

Temperature Studies of Glyceraldehyde-3-Phosphate Dehydrogenase Binding to Liposomes Using Fluorescence Technique

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Abstract. Interaction of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase with negatively charged liposomes was investigated as a function of temperature. This interaction affects the temperature-dependent conformational transition in the enzyme and exerts stabilizing effect on the protein structure. It can be seen from the fluorescence quenching experiments that the accessibility of tryptophanyl residues and isoindol probe fluorophores (covalently bound with the protein amino groups) for a dynamic quencher, acrylamide, is altered upon binding. This accessibility represented by effective quenching constant (K_{eff}) strongly depends on temperature for unmodified enzyme and for the enzyme adsorbed on liposomes, it is nearly constant over a wide range of temperatures.

Key words: Glyceraldehyde-3-phosphate dehydrogenase — Lipid vesicles — Fluorescent probe — Fluorescence quenching — Enzyme-lipid interaction

Introduction

Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) has been the most reported glycolytic enzyme bound to cellular membranes (see for review Steck 1974; Salhany and Gaines 1981). In erythrocytes it is bound to plasma membrane likely via one of the intrinsic protein, band 3 protein (Yu and Steck 1975; Kliman and Steck 1980). Since the fragment of the band 3 protein being the binding site for glyceraldehyde 3-phosphate dehydrogenase is strongly negatively charged at physiological pH and the binding is very sensitive to pH and ionic strength, it is generally believed that the binding is controlled by electrostatic interactions.

The binding of glycolytic enzymes to membranes has been found to usually affect their kinetic properties and this may play an important role in the regulatory processes in glycolysis.

In our laboratory phospholipid liposomes with well defined surface charge have

been used as a useful model for studying the interaction of some glycolytic enzymes with membranes. The simplicity of this adsorption system allows to apply the fluorescence method to investigate the mechanism of the binding and the influence of binding on some properties of the enzyme. Lipid monolayers and liposomes have been known to be able to adsorb glyceraldehyde-3-phosphate dehydrogenase and to modify its specific activity and conformation (Wooster and Wrigglesworth 1976a, b, Gutowicz and Modrzycka 1978, 1986, Michalak et al 1987).

In the present paper results of our investigation of glyceraldehyde-3-phosphate dehydrogenase association with phosphatidylinositol (PI) liposomes as a function of temperature are presented. The association was investigated by measurements of tryptophanyl intrinsic fluorescence and isoindole probe fluorescence as well as using fluorescence quenching of both kinds of fluorophores in the protein by a dynamic quencher, acrylamide.

Materials and Methods

Materials

Glyceraldehyde-3-phosphate dehydrogenase was prepared from rabbit skeletal muscle according to Kochman and Rutter (1968). Enzyme concentration was determined using $E_{280}^{0.1\%} = 1$ as absorbance coefficient (Dandliker and Fox 1955). Specific activity of glyceraldehyde-3-phosphate dehydrogenase was determined by measurements of NADH absorbance at 340 nm as a function of time (Velick 1955).

Bovine brain phosphatidylinositol (PI) was purchased from Koch-Light Laboratories. Its purity was checked by TLC. *o*-phthaldialdehyde, 2-mercaptoethanol and acrylamide were purchased from Sigma, Fluka and Bruxelles-r c b (Belgium), respectively. Other chemicals of analytical grade were purchased from POCH (Poland).

Vesicle preparation

Phospholipid liposomes were prepared by mechanical shaking of phospholipid suspension in 50 mmol/l triethanolamine buffer, pH 8.6, for 30 min. Then, the suspension was centrifuged at $12,500 \times g$ for 45 min to remove large lipid aggregates. The phospholipid concentration was estimated by phosphorus determination according to Bartlett (1959). This procedure produces phospholipid vesicles since in all our experiments the lipid concentration was higher than critical micellar concentration which does not exceed 10^{-10} mol/l for phospholipids (Kagava 1985).

Protein labelling

Accessible amino groups of the enzyme were fluorescently labelled by their reaction with *o*-phthaldialdehyde in the presence of 2-mercaptoethanol. This procedure for labelling of phosphatidylserine amino group was described in details elsewhere (Sidorowicz and Michalak 1984). As a result of this procedure, highly fluorescent isoindole derivatives were formed (Simons and Johnson 1978, Chang and Huang 1979). Covalent binding of the isoindole fluorophore with G3PDH results in a new absorption band with a maximum at 337 nm as well as a new fluorescence band with emission maximum at 445 nm, the

labelling does not strongly alter enzyme activity (Michalak et al. 1987). During the labelling 4–6 moles of the probe per mole of the enzyme were not exceeded. Decrease in enzyme activity did not exceed 14% at this degree of labelling.

