Short communication

Inhibition of Fructose Diphosphate Aldolase by Phosphatidylserine Liposomes

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Phosphatides and their cleavage products modulate the activities of a variety of proteins, including glycolytic enzymes. Very abundant glycolytic enzyme – fructose bis-phosphate aldolase was found to be strongly inhibited by phosphatidylinositol (PI) in the form of liposomes, which was interpreted as a result of electrostatic interaction of the protein with the negatively charged liposomes (Gutowicz and Modrzycka 1979). The inhibitory effect of PI was associated with quenching and batochromic shift of the protein fluorescence. More recently Koppitz et al. (1986) have shown that also water soluble inositol phosphates, which are cleavage products of phosphoinositides, act as potent inhibitor of aldolase. Thus, the inactivation by PI may result from interaction of the enzyme either with the liposomes, or with single molecules of an inhibitor present in the lipid phase. A simultaneous operation of both mechanisms seems also possible.

Since aldolase belongs to membrane bound proteins (Strapazon and Steck 1976, Harris and Winzor 1990) it was of interest to study the effect of another anionic phospholipid, phospatidylserine (PS), an important structural component of biological membranes. It was shown to be a potent inhibitor of glyceraldehyde-3-phosphate dehydrogenase (Gutowicz and Modrzycka 1978; Sidorowicz et al. 1990) and 3-phosphoglycerate kinase (Sidorowicz et al. 1986), but it also acts as an activator of other enzymes, e.g. pyruvate kinase (Dąbrowska et al. 1988) and protein kinase C (Hannun and Bell 1986).

The aim of the present communication was to study the effect of PS on the activity and fluorescence of rabbit muscle aldolase. In order to show a possible effect of the lipid impurities, three different preparations of PS were examined, namely synthetic dipalmitoyl PS-1, highly purified bovine brain PS-2 (both were from Sigma

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Chemical Co.) and crude Folch fraction III (PS-3) from Koch-Light Laboratories. Possible conformational changes of the enzyme were studied by measuring fluorescence parameters of the isoindole probe covalently bound to the enzyme. This probe was shown to be a good acceptor of excitation energy transferred from tryptophanyl residues of several proteins (Sidorowicz and Michalak 1985; Michalak et al. 1987).

Rabbit muscle aldolase was prepared according to Penhoet et al. (1969). Its specific activity, determined according to Blostein and Rutter (1993), ranged within 8–10 U/mg protein. The enzyme concentration was determined spectrophotometrically, using $E_{280}^{1\%} = 0.91$ as an extinction coefficient (Baranowski and Niederland 1949). The isoindole labelled aldolase was prepared using *o*-phthaldialdehyde reagent described previously (Sidorowicz and Michalak 1984).

The phospholipid suspensions were prepared in 10 mmol/l triethanolamine – 1 mmol/l EDTA buffer, pH 8.0, by sonication over 20 min, using a MSE 150 W sonifier. Then the suspensions were centrifuged for 45 min at 12,500 × *g* to remove large lipid aggregates. Such prepared suspensions contained mainly single – shelled vesicles with a mean diameter of about 25 nm (Hauser and Phillips 1973). Phospholipid concentration in the suspensions was estimated by phosphorus determination according to Bartlett (1959). The lipid suspensions were mixed with a solution of aldolase in the same buffer. The final concentration of aldolase was kept at 1 μmol/l and the lipid concentration ranged between 0.2 and 0.8 mmol/l.

The activity of aldolase was measured immediately after mixing with PS suspension and after incubation of the mixture over 30, 60 and 120 min at room temperature. The reaction was started by addition to the assay mixture of about 1.6 μg aldolase without PS, or with 0.66; 1.32; 2.0 and 2.65 μmol PS (total volume 10 μl, lipid/protein molar ratio 200; 400; 600 and 800, respectively). In control samples corresponding amounts of PS were present in the assay mixture before addition of aldolase in order to exclude interaction of PS with substrates.

The spectrophotometric measurements were done on a SPECORD-UV-vis spectrophotometer (Karl Zeiss Jena). Fluorescence emission spectra were measured on a Perkin-Elmer MPF-3 spectrofluorimeter, with an excitation wave-length of 290 nm. Fluorescence polarization degree of the isoindole probe was measured at 25 °C with 350 nm excitation (UV-D25 interference filter).

