

**ULTRACENTRIFUGATION STUDIES OF THE LOCATION OF THE
SITE INVOLVED IN THE INTERACTION OF PIG HEART LACTATE
DEHYDROGENASE WITH ACIDIC PHOSPHOLIPIDS AT LOW pH.
A COMPARISON WITH THE MUSCLE FORM OF THE ENZYME**

GRZEGORZ TERLECKI*, ELŻBIETA CZAPIŃSKA
and KATARZYNA HOTOWY

Department of Medical Biochemistry, Wrocław Medical University, Wrocław,
Poland

Abstract: Lactate dehydrogenase (LDH) from the pig heart interacts with liposomes made of acidic phospholipids most effectively at low pH, close to the isoelectric point of the protein (pH = 5.5). This binding is not observed at neutral pH or high ionic strength. LDH-liposome complex formation requires an absence of nicotinamide adenine dinucleotides and adenine nucleotides in the interaction environment. Their presence limits the interaction of LDH with liposomes in a concentration-dependent manner. This phenomenon is not observed for pig skeletal muscle LDH. The heart LDH-liposome complexes formed in the absence of nicotinamide adenine dinucleotides and adenine nucleotides are stable after the addition of these substances even in millimolar concentrations. The LDH substrates and studied nucleotides that inhibit the interaction of pig heart LDH with acidic liposomes can be ordered according to their effectiveness as follows: NADH > NAD > ATP = ADP > AMP > pyruvate. The phosphorylated form of NAD (NADP), nonadenine nucleotides (GTP, CTP, UTP) and lactate are ineffective. Chemically cross-linked pig heart LDH, with a tetrameric structure stable at low pH, behaves analogously to the unmodified enzyme, which excludes the participation of the interfacing parts of subunits in the interaction with acidic phospholipids. The presented results indicate that in lowered pH conditions, the NADH-cofactor binding site of pig heart LDH is strongly involved in the interaction of the enzyme with acidic phospholipids.

* Author for correspondence; e-mail: terlecki@bioch.am.wroc.pl, tel: +48 (71) 784-13-81

Abbreviations used: CL – cardiolipin; EDTA – (ethylenedinitrilo) tetraacetic acid; LAC – lactate; LDH – lactate dehydrogenase; MES – 2-morpholinoethanesulfonic acid; PS – phosphatidylserine; PYR – pyruvate; TRIS – 2-amino-2(hydroxymethyl)propane-1,3-diol

The contribution of the ATP/ADP binding site to this process can also be considered. In the case of pig skeletal muscle LDH, neither the cofactor binding site nor the subunit interfacing areas seem to be involved in the interaction.

Key words: Lipid-protein interaction, Lactate dehydrogenase isoenzymes, Acidic phospholipids, Cardiolipin, Phosphatidylserine.

INTRODUCTION

Lactate dehydrogenase (LDH) is one of the glycolytic enzymes that are present in all the tissues of all eukaryotic organisms and microorganisms and also in prokaryotic cells/organisms. It catalyses the reversible conversion of pyruvate to lactate with the simultaneous, reversible conversion of NADH to NAD. In eukaryotic cells with anaerobic metabolisms, this is the key reaction responsible for the regeneration of the NAD form. Recent studies on the energy-producing metabolic processes in the brain clearly showed that lactate, the product of the LDH-catalyzed reaction, is the main substrate for energy production in neurons [1]. Besides its catalytic function, LDH also plays other roles in cells. Crawford *et al.* [2] proved that M-LDH is an integrated part of the sarcolemmal K_{ATP} channel protein complex *in vivo*, where, by virtue of its catalytic activity, it couples the metabolic status of the cell with the K_{ATP} channel activity that is essential for cell protection against ischemia. Other investigations by Pioli *et al.* [3] showed that LDH is an Au-rich element-binding protein participating in the post-translational regulation of gene expression. So far, three isoenzymatic forms of LDH have been found in mammals: the muscle form M, the heart form H, and the testes form X, respectively corresponding to A, B, and C genes [4, 5]. Different tissues contain different relative concentrations of M and H, depending on their metabolism: anaerobic tissues (skeletal muscles, blood erythrocytes) contain mainly the M form, while aerobic tissues (the heart) mainly the H form. The third form was discovered most recently, and is only found in the testes. The M and H isoenzymatic forms differ strongly with respect to their kinetic properties, but their amino acid sequence identity is high (75% for the isoenzymatic forms from pig tissues) [6]. LDH is a tetramer under physiological conditions. Tetrameric forms of the enzymes from various tissues consist either of four identical subunits (M_4 or H_4) or of M and H subunits in different proportions (M_3H , M_2H_2 , MH_3) [4, 5]. Enzymatic activity has been confirmed for the tetrameric and dimeric forms of LDH [7], while the activity of the monomer of the enzyme is contradictory [7, 8]. Each subunit (monomer) of the enzyme consists of about 330 amino acids. The whole is formed in a bilobal structure of two domains: the larger domain, with a Rossmann-type fold, provides the NADH-cofactor binding site, and the smaller, the mixed α/β substrate binding domain. The pyruvate/lactate binding pocket is located at the interface of the two domains [6]. Despite the amino acid sequence diversity between the M and H forms, the two isoenzymes are indistinguishable in terms of the enzyme, its domain structure, subunit association and active-site region structure [6].

Our preliminary studies showed that both LDH forms interact with liposomes made of lipids extracted from erythrocyte membranes [9, 10]. Detailed investigation proved that LDH from pig skeletal muscles (LDH-M₄) interacts with acidic phospholipids at low pH. This interaction results in the adsorption of the protein on liposomes made of the lipids and in the subsequent inhibition of its enzymatic activity, and the quenching of its tryptophanyl fluorescence [11]. The binding of LDH to the liposomes has an electrostatic nature and depends strongly on the pH and ionic strength [11]. This binding is restricted to the conditions in which acidic phospholipids form bilayer structures [11-13]. The interaction of muscle LDH with acidic liposomes and the resulting inhibition of the enzyme activity are most effective at pH < 6.5. For cardiolipin (CL) and phosphatidylserine (PS) liposomes, the respective IC₅₀ values at pH = 5.5 are 0.050 μM and 1.3 μM [11]. Such behaviour of muscle LDH (the M₄ isoform) seems to be obvious since at pH ≥ 3.5, acidic phospholipids are net negatively charged [14], and the pI value of LDH from pig skeletal muscles equals 8.6 [2]. In this article, we show that pig heart LDH (the H₄ isoform) also interacts most effectively with acidic phospholipids (most effective adsorption on liposomes) in an acidic environment (pH < 6.5), particularly if the pH value is close or equal to the pI of the protein (pI = 5.5 [2]). These are also the conditions in which the activity of pig heart LDH is most effectively inhibited by acidic phospholipids. However, unlike pig muscle LDH, the heart form of the enzyme at pH = 5.5 has a net charge close to zero. This means that for the interaction with the phospholipids, which in such conditions bear a negative net charge, the enzyme would require a site possessing some structural individuality and a local positive charge. Here, we aim to roughly define the part/parts of the enzyme molecule which are probably involved in the interaction with acidic phospholipids.

