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Fluorescent probe studies on binding of glyceraldehyde-3-phosphate dehydrogenase to phosphatidylinositol liposomes

Further evidence for conformational changes

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Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle can be adsorbed on charged lipid bilayers by electrostatic forces. Upon binding to phosphatidylinositol liposomes the enzyme modifies its conformational state as it is shown by resonance energy transfer experiments. In the presence of 2-mercaptoethanol *o*-phthaldialdehyde reacts with amino groups of the protein and the covalently bound fluorophore is an acceptor of excitation energy transferred from tryptophanyl residues of the protein. The observed decrease of energy transfer efficiency upon binding to phosphatidylinositol liposomes is compared with the influence of the urea on the fluorescence spectra of the labelled protein. Significance of conformational changes of the enzyme upon adsorption on liposomes in the regulating function of cell membranes is discussed.

Glyceraldehyde-3-phosphate dehydrogenase; Membrane enzyme; Liposome; *o*-Phthaldialdehyde; Fluorescence probe; Resonance energy transfer

1. INTRODUCTION

It is a well established fact that glyceraldehyde-3-phosphate dehydrogenase belongs to peripheral membrane proteins [1–6]. Its association with membranes is affected by the environmental parameters such as: pH, ionic strength, ionic metabolites and detergents [7–9]. It indicates that the binding is controlled by nonspecific, electrostatic interactions. Hence, the electrically charged surface of lipid bilayers is a good model for investigation of this kind of interactions. It has been found that electrically charged surfaces of such model systems (monolayers, liposomes) can adsorb the enzyme and also modify its specific activity [10–12]. Recently, using the measurements of changes in intrinsic fluorescence spectra and the fluorescence quenching method some rearrangements in the enzyme conformation were detected [13]. This report presents further evidence for changes in conformational state of the enzyme during adsorption on liposomes. Since the changes in conformational state of the enzyme upon the adsorption on membranes may have important biological implications, the support of the fact by independent methods seems to be necessary. Here we applied fluorescence probes bound to enzyme molecules. This was formed during the reaction of the *o*-phthaldialdehyde with amino groups of the

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Abbreviations: G3PDH, glyceraldehyde-3-phosphate dehydrogenase; OPA, *o*-phthaldialdehyde; PI, phosphatidylinositol; NADH, nicotinamide adenine dinucleotide, reduced form

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protein. Roth [14] has found that the reaction of the *o*-phthaldialdehyde with the amino group of amino acids results in the formation of a strongly fluorescent product. The probe connected with protein molecules can be an acceptor of the excitation energy transferred from tryptophanyl residues. Resonance energy transfer efficiency strongly depends on the distance between donor and an acceptor of the excitation energy. Hence, any changes in the distance connected, for example, with conformational rearrangement in protein molecule, must affect energy transfer.

Preliminary results of this study were presented elsewhere [15].

2. MATERIALS AND METHODS

Glyceraldehyde-3-phosphate dehydrogenase was prepared from rabbit muscle according to Kochman and Rutter [16]. Phosphatidylinositol from bovine brain, *o*-phthaldialdehyde and 2-mercaptoethanol were purchased from Koch-Light Laboratories, Sigma and Fluka, respectively. All other reagents were of analytical grade.

Specific activity of the enzyme was determined by measurements of the NADH extinction at 340 nm as a function of time [17]. The extinction was recorded with a Specord UV/VIS (Karl Zeiss Jena) spectrophotometer. The enzyme concentration was determined spectrophotometrically using $E_{280}^{0.1\%} = 1.0$ as an extinction coefficient [18]. Amino groups of G3PDH were labelled with OPA in the same manner as phosphatidylserine amino groups described elsewhere [19]. Degree of labelling, i.e., the molar ratio of the probe to protein was within the range of 4–6 in our experiments.