Incubation of the lipid – aldolase mixtures results in progressive decrease of enzymatic activity with lipid concentration and with incubation time (Table 1). The activity of free enzyme measured at zero time (8.6 U/mg) was considered 100% and the other results were related to this value. Incubation of the enzyme with PS-1 or with PS-2 for 30 min yielded a decrease of the activity up to about 70% of its initial value, which remained unchanged after longer incubation time (data not shown). In contrast, the inactivation by PS-3 was much stronger and increased with incubation time. Control experiments gave little inactivation when

Table 1. Influence of phosphatidylserine liposomes on the activity of rabbit muscle aldolase at different incubation times

Lipid/protein molar ratio	% of enzymatic activity after different times of incubation				
	30 min			60 min	120 min
	PS-1	PS-2	PS-3	PS-3	PS-3
200	88	92	90	83	58
400	81	85	65	58	50
600	73	76	56	50	33
800	68	70	50	33	15

the activity was measured immediately after addition of the lipid. No significant change of the activity was also observed when the lipid was added to the assay mixture, which presumably resulted from large excess of substrates and coupling enzymes. The results obtained for PS-3 suggest that it contains an additional inhibitor, acting either directly with the enzyme or as a component of the lipid membrane.

Table 2. Influence of 24 hour dialysis of PS-3 suspension on the inactivation of aldolase (per cent of initial activity)

Lipid/protein molar ratio	Before dialysis	After dialysis	Dialysate
400	50.2	52.3	84.0
600	40.5	45.5	74.0

In the next experiment PS-3 suspension was used after 24 hours of dialysis against the buffer and the inhibitory effect of the dialysed suspension and dialysate was determined. The results shown in Table 2 (again as per cent of the initial activity) indicate insignificantly higher enzyme activity in the presence of the dialysed suspension as compared to the undialysed one. Simultaneously, inactivation was observed when the same volume of dialysate was used instead of the lipid suspension. This result again supports the presence of a water soluble inhibitor in PS-3. This may account for phosphatidylinositol (detected in PS-3 by thin-layer chromatography) and/or its cleavage products.

Neither PS-1 nor PS-2 altered aldolase fluorescence. In contrast, in the presence of PS-3 apparent quenching of the protein fluorescence was observed, similar

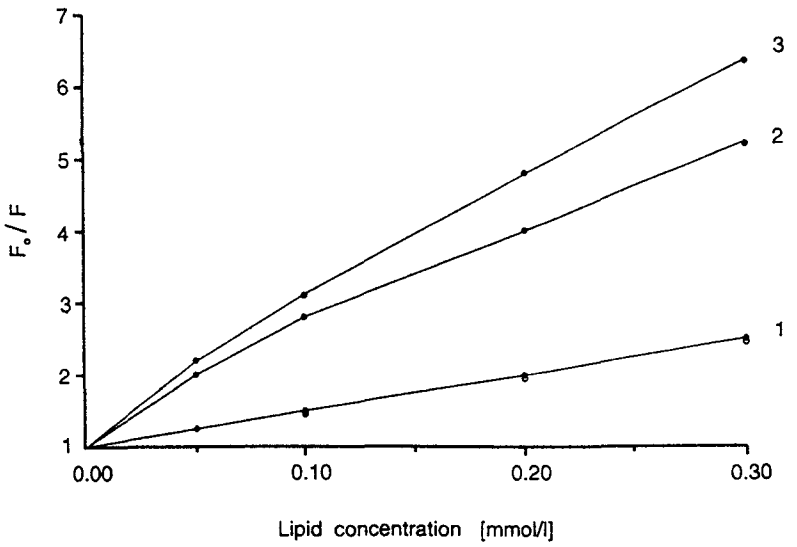

Figure 1. Stern-Volmer plots for the quenching of aldolase fluorescence by PS-3 in 10 mmol/l triethanolamine buffer, pH 8 (curve 1) and in the presence of 3 mol/1 and 5 mol/1 urea (curves 2 and 3, respectively). Open symbols represent values of F_0/F corrected for the filter effect according to Parker and Barnes (1957).

for the native and isoindole labelled enzyme. Since PS-3 showed strong absorption of light at 290 nm and at 320 nm (maximum of excitation and emission, respectively) it seemed us that the quenching resulted from an "inner filter effect". Correction of experimental fluorescence readings for absorption of light by the lipid suspension showed that the "quenching" was caused solely by the filter effect. This is shown in Fig. 1, where in curve 1 the full symbols represent Stern-Volmer plot for the experimental fluorescence readings and the open symbols represent values of F_0/F calculated from the filter effect according to Parker and Barnes (1957). On the other hand, the quenching by PS-3 indeed takes place after defolding of the enzyme in 3 mol and 5 mol urea (Fig. 1, curves 2 and 3, respectively).