MATERIALS AND METHODS

Chemicals

NADH, NAD, NADP, ATP and ADP were purchased from Roche Molecular Biochemicals (Germany). 2-morpholinoethanesulfonic acid (MES) and glutaraldehyde were from SERVA (Germany). All the other chemicals were purchased from Sigma-Aldrich and were of analytical grade. All the solutions were prepared in nanopure water.

Enzymes

Pig heart lactate dehydrogenase (LDH), and skeletal muscle LDH from pig muscles and from rabbit muscles were obtained from Roche Molecular Biochemicals and were in the form of a suspension in 3.2 M ammonium sulfate solution. Solved, apo-forms of the enzymes were prepared using exhaustive dialysis against 100 mM TRIS/HCl, 0.2 mM EDTA buffer (pH 7.5) at a temperature of 5°C. The enzymes were used for experiments if, after dialysis, their specific activity in standard conditions (25°C, pH 7.5) was not lower than 250 U/mg for heart LDH and 500 U/mg for muscle LDH.

Protein content determination and the enzyme assay

The protein content was determined as per the method of Bradford [15] using a Bio-Rad Protein Assay kit, Bio-Rad (Germany) and bovine serum albumin as a standard protein. The LDH activity was measured in a reagent solution containing 100 mM MES/NaOH or TRIS/HCl, 0.2 mM EDTA buffer of the appropriate pH, 3.0 mM pyruvate and 0.2 mM NADH, using a spectrophotometer (Jasco V-530, Japan) set at a wavelength of 340 nm and at a temperature of 25°C. The reaction was started either by adding a mixture of both substrates or by adding the enzyme. In all the assays, the concentrations of the enzymes equaled 3 nM for heart LDH and 1.5 nM for muscle LDH. One unit of LDH activity (U) represents the amount of the enzyme which converts 1 μ mole of coenzyme per min. under the assay conditions. Each point in the presented plot in Fig. 1 represents a mean \pm SD from three separate experiments performed in duplicate.

Stabilization of LDH in its tetrameric state

Pig heart lactate dehydrogenase was stabilized in its tetrameric state by crosslinking with glutaraldehyde via the method established for pig heart LDH by Gottschalk and Jaenicke [16]. Before the experiments, the stabilized enzyme was dialysed against the appropriate buffer.

Lipids and liposomes

The chloroform solutions of natural bovine phosphatidylserine (PS) and cardiolipin (CL) were obtained commercially from Avanti Polar Lipids, Inc. (USA). The phospholipid concentration was determined via a phosphorus assay [17]. Liposome suspensions were prepared according to the procedure presented in our previous paper [11]. The liposomes were stable under the experimental conditions and during seven-day storage (at 5°C, in the dark). Freshly prepared samples (up to 2 days old) were used for the experiments.

Ultracentrifugation

Centrifugation studies of the adsorption of LDH on PS or CL liposomes were performed using 100 mM MES/NaOH, 0.2 mM EDTA or 100 mM TRIS/HCl, 0.2 mM EDTA buffers of the appropriate pH. The components of each sample were mixed in centrifuge tubes in the sequence established for the experiment, pre-incubated for 5 min. at 25°C and then centrifuged at 105,000 \times g for 30 min. at 5°C. Under these conditions, liposomes and a liposome-protein complex were pelleted while the protein alone (unmodified or stabilized in its tetrameric state) was not. Samples of the supernatants were carefully transferred to separate tubes and the protein and phospholipid contents were determined. The final concentration of LDH in the centrifugation tube was 0.5 μ M. 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, 2 and 6 and each bar in the Figs 4 through 9 represents a mean \pm SD from three separate experiments performed in duplicate.

All the experiments prepared for the observation of the interaction of LDH with acidic liposomes (activity studies, ultracentrifugation studies) were performed in conditions appropriate for interaction (low pH and low ionic strength). However, these conditions were not denaturing for LDH used alone or in the mixture with CL- or PS-liposomes. This was evidenced using the UV circular dichroism method. The obtained spectra were published in our previous paper [11].

RESULTS AND DISCUSSION

Pig heart LDH (H_4 isoform) is inhibited by liposomes made of acidic phospholipids such as phosphatidylserine (PS) or cardiolipin (CL) if the pH of the environment drops below 6.5. The most effective inhibition is observed when the pH value is equal or close to pI of the protein, i.e. 5.5 (Fig. 1).

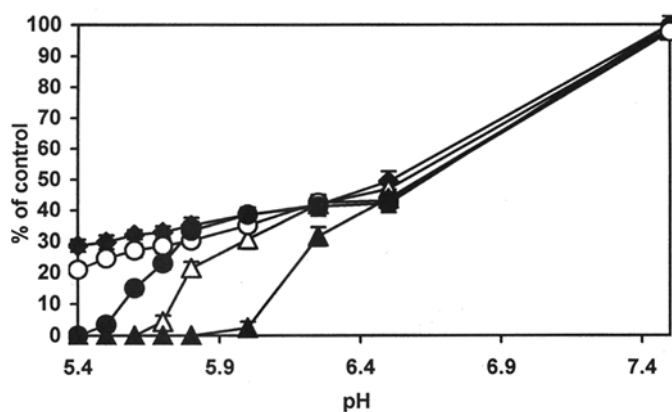


Fig. 1. The pH dependence of the inhibition of pig heart LDH activity by PS and CL liposomes; (◆) enzyme alone, (○) enzyme and 2.5 μ M PS, (●) enzyme and 25 μ M PS, (Δ) enzyme and 2.5 μ M CL, (▲) enzyme and 25 μ M CL. The activity of a sample without any phospholipid at pH 7.5 was used as a control. Buffers used: 100 mM MES/NaOH, 0.2 mM EDTA for pH 5.4-6.5 and TRIS/HCl, 0.2 mM EDTA for pH 7.5. For other details see the Materials and Methods section.

