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Effect of NAD coenzyme on the inactivation of glyceraldehyde-3-phosphate dehydrogenase by anionic phospholipids

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The inactivation of bovine heart glyceraldehyde-3-phosphate dehydrogenase by phosphatidylinositol (PI) and phosphatidylserine (PS) in the form of liposomes was investigated in the presence and absence of NAD excess. In the absence of NAD, the enzyme activity decreased to about 50% of its initial value at 0.6 mM PI and 0.8 mM PS (lipid-to-protein molar ratio 600 and 800, respectively). In the same lipid concentration range almost full regainment of the activity was observed in the presence of 80 μM NAD. It was shown that the excess of NAD protects the enzyme against conformational change induced by the phospholipids. Centrifugation experiments showed that both PI and PS bind significant amounts of NAD.

Glyceraldehyde-3-phosphate dehydrogenase; Phosphatidylinositol; Phosphatidylserine; Lipid-protein interaction

1. INTRODUCTION

The interaction of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPD) with negatively charged liposomes prepared from natural phospholipids decreases its enzymatic activity [1,2]. This process is associated with conformational change of the enzyme [3]. Since GAPD is known as membrane bound protein [4,5], the question arises about a possible participation of membrane components in the regulatory processes of cells. However, until now little is known about the molecular mechanism of the inactivation. Conformational change of the enzyme may result from several different processes, i.e. inductive effect of the phospholipid membrane, dissociation of NAD coenzyme from the protein, binding of single molecules of a lipid component to the protein, etc.

Preliminary experiments showed that an excess of NAD protects GAPD against inactivation by phosphatidylinositol (PI), which was shown to be the most effective inhibitor of the enzyme among other phospholipids [2]. It was also possible to regain a part of the activity after subsequent addition of NAD to the lipid-enzyme mixture. This result suggested that the inactivation of GAPD by phospholipids may be, at least partially, caused by displacement of a part of NAD from the enzyme. GAPD binds 4 NAD molecules with different dissociation constants: the first two are bound with $K_d < 0.05$ μM, while the third and fourth are bound with $K_d = 4$ and 35 μM, respectively [6,7]. It seems likely that adsorption of GAPD on the liposomes is followed by dissociation of the two weakly bound NAD molecules and their binding to the lipid bilayer. Since all four NAD binding sites in GAPD are catalytically active [8], the above process leads to a decrease of the activity, which should be fully restored in the presence of excess NAD. Alternatively, in case of competition for the same site between NAD and a lipid component, at least partially irreversible inactivation should be expected.

In the present paper we show the effect of NAD on reversible inactivation of bovine heart GAPD by two acidic phospholipids of the same origin (bovine brain): phosphatidylserine (PS) and phosphatidylinositol (PI). We present also evidence for the binding of NAD with the phospholipids.

2. MATERIALS AND METHODS

Bovine heart GAPD (sp. act. 80 U/mg at 25°C) was isolated and purified according to Siemieniewski et al. [9]. Its $A_{280}:A_{260}$ ratio was found to be 1.18, which corresponds to about 2.8 mol of NAD bound with one mole of the protein [7]. Glycerol-3-phosphate, NAD and NADH were purchased from Boehringer (FRG). Phosphoglycerate kinase and ATP (disodium salt) were from Polish Chemical Reagents. Other common chemicals used in the assay procedure were of analytical grade. PI (Folch fraction I) and PS were purchased from Koch-Light Laboratories (UK) and Sigma Chemical Co. (USA), respectively.

Phospholipids were suspended in 10 mM Tris-HCl buffer, pH 7.6 (unless specified otherwise) and sonicated over 10 min (PI), or 20 min

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Abbreviations: GAPD, glyceraldehyde-3-phosphate dehydrogenase; PI, phosphatidylinositol; PS, phosphatidylserine; NAD, nicotineamide adenine dinucleotide; ATP, adenosine-5'-triphosphate; OPA, *o*-phthaldialdehyde

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(PS), using MSE 150 W sonifier. Then the suspension was centrifuged for 45 min at 12500×g to remove large lipid aggregates. As follows from electron micrographs and literature data [10], such prepared dispersions contain mainly single shelled vesicles with a mean diameter of about 25 nm. Phospholipid concentration in the suspensions was estimated by phosphorus determination according to Bartlett [11].

