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Investigation of the interaction of pig muscle lactate dehydrogenase with acidic phospholipids at low pH

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Abstract

Interaction of pig muscle lactate dehydrogenase (LDH) with acidic phospholipids is strongly dependent on pH and is most efficient at pH values <6.5. The interaction is ionic strength sensitive and is not observed when bilayer structures are disrupted by detergents. Bilayers made of phosphatidylcholine (PC) do not bind the enzyme. The LDH interaction with mixed composition bilayers phosphatidylserine/phosphatidylcholine (PS/PC) and cardiolipin/phosphatidylcholine (CL/PC) leads to dramatic changes in the specific activity of the enzyme above a threshold of acidic phospholipid concentration likely when a necessary surface charge density is achieved. The threshold is dependent on the kind of phospholipid. Cardiolipin (CL) is much more effective compared to phosphatidylserine, which is explained as an effect of availability of both phosphate groups in a CL molecule for interaction with the enzyme. A requirement of more than one binding point on the enzyme molecule for the modification of the specific activity is postulated and discussed. Changes in CD spectra induced by the presence of CL and PS vesicles evidence modification of the conformational state of the protein molecules. In vivo qualitative as well as quantitative phospholipid composition of membrane binding sites for LDH molecules would be crucial for the yield of the binding and its consequences for the enzyme activity in the conditions of lowered pH.

Keywords: Lipid–protein interaction; Lactate dehydrogenase; Acidic phospholipids; Phosphatidylserine; Cardiolipin

1. Introduction

Lactate dehydrogenase (LDH, EC 1.1.1.27) catalyzes the reduction/oxidation reaction between pyruvate and lactate and is involved in the glycolytic pathway, especially in the situation when anaerobic conditions arise. Until recently, LDH used to be considered as a representative of cytosolic enzymes, which have been shown to be able in vitro to bind reversibly to cellular membrane fractions and to some components of cytoskeleton [1–9]. Quite recently, membrane location of this enzyme in intact human red blood cells has been evidenced by immunostaining and confocal microscopy studies [10]. Assuming the domain structure of membranes, the surfaces of specific phospholipid domains in cell membranes are considered as possible effective binding sites for the enzyme. It is particularly interesting in the light of the generally accepted fact that the cytosolic surface of plasma membranes is enriched in acidic phospholipids, which have been suggested to form phospholipid binding sites for peripheral membrane proteins via electrostatic interactions [11–13]. Possible physiological relevance of LDH (as well some other glycolytic enzymes) association with membranes is still unclear and controversial. The binding may provide a further post-translational mechanism of enzyme regulation sensitive to local conditions if the bound and unbound forms of an enzyme differ in the catalytic activity. Acidification of the environment would promote the binding of non-acidic proteins. Local pH in cell compartments differs markedly from measurable bulk pH. For example, acidification of the environment has been evidenced locally in cytosol close to the membrane in yeast cells [14]. Well-known examples of non-enzymatic proteins of different functions able to interact with acidic phospholipids below pH 6.0 are insulin [15] and alfa-lactalbumin [16].

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The interaction of various peripheral membrane proteins with phospholipid sites may play a role in molecular mechanisms in some pathological processes where the metabolism of a cell and its internal environment are altered. This is a case in cancer cells where the phospholipid and fatty acid residue composition of their membranes is changed [17]. Tumor cell membranes usually contain a higher content of anionic phospholipids. In human breast cancer cells the amount of phospholipids increases 3.6 times compared to that in healthy cells [18]. Also, in plasma membranes of Alzheimer disease brain cells from different regions of the brain, the level of serine glycerolphospholipids is significantly higher than that in control brains [19]. It is a well-known fact that in cancer tissue a dramatic increase in glycolysis is observed, which is likely induced by increased hypoxia and increase need for ATP generation [20,21]. The mechanisms of tumor acidity have not been solved in detail. However, it is known that in hypoxia the enhanced production of lactate, catalyzed by LDH, is one of the main metabolic paths [22], as well as that oxidative stress decreases the Na^+/H^+ antiporter activity of a cell [23], which results in the lowering the pH of the cytosol [24].

Also, acidification of the environment is related to virus infection. It has been shown, for example, that the infection of host cells by the Sendai virus occurs by a direct fusion of its envelope with the cell plasma membrane, which is most effective at a high H^+ concentration in intercellular medium [25]. The infection causes a reversible impairment of the Na^+/H^+ antiporter lowering of pH in cytosol [26]. LDH has already shown to bind to lipid bilayers containing anionic phospholipids [27–30]. In addition, LDH is an enzyme retaining high activity also at low pH values [29].

Our work was aimed at the investigation of its interaction with phospholipids in such conditions. Lipid bilayers containing anionic phospholipids, and thus bearing net negative charge in a broad pH range, provide a good simplified adsorptive model system for studies of the mechanisms of such types of binding and its consequences. For a detailed investigation of the influence of different structures and different numbers of negatively ionized groups of the used lipids on the binding, in this work, we compared interaction of pig muscle LDH with phosphatidylserine- and cardiolipin-containing liposomes. The interaction was studied using ultracentrifugation and the enzyme activity and its native tryptophanyl fluorescence measurements. Possible occurrence of conformational changes in the protein molecule upon the interaction has been checked by using UV circular dichroism spectroscopy. Phosphatidylserine (PS) and cardiolipin (CL) are acidic membrane phospholipids which differ markedly from each other in their functional role, structure and number of ionic groups and hydrocarbon chains. A cardiolipin (diphosphatidylglycerol) molecule contains three moieties of glycerol, four fatty acid chains and two phosphate groups. The molecular mass and size of CL are approximately two times bigger than those of PS. In aqueous dispersions both phospholipids form bilayers, although cardiolipin shows an ability to form non-bilayer hexagonal phases depending on specific environmental conditions (molar concentration of NaCl, or millimolar Ca^{2+}) and on the saturation degree of its fatty acids [31–34].

2. Materials and methods

2.1. Chemicals

Pyruvate, NADH and Tween-20 were purchased from Roche Molecular Biochemicals (Germany). 2-Morpholinoethanesulfonic acid (MES) was from SERVA (Germany). All other chemicals were from Sigma-Aldrich and were of analytical grade.

2.2. Enzyme

Skeletal muscle lactate dehydrogenase preparation, from pig muscle, was obtained commercially from Roche Molecular Biochemicals as a suspension in 3.2 M ammonium sulphate solution. For the experiments the suspension was centrifuged down and the pellet was dissolved in 100 mM Tris–HCl, 0.2 mM EDTA buffer (of adjusted pH) and dialysed exhaustively against the buffer at a temperature of 5 °C. 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 whose specific activity in the standard conditions (pH=7.5) was not lower than 500 U/mg protein were used for the study.

2.3. Protein determination and the enzyme assay

Protein concentration was determined by the method of Bradford [35] using a Bio-Rad Protein Assay kit, Bio-Rad (Germany). Bovine serum albumin was used as a standard protein. Lactate dehydrogenase activity was assayed by the modified method of Bergmeyer et al. [36], using sodium pyruvate as a substrate and spectrophotometrical monitoring of the decrease in absorbance at 340 nm (changes of NADH concentration). The assay sample contained 0.2 mM NADH and 3 mM sodium pyruvate in 100 mM MES/NaOH, 0.2 mM EDTA or 100 mM Tris–HCl, 0.2 mM EDTA buffer of appropriate pH values. The reaction was started by addition of the mixture of both substrates (NADH and pyruvate) to the sample. All assays were performed at a temperature of 25 °C. The assays in the presence of phospholipids were started after a 5-min pre-incubation at 25 °C and run at the same temperature. In all assay samples, the concentration of the enzyme was 1.5 nM. One unit of LDH activity (U) represents the amount of the enzyme which converts 1 μmole of coenzyme per min under the assay conditions. Each point in the presented plots represents an average of the assays of at least four separate samples. Determinations with variations less than 10% were taken into consideration.