The inhibition of pig muscle LDH (M_4 isoform) by such liposomes is also observed at $\text{pH} < 6.5$, but it requires a lower concentration of acidic phospholipids [11]. Moreover, the pI of the muscle enzyme is equal to 8.6 [2], and thus at low pH, the net charge of the enzyme molecule is positive. This enables the effective interaction with acidic phospholipids bearing a negative net charge at $\text{pH} \geq 3.5$ [14]. The net charge of pig heart LDH at pH 5.5 is close to zero, which should exclude or minimize the electrostatic interaction with acidic phospholipid molecules. However, the adsorption of pig heart enzyme on PS- or CL-liposomes is most efficient at a pH of about 5.5, and CL-liposomes are more effective than PS-liposomes (Fig. 2). This indicates that, as in the case of muscle LDH, the number of potentially ionic groups in an acidic phospholipid molecule

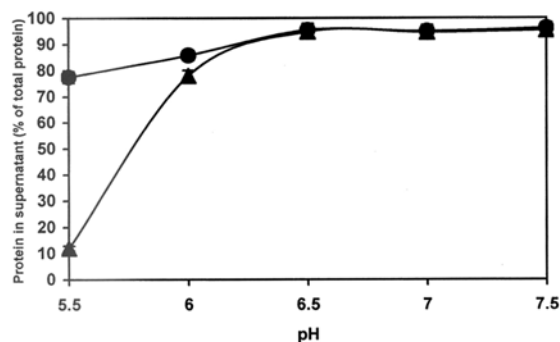


Fig. 2. Centrifugation studies of the adsorption of pig heart LDH on PS (●) or CL (▲) liposomes at different pH values. The LDH concentration was 0.5 μ M and the enzyme:phospholipid molar ratio was 1:150 for PS and 1:100 for CL. Buffers used: 100 mM MES/NaOH, 0.2 mM EDTA for pH 5.5-6.5 and TRIS/HCl, 0.2 mM EDTA for pH 7-7.5. After a 5-min incubation at 25°C, the mixture was centrifuged for 30 min. (105,000 \times g) at 4°C. For other details, see the Materials and Methods section.

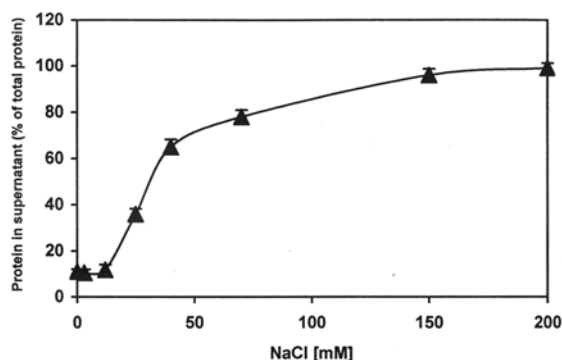


Fig. 3. Centrifugation studies of the adsorption of pig heart LDH on CL liposomes at different NaCl concentrations. 100 mM MES/NaOH, 0.2 mM EDTA, pH 5.5 buffer was used for the experiment. The LDH concentration was 0.5 μ M and the enzyme:phospholipid molar ratio was 1:150. After a 5-min incubation at 25°C, the mixture was centrifuged for 30 min. (105,000 \times g) at 4°C. For other details see the Materials and Methods section.

is important for the interaction [11]. Full dissociation of the pig heart LDH-acidic phospholipid liposome complexes is observed in the presence of NaCl at concentrations \geq 150 mM (Fig. 3), which suggests the binding has an electrostatic nature.

The presented results also indicate that the interaction of pig heart LDH with acidic phospholipids would most likely require the presence of a local structure, bearing a positive net charge at pH < 6.5, on the surface of the enzyme. The value of the charge and/or the availability of the structure for the interaction could be pH dependent, and thus explain the increase of the adsorption on the liposomes and inhibition of the enzyme activity in response to the lowering of the pH of the environment (Figs 1 and 2).

The inhibition of the pig heart LDH activity by PS- or CL-liposomes displays a strong dependence on the sequence of the addition of reagents into the assay sample (data not shown). If pig heart LDH is first incubated with PS- or CL-liposomes, and then the reaction is started by adding a mixture of its substrate and cofactor, inhibition is observed. However, if the sample contains liposomes and substrates and the reaction is started by the adding the enzyme, the effect is not observed. Such a phenomenon is not detected for pig muscle LDH, the inhibition of which does not depend on the sequence of addition of the assay sample elements. The results obtained for pig heart LDH suggest that the interaction of the enzyme with acidic phospholipids is strongly limited or is impossible in the presence of its cofactor and/or substrate. To verify this suggestion, we tested the influence of the sequence of addition of elements into the sample on the adsorption of pig heart LDH on PS- or CL-liposomes using the ultracentrifugation method.

The control samples contained an appropriate buffer and the enzyme (no adsorption) or the buffer, the liposomes and the enzyme (maximal adsorption). Other samples were prepared in two different ways: A) the enzyme was mixed with the sample containing the buffer and cofactor/substrate or other studied substance before the addition of the liposomes; or B) the enzyme was mixed with the sample containing the buffer and liposomes before the addition of the substrate/cofactor or other studied substance. The results of the ultracentrifugation studies of the influence of LDH cofactors/substrates on the adsorption of the enzyme on PS- or CL-liposomes are presented in Figs 4 and 5.

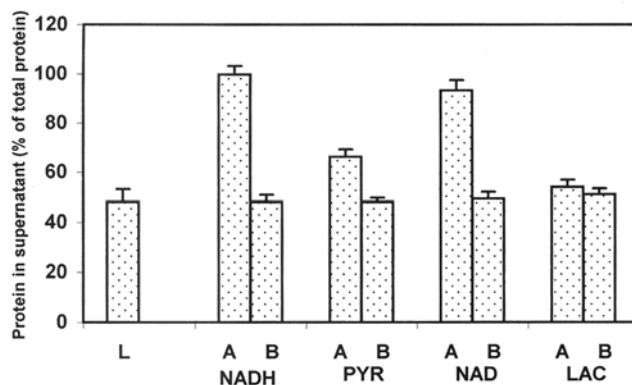


Fig. 4. Centrifugation studies of the adsorption of pig heart LDH on PS-liposomes. The influence of the sequence of addition of the sample components. A – The enzyme was mixed with a sample containing the buffer and a substrate before the addition of the liposomes. B – The enzyme was mixed with a sample containing the buffer and liposomes before the addition of a substrate. L – The control sample, containing the buffer, the liposomes and the enzyme. The concentrations of the cofactors and substrates were: NADH – 0.2 mM, NAD – 0.2 mM, pyruvate – 3.0 mM, and lactate – 3.0 mM. The LDH concentration was 0.5 μ M and the enzyme:PS molar ratio was 1:400. 100 mM MES/NaOH, 0.2 mM EDTA, pH 5.5 buffer was used for the experiment. After a 5-min incubation at 25°C, the mixture was centrifuged for 30 min. (105,000 \times g) at 4°C. For other details, see the Materials and Methods section.