The lipid suspensions were mixed with a solution of GAPD and NAD in the buffer and incubated for 30 min before the enzyme activity was measured. Final concentrations in the mixtures were as follows: GAPD 1 μM, NAD 0, 40 or 80 μM, and phospholipid 0.05–0.8 mM. Specific activity of GAPD was determined at 25°C, using glycerol-3-phosphate as substrate and 3-phosphoglycerate kinase as a coupling enzyme. In a volume of 3 ml of the buffer the substrate concentrations were (in mM): glycerol-3-phosphate 6.2, NADH 0.2, ATP 1.1, $MgCl_2$ 2.0 and 3-phosphoglycerate kinase 26 U/mg. The reaction was started by addition of 0.3–0.4 μg GAPD with or without phospholipid and followed by the measurement of absorption decrease at 340 nm. Concentration of GAPD was determined by measuring absorbance at 280 nm, using $E_{280}^{0.1\%} = 1.0$ as an extinction coefficient [12]. Fluorescence labeling of GAPD by the reaction with OPA was performed in 10 mM triethanolamine buffer, pH 8.0, as described elsewhere [3].

To measure binding of NAD to the liposomes, various amounts of unsonicated lipid suspensions were mixed with a solution of NAD in the buffer (final concentration 30 μM), stored overnight at room temperature and then centrifuged for 60 min at 100000×g, using Janetzky 602-1 centrifuge. The same lipid suspensions without NAD were centrifuged simultaneously and used as a control. NAD was determined in the supernatant by measuring difference of absorption at 260 nm (absorption of NAD + lipid minus absorption of the control), using the molar extinction coefficient of 17.8×10^3 $M^{-1} \cdot cm^{-1}$ [7].

The spectrophotometric measurements were done on SPECORD-UV-vis spectrophotometer (Karl Zeiss Jena). Fluorescence emission spectra were measured on a Perkin-Elmer MPF-3 spectrofluorimeter.

3. RESULTS AND DISCUSSION

Fig. 1 presents the inactivation of GAPD by PI and PS without NAD excess (full symbols) and in the presence of 80 μM NAD (open symbols) as a function of the lipid concentration. The initial enzyme activity (80 U/mg) was taken as 100% and the other results were related to this value. As can be seen, in the absence of NAD the specific activity decreases progressively with increasing concentration of the phospholipids. At 0.6 mM PI and 0.8 mM PS (lipid/protein molar ratio 600 and 800, respectively) the activity drops to about 50% of its initial value and the curves tend to show a saturation effect. This effect was not observed in case of the rabbit muscle enzyme, which was completely inactivated by the same phospholipids [2]. Addition of NAD either to the lipid/protein mixture or together with the lipid leads to large regainment of the activity. In the presence of 80 μM NAD almost full protection of the activity was attained up to 0.8 mM PS and 0.4 mM PI. Addition of 40 μM NAD allows to protect ca 80% activity at the same lipid concentration range (not shown). The incomplete inhibition of GAPD in absence of NAD as well as full regainment of the activity in the presence of NAD excess seem to support the proposed above

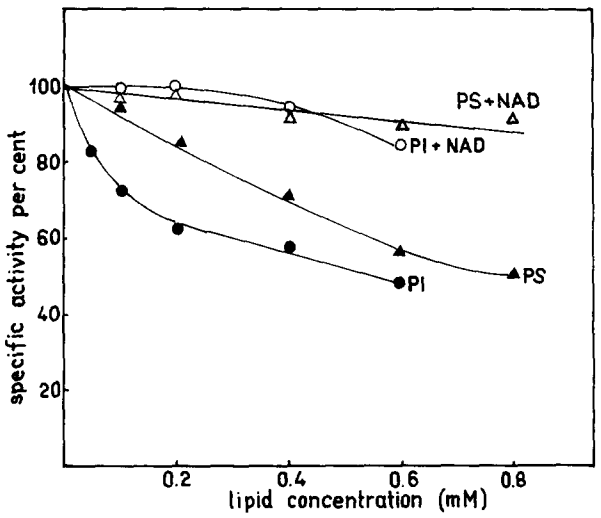

Fig. 1. Inactivation of GAPD by PS (△ ▲) and PI (○ ●) liposomes without NAD (full symbols) and in the presence of 80 μM NAD (open symbols). GAPD concentration: 1.0 μM in 10 mM Tris-HCl buffer, pH 7.6.

mechanism of the inactivation, i.e. dissociation of the weakly bound coenzyme molecules from the protein.

The question arises about a possible effect of NAD excess on conformational change of GAPD, which takes place in the presence of phospholipids. The conformational change of rabbit muscle GAPD induced by PI was clearly demonstrated by investigation of fluorescence quenching of the protein-bound isoindole probe by the phospholipid [3]. The covalently bound fluorophore, obtained by reaction of protein amino group with OPA, is an acceptor of the excitation energy