2.4. Lipids and liposomes

The preparations of natural, bovine phosphatidylserine (PS), cardiolipin (CL) and phosphatidylcholine (PC) were purchased from Avanti Polar Lipids, Inc. (USA), and were stored as chloroform solutions. The concentration of phospholipid was assayed by the procedure according to Bartlett [37]. Aqueous suspensions of the phospholipids or of the mixtures with PC were prepared according to the following protocol. An appropriate volume of the lipid solution or lipid mixture was dried under a stream of nitrogen to the moment when a lipid film appeared on the tube wall. Next, drying of the sample/s was continued under vacuum for at least two h. The dried phospholipids were hydrated with an appropriate volume of 100 mM MES/NaOH, 0.2 mM EDTA buffer, pH 5.5, and mechanically shaken. Finally, the suspension was subjected to 25 passes through two polycarbonate membrane filters with 100 nm size pores (Corning Costar Corporation, MA, USA), installed in tandem. This procedure produces transparent samples of anionic phospholipid liposomes while the liposomes made of PC alone or of PL/PC mixture shows some turbidity dependent on PC content. According to the method described elsewhere [38,39] unilamellar vesicles of an approximately 100 nm diameter are formed.

The preparations of liposomes were stable in entire pH range studied. No sedimentation was observed during their storage up to several days as well as no

changes in turbidity and control centrifugation were found. For the experiments, the samples freshly prepared (up 2 days) were used.

2.5. Ultracentrifugation

Centrifugation of mixtures of liposomes and lactate dehydrogenase buffered with 100 mM MES/NaOH or 100 mM Tris–HCl with 0.2 mM EDTA was used for the binding studies. The phospholipid liposomes and the enzyme solution were mixed in centrifuge tubes, pre-incubated for 5 min at 25 °C and then centrifuged at 105,000×*g* for 30 min at 5 °C. At these conditions, liposomes and a liposome–protein complex were pelleted while the protein alone was not. Samples of the supernatants were carefully transferred to separate tubes and the protein and phospholipids were determined. The final concentration of LDH in the centrifugation tube was 0.5 μM or variable in the titration experiment. In the control samples of the enzyme without liposomes no decrease in protein concentration was observed after centrifugation for each pH value studied. Each point in the presented plots in Figs. 1–3 represents an average of the assays of at least four separate samples. Determinations with variations less than 10% were taken into consideration.

2.6. Fluorescence measurements

In our previous studies, we had shown that the overall tryptophanyl fluorescence was a parameter sensitive to the interaction of LDH with phospholipids [30]. The protein intrinsic fluorescence excited at 295 nm was used to monitor the interaction. Excitation at this wavelength value arises practically only from tryptophanyl residues in a protein since the light absorption bands of other natural protein fluorophores (tyrosine and phenyloalanine residues) do not overlap with the band for tryptophanyl residues in the wavelength value [40]. All fluorescence measurements were performed at 25 °C after a 5-min pre-incubation of the samples also at 25 °C, on a luminescence spectrophotometer Hitachi F-4500 equipped with a thermostatted sample holder. In all fluorescence measurements the concentration of LDH was 0.2 μM. Buffers used in the fluorescence experiments were the same as for activity and centrifugation studies. Some weak turbidity of the liposomes yielded a small background in the fluorescence spectra at the concentrations used. These background values were deducted from the spectra.

Each point in the presented plots represents an average of the assays of at least four separate samples. Determinations with variations less than 10% were taken into consideration.

2.7. CD measurements

CD spectra were recorded on a Jasco J-600 spectropolarimeter (Japan), at 25 °C, in 15 mM phosphate buffer, pH=5.5. Concentration of the protein was 0.5 μM. Spectra were measured for the protein alone and in the presence of CL and PS liposomes, with the protein:lipid molar ratio of 1:100. Each spectrum represents the average of at least three scans. The data are presented as mean residue elipticity [θ].

3. Results and discussion

Binding of LDH with liposomes was indirectly evidenced using ultracentrifugation. When PS and CL liposomes in the presence of LDH were centrifuged at 105,000×*g*, the enzyme co-precipitated with the liposomes effectively below pH=6.5 and at NaCl concentrations of 50 mM and 150 mM for PS and CL, respectively (at pH=5.5), as shown in Figs. 1 and 2. The data were not affected when NADH at a concentration range 0.2–2 mM was present in the mixture (Fig. 1). The enzyme did not co-precipitate with PC liposomes (not shown). PS and CL in a mixture with neutral PC went to the pellet almost completely at pH ≤ 6.0 for the liposomes containing more than 10 and 20 mol % of CL and PS, respectively (Fig. 3). To obtain a titration curve, the given amounts of the phospholipids with increasing concentration of LDH were centrifuged. From the titration curve for pH=5.5 the binding data: K_d and stoichiometry of centrifugable phospholipid/protein complex were calculated. The obtained values were: $K_d = 0.7 \pm 0.58 \times 10^{-7}$ M for CL and

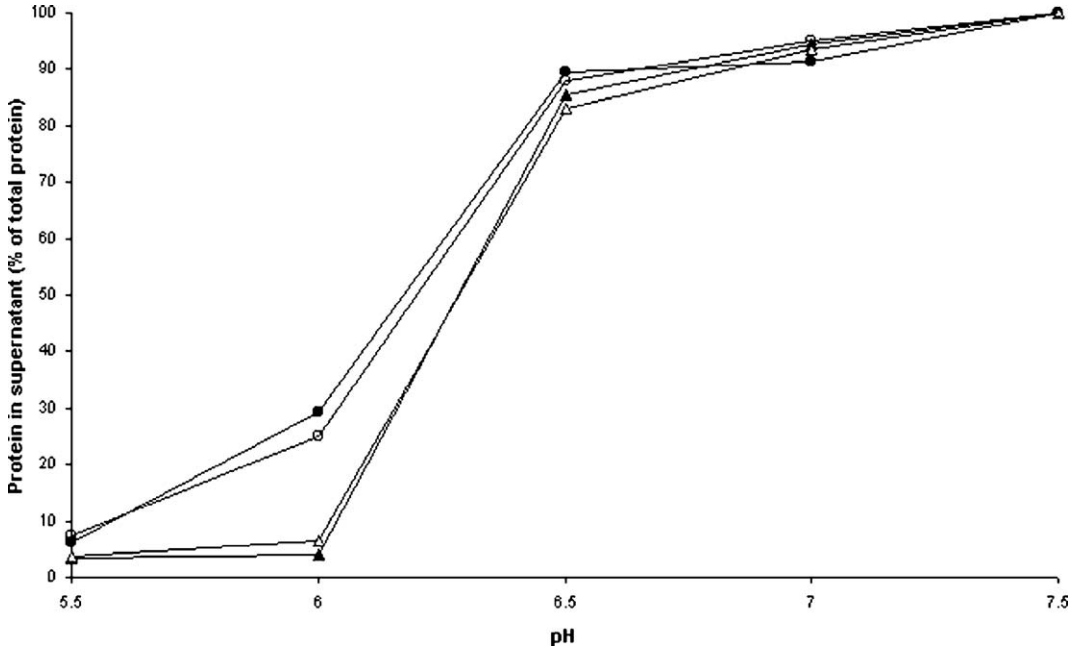

Fig. 1. Centrifugation studies of adsorption of LDH on PS (○, ●) or CL (△, ▲) liposomes at different pH values in the absence (opened symbols) and in the presence (closed symbols) of 0.2 mM NADH. LDH concentration was 0.5 μM and the enzyme/phospholipid molar ratio was 1:100. For the experiment 100 mM MES/NaOH, 0.2 mM EDTA and Tris–HCl, 0.2 mM EDTA buffers were used. After a 5-min incubation at 25 °C, the LDH/liposomes mixture was centrifuged for 30 min (105,000×*g*) at 4 °C.