Fluorescence measurements

Samples of the enzyme were incubated with phospholipid liposomes for at least 30 min. at room temperature before fluorescence spectra or polarization degree were taken of. These measurements were performed with a Perkin Elmer MPF-3L spectrofluorimeter equipped with a thermostatic device. Temperature of the samples was monitored by a digital thermometer with a platinum resistance sensor immersed in cuvettes. Excitation at 290 nm and 340 nm for the tryptophanyl and the probe fluorophores, respectively, was used. In all experiments the bandwidth of 7 nm was applied for both excitation and emission monochromators. The degree of fluorescence polarization was measured at 20°C. Some weak turbidity of PI liposomes yielded a small background in the fluorescence spectrum at the concentrations used. This background was deducted from the spectra. Changes in the background with increasing temperature were negligible.

Results

The intrinsic fluorescence intensity for the unlabelled enzyme excited at 290 nm was measured as a function of temperature in the presence and absence of PI liposomes. In the absence of the lipid, fluorescence intensity of the protein gradually decreased with the increasing temperature, up to approx. 39°C. At this point, the samples became turbid and an artificial increase in fluorescence intensity was observed, probably due to light scattering (Fig. 1). With the enzyme incubated with liposomes (lipid/protein molar ratio 1 : 200), no turbidity was observed up to 60°C (Fig. 1). Similar results were obtained with the fluorescence of the isoindole probe bound to protein molecule followed as a function of temperature (data not shown).

Acrylamide, the well known dynamic quencher (Eftink and Ghiron 1976a) was used as a probe for mean exposure of tryptophanyl residues and of isoindole bound to the surface amino groups of the enzyme. Stern-Volmer plots of fluorescence quenching for the two kinds of fluorophores (free and adsorbed enzyme) are shown in Fig. 2. Adsorption of G3PDH to liposomes results in enhanced quenching of tryptophanyl residues fluorescence and in a strong decrease of quenching of the probe fluorescence. Under conditions of the present experiments, the Stern-Volmer plots for the two types of fluorophores are not linear. Fluorescence quenching data can be analyzed by the modified Stern-Volmer equation:

$$F_0/F = (1 + K_{sv}[Q]) \exp\{V \times [Q]\},$$

where $[Q]$ is the quencher concentration, F and F_0 are the fluorescence emission intensities in the presence and in the absence of the quencher, respectively, K_{sv}

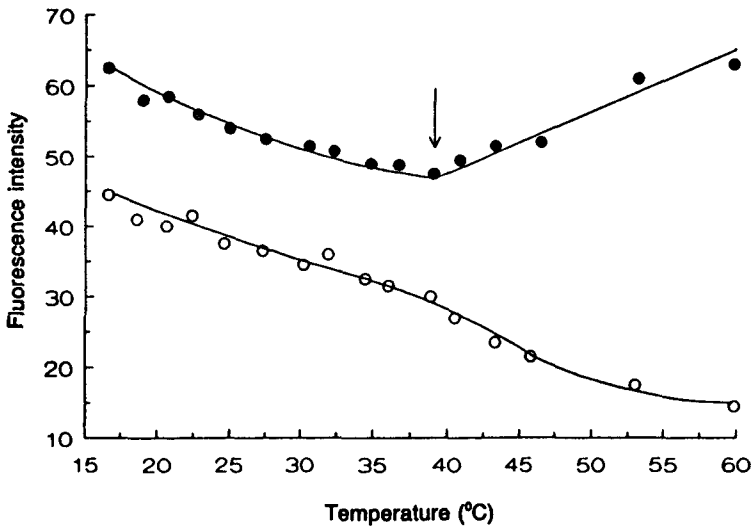


Figure 1. Temperature dependence of the enzyme tryptophanyl fluorescence intensity at λ_{\max} in the absence (—●—) and in the presence (—○—) of phospholipid vesicles. Lipid/protein molar ratio 1 : 200. The arrow indicates temperature at which the samples became turbid.

