PROPERTIES OF LACTATE DEHYDROGENASE IMMOBILIZED IN A LIPID–PROTEIN MATRIX

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1. Introduction

In the important field of organization and functional properties of biological membranes, adsorbed proteins on monolayers of phospholipids are useful model systems for characterizing lipid–protein interactions [1–3]. In such supports, the existence of diffusion limitations can be approached and enzyme kinetics could be followed at the interface [4].

Recent developments in enzyme technology [5,6] make possible a study of the interaction between diffusion and enzyme reaction within a well-defined context, but little attention was paid to the effect of hydrophilic–hydrophobic micro-environment. Introduction of phospholipids in the matrix can constitute a new approach showing the influence of lipidic micro-environment on the enzyme activity. In the present paper we tend to compare catalytic properties of lactate dehydrogenase in solution, immobilized in an albumin membrane and immobilized in a phosphatidyl serine–albumin membrane. Lactate dehydrogenase was chosen for this investigation since it has long been the subject of extensive kinetic data in free [7] and immobilized form [8–10].

2. Materials and methods

Lactate dehydrogenase (Type XII) from rabbit muscle was purchased from Sigma, Phosphatidyl L-serine (bovine brain) was obtained from Koch Light Laboratories and human serum albumin was obtained from CRTS (Lille).

Liposomes were prepared by ultrasonic microdispersion (Branson B_{12} at 30 W during 90 min) of phospholipid in phosphate buffer 0.02 M, pH 6.8.

Membranes with or without liposomes were produced by a previously described method [6–11]. A solution containing human serum albumin (40 $mg.ml^{-1}$) phosphatidyl serine (20 $mg.ml^{-1}$) glutaraldehyde (2 $mg.ml^{-1}$) lactate dehydrogenase (0.2 $mg.ml^{-1}$) and NADH (1 $mg.ml^{-1}$) in 0.02 M phosphate buffer, pH 6.8, was spread on a plane glass surface. After polymerization total insolubilization occurred, and a 50 μm thick membrane was produced.

Lactate dehydrogenase (E.C.1.1.1.27) was assayed at 30°C in 0.05 M phosphate buffer, pH 7.5, containing 1 mM pyruvic acid and 0.5 mM NADH. The activity was measured by the optical density variations at 340 nm continuously recorded through a Beckman DBT Spectrophotometer.

Experimental device used for measuring fluxes has already been described [11]. NADH fluxes are continuously recorded at 340 nm. For electron microscopy, liposomes were negatively stained with Potassium Phosphotungstate (1%) pH 7.2. Ultra-thin sections of membranes were obtained using a previously described method [12]. All the observations were made with a Jeol 100 C electron microscope.

3. Results and discussion

3.1. *Electron microscope examinations*

Ultrastructural organization of sonicated phosphatidyl serine was controlled before their incorporation in albumin membranes. Figure 1a corresponds to a negatively stained preparation showing numerous spherules with homogeneous size. After 5 min sonication a small proportion of the material is present as 1000 Å diameter vesicles, but prolonged sonication

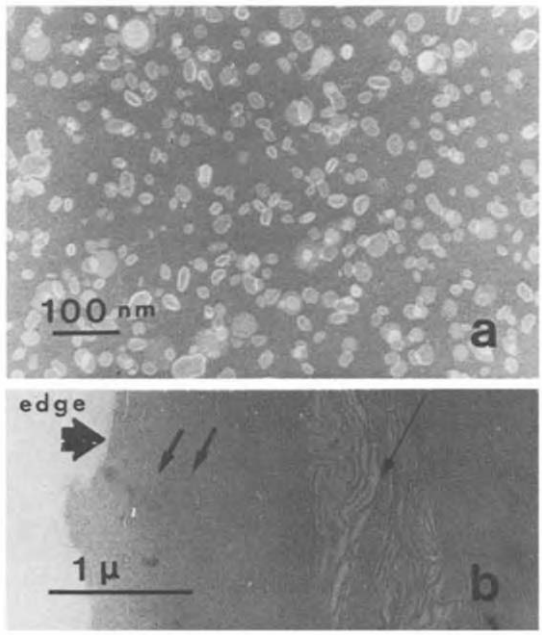

Fig.1. (a) Negative-stain electron microscopy of phosphatidyl serine liposomes observed after 90 min sonication. Samples were prepared as described in Materials and methods. (b) Phosphatidyl serine–albumin membrane ultra-thin section stained with uranyl acetate and lead citrate. Arrows indicate lamellar and multilamellar structures.