Phospholipid vesicles were prepared by shaking a lipid suspension in 50 mM triethanolamine buffer, pH 8.6, for 30 min. Then the suspension was centrifuged for 45 min at 12500 × *g* to remove large lipid aggregates. The lipid concentration in suspension was calculated from phosphorus determination according to Bartlett [20]. The fluorescence was always measured after the incubation of the labelled enzyme together with the phospholipid vesicles for 30 min at room temperature. Fluorescence spectra were recorded with Perkin Elmer MPF-3L spectrofluorimeter. Fluorescence of the probe was excited at 340 nm. Relative quantum yields of tryptophanyl residues and of the isoindole probe were calculated from corrected fluorescence spectra. Two fluorescence quantum yield standards were used: tryptophan in water for ultraviolet region ($q = 0.12$) and quinine in 0.1 N H_2SO_4 for isoindole fluorescence excited at 340 nm ($q = 0.55$).

3. RESULTS AND DISCUSSION

Covalent binding of the isoindole fluorophore with G3PDH from rabbit muscle results in a new absorption band with maximum at 337 nm and a new fluorescence band with emission maximum at 445 nm. Intensities of these bands depend on the degree of labelling (R). Uncorrected emission spectra of the unlabelled and labelled enzyme for some values of the degree of labelling are shown in fig.1. Upon binding of the probe, the enzyme specific activity gradually decreased with increase in the degree of labelling (R). For example for $R = 1.4$, the activity was 86.6%; for $R = 5.5$, 77.7%; and for $R = 14$, 65.7%, value for unlabelled enzyme. Hence, to avoid the marked inactivation of the enzyme, in our further experiments R did not exceed 4–6 molecules of OPA per tetrameric molecule of the enzyme. In addition light absorption of the protein at 290 nm was unchanged under these conditions. As can be seen in fig.1 the labelled enzyme fluorescence, excited at 290 nm, is quenched in the

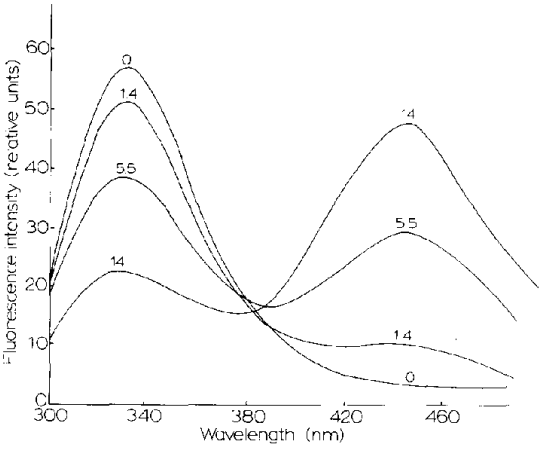

Fig.1. Fluorescence emission spectra of the glyceraldehyde-3-phosphate dehydrogenase labelled with *o*-phthaldialdehyde. λ_{ex} = 290 nm. Molar ratio of OPA to protein: 0, 1.4, 5.5, 14, respectively. Other conditions are given in the text.

tryptophan emission band and enhanced in the probe emission band (445 nm). It can only be explained by resonance energy transfer between tryptophanyl residues and the isoindole fluorophore. The existence of the additional band in the excitation fluorescence spectrum of the probe (fig.2) in the range of donor absorption (295 nm) confirms that the resonance energy transfer between Trp residues and the probe occurs indeed.

To test if conformational changes in the enzyme molecule may affect isoindole fluorescence and Trp $\longrightarrow$ probe energy transfer efficiency we investigated the effect of a well known modifier of conformation, urea, on emission spectra of the labelled protein. Addition of 6 M urea to the G3PDH solution caused an increase of tryptophanyl fluorescence intensity of the unlabelled protein (fig.3). Urea at the same concentration added to the labelled protein caused greater enhancement of the Trp residues' fluorescence intensity and quantum yield than for the unlabelled one and a simultaneous decrease of the isoindole fluorescence (fig.3 and table 1). The wavelength of excitation was 290 nm in this experiment.