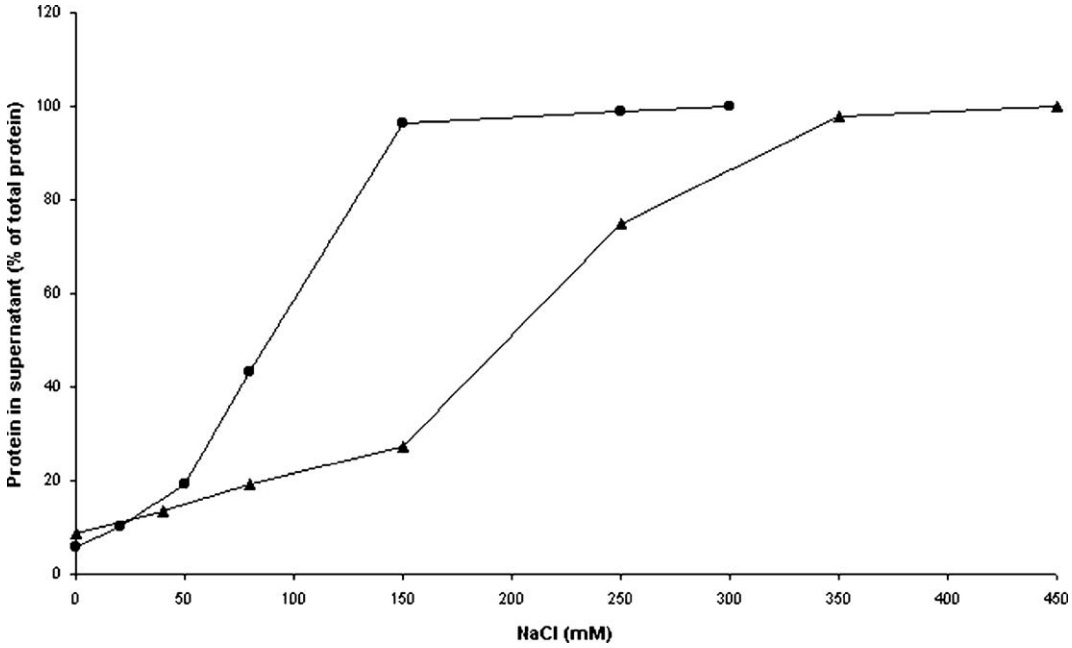

Fig. 2. Centrifugation studies of adsorption of LDH on PS (●) or CL (▲) liposomes at different NaCl concentrations. LDH concentration was 0.5 μM and the enzyme/phospholipid molar ratio was 1:100. For the experiment 100 mM MES/NaOH, 0.2 mM EDTA, pH = 5.5 buffer was used. Other conditions as in Fig. 1.

$K_d = 2.34 \pm 0.99 \times 10^{-7}$ M for PS. These values indicate that the affinity of the binding is relatively high. They are situated in the range of values between that for phospholipase A_2 bound to DPPC large unilamelar vesicles (10^{-6} M) [41] and the values for glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase bound to red blood cell membrane band 3 protein (10^{-8} M) [42]. Molar lipid/protein ratios in pellet in saturation conditions were 13.77 ± 2.55 and 6.25 ± 1.29 for PS/LDH and CL/LDH, respectively. The standard deviations were calculated from five repeated experiments. The stoichiometry correlates with the double negative charge of the CL molecule. If it is assumed that the complex is formed by electrostatic adsorption on the surface of bilayer walls of the vesicles the obtained ratio values are unexpectedly low. In liposome–protein complexes, their

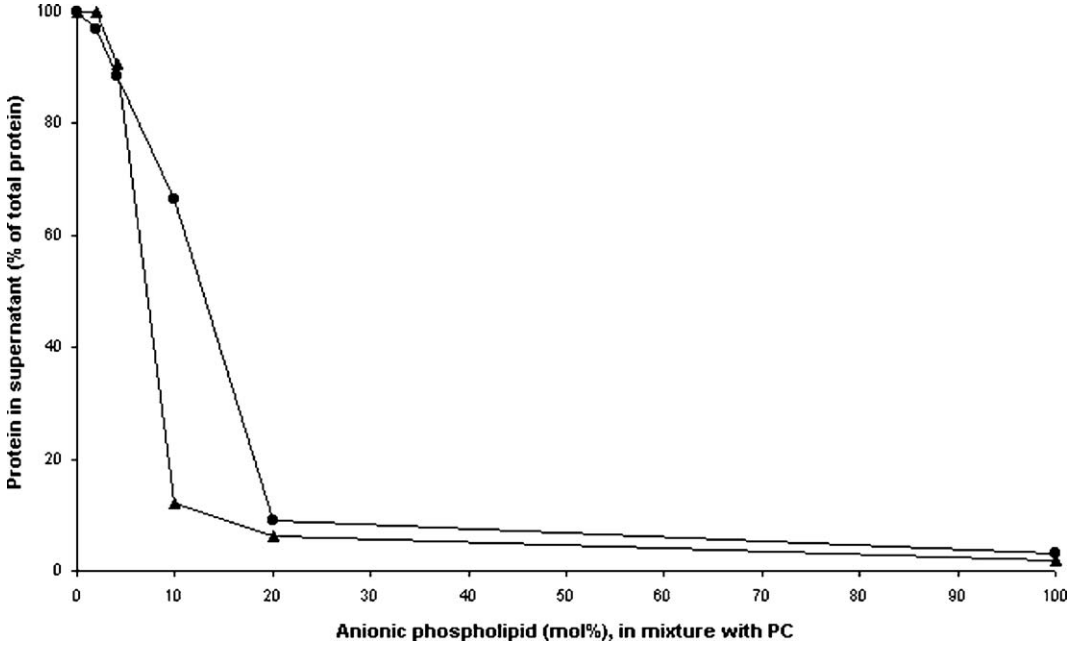

Fig. 3. Centrifugation studies of adsorption of LDH on liposomes containing PC and acidic phospholipid in different proportions. (●) Liposomes made of PC/PS mixtures. (▲) Liposomes made of PC/CL mixtures. LDH concentration was 0.5 μM and the enzyme/acidic phospholipid molar ratio was 1:100. In the sample liposomes made of PC, only the ratio was 1:5000. For the experiment 100 mM MES/NaOH, 0.2 mM EDTA, pH 5.5 buffer was used. After a 5-min incubation at 25 °C the mixtures of LDH with liposome suspension were centrifuged for 30 min (105000×g) at 4 °C.

stoichiometry is supposed to be dependent on the kind and properties of liposomes and does not reflect any specific lipid–protein stoichiometry. Phospholipids in water form vesicles with bilayer walls, therefore some molecules are not accessible for the interaction and the number of accessible molecules depends on the type and size of liposomes. Aggregation of the protein, induced by the interaction with the phospholipids would be a possible explanation of the results. The phenomenon of the aggregation of various proteins induced by the interaction with PS has been shown by Zhao et al. [43].