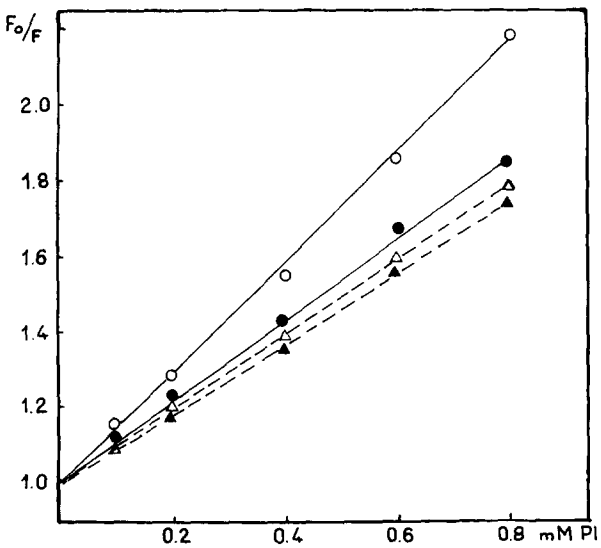

Fig. 2. Fluorescence quenching of the isoindole probe in GAPD by PI without NAD (open symbols) and in the presence of 80 μM NAD (full symbols). The probe was excited directly (340 nm, dashed plots) and at 290 nm (full plots). $\lambda_{em} = 445$ nm. F_o,F = fluorescence intensities in absence and presence of PI, respectively. GAPD concentration: 1 μM in 10 mM triethanolamine buffer, pH 8.0.

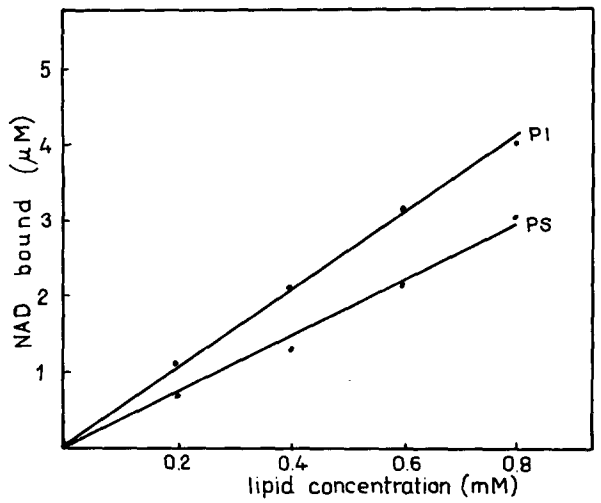

Fig. 3. Binding of NAD to PS and PI liposomes as determined by centrifugation of the lipid suspensions from a solution of 30 μM NAD in Tris-HCl buffer, pH 7.6.

transferred from the tryptophanyl residue of the enzyme. Direct excitation of the probe ($\lambda_{ex}=340$ nm) resulted in minor fluorescence quenching by PI in comparison with the quenching measured after excitation of the energy donor ($\lambda_{ex}=290$ nm). The difference in quenching was interpreted as a result of reduction of the energy transfer efficiency caused by an increase of donor/acceptor distance [3].

We performed a similar experiment in the presence and absence of 80 μM NAD to show its effect on the conformational change. The result is presented in Fig. 2 in the form of Stern-Volmer plots. As can be seen, direct excitation of the isoindole probe bound to GAPD ($\lambda_{ex}=340$ nm; dashed plots) results in similar fluorescence quenching by PI regardless of the presence or absence of NAD. On the other hand, excitation of the protein tryptophan ($\lambda_{ex}=290$ nm; full plots) results in a significantly smaller quenching in the presence of NAD (full symbols). Assuming that the additional quenching of the fluorescence excited at 290 nm is a result of reduction in the energy transfer efficiency, one can conclude that the excess of NAD protects the enzyme against conformational change induced by the phospholipid.

The next important question was whether the protective effect of NAD results from its interaction with the enzyme or with the phospholipid. Binding of NAD to PS and PI was determined directly by centrifugation of the lipid suspensions from a solution of NAD, as described in the experimental section. The results shown in Fig. 3, which represent the mean values of at least 3 replicates, indicate that the binding is proportional to the lipid concentration. At the concentrations applied in the activity measurements (0.2 to 0.8 mM) the amounts of NAD bound to the liposomes varied from 0.6 yo 3 μM for PS and from 1.1 to 4 μM for PI, which gives ca 200 and 270 lipid molecules per one NAD molecule, respectively. There is correlation between the binding and the enzyme inactivation - both are somewhat more effective for PI than for PS. Recent studies indicated that the binding of NAD to PS affects physical properties of the lipid bilayer - an increase of phase transition temperature and change of surface potential were observed (to be published).

It is well known that dissociation of the weakly bound NAD molecules from GAPD is followed by conformational change [13,14] and partial inactivation [6,8] of the enzyme. The general conclusion which can be drawn from the results presented here is that the binding of NAD coenzyme to the phospholipid membranes may be responsible for partial inactivation of GAPD. Since the enzyme belongs to peripheral membrane proteins [4,5], the interaction of NAD with phospholipids may have important biological implications.

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