For control, it was established that 6 M urea did not affect absorption and fluorescence spectra of the isoindole chromophore bound, for example, to amino acid and excited with 340 nm (not shown). 6 M urea caused a slight decrease (15%) of the isoindole fluorescence quantum yield when the probe was connected with protein molecules and an exciting wavelength of 340 nm was used. This was probably due to the modification of the probe environment in protein caused by urea. When the

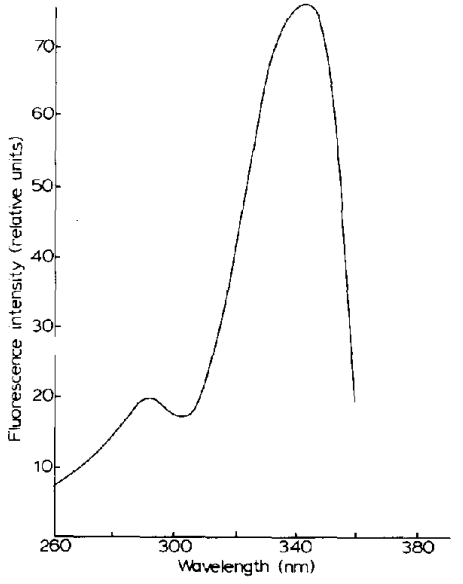

Fig.2. Fluorescence excitation spectrum of isoindole fluorophore bound with glyceraldehyde-3-phosphate dehydrogenase. λ_{em}, 445 nm; other conditions as in fig.1.

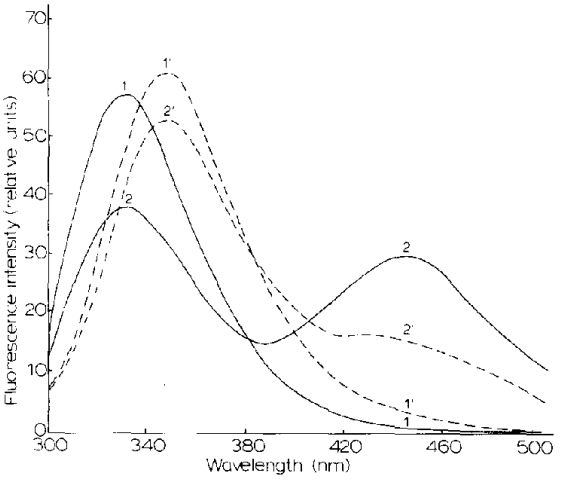

Fig.3. The effect of urea on the emission spectra of the labelled and unlabelled enzyme. 1,2, emission spectra of the unlabelled and labelled enzyme, respectively; 1',2', spectra 1 and 2 after the addition of the 6 M urea; λ_{ex}, 290 nm.

Table 1

Fluorescence quantum yields of Trp residues in unlabelled and labelled G3PDH and isoindole probe in the labelled enzyme in experiments with urea

Protein q_0	Protein + urea q	$q/q_0 \times 100$ (%)	Fluorophore
0.143	–	100 unlabelled	tryptophanyl residues
–	0.26	183 protein	
0.096	–	100 labelled	
–	0.21	221 protein	
0.065	–	100 labelled	probe
–	0.032	50 protein	

q_0, q, quantum yields in the absence and presence of urea; λ_{ex} = 290 nm

probe fluorescence was excited with a wavelength of 290 nm, the decrease of the isoindole quantum yield was much greater (50%) after addition of urea (table 1).

Changes in the spectrum of the labelled protein shown in fig.3 and data presented in table 1 can be explained only as a result of reduction of Trp → probe energy transfer efficiency due to the increase of donor-acceptor distance during unfolding of the protein molecule. The experiment with urea, used as an unfolding substance for proteins, demonstrates that incorporation of the isoindole probe into the dehydrogenase molecule enables one to monitor conformational modification of the enzyme.

Addition of the lipid vesicles to the labelled protein solution results in quenching of the probe fluorescence when the probe is directly excited (λ_{ex} = 340 nm), as well as when the donor is excited (λ_{ex} = 290 nm) (fig.4). Presumably two mechanisms are simultaneously responsible for quenching of the probe fluorescence: (i) quenching by direct interaction of phospholipid molecules with the isoindole chromophore; (ii) reduction of the energy transfer efficiency between the two types of fluorophores in the protein, as a result of the change in the distance between them which means conformational rearrangements in the protein molecules.