The specific activity of LDH is sensitive to pH as well. The pH optimum for the activity is around a value of 7.5. The activity of LDH decreases with decreasing pH (curves 1 in Fig. 4A and B). Besides titrating of ionized groups in protein molecules, the decrease in pH has been found to result in destabilization of LDH quaternary structure by dissociating the tetramers to dimers. Tetramer–dimer conversion occurs within a min of adjusting the pH of LDH solution from 7 to 5 [44]. The dimers retain full activity [45]. Under the conditions of the assay, we have tetramers, as nicotinamide-adenine dinucleotide (NADH or/and NAD) which must be present in the activity assay solution, have been shown to stabilize the tetramers even at pH=5.0 [44]. The presence of phosphatidylserine (PS) and cardiolipin (CL) aqueous suspensions alters the dependence of the activity on pH as shown in Fig. 4A and B (lines 2–4), while again the presence of phosphatidylcholine (PC) is ineffective in the studied range of pH (not shown). The highest decrease of the enzyme specific activity is observed at pH=5.5 for the liposomes of both anionic phospholipids. The observed effect induced by the phospholipids is diminished with increasing pH. Above pH=6.5 for PS and pH=7.0 for CL, for the studied phospholipid range of concentrations, the inhibition can be practically neglected. The effect of CL, however similar, is much stronger and the fully inhibiting concentrations of CL and PS differ almost one order of magnitude from each other. Such a big difference in effects of the two types of phospholipids is not explainable simply by the difference in the charge values of PS and CL. In an aqueous environment at pH values ≥3.5 both phospholipids become net negatively charged because of the protonation/deprotonation balance of ionizable groups [46], but the charge of CL is not double compared to PS. The two phosphate groups of CL have different chemical environments and hence different values of acidity: $pK_a^1=2.8$ and $pK_a^2>7.5$ [47]. The high value of pK_a (weak acidity) of the latter phosphate group is a result of formation of a hydrogen bond with the central hydroxyl group and formation of a specific configuration [47,48]. Therefore, no electrostatic binding produced by this group should be expected unless the group is modified by interaction/binding with the other one. The differences in effectiveness between the two classes of phospholipids are more likely due to differences in chemical structure. The isoelectric point of the enzyme is within the range of 8.6–9.6 [49–51]. Therefore the enzyme molecules bear negative net charge within the entire range of pH studied here. It is clear from this experiment that the observed changes in the enzyme activity correlate well with the binding of the protein to the liposomes evidenced from the centrifugation experiments, and the reversibility of the changes with increasing pH reflects the reversibility of the protein–liposome binding. This is in agreement with the generally accepted idea that electrostatic

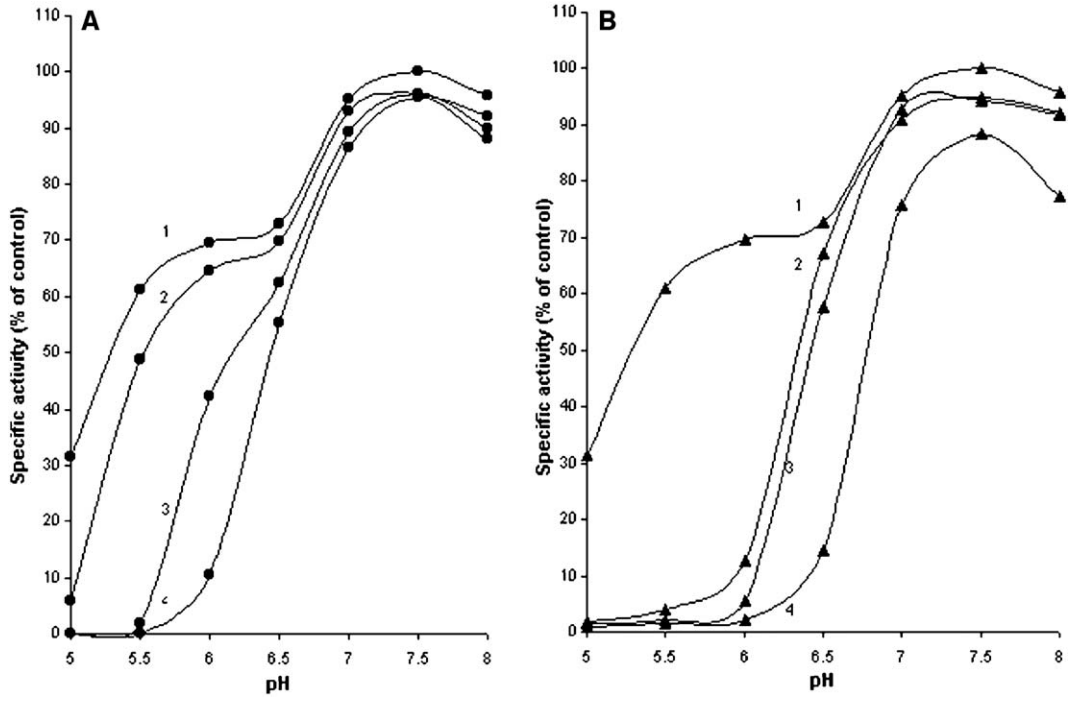

Fig. 4. The dependence of the inhibition of LDH specific activity by phosphatidylserine (A) and cardiolipin (B) on pH. Concentration of LDH in each sample was 1.5 nM. Phospholipid concentrations: (1) no phospholipid; (2) 0.25 μM; (3) 2.5 μM; (4) 25 μM. Buffers used: 100 mM MES/NaOH, 0.2 mM EDTA for pH 5–6.5 and 100 mM Tris–HCl, 0.2 mM EDTA for pH 7–8. The control was the activity of the sample without phospholipid at pH 7.5.

interactions are those forming the liposome–enzyme complex. Negative charge of phospholipid increases with increasing pH and the balance between positive and negative charge on protein molecules is shifted toward negative charge as well. For further experiments, we chose a value of pH = 5.5, as in the range of pH 6.5–5.5 the enzyme activity is constant and at these pH values the highest effect of liposomes of both anionic phospholipids is observed. In this pH range V_{max} is lowered while K_m is unchanged in the presence of PS or CL liposomes for the both substrates of the enzyme (NADH and pyruvate).

As expected for ionic interactions, the phospholipid–LDH interaction is also sensitive to ionic strength (Fig. 5). However, some specific effect of salt on the enzyme overlaps the effect of dissociation of the binding. The enzyme alone at a low salt concentration shows about half of its full specific activity at pH = 5.5 and reaches the optimal activity at the concentration of NaCl higher than 100 mM. At low ionic strength, the studied phospholipids effectively lower the enzyme activity and also prevent this activity from being recovered by increasing ionic strength to threshold values. Then, the inhibition is diminished with increasing ionic strength and the enzyme activity is fully recovered at an NaCl concentration of about 150 mM and 400 mM for PS and CL liposomes, respectively. The curves likely reflect overlapping of two effects: 1) increasing activity with the increase in ionic strength and 2) dissociation of the ionic bonds with increasing ionic strength.