The cofactor and substrate of the reaction catalyzed by the enzyme were used for the experiments at concentrations as in the enzyme assay (0.2 mM for NADH and NAD, 3.0 mM for pyruvate and lactate). The samples containing only pig heart LDH and the phospholipids at molar ratios of 1:400 and 1:150 (for the PS-liposomes and CL-liposomes, respectively) contained about 50% and 10% of the protein in the supernatant after centrifugation (Figs 4 and 5 – L). If the enzyme was incubated with NADH or NAD before the addition of the liposomes, the enzyme-liposome complexes were not formed (Figs 4 and 5 – NADH A) or formed in a low number (Figs 4 and 5 – NAD A). Pyruvate inhibited the complex formation very weakly (Figs 4 and 5 – pyruvate A), and no influence of lactate was observed (Figs 4 and 5 – lactate A). If the sequence of the addition of the sample components was reversed (B variant), the formation of pig heart LDH complexes with the liposomes was not inhibited (Figs 4 and 5 – NADH B and NAD B). The formation of the complexes of pig muscle LDH (M_4 isoform) was independent of the sequence of the addition of the sample elements, and over 90% of the protein was pelleted (data not shown).

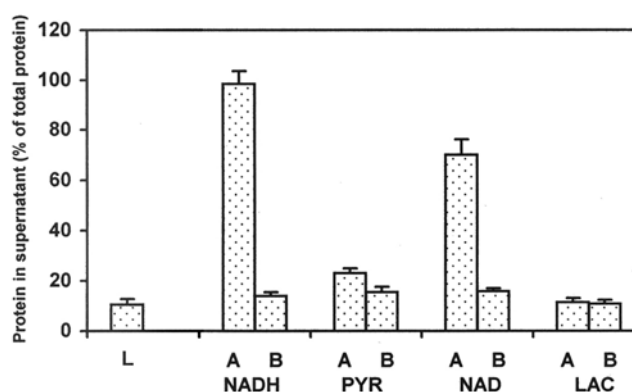


Fig. 5. Centrifugation studies of the adsorption of pig heart LDH on CL-liposomes. The influence of the sequence of addition of the sample components. A – The enzyme was mixed with a sample containing the buffer and a substrate before the addition of the liposomes. B – The enzyme was mixed with a sample containing the buffer and the liposomes before the addition of a substrate. L – The control sample, containing the buffer, the liposomes and the enzyme. The concentrations of cofactors and substrates were: NADH – 0.2 mM, NAD – 0.2 mM, pyruvate – 3.0 mM, and lactate – 3.0 mM. The LDH concentration was 0.5 μ M and the enzyme/CL molar ratio was 1:150. 100 mM MES/NaOH, 0.2 mM EDTA, pH 5.5 buffer was used for the experiment. After a 5-min incubation at 25°C, the mixture was centrifuged for 30 min. (105,000 \times g) at 4°C. For other details, see the Materials and Methods section.

According to the results presented above, the formation of pig heart LDH-liposome complexes is most effectively inhibited by NADH, which is a cofactor of the enzyme. In the conditions of the experiment (0.5 μ M LDH and 75 μ M CL), after preincubation of the enzyme with NADH at concentrations \geq 0.020 mM, less than 20% of the protein was bound to CL-liposomes. Preincubation of the enzyme with the liposomes resulted in the participation of about 90% of the protein in enzyme-liposome complexes. The interaction was stable even in the

presence of 5 mM NADH (Fig. 6A and B). It cannot be excluded that this is the consequence of a very tight to the liposomal surface by this part of LDH molecule, which contains the NADH-binding site that could make it impossible for NADH/NAD molecules to penetrate into the site.

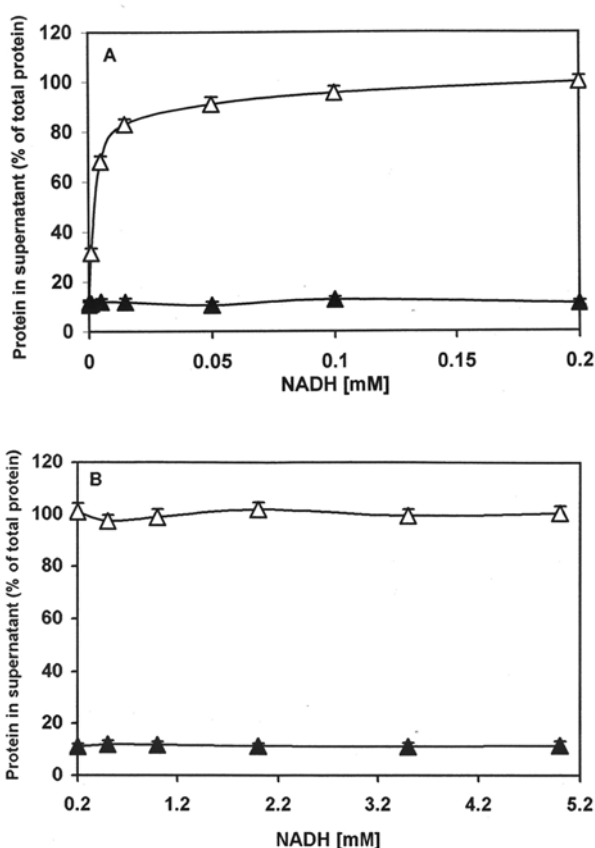


Fig. 6. Centrifugation studies of the adsorption of pig heart LDH on CL-liposomes. The influence of variable concentrations of NADH and the sequence of addition of the sample elements. A – NADH concentrations: 0-0.2 mM. B – NADH concentrations: 0.2-5.0 mM. (Δ) The enzyme was mixed with the samples containing NADH before the addition of the liposomes. (▲) The enzyme was mixed with the samples containing the liposomes before the addition of NADH. The control sample contained the buffer, the liposomes and the enzyme. The LDH concentration was 0.5 μ M and the enzyme:CL molar ratio was 1:150. 100 mM MES/NaOH, 0.2 mM EDTA, pH 5.5 buffer was used for the experiment. After a 5-min incubation at 25°C, the mixture was centrifuged for 30 min. (105,000 \times g) at 4°C. For other details, see the Materials and Methods section.

ATP and ADP (and other nucleotides) are not among the substrates or inhibitors/activators of mammalian LDH, and thus do not influence the pig heart LDH activity (even at low pH conditions). However, each of the two nucleotides used at the same concentration as NADH or NAD (0.2 mM) inhibited complex formation with an effectiveness comparable to NAD (Fig. 7). AMP was much

less efficient (Fig. 7) and GTP, CTP or UTP did not exert any inhibitory effect on the enzyme-liposome complex formation. When pig muscle LDH was used for the experiments, the effect of ATP, ADP or AMP was not observed.