In addition, it is noteworthy that the quenching of isoindole fluorescence by lipid vesicles was more

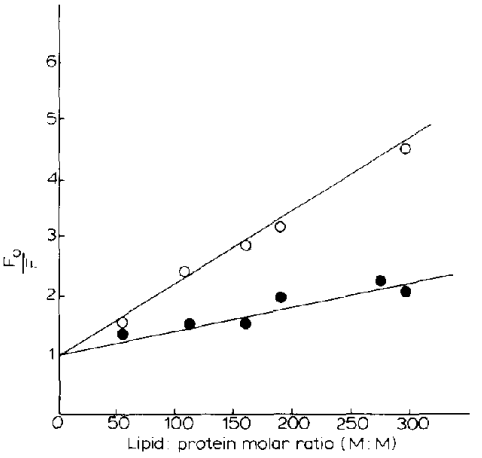

Fig.4. Stern-Volmer plots of the quenching of the probe fluorescence in enzyme as a function of PI to protein molar ratio at the excitation wavelength λ_{ex} = 290 nm (○) and 340 nm (●). λ_{em}, 445 nm; F_0, F, intensity of the probe fluorescence in the absence and in the presence of the lipid, respectively.

efficient when the fluorescence was excited at 290 nm than that excited at 340 nm (fig.4). The ratio of the isoindole quantum at the excitation wavelength of 340 nm (q_{340}) to quantum yield at that of 290 nm (q_{290}) increased with increase of the lipid to protein molar ratio (table 2). This result may be due to occurrence of the second mechanism. This was confirmed by calculation of energy transfer efficiency at different lipid to pro-

Table 2

Fluorescence quantum yields of the isoindole probe in labelled G3PDH at different lipid to protein molar ratios with excitation wavelengths of 340 and 290 nm

Lipid:protein molar ratio (M:M)	λ_{ex} = 340 nm		λ_{ex} = 290 nm		q_{340}/q_{290}
	q	$q/q_0 \times 100$ (%)	q	$q/q_0 \times 100$ (%)	
0	0.2	100	0.065	100	3.1
55	0.16	80	0.044	68	3.6
110	0.12	60	0.03	48	4.0
200	0.09	45	0.018	28	5.0
275	0.07	35	0.01	16	7.0

q_0, q, quantum yields in the absence and presence of liposomes at indicated excitation wavelength; q_{340}, q_{290}, quantum yield when protein fluorescence was excited with 340 nm and 290 nm, respectively

Table 3

Quantum yields of tryptophanyl fluorescence in unlabelled and labelled G3PDH and energy transfer efficiency at different lipid to protein molar ratios

Lipid:protein molar ratio (M:M)	Unlabelled protein q_D^0	Labelled protein q_D	$E \times 100$ (%)
0	0.143	0.096	33
55	0.078	0.052	33
110	0.052	0.039	25
200	0.026	0.023	12

q_D^0, quantum yields of donors (Trp residues) in the absence of acceptors (isoindole chromophores) in unlabelled protein; q_D, quantum yields of donors in the presence of acceptors in labelled protein; λ_{ex}, 290 nm

tein molar ratios. For the calculation we used the relation [21]

$$E = 1 - \frac{q_D}{q_D^0}$$

where q_D^0 was quantum yields of donors (Trp residues) without acceptors (unlabelled protein) and q_D was quantum yields of donors in the presence of acceptors (labelled protein). Energy transfer efficiency decreased with increase of the lipid to protein molar ratio (from 33% to 12%; see table 3).

So, in our opinion, data presented here indicate the modification of the conformational state of the enzyme studied. The previously observed increase in quenching of the enzyme tryptophanyl fluorescence by a dynamic quencher may be interpreted as a dynamic exposure of the fluorophores (changes in frequency of conformational fluctuations) [13]. This would mean the formation of new regions of greater flexibility in the conformation, but the data obtained here rather suggest changes in a static conformational state.

Capability of membranes for modification of the enzyme conformation may have some implications for the enzyme properties and function in vivo. The modification may result in, and supposedly does, changes of such properties as: catalytic activity, thermostability, resistance for action of activity modifiers, etc. The effects are the subject of further studies.

The modification of enzyme properties by the adsorption and subsequent rearrangement in their conformations seems to be a more general rule. Adsorption on lipid membranes affecting the conformation and/or other properties have been detected for aldolase [22], phosphoglycerate kinase [23], and trypsin [24].

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