To compare the inhibitory effectiveness of the studied phospholipids, we measured the specific activity of the enzyme as a function of the concentration of phospholipids, and concentration values for the half-inhibition (IC_{50}) were determined from the curves 1 in Fig. 6A and B. The IC_{50} values are shown in Table 1. Again, it can be seen that CL liposomes are more effective than those of PS. The small value of IC_{50} for CL alone suspensions may suggest a possibility of contribution of non-vesicle particles (micelles, single molecules) to this effect. The possibility that phospholipid structures other than liposomes can be agents inactivating the enzyme was excluded by an experiment with Tween 20. Gradual addition of Tween 20 resulted in shifts of the equilibrium of phospholipid structures toward more soluble micelles, and this restored the enzyme specific activity depending on the detergent/lipid molar ratio for PS and CL in the same manner (Fig. 7). The activity of LDH in the presence of Tween 20 (without the phospholipids) was not altered at the used concentration of the detergent (control). These results clearly indicate that bilayer-type structures are crucial for the interaction for both phospholipids. The necessity of the bilayer structure for the observed effects was also evidenced for PS by similar experiments in our previous work [29,30]. Liposomes made of PC (neutral in a broad range of pH) do not change the enzyme activity up to millimolar lipid concentrations in a range of pH 5.0–8.0. Liposomes made of PC/PS and PC/CL mixtures of various molar proportions represent various charge density values on their surface. They decrease the activity as shown in Fig. 6A and B, curves 2–8. The individual curves in these figures represent various PC:CL and PC:PS molar ratios. First of all, as expected, the PC/CL liposomes were much more effective compared to the PC/PS ones. CL concentrations produced comparable effect at more than an order of magnitude lower concentration. The "dilution" of PS or CL by increasing PC contents in the bilayers

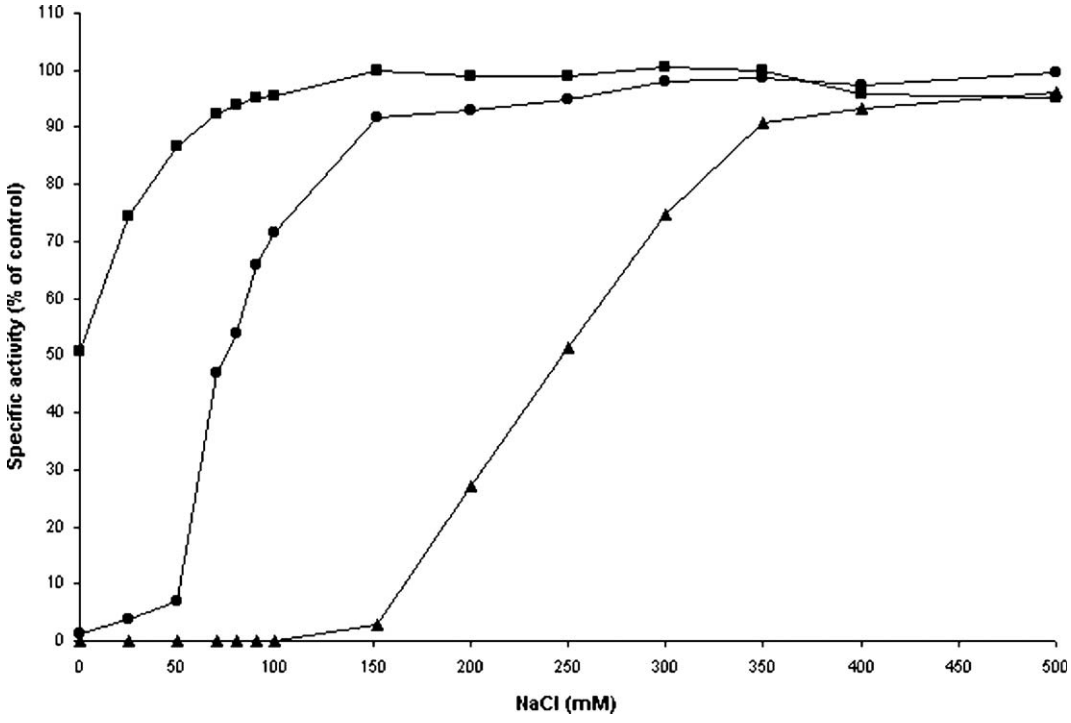

Fig. 5. The dependence of the specific activity of LDH on salt concentration. LDH alone (■), LDH+PS (●), LDH+CL (▲). LDH concentration was 1.5 nM. Phospholipid concentrations: (■) no phospholipid; (●) 5 μM PS; (▲) 5 μM CL. Buffer: 100 mM MES/NaOH, 0.2 mM EDTA, pH = 5.5. The value of the activity of the sample without phospholipid, containing 150 mM NaCl, was assumed as 100%.

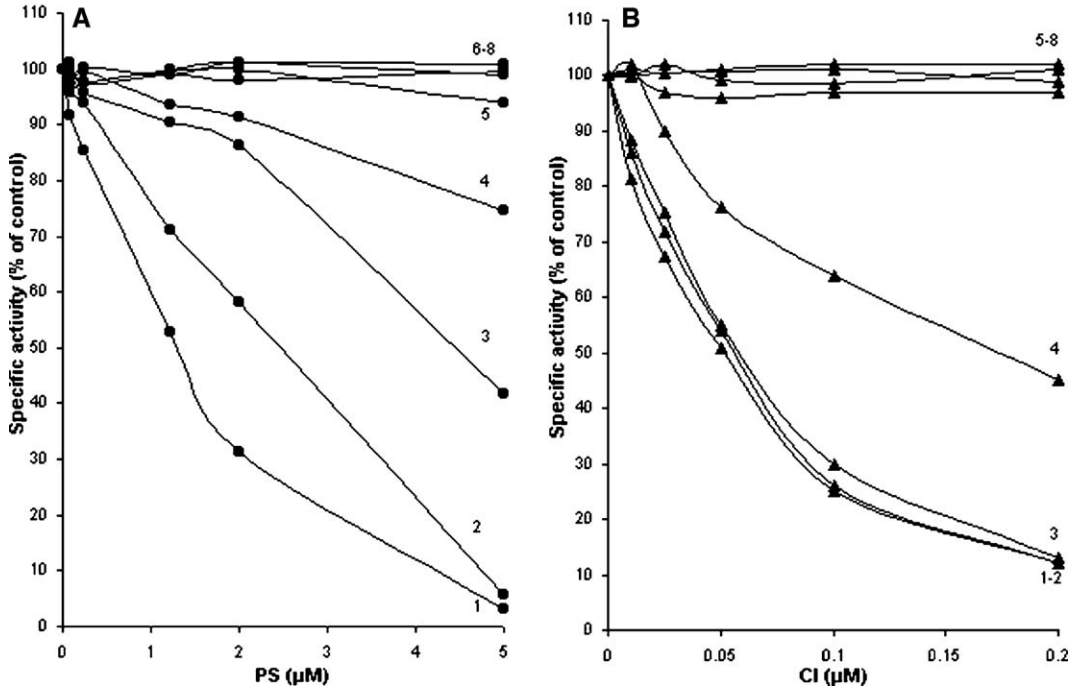

Fig. 6. The dependence of LDH specific activity on the concentration of PS in the PS/PC mixture (A) and of CL in the CL/PC mixture (B). PS (A) or CL (B) concentrations: (1) control sample, 100 mol% PL (no PC); (2) molar ratio PC/PL = 3 (25 mol% PL); (3) molar ratio PC/PL = 4 (20 mol% PL); (4) molar ratio PC/PL = 5 (16.7 mol% PL); (5) molar ratio PC/PL = 7 (12.5 mol% PL); (6) molar ratio PC/PL = 10 (9.1 mol% PL); (7) molar ratio PC/PL = 20 (4.76 mol% PL); (8) molar ratio PC/PL = 100 (0.99 mol% PL). (PL = PS or CL). The concentration of LDH in each sample was 1.5 nM, and the samples were buffered with 100 mM MES/NaOH, 0.2 mM EDTA, pH 5.5. For other details, see Materials and methods.