The very effective inhibition of the adsorption of pig heart LDH on CL- or PS-liposomes by NADH suggests that the cofactor-binding site of the enzyme can be strongly involved in the interaction with acidic phospholipids. The results of our experiments with the phosphorylated form of NAD, i.e. NADP, show that the phosphorylation of the coenzyme precludes the inhibition of pig heart LDH-CL-liposome complex formation (Fig. 7). It is well known that NADPH/NADP cannot be a cofactor of LDH and cannot replace NADH/NAD in the catalysis, which would explain their different behaviour. However, the cofactor-binding site, present in each subunit of the LDH tetramer, does not need to be directly responsible for the complex formation. Alternatively, it is possible that binding the cofactor stabilizes the tetrameric structure of the enzyme at low pH, thus preserving the monomer interfaces from the interaction with acidic phospholipids. If the interfacing parts of the LDH monomers were responsible for the binding of these phospholipids, the stabilization of the tetrameric state of the enzyme should preclude the availability of these areas for the interaction with the phospholipid bilayer.

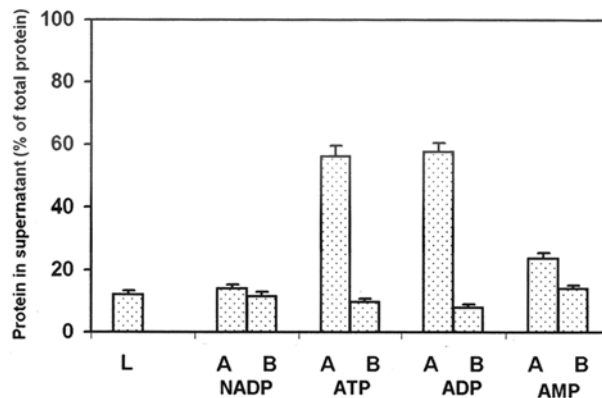


Fig. 7. Centrifugation studies of the adsorption of pig heart LDH on CL-liposomes. The influence of NADP, ATP, ADP, and AMP on the adsorption relative to the sequence of addition of the sample elements. A – The enzyme was mixed with samples containing the studied substance before the addition of the liposomes. B – The enzyme was mixed with samples containing the liposomes before the addition of the studied substance. L – The control sample, containing the buffer, the liposomes and the enzyme. The LDH concentration was 0.5 μ M and the enzyme:CL molar ratio was 1:150. 100 mM MES/NaOH, 0.2 mM EDTA, pH 5.5 buffer was used for the experiment. After a 5-min incubation at 25°C the mixture was centrifuged for 30 min. (105,000 x g) at 4°C. For other details, see the Materials and Methods section.

To resolve this ambiguity, we performed experiments with pig heart LDH that was enzymatically active and tetrameric at low pH. To obtain such a preparation of the enzyme, we stabilized the tetrameric state of the enzyme by crosslinking with glutaraldehyde. This method, established for the heart form of pig LDH by Gottschalk and Jaenicke [16], guarantees the final properties of the enzyme preparation exactly as needed. This stabilized enzyme essentially preserved the behaviour of the unstabilized enzyme and was inhibited by acidic phospholipids depending on the sequence of the addition of the sample components. Ultracentrifugation studies confirmed that in its stable tetrameric structure, the enzyme interacts with acidic phospholipids at low pH in the same manner as its natural, dissociating form (Fig. 8). The L position (the control sample, containing only a buffered mixture of the enzyme and CL-liposomes) in the figure shows that about 85% of the stabilized protein is bound to CL-liposomes, which would be impossible if the interfacing areas of its subunits were responsible for the adsorption. Moreover, as for the unstabilized enzyme, the complex formation depends strongly on the presence of NADH in the sample. When CL-liposomes were added to the sample containing a buffered mixture of the stabilized enzyme and NADH, 98% of the protein remained unbound (Fig. 8 – NADH A). The inhibition of the adsorption of the stabilized pig heart LDH on CL-liposomes by ATP was also observed, but it was not as effective as in the case of the unmodified enzyme (Fig. 8). The results obtained for PS-liposomes and the

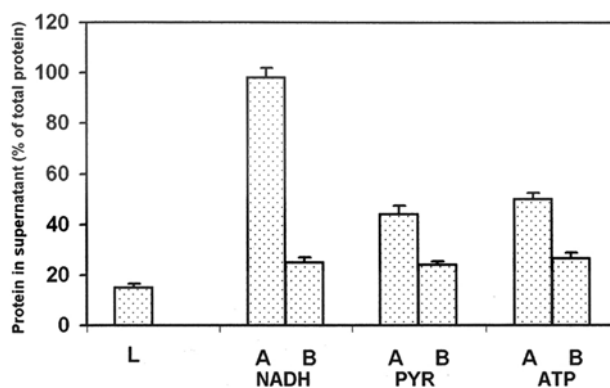


Fig. 8. Centrifugation studies of the adsorption on CL-liposomes of chemically crosslinked pig heart LDH with a tetrameric structure stable (undissociating) at acidic pH. The influence of NADH, pyruvate and ATP on the adsorption relative to the sequence of addition of the sample elements. A – The enzyme was mixed with samples containing the studied substance before the addition of the liposomes. B – The enzyme was mixed with samples containing the liposomes before the addition of the studied substance. L – The control sample containing the buffer, the liposomes and the enzyme. The LDH concentration was 0.5 μ M and the enzyme: CL molar ratio was 1:150. 100 mM MES/NaOH, 0.2 mM EDTA, pH 5.5 buffer was used for the experiment. After a 5-min incubation at 25°C the mixture was centrifuged for 30 min. (105,000 x g) at 4°C. For other details, see the Materials and Methods section.

preparations of stabilized and unmodified pig heart LDH also shared very high similarity (Fig. 9). The data presented above definitely excludes the possibility of the involvement of the subunit interfacing areas in the pig heart LDH interaction with acidic phospholipids and supports the hypothesis that NADH-binding site/sites (one or more per tetrameric molecule) plays/play key role in the interaction. Moreover, the very similar, almost complete inhibition of the interaction of unstabilized and stabilized forms of the enzyme by NADH (Figs 4, 5, 8, 9) could indicate that the availability of the key area for the interaction with acidic phospholipids is rather directly blocked by the coenzyme than indirectly by conformational changes produced by the binding of the cofactor to its binding site.

