetic DPPS respectively reveal gel/liquid crystal phase transition at 28ºC and above 50ºC [23]. Both phospholipid bilayers

are below, although not to an equal extent, their gel/liquid crystal phase transition (gel phase) under the studied conditions.

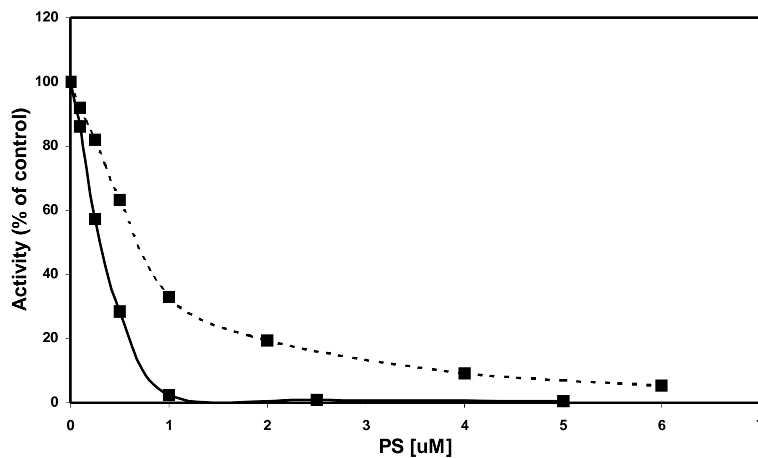

Fig. 2. The dependence of LDH activity on PS concentration. —— bovine brain phosphatidylserine (PS), – – – synthetic dipalmitoylphosphatidylserine (DPPS). Buffer: MES/NaOH, pH 5.5.

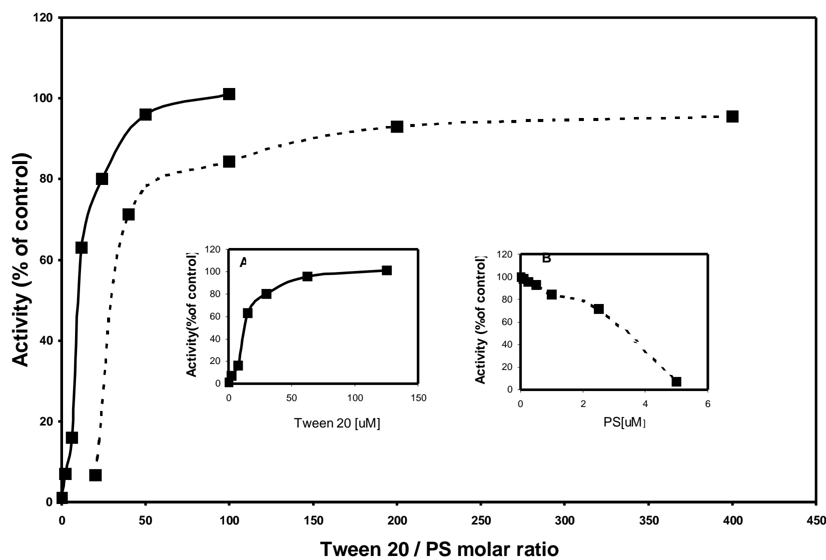

Fig. 3. Dependence of lactate dehydrogenase activity on the Tween 20/lipid molar ratio. —— data from A, – – – data from B. Buffer: MES/NaOH, pH 5.5. A - constant concentration of PS = 1.25 μM, B - constant concentration of Tween 20 = 100 μM.

The addition of Tween 20, which is a nonionic detergent, results in a gradual diminishing of the lipid inhibition, dependently on the detergent/lipid ratio (Fig. 3). The curves in the figure represent the two ways of changing the ratio: 1) by adding an increasing concentration of Tween 20 keeping the lipid concentration constant; and 2) by changing the lipid concentration at a constant concentration of the detergent (Fig. 3A and 3B).
The two experiments were performed to verify whether the sequence of addition is important for the formation of binding between the three studied components. The Tween/phospholipid molar ratio values at which the complete recovery of inhibition is reached are somewhat different for both series of measurements. The concentration of Tween 20 needed for complete disruption of the vesicles probably depends on the lack or the presence of interaction with the protein and on absolute values of the concentration of the lipid and the detergent.
In the control experiments, we checked that the activity of LDH in the presence of Tween 20 is not altered at detergent concentration much higher than those used in the studies (Fig. 4).