(1.5 h) results in a much higher proportions of this material and 300 Å diameter globules. The mechanical properties of artificial membranes are better when realized with liposomes of 200–500 Å diameter. On fig.1b the thin section micrograph shows lamellar and multilamellar organization of a phosphatidyl serine–albumin membrane. Hydrophobic interactions between phospholipid and serum albumin [13,14] can explain such structures. However unsticking appearance is probably due to ethanol dehydration during the specimen treatment, measurement of fluxes giving evidence for the absence of holes in such membranes.

3.2. *Lactate dehydrogenase (LDH) properties*

3.2.1. Stability of LDH activity

Figure 2 shows as previously described [6], that insolubilization stabilizes the enzyme activity. However the crosslinked LDH in a phosphatidyl serine–albumin membrane is better protected against denaturation than in a classical proteic membrane. In

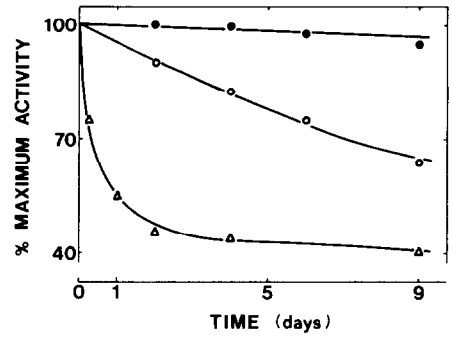

Fig.2. Effect of the insolubilization of lactate dehydrogenase on the stability of the enzyme as a function of time. Between experiments, samples were stored at 4°C, membranes being kept in distilled water and native enzyme in 0.05 M phosphate buffer, pH 7.5. LDH in phosphatidyl serine–albumin membrane (•–•), LDH in albumin membrane (○–○), native LDH (△–△).

both cases no leakage of enzyme has been observed. The activity measured after binding per mg of LDH compared to initial activity in the bulk solution per mg of LDH can be relatively low, but this yield may increase to 12% (with phosphatidyl serine) or 5% (without phosphatidyl serine) by introduction, before immobilization, of NADH as protector [15]. The protection to inhibition after immobilization was already described [11].

3.2.2. pH activity behaviour

The data presented in fig.3 for pyruvate ⟶ lactate reaction shows a broadening of the curve in the case of immobilized enzyme. LDH in a membrane, with or without liposomes, is less sensitive to the effect of pH variations than in the bulk solution. For basic pH it seems that the protection is higher with phosphatidyl serine. All the results were obtained with immobilization at pH 6.8. Negligible shift in the pH activity curve is reported in this case.

3.2.3. Determination of K'_m

To assess the effect of insolubilization on the LDH behavior K'_m values were determined, the results are summarized in table 1. Due to co-crosslinking reaction during immobilization and diffusion limitations during enzymatic reaction, Michaelis constants determined for immobilized enzymes are necessarily only apparent constants (K'_m). For pyruvate, the K'_m of immobiliz-

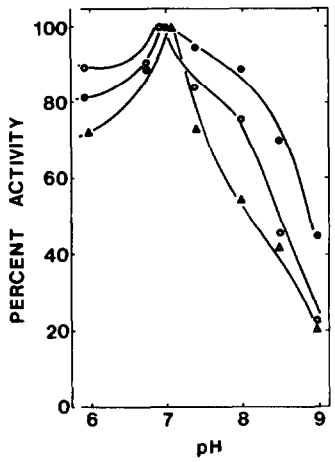

Fig.3 Effect of variation of pH on LDH activity. Results are given for LDH in phosphatidyl serine–albumin membrane (•–•), LDH in albumin membrane (○–○), free LDH (△–△).