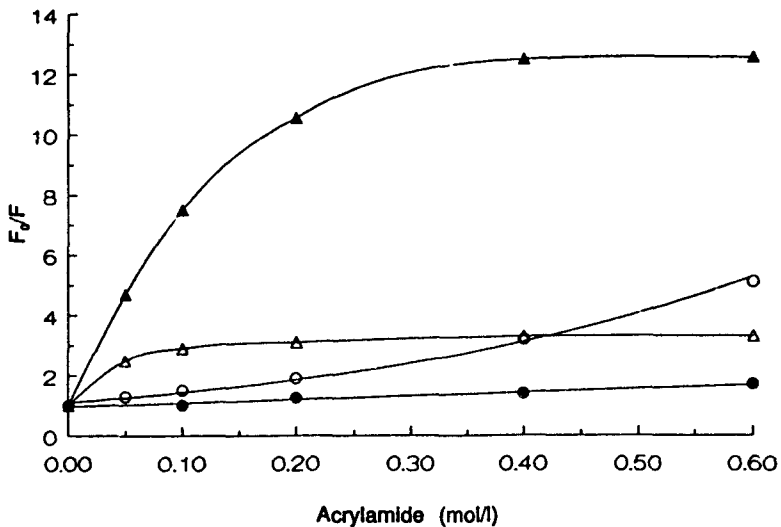
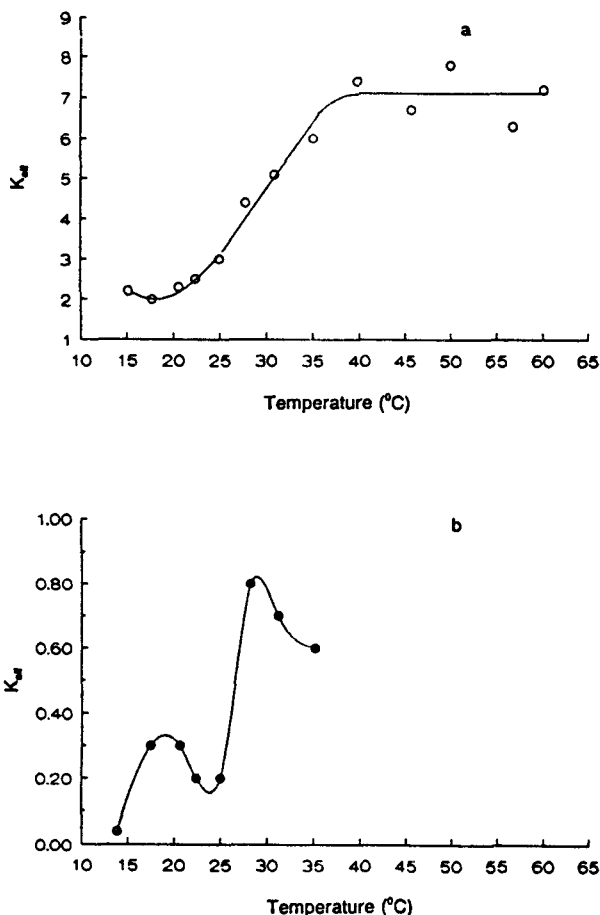


Figure 2. Stern-Volmer plots of quenching of the tryptophan (—●—, —○—) and the probe (—▲—, —△—) fluorescence by acrylamide at 20°C. Filled symbols indicate the absence of PI liposomes. F_0 , F - fluorescence intensity in the absence and in the presence of the quencher, respectively.

is dynamic quenching constant, and V is static quenching constant. For proteins containing more than one tryptophan residue in a molecule, fluorescence emission is heterogenous and analysis of quenching data with the above equation is not feasible. Then, initial slope of F_0/F as a function of $[Q]$ can be taken as effective quenching constant (K_{eff}); this was also done in our calculations. Using the nonionic quencher, acrylamide, the exposure of the fluorophores can be monitored, since the effective quenching constant is a good measure of the accessibility of the fluorophores for the quencher (Eftink and Ghiron 1976b).

Figure 3. Dependence of the effective quenching constant K_{eff} on temperature for the enzyme tryptophanyl fluorescence quenching by acrylamide in the presence (a) and absence (b) of the liposomes. Lipid/protein molar ratio 1 : 200.