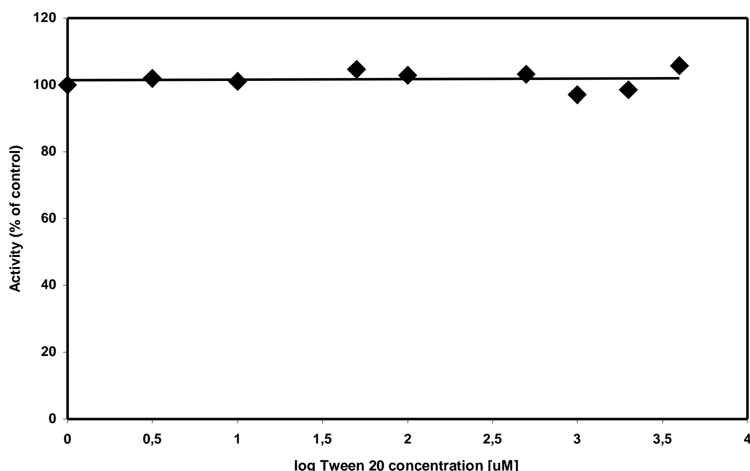

Fig. 4. The effect of Tween 20 on the activity of lactate dehydrogenase (control). Enzyme concentration as in the assay, buffer: MES/NaOH, pH 5.5

The experiments prove that the presence of the studied phospholipid molecules is not enough to inhibit the enzyme. Since lipid-detergent micelles are the dominating forms at saturating concentration values of the detergent, it is obvious that neither micelles nor single molecules affect of the activity.
A reasonable model of the interaction emerging from the obtained data would assume that the enzyme adsorbs onto the surface of lipid bilayer due to electrostatic attraction between the negatively charged surface and cationic site(s) on the protein molecule. Since pretty rather non-specific interactions cause the adsorption (association), it is possible that it may occur on any

negatively charged surface (domain) provided by the membrane. Since the lipid micelles produced by the detergent did not affect the enzyme's activity, a surface of appropriate size and curvature is probably necessary for effective adsorption. Both the lipid and protein components of cell membranes can provide such domains [7, 24, 25, 26]. The diversity and specificity of the interaction are probably due to the nature of the cationic site on the protein molecule, and the three-dimensional fit between the membrane domains and the binding sites. For example, the inhibition of the heart isoform of LDH by PS is markedly weaker than that of the LDH muscle form (not shown). Our results indicate that phosphatidylserine domains of the membrane are very effective adsorptive sites at appropriate local conditions and other anionic phospholipids may also be involved. There are several possible mechanisms of enzyme activity alteration in such a model: 1) changes in the static or dynamic conformational state; 2) the alteration of accessibility of binding and active sites for substrate and cofactor molecules by steric hindrance generated by this association; or 3) the alteration of the local concentration of effector molecules (local concentration of H^+, salt ions). Also, a combination of the mechanisms is obviously possible. Further studies on the structural consequences of the interaction are necessary to determine which mechanism operates here. Despite its molecular mechanism, the inhibition of the glycolytic enzymes binding to membranous structures may play an important physiological role if it occurs *in vivo*. Local regulation of glycolysis pathway in a cell and ATP compartmentation are hypothesized to be coupled to the function of organelles within the cell [27].

Still, the results presented above do not exclude the possibility of the formation of molecular lipid-protein or lipid micelle-protein complexes with the catalytic activity of the enzyme not decreased. Such a mechanism of lipid binding was found for pyruvate kinase [13]. Further research is necessary to assess if this possibility is also true for LDH.

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