diminished the loss of specific activity even though the same overall concentration of the effective phospholipid was retained. Even though very effective overall concentration values of anionic phospholipids (5 μM and 0.2 μM for PS and CL, respectively) were used, no marked effects were observed when PC:PS and PC:CL values exceeded 7:1. Probably two or more binding sites per protein molecule are necessary for the activity modification, and that is why an appropriate charge density on the bilayer surface is important. Possibly, CL could provide two negatively charged groups in an appropriate configuration and distance for such a two-point interaction, if we assume that the interaction induces acidity of the second phosphate group in CL. This would explain the high effectiveness of cardiolipin.

The interaction was confirmed by our measurements of the enzyme's native fluorescence spectrum (not shown). In the fluorescence study we could not use exactly the same conditions for the following reasons. The native fluorescence of the enzyme is mostly reduced because of quenching by NADH/NAD as a result of excitation energy transfer between tryptophanyl and nicotinamide fluorophores and conformational changes [40], and the native fluorescence of such a small amount of the enzyme as used in the assay is not detectable. Therefore, during the fluorescence experiments the apoenzyme was used. Addition of anionic phospholipid liposomes resulted in the lowering of the fluorescence intensity without changes in λ_{max} of the fluorescence emission spectra of the enzyme.

Table 1
Values of the phospholipid concentration that decrease the enzyme specific activity to half of the value for the enzyme alone (IC_{50}) at pH 5.5

Phospholipid	IC_{50} (μM)
PS	1.30
CL	0.05

Experimental conditions as in the legend to Fig. 6.

The dependence of the enzyme fluorescence on pH is shown in Fig. 8. It correlates well with the data for centrifugation (Fig. 1) and activity experiments (Fig. 4). Tryptophanyl fluorescence of the apoenzyme is quenched in the presence of both phospholipids alone and in the mixture with PC, and again the cardiolipin suspension is more effective but the difference is not as high as for the specific activity. Suspensions of PC do not affect the enzyme fluorescence. The quenching monotonically increases with increasing anionic phospholipid concentration (the anionic phospholipid/protein ratio as well). The effectiveness of fluorescence quenching is lower in the case of anionic phospholipid mixed with PC. However, the shape of the curves is quite similar (not shown). The quenching can be reversed similarly to the inhibition of the activity when the pH (Fig. 8) and ionic strength increase. Thus, the alteration of both the activity and native tryptophanyl fluorescence of the protein proves and reflects the binding of the holo- and apoenzyme.

The comparison of the effects on the activity and fluorescence of LDH for the liposomes made of a PC/anionic phospholipid mixture is shown in Fig. 9A and B. The concentration of PS and CL in all the samples was kept constant and it was high enough for effective inhibition and fluorescence quenching of the enzyme in the case when the anionic phospholipids were only component of the bilayer. In

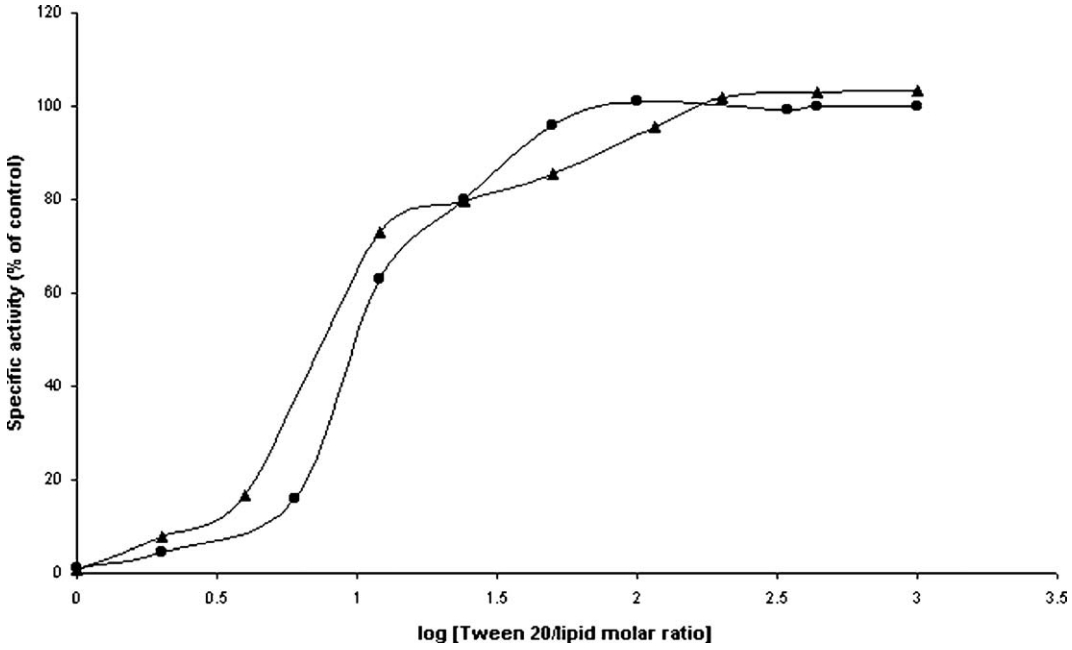

Fig. 7. The effect of the nonionic detergent Tween 20 on the inhibition of LDH specific activity by PS (●) and CL (▲). Phospholipid concentrations in each sample were 1.25 μM and 0.130 μM for PS and CL, respectively. Tween 20 concentration varied. LDH concentration was 1.5 nM. Samples were buffered with 50 mM MES/NaOH, 0.2 mM EDTA, pH 5.5.

our interpretation of the data, we exclude phase segregation that might occur in phospholipid mixtures as a reason of the observed sharp changes in slope of the curves. The preparations of natural PC, PS and CL that were used were miscible. No phase segregation in similar phospholipid mixtures of broad range of molar ratio was observed in the studies elsewhere [16,52]. Besides, the fluorescence quenching and activity change curves (1 and 2 in the figures) for each of anionic phospholipids studied are not exactly parallel to each other. The activity of the enzyme, contrary to the fluorescence, needs to