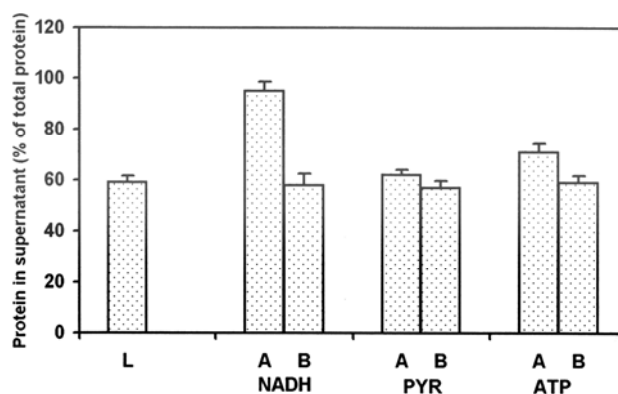


Fig. 9. Centrifugation studies of the adsorption on PS-liposomes of chemically crosslinked pig heart LDH with a tetrameric structure stable (undissociating) at acidic pH. The influence of NADH, pyruvate and ATP on the adsorption relative to the sequence of addition of the sample elements. A – The enzyme was mixed with samples containing the studied substance before the addition of the liposomes. B – The enzyme was mixed with samples containing the liposomes before the addition of the studied substance. L – The control sample, containing the buffer, the liposomes and the enzyme. The LDH concentration was 0.5 μ M and the enzyme:PS molar ratio was 1:400. 100 mM MES/NaOH, 0.2 mM EDTA, pH 5.5 buffer was used for the experiment. After a 5-min incubation at 25°C the mixture was centrifuged for 30 min. (105,000 x g) at 4°C. For other details, see the Materials and Methods section.

In the second case, the stabilization of the enzyme structure with glutaraldehyde would make the changes difficult or impossible, which in consequence would lead to a significant decrease or disappearance of the inhibition of the enzyme interaction with acidic liposomes by NADH. Thus, direct involvement of the coenzyme binding site in the interaction of pig heart LDH with acidic phospholipid seems to be necessary for the interaction. An involvement of the NAD-binding site in an interaction of diphtheria toxin with lipid vesicles was suggested by Cabiaux *et al.* [18] as an explanation of the aggregation and fusion of the vesicles at low pH under influence of the toxin [18, 19].

As we showed, the stabilization of pig heart LDH has no influence on the inhibition by NADH of the enzyme-anionic liposome complex formation, but significantly reduces the analogous influence of ATP (Fig. 8). In our opinion, this could be the evidence that the cofactor binding site does not overlap with the nucleotide binding site. However, if the sites were two independent, acidic phospholipid binding sites located away from one another, the observed almost complete inhibition of the enzyme-acidic liposome complex formation by NADH (Figs 4, 5, 8 and 9) would be impossible. Therefore, we assume that the nucleotide-binding site is located close to the active site of the pig heart LDH. Thus, the area responsible for direct interaction with anionic phospholipids could cover the cofactor binding site and the nucleotide binding site, and be adjacent to substrate-binding pocket, which would explain some of the effectiveness of pyruvate in the inhibition of the enzyme-acidic liposome complex formation (Figs 4, 5, 8 and 9).

The influence of adenine nucleotides and/or their analogues on the activity of lactate dehydrogenases has been documented on a few occasions. Kinetic studies of the inhibition of a D(-)-specific LDH purified from *Butyrivacterium rettgeri* by ATP were performed [20], and the influence of ATP on purified L (+) LDH from the electric organ of *Electrophorus electricus* (L) [21] and regulatory effect of ATP, ADP and AMP on the LDH activity of *Phycomyces blakesleenus* [22] were investigated. Numerous kinetic studies of mammalian M and H lactate dehydrogenases did not reveal any influence of adenine nucleotides on their activity, and this is the probable reason why the presence of an ATP-binding site in their molecules was not suggested.

However, examples of the involvement of such a site in the interaction of some proteins with anionic phospholipids are known and studied. Rat brain hexokinase (ATP: D-hexose 6-phosphotransferase) is inhibited by acidic phospholipids such as phosphatidylinositol, phosphatidylserine and cardiolipin. The effectiveness of ATP in protecting against the inhibition is attributed to direct competition between ATP and the phospholipid for a common binding site [23]. Cytochrome c is a well characterized peripheral membrane protein of the inner mitochondrial membrane, and the presence of an ATP-binding site in its molecule is well established [24]. The protein interacts with lipid monolayers and liposomes containing acidic phospholipids [25-28]. The interaction is mainly electrostatic in nature and is sensitive to changes in ionic strength [29, 30]. The involvement of the ATP-binding site in the interaction of cytochrome c with acidic phospholipids was described by Rytomaa *et al.* [31, 32].

The results of the experiments performed on pig skeletal muscle LDH (M₄-isoform) clearly showed that the enzyme strongly interacts with CL- and PS-liposomes at low pH in the absence and in the presence of NADH/NAD or adenine nucleotides. The sequence of the addition of the sample components had no influence on the results of the ultracentrifugation (data not shown). Such behaviour of the enzyme excludes the possibility of the participation of its cofactor-binding sites in direct interaction with acidic phospholipids. Therefore, we decided to check whether the interfacing areas can play the key role in

complex formation with anionic liposomes in the case of skeletal muscle LDH. The M₄ isoform of LDH from rabbit skeletal muscles was chosen for the experiments because it has been evidenced that this enzyme is stabilized in its tetrameric form at acidic pH (around 5.0) by NADH/NAD coenzyme [33]. The performed experiments proved that there is no difference between the interaction of the two muscle enzymes with acidic phospholipids in the presence and in the absence of the cofactor. Therefore, in our opinion, it is possible that in the case of mammalian LDHs from skeletal muscles (i.e. M₄-isoforms), the area responsible for the interaction with acidic phospholipids is located away from the NADH-binding site and from the interfacing area of the enzyme subunit, and that both the structures are rather not involved in the interaction.

Our studies showed clear differences between the interaction of the H₄ and M₄ isoforms of mammalian LDH with acidic phospholipids at low pH conditions. Their importance for physiological and/or pathological processes in the cell was not studied. However, in the light of the fact that LDH-M₄ is the most widespread isoform of the enzyme in mammalian tissues, while the H₄ isoform dominates in the heart and is present in other tissues together with the M₄ isoform (also in skeletal muscles where LDH-M₄ dominates), our findings indicate that the presence of NADH/NAD and/or ATP/ADP in the direct environment of the enzyme in the cell, even at relatively low concentrations (about 0.2 mM), could protect LDH-H₄ but not LDH-M₄ from interaction with membranes containing acidic phospholipids, mainly with the plasma membrane [34], in the conditions of lowered pH, and in this way change the availability of the isoenzymatic forms in the cell. For example, extreme acidification of the cytosol of skeletal muscle cells takes place during intensive contractions due to the anaerobic conditions [35, 36]. Besides, in physiological conditions, the local pH in cell compartments differs markedly from the measurable bulk pH, and acidification of the environment has been evidenced in the cytosol close to the plasma membrane [37]. Moreover, Zhao *et al.* [38] suggest that PS and other acidic phospholipids could provide a physiological low-pH environment on cellular membranes, strongly influencing proteins interacting with the membranes.