To determine the influence of temperature on conformational states on the liposome-adsorbed and nonadsorbed enzyme, quenching of tryptophan and of the probe fluorescence by acrylamide was studied as a function of temperature within

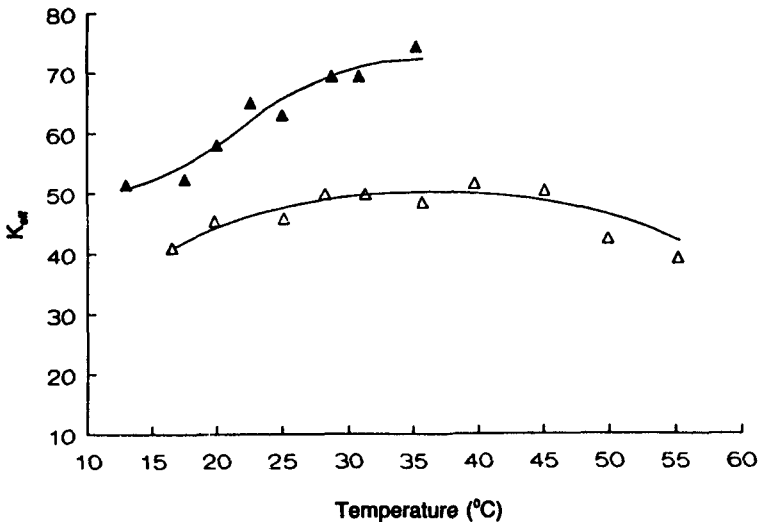


Figure 4. Dependence of the effective quenching constant K_{eff} on temperature for the probe fluorescence quenching by acrylamide in the presence (—▲—) and absence (—Δ—) of liposomes Lipid/protein molar ratio 1 : 200

14–60°C K_{eff} was calculated from initial slopes of the Stern-Volmer plots for each temperature value, and the data are shown in Figures 3 and 4. For intrinsic fluorescence quenching, within the range of the temperatures studied, K_{eff} values are much higher for the adsorbed than for the nonadsorbed enzyme (Fig 3). In both cases, quenching was enhanced with the increasing temperature, with a rather sharp increase for the enzyme alone around 25°C. Samples of unbound enzyme became turbid at 39°C and the experiment was not continued for higher temperatures. With the enzyme in the presence of liposomes, the samples were more stable at higher temperatures and K_{eff} values were almost constant within 40–60°C. Quenching of the extrinsic probe was stronger for unbound enzyme. It gradually increased with the increasing temperature up to a point where turbidity appeared (Fig 4). Contrary to the unbound enzyme, no significant changes in K_{eff} are observed for the enzyme interacting with liposomes in the temperature range studied (15–55°C). The interaction of G3PDH with liposomes also affects some spectroscopic properties of the isoindole probe covalently bound with the enzyme. The wavelength of the emission maximum (λ_{max}) was shifted from 445 nm in the absence of lipids to 432 nm for the lipid-to-protein molar ratio of 800 (Fig 5). Addition of lipid vesicles to the labelled enzyme resulted in quenching of the isoindole fluorescence up to a lipid/protein molar ratio of approx. 500. At higher molar ratios, fluorescence of the probe emission was more intense in the

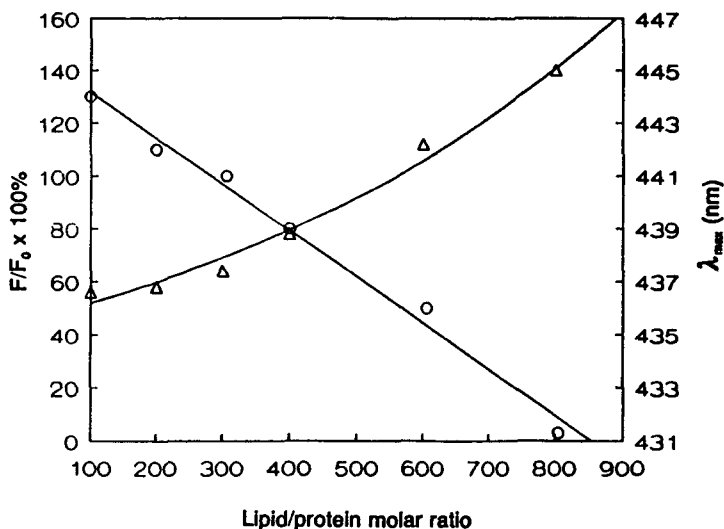


Figure 5. Dependence of the emission maximum wavelength ($- \circ -$) and the fluorescence intensity ($- \Delta -$) on lipid/protein molar ratio. F_0, F - intensity of the probe fluorescence in the absence and presence of PI liposomes, respectively.

presence of lipids than in their absence (Fig. 5). Probably, at higher molar ratios the increase in fluorescence intensity due to the movement of the probe to less polar environment ("blue" shift) is much more significant than the quenching effect of the lipid vesicles.