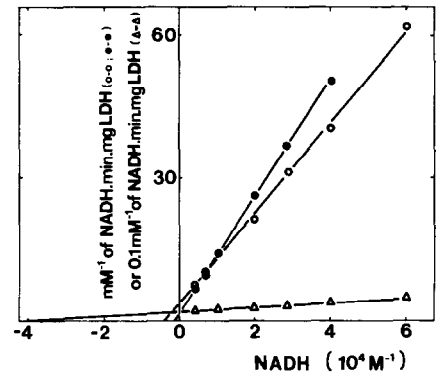

Fig.4. Activity of LDH as a function of NADH concentrations (with pyruvate 1 mM). Plot of $1/V$ versus 1/(NADH) for determination of V_m and apparent K_m (see table 1). LDH in phosphatidyl serine–albumin membrane (•–•), LDH in albumin membrane (○–○), native LDH (△–△).

ed LDH is slightly smaller than the K_m of native water-soluble enzyme. Such results were already observed by Levi [10] with a Sepharose-bound muscle LDH. On the other hand, for NADH, there is an increasing of K'_m values in the case of membrane kinetics (fig.4). This kind of variation has been observed with or without phosphatidyl serine liposomes inside the membrane, suggesting that the apparent change in catalytic properties of immobilized LDH does not depend entirely on the nature of the matrix. For NADH, the increasing of K'_m is higher in the case of phosphatidyl serine membrane, may be due to diffusion limitations.

3.2.4. Determination of true K_m values of the insolubilized LDH

As already described [16] it was possible using a diffusion reaction system to measure experimentally the true value of K_m. This value characterizes the reaction kinetics without the diffusion terms. It is expected to be similar to that of the soluble enzyme. Equation (1) which was established in [16] links

Table 1

	K'_m Pyruvate (0.5 mM NADH)	K_m Pyruvate	K'_m NADH (1 mM Pyruvate)	K_m NADH	Maximum activity (μM NADH min.$^{-1}$ mg^{-1})
Native LDH		4×10^{-4} M	–	2.5×10^{-5} M	~ 5000
Matrix-bound LDH with phosphatidyl serine	1.6×10^{-4} M	not measured	8×10^{-4} M	~2 $\times 10^{-5}$ M[a]	~ 600
Matrix-bound LDH without phosphatidyl serine	1.2×10^{-4} M	not measured	2.5×10^{-4} M	~2 $\times 10^{-5}$ M[a]	~ 250

[a]see text § 3.2.4.

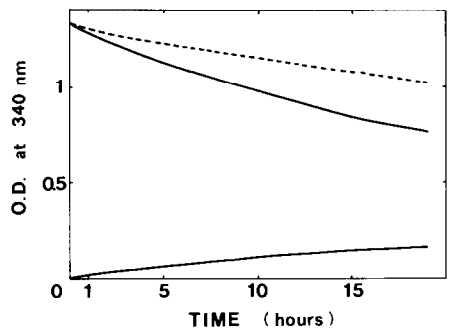

Fig.5. Continuously recorded optical density of NADH of donor and receptor compartments of a diffusion cell as a function of time for a phosphatidyl serine–albumin membrane bearing LDH (——), in presence of pyruvate. Simple diffusion, without enzyme activity is indicated (- - - -).

the true K_m value of the bound enzyme to measurable parameters

$$J_1^2 - J_2^2 = K_2 E_0 D^*$$

$$[(S_1 - S_2) - K_m \log \frac{S_1 + K_m}{S_2 - K_m}] \qquad (1).$$

With J_1 and J_2, ingoing and outgoing fluxes of substrate (S). K_2E_0, enzyme activity. D^*, mean effective diffusion coefficient.

An experimental illustration is given on fig.5 for NADH diffusion in a phosphatidyl serine–albumin membrane. By such measurements true K_m values have been calculated and are given on table 1. The affinity of LDH for NADH remains therefore unchanged after immobilization with or without liposomes.

4. Conclusion

Mechanical properties and LDH activity are better for phosphatidyl serine–albumin membranes than those described for phosphatidyl inositol–cytochrome *c* membrane bearing LDH [17]. We have shown that when LDH is immobilized in presence of its cofactor and phosphatidyl serine liposomes, the enzyme is highly stabilized. Use of such membranes as electrochemical sensors could offer some advantages from the stability point of view, for continuous regeneration of cofactors [15].

Our results are in good agreement with the fact that enzyme liposomes have a very high degree of stability [18]. This paper deals also with the modulation of an enzymatic activity due to the introduction of phospholipids. Interaction studies of lipids and some mitochondrial–NAD linked dehydrogenases have been described [18–22] for explaining effects of lipids should be extensively tested with our matrix and histochemical methods can be applied [12] for the localization of enzyme activity.

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