LDH was defined for years as a cytoplasmic protein that was easy to isolate. However, Baba and Sharma [39] also evidenced the presence of LDH in the mitochondria of the rat heart and skeletal muscle using histochemical techniques. Later studies, performed by Kline *et al.* [40] and Brandt *et al.* [41], who employed different techniques, gave similar results.

The importance of the findings is strongly connected with the hypothesis of the intracellular (intramuscular) lactate shuttle being a key element of the idea presented and discussed by Gladden [42], that lactate is an important intermediary in numerous metabolic processes, a particularly mobile fuel for aerobic metabolism, and perhaps a mediator of the redox state among various compartments both within and between cells. Because LDH is the enzyme responsible in the cell for lactate↔pyruvate transformation, the knowledge of the location of the protein in intact cells is one of basic elements of this new and

very important idea. Brooks *et al.* [43] and Hashimoto *et al.* [44] evidenced the localization of LDH on/in the mitochondrial inner membrane and colocalization of the enzyme in the membrane of L6 muscle cells with the lactate-pyruvate transporter MCT1 and CD147, a purported chaperone protein for MCT1 [44]. In the opinion of those authors [44], the results of their experiments allow them to propose the existence of a mitochondrial lactate oxidation complex involving MCT1, CD147, LDH and cytochrome oxidase. Our findings that LDH interacts with cardiolipin with particular efficiency at acidic pH ([11] and this article) very well correspond with at least two well known facts: firstly, that cardiolipin is the main lipid compound of the mitochondrial inner membrane and is present in eukaryotic cells exclusively in the mitochondrial membranes; and secondly, that the electron transport chain, which is exclusively localized in the mitochondrial inner membrane, produces a proton gradient across the membrane resulting in lowered pH in the compartment between the inner and outer membrane. Hashimoto *et al.* [44] indicated the possibility of mitochondrial LDH being anchored to the outer side of the inner mitochondrial membrane, making the enzyme susceptible to loss during isolation. However, in our opinion, the results of our studies ([11] and this paper) could suggest that the binding of LDH to the mitochondrial inner membrane is the result of the interaction with cardiolipin, rather than an anchoring. Changes in pH determined by the intensity of the influx of electrons into the electron transport chain and also changes in the concentrations of adenine nucleotides and nicotinamide adenine dinucleotides in the direct environment of the enzyme could regulate the interaction. Therefore, in our explanation, the high susceptibility of mitochondrial LDH to its loss during the isolation of mitochondria could be the consequence of the increase of pH in the intermembrane space (a deficit of oxygen for the functioning of electron transport chain) and the subsequent dissociation of the enzyme from the inner membrane surface into the intermembrane space and leakage from the mitochondria through the damaged outer membrane.

REFERENCES

1. Aubert, A., Costalat, R., Magistretti, P.J. and Pellerin, L. Brain lactate kinetics: Modeling evidence for neuronal lactate uptake upon activation. **Proc. Natl. Acad. Sci. USA** 102 (2005) 16448-16453.
2. Crawford, R.M., Budas, G.R., Jovanovic, S., Ranki, H.J., Wilson, T.J., Davies, A.M. and Jovanovic, A. M-LDH serves as a sarcolemmal $K_{(ATP)}$ channel subunit essential for cell protection against ischemia. **EMBO J.** 21 (2002) 3936-3948.
3. Pioli, P.A., Hamilton, B.J., Connolly, J.E., Brewer, G. and Rigby, W.F. Lactate dehydrogenase is an AU-rich element-binding protein that directly interacts with AUF1. **J. Biol. Chem.** 277 (2002) 35738-35745.
4. Li, S.S. Lactate dehydrogenase isoenzymes A (muscle), B (heart) and C (testis) of mammals and the genes coding for these enzymes. **Biochem. Soc. Trans.** 2 (1989) 304-307.

5. Li, S.S. Human and mouse lactate dehydrogenase genes A (muscle), B (heart), and C (testis): protein structure, genomic organization, regulation of expression, and molecular evolution. **Prog. Clin. Biol. Res.** 344 (1990) 75-99.
6. Read, J.A., Winter, V.J., Eszes, C.M., Sessions, R.B. and Brady, R.L. Structural basis for altered activity of M- and H-isozyme forms of human lactate dehydrogenase. **Proteins** 43 (2001) 175-185.
7. Wang, X.C., Jiang, L. and Zhou, H.M. Minimal functional unit of lactate dehydrogenase. **J. Protein Chem.** 3 (1997) 227-231.
8. King, L. and Weber, G. Conformational drift of dissociated lactate dehydrogenases. **Biochemistry** 25 (1986) 3632-3637.
9. Dabrowska, A. and Gutowicz, J. Interaction of bovine heart lactate dehydrogenase with erythrocyte lipids. **Biochim. Biophys. Acta** 855 (1986) 99-104.
10. Dabrowska, A., Terlecki, G. and Gutowicz, J. Interaction of bovine skeletal muscle lactate dehydrogenase with liposomes. Comparison with the data for the heart enzyme. **Biochim. Biophys. Acta** 980 (1989) 357-360.
11. Terlecki, G., Czapińska, E., Rogozik, K., Lisowski, M. and Gutowicz, J. Investigation of the interaction of pig muscle lactate dehydrogenase with acidic phospholipids at low pH. **Biochim. Biophys. Acta** 1758 (2006) 133-144.
12. Terlecki, G., Czapińska, E. and Gutowicz, J. The role of lipid phase structure in the interaction of lactate dehydrogenase with phosphatidylserine. Activity studies. **Cell. Mol. Biol. Lett.** 7 (2002) 895-903.
13. Terlecki, G. and Gutowicz, J. Further evidence for the importance of lipid bilayers in the interaction between lactate dehydrogenase and phosphatidylserine. **Cell. Mol. Biol. Lett.** 7 (2002) 905-910.
14. Marsh, D. **Handbook of Lipids Bilayers**, CRC Press, Boca Raton, FL, 1990.
15. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal. Biochem.** 72 (1976) 248-254.
16. Gottschalk, N. and Jaenicke, R. Chemically crosslinked lactate dehydrogenase: stability and reconstitution after glutaraldehyde fixation. **Biotechnology and Applied Biochemistry** 9 (1987) 387-400.
17. 18. Rouser, G., Siakatos, A.N. and Fleischer, S. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. **Lipids** 1 (1966) 85-86.
18. Cabiliaux, V., Vandenbranden, M., Falmagne, P. and Ruyschaert, J.M. Aggregation and fusion of lipid vesicles induced by diphtheria toxin at low pH: possible involvement of the P site and the NAD⁺ binding site. **Biosci. Rep.** 3 (1985) 243-250.
19. Cabiliaux, V., Vandenbranden, M., Falmagne, P. and Ruyschaert, J.M. Diphtheria toxin induces fusion of small unilamellar vesicles at low pH. **Biochim. Biophys. Acta** 775 (1984) 31-36.
20. Wittenberger, C.L. Kinetic studies on the inhibition of a (D-)-specific lactate dehydrogenase by adenosine triphosphate. **J. Biol. Chem.** 243 (1968) 3067-3075.