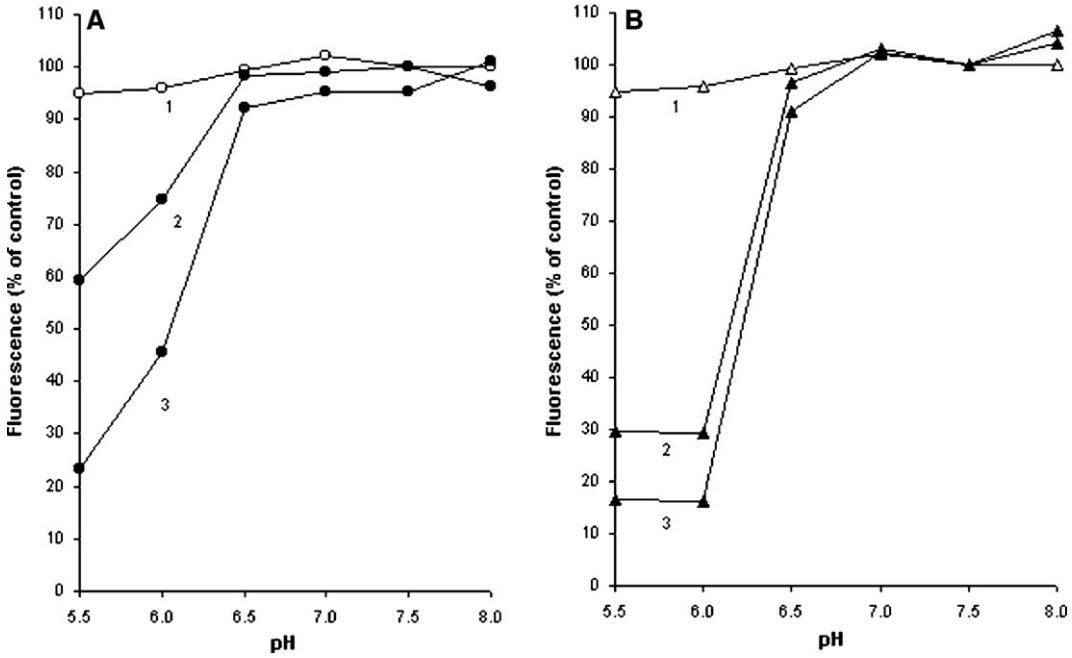

Fig. 8. The dependence of the quenching of tryptophanyl fluorescence of LDH on pH in the absence and in the presence of an acidic phospholipid. Plots: (A) 1-LDH alone, 2, 3-PS/LDH molar ratio: 40, 200; (B) 1-LDH alone, 2, 3-CL/LDH molar ratio: 40, 200. The experiment was performed at 25 °C, using 100 mM MES/NaOH, 0.2 mM EDTA and Tris–HCl, 0.2 mM EDTA buffers pH 5.5, and LDH at a concentration of 0.20 μM. The values of the maximal fluorescence at 345 nm (λ_{exc}=295 nm) were shown as the percent of a control. The control was the fluorescence of the sample containing LDH alone in the buffer pH 7.5.

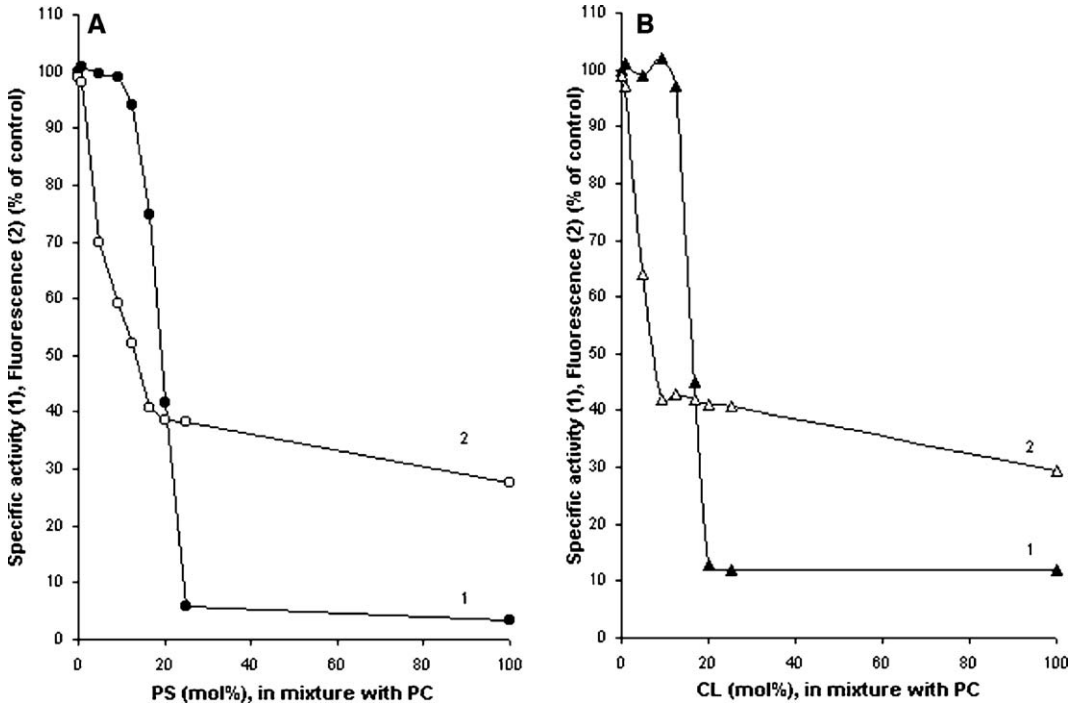

Fig. 9. The dependence of the specific activity and the tryptophanyl fluorescence of LDH on the PC/PS (A) and PC/CL (B) molar ratio at a constant concentration of PS or CL. The concentration values of PS in all the samples were 5 μM and 40 μM, for the activity and fluorescence studies, respectively (A). The concentration values of CL in all the samples were 0.20 μM and 8 μM, for the activity and fluorescence studies, respectively (B). The anionic phospholipid/protein molar ratios were the same in all the samples for the fluorescence studies, and were 200 and 40 for PS and CL, respectively. The protein (LDH) concentrations in all the samples for the activity and fluorescence studies were 1.5 nM and 0.20 μM, respectively. Buffer: 100 mM MES/NaOH, 0.2 mM EDTA, pH 5.5. For other details see Materials and methods.

reach a threshold value of the density of anionic phospholipid in the mixture for alteration. The fluorescence is affected even though the molar proportion PC/anionic phospholipid used here is high enough not to affect the activity. These results, together with those for ultracentrifugation studies (Fig. 3), suggest that binding of a protein molecule by every single phospholipid anionic group can exist but is not sufficient to change the activity. The results are in agreement with the idea that the activity alteration needs two- or multipoint binding.

Probably, a change in the enzyme activity requires a more specific steric fit (maybe mutual action of more than one binding ionic pair, conformational change, substrate availability, etc.), which can be achieved above the appropriate surface charge density. Such conformational changes induced by binding have already been shown for aldolase and glyceraldehyde-3-phosphate dehydrogenase interacted with lipid bilayer [53,54]. Despite the difference in the structure and number of potentially ionic groups between PS and CL, the difference in the threshold values and effective molar ratio values for the activity modification produced by PS/PC and CL/PC, observed here, does not exceed several mol%.

LDH is a multitryptophan protein. Its molecule contains 6 tryptophans per subunit [55]. The observed steady state fluorescence excited at 295 nm is an overall emission from all the variously located tryptophanyl chromophores. This emission gives a symmetric, broad band with its maximum at a wavelength of 345 nm. Such a position of the emission band maximum locates the protein in the middle of the scale of Burstein's classification of protein according to the tryptophan location in the interior of the protein molecule [56]. Quenching by the liposomes of the tryptophan residues exposed to aqueous environment and to the ionic bonds would cause a "blue" shift of the band maximum because the short-wavelength fluorescence of the hidden chrompohores, not accessible to the interaction, would dominate. Here we do not observe any shift of the band maximum. This suggests that the mechanism of the quenching is complex (simultaneous quenching by direct interaction and possible modification of the conformation). We measured the quenching of the tryptophanyl fluorescence of LDH by acrylamide. It is an effective dynamic quencher of protein fluorescence and can be used as a probe of fluorophores exposure changes [40]. In our case Stern–Volmer plots of the quenching did not differ markedly from those in the presence the studied phospholipids (not shown). Conformational changes producing modifications in exposure to Trp residues still cannot be excluded, since there may be a case where the net effect of exposure modifications of many variously located fluorophores is zero.