The degree of polarization of the enzyme tryptophanyl fluorescence increases as a result of interaction of the enzyme with phospholipid vesicles, from 0.04 for the enzyme alone to 0.15 for lipid/protein molar ratio of 1 : 800. Quantitative interpretation of the increase in fluorescence polarization degree is not possible since it was determined from total probe fluorescence, but qualitative explanation can be offered for the increase as being a result of a decreased mobility and/or change in interactions of isoindole with other chemical group(s).

Discussion

First of all, the data presented above suggest that the fluorescence properties of covalently bound isoindole chromophore are sensitive to the interaction of the enzyme studied with PI liposomes. Fluorescence emission intensity, emission maximum wavelength, and polarization degree of the probe were altered after addition of liposomes to labelled enzyme solution. Also, the temperature dependence of tryptophanyl fluorescence intensity for the enzyme appears to be quite different

in the presence and in the absence of the liposomes. In addition, no aggregation (turbidity) was observed up to 60°C in the presence of liposomes. Exposure of tryptophan residues is larger and that of the extrinsic probe is smaller with the liposome-modified enzyme as compared to the unmodified enzyme (Figs 3 and 4). Since during the reaction of *o*-phthaldialdehyde probe fluorophores were formed first with well exposed surface amino groups, they are located on the surface of the enzyme molecules and are well accessible to the quencher. The decrease in the probe exposure (decrease of the effective quenching constant) can be interpreted as being a result of a screening by liposomes, but the increased accessibility of tryptophans, normally buried in this enzyme (ineffective quenching by acrylamide for unmodified enzyme, Fig 2) can only be due to some conformational rearrangement. The temperature dependence of the exposure of the two kinds of fluorophores, as represented by the temperature dependence of effective quenching constant, is also quite different depending on the presence of liposomes. The modified conformational state appears to be stable between 39°C and 60°C because in this range, tryptophan and the probe accessibility to the quencher is not further changed with the increasing temperature.

All these results strongly support our previous finding that interaction produces conformational changes in glyceraldehyde-3-phosphate dehydrogenase molecule (Gutowicz and Modrzycka 1986, Michalak et al 1987). Also, we suggested that such a conformational modification of the enzyme may have important implications for the enzyme properties. It can be concluded from the present results that this actually is the case. The nature of the temperature dependence of tryptophan and the probe fluorescence quenching and their modification by liposomes seems very interesting. The plot for unmodified enzyme is not linear, and it shows a sharp increase of mean exposure of tryptophanyl residues upon increasing the temperature between 25°C and 30°C. In the presence of liposomes, k_{eff} also increases with the temperature but the increase is monotonically extended over a wide temperature range (25–40 °C), and is nearly constant for higher temperature values (Fig 3). With the isoindole probe located on the enzyme molecule surface this temperature transition is also visible though less distinctly (Fig 4). In the presence of liposomes, initially the exposure slightly increases subsequently reaching a nearly constant level. The physiological role of the temperature transition in this enzyme is unknown. In an organism the enzyme always works above the transition. The implications of the transition and of its modification by liposomes for the catalytic properties of the enzyme remain unknown and will be subject of our further studies. A similar effect occurring upon the binding of the enzyme to membranes *in vivo* may have important physiological implications.

It is noteworthy that quite similar temperature conformational transition below the physiological temperature has been found for aldolase (Lehrer and Barker 1971, Heyduk and Kochman 1985) with an alteration upon adsorption of the en-

zyme to liposomes (Gutowicz and Kośmider-Schmidt 1987).

Modification of the static conformational state is not the only possible explanation for the data from the fluorescence quenching experiments. Protein molecules show different types of motion such as conformational fluctuations, segmental, interdomain and intersubunit mobilities. Dynamic quenchers are also sensitive probes for changes in the dynamics of a protein structure. Another possible interpretation of similar data in terms of changes in protein dynamics has been discussed in details by Gutowicz and Kośmider-Schmidt (1987).

Acknowledgements. We wish to thank Mrs. H. Dudziak for technical assistance. This work was supported by research Grant C.P.B.P. 04.01.1.12. of Polish Academy of Sciences.

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Final version accepted September 24, 1992