Aldolase – liposome interaction was studied after labelling of the protein by the reaction with *o*-phthaldialdehyde, which yields covalently bound isoindole probe. The probe can act as an acceptor of excitation energy emitted from tryptophanyl residues. It is also sensitive to the polarity and microviscosity of its environment (Sidorowicz and Michalak 1984). Fig. 2 shows the fluorescence emission spectra of native aldolase (1) and isoindole-aldolase (2) excited at 290 nm and at the probe absorption maximum (3). The isoindole-aldolase exhibits an additional fluorescence maximum at 450 nm, which corresponds to water-exposed isoindole. The fluorescence of the labelled enzyme is quenched in the tryptophan emission band (320 nm)

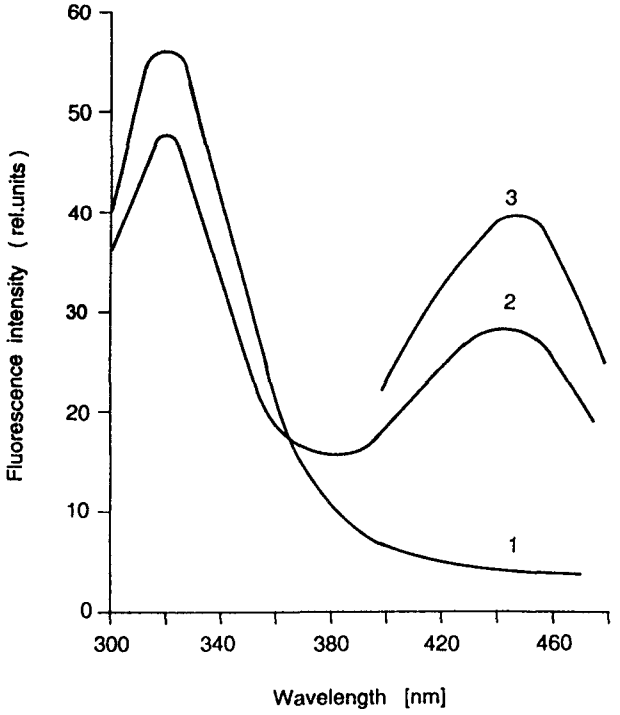

Figure 2. Fluorescence emission spectra of native aldolase (1) and isoindole-aldolase in triethanolamine buffer, pH 8 (2) excited at 290 nm and at 340 nm (3). Protein concentration: 1 μmol/l.

indicating transfer of energy to the isoindole acceptor. This was confirmed by the existence of an additional maximum in the excitation spectrum (data not shown). No change in the energy transfer efficiency was observed in the presence of any PS. This result and the lack of fluorescence quenching of native aldolase suggest that the interaction with PS is not followed by pronounced change in the protein conformation. On the other hand, addition of any PS to the isoindole-aldolase was followed by progressive increase of the probe polarization degree. In absence of PS this parameter was found to be only 0.07 (mean of three measurements), which reflects fast rotational motion of the isoindole moiety and confirms its localization near the protein surface. Addition of any PS to the isoindole-aldolase yields progressive increase of the polarization degree. The results are shown in Table 3 as a function of lipid/protein molar ratio. The observed increments of the polarization degree are comparable for PS-1 and PS-2 whereas corresponding values for PS-3 are somewhat lower. Since inhibitory effect of PS-1 and PS-2 is also comparable (Table 1), this result suggests the existence of certain correlation between the

Table 3. Changes of the fluorescence polarization degree of the isoindole in aldolase in the presence of phosphatidylserine liposomes

Lipid/protein molar ratio	0	200	400	600	800
PS-1	0.07	0.19	0.21	0.24	0.26
PS-2	0.07	0.12	0.20	0.23	0.27
PS-3	0.07	0.11	0.16	0.20	0.21

liposome-aldolase interaction and the enzyme inhibition. In contrast, there is no such correlation in the case of PS-3, where molecular mechanism participates in the enzyme inhibition.

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