To verify with another method whether the conformational change occurred the UV circular dichroism (UV CD) spectra were measured. The CD spectra of LDH alone and in the presence of a 100-fold molar excess of phospholipids at pH=5.5 are presented in Fig. 10. Addition of PS or CL liposomes resulted in differentiated modification of the spectrum of LDH. The result strongly supports our conclusion of conformational changes. Again, CL was more effective lipid in the modification than PS.

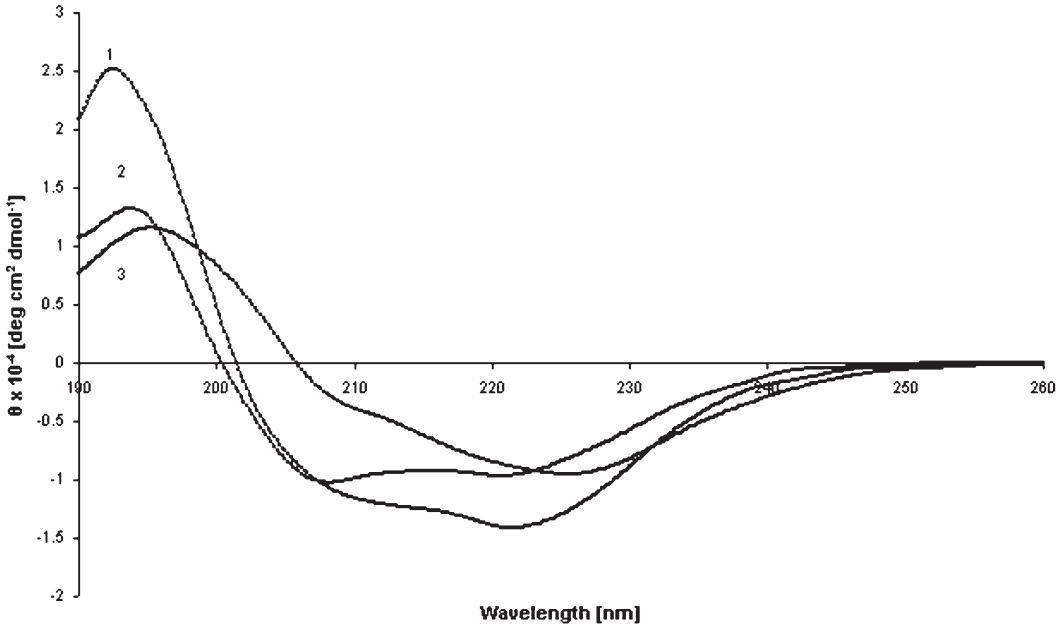

Fig. 10. UV circular dichroism spectra of LDH (0.5 μM) alone and in the presence of phospholipids in 15 mM phosphate buffer, pH = 5.5; (1) LDH; (2) LDH + PS, (3) LDH + CL, protein:lipid molar ratio 1:100.

From all the results obtained, it is clear that anionic phospholipid bilayers are those which are capable of functional binding of the studied enzyme resulting, above all, from the ionic bonds. Lack of any difference between the data obtained in centrifugation experiments in the absence and in the presence of nicotinamide-adenine dinucleotide indicates that both dimers and tetramers are likely bound. The data from the three methods used correlate pretty well. Nevertheless, a simple model of unspecific electrostatic adsorption on the charged surface of the bilayer is not adequate and sufficient here. The interaction is more complex. Conformational properties (flexibility) of the interacting protein and steric fit of the ionic groups of the protein and phospholipid are involved, and make the binding more specific. They are responsible for binding strength, functional consequences and the diversity of the binding among the different protein forms as well as phospholipids. Specific protein behaviour (binding) at the lipid bilayer surface is also determined by the type of lipid present and local properties (depending on the lipid composition) of the surface [57]. While the negative charges provided by phospholipid ionizable groups can produce some binding, an appropriate surface density and/or their specific configuration are necessary to induce changes in the activity in the studied case. Despite the differences in molecular structure between PS and CL, the two kinds of phospholipids produced similar binding characteristics (the observed differences are of a quantitative type). However, the interaction with them produces markedly different conformational modifications. In our opinion, the interaction with the protein may influence the cardiolipin phosphate protons mobility changing the net value of pK_a. If both phosphate groups of CL were fully ionized, and if they were in an appropriate configuration for two-point binding with the protein molecule, the binding would be expected to be insensitive to the "dilution" in PC. In fact, the effect of CL on the activity, i.e., in the case of the holoenzyme (Fig. 6), reveals a markedly different function of the "dilution" than this of PS. While the effect of PS is gradually diminished with the dilution with PC, the effect of CL is much less sensitive up to a threshold value of the added PC, and above that value the inhibition of the enzyme by CL drastically decreases. Maybe the acidification of the non-acidic phosphate group in CL depends on the environment parameters, the interaction with particular proteins included.

Obviously, importance of such interactions and their consequences for the enzyme properties in vivo cannot be solved from the studies in vitro. Physiological bulk pH values in cytosol of most animal cells are not favourable for the interaction, but there is a possibility that the interaction can be controlled by local and/or pathological conditions. PS and CL are natural, acidic phospholipids which occur in mammalian and human cell membranes. PS is widely distributed in cytoplasmic membranes of various kinds of cells, mainly on the cytosolic side, where it is most abundant among acidic phospholipids [11,58]. Therefore, it is the most probable phospholipid to form electrostatic membrane binding sites for proteins in normal and pathological conditions [13]. Cardiolipin is also a strongly acidic, exclusively mitochondrial membrane phospholipid in normal animal cells. It is primarily localized in the inner membrane of mitochondria [59]. However, small amounts of this phospholipid were detected in the outer membrane [60,61], mainly in its outer leaflet [62]. The membrane sites abundant in such acidic phospholipids create near-membrane zones a couple of nanometers thick, where pH is below 6.5 in physiological states [14]. Strong pH effect of near-membrane zones created by acidic phospholipids has been shown for insulin and alpha-lactalbumin [43], which interact in vitro with acidic phospholipids at pH < 6.0 [15,16]. Those proteins are able to form amyloid fibers when incubated with PC/PS liposomes at pH 7.4 likely because of increasing

hydrogen ion concentration locally by anionic phospholipids despite physiological bulk pH [43]. These results suggest that PS and other acidic phospholipids could provide a physiological low-pH environment on cellular membranes, enhancing protein fibril formation in vivo [43]. To date, there has been no sufficient explanation of the problem what is local pH in the zone adjacent to the membrane in various pathological states and how deep is the penetration of such zones into cytosol. Similarly, little is known about the adjacent zones of a changed environment around cell organelles. As a matter of fact, Okeley and Gelb have shown that the amount of anionic phospholipid in cytosol-facing internal membranes is smaller than in the cytosol-facing leaflet of the plasma membrane, but still the presence of anionic phospholipid forming such zones has not been excluded [13].

Existence, stability and parameters of the local, adjacent to the membrane, zones of changed pH depend on the lipid composition of the membrane and can change with time. The changes are slow enough to control the lipid–protein interaction, which is a very fast process. Changes in pH in a cell infected by a virus, for example, last up to several tenths of a minute and while the bulk values in cytosol do not go down below 7.0 [26], much lower local and temporary pH values must be taken into account.

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