21. Torres-da Matta, J., Batista e Silva, C. and Hasson-Voloch, A. Effect of ATP on purified L(+) lactate dehydrogenase from electric organ of *Electrophorus electricus* (L.). **Int. J. Biochem.** 18 (1986) 191-194.
22. Busto, F., de Arriaga, D. and Soler, J. ATP, ADP and AMP on the regulation of lactate dehydrogenase activity of *Phycomyces blakesleeanus*. **Int. J. Biochem.** 15 (1983) 73-78.
23. Nemat-Gorgani, M. and Wilson, J.E. Acidic phospholipids may inhibit rat brain hexokinase by interaction at the nucleotide binding site. **Arch. Biochem. Biophys.** 236 (1985) 220-227.
24. Craig, D.B. and Wallace, C.J. ATP binding to cytochrome c diminishes electron flow in the mitochondrial respiratory pathway. **Protein Sci.** 2 (1993) 966-976.
25. Kimelberg, H.K. and Lee, C.P. Binding and electron transfer to cytochrome c in artificial phospholipid membranes. **Biochem. Biophys. Res. Commun.** 34 (1969) 784-790.
26. Vanderkooi, J., Erecinska, M. and Chance, B. Cytochrome c interaction with membranes. II. Comparative study of the interaction of c cytochromes with the mitochondrial membrane. **Arch. Biochem. Biophys.** 152 (1973) 531-540.
27. Mustonen, P., Virtanen, J.A., Somerharju, P.J. and Kinnunen, P.K. Binding of cytochrome c to liposomes as revealed by the quenching of fluorescence from pyrene-labeled phospholipids. **Biochemistry** 26 (1987) 2991-2997.
28. Demel, R.A., Jordi, W., Lambrechts, H., van Damme, H., Hovius, R. and de Kruijff, B. Differential interactions of apo- and holo-cytochrome c with acidic membrane lipids in model systems and the implications for their import into mitochondria. **J. Biol. Chem.** 264 (1989) 3988-3997.
29. Nicholls, P. Cytochrome c binding to enzymes and membranes. **Biochim. Biophys. Acta** 346 (1974) 261-310.
30. Brown, L.R. and Wuthrich, K. NMR and ESR studies of the interactions of cytochrome c with mixed cardiolipin-phosphatidylcholine vesicles. **Biochim. Biophys. Acta** 468 (1977) 389-410.
31. Rytomaa, M., Mustonen, P. and Kinnunen, P.K. Reversible, nonionic, and pH-dependent association of cytochrome c with cardiolipin-phosphatidylcholine liposomes. **J. Biol. Chem.** 267 (1992) 22243-22248.
32. Rytomaa, M. and Kinnunen, P.K. Evidence for two distinct acidic phospholipid-binding sites in cytochrome c. **J. Biol. Chem.** 269 (1994) 1770-1774.
33. Lovell, S.J. and Winzor, D.J. Effects of phosphate on the dissociation and enzymatic stability of rabbit muscle lactate dehydrogenase. **Biochemistry** 13 (1974) 3527-3531.
34. Okeley, N.M. and Gelb, M.H. A designed probe for acidic phospholipids reveals the unique enriched anionic character of the cytosolic face of the mammalian plasma membrane. **J. Biol. Chem.** 279 (2004) 21833-21840.
35. Korzeniewski, B. and Zoladz, J.A. Influence of rapid changes in cytosolic pH on oxidative phosphorylation in skeletal muscle: theoretical studies. **Biochem. J.** 365 (2002) 249-258.
36. Korzeniewski, B. AMP deamination delays muscle acidification during heavy exercise and hypoxia. **J. Biol. Chem.** 281 (2006) 3057-3066.

37. Kraayenhof, R., Sterk, G.J. and Sang, H.W. Probing biomembrane interfacial potential and pH profiles with a new type of float-like fluorophores positioned at varying distance from the membrane surface. **Biochemistry** 32 (1993) 10057-10066.
38. Zhao, H., Tuominen, E.K. and Kinnunen, P.K. Formation of amyloid fibers triggered by phosphatidylserine-containing membranes. **Biochemistry** 43 (2004) 10302-10307.
39. Baba, N. and Sharma, H.M. Histochemistry of lactic dehydrogenase in heart and pectoralis muscles of rat. **J. Cell. Biol.** 51 (1971) 621-635.
40. Kline, E.S., Brandt, R.B., Laux, J.E., Spainhour, S.E., Higgins, E.S., Rogers, K.S., Tinsley, S.B. and Waters, M.G. Localization of L-lactate dehydrogenase in mitochondria. **Arch. Biochem. Biophys.** 246 (1986) 673-680.
41. Brandt, R.B., Laux, J.E., Spainhour, S.E. and Kline, E.S. Lactate dehydrogenase in rat mitochondria. **Arch. Biochem. Biophys.** 259 (1987) 412-422
42. Gladden, LB. Lactate metabolism: a new paradigm for the third millennium. **J. Physiol.** 558 (2004) 5-30.
43. Brooks, G.A., Dubouchaud, H., Brown, M., Sicurello, J.P. and Butz, C.E. Role of mitochondrial lactate dehydrogenase and lactate oxidation in the intracellular lactate shuttle. **Proc. Natl. Acad. Sci. USA** 96 (1999) 1129-1134.
44. Hashimoto, T., Hussien, R. and Brooks, G.A. Colocalization of MCT1, CD147, and LDH in mitochondrial inner membrane of L6 muscle cells: evidence of a mitochondrial lactate oxidation complex. **Am. J. Physiol. Endocrinol. Metab.** 290 (2006) 1237-1244.