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Incorporation of mitochondrial L-glycerol-3-phosphate dehydrogenase into liposomes; effect of sodium oleate and calcium ions

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FAD-linked L-glycerol-3-phosphate dehydrogenase purified from liver mitochondria of hyperthyroid rats was incorporated into unilamellar phospholipid liposomes. The incorporation influenced both $V_{max,app}$ and $K_{m,app}$ values of the enzyme for its substrate, L-glycerol 3-phosphate. The $K_{m,app}$ for the electron acceptor remained unchanged with a simultaneous slight enhancement of the corresponding $V_{max,app}$ value. The steady-state fluorescence anisotropies of the fluorescein isothiocyanate and trimethylammoniumdiphenylhexatriene labels were affected by sodium oleate and calcium ions in the case of both solubilized and liposome-incorporated L-glycerol-3-phosphate dehydrogenase. These results indicate that calcium ions cause a significant alteration of the enzyme conformation. Sodium oleate (used as a model of free fatty acids), besides its direct action on the enzyme itself, affects the enzyme indirectly as well, via alteration of the physical properties of the membrane.

Introduction

FAD-linked L-glycerol-3-phosphate dehydrogenase (EC 1.1.99.5), which is embedded in the inner mitochondrial membrane, provides electrons for the respiratory chain. As a component of the glycerol-3-phosphate shuttle, it plays a role in transferring reducing equivalents from the cytosol into the mitochondria. The activity of the enzyme is influenced by free fatty acids and divalent cations (Ca^{2+} and Mg^{2+}).

In mitochondria from brown adipose tissue, L-glycerol-3-phsphate cytochrome *c* oxidoreductase and L-glycerol-3-phosphate oxidase were shown to be inhibited by endogenous and exogenous free fatty acids in a reversible, noncompetitive manner [1,2]. Addition of serum albumin abolishes this inhibitory effect. Good correlation has been found between the inhibition of L-glycerol 3-phosphate oxidation by sodium oleate and the alteration of the structural and dynamic parameters of the lipid bilayer as monitored by the limiting anisotropy and the rotation relaxation time of diphenylhexatriene [3].

L-Glycerol-3-phosphate oxidase activity of mitochondria can be stimulated by calcium ions (demonstrated in blowfly flight muscle [4–6], brown adipose tissue [7], liver [8] and lung [9]), through activation of the FAD-linked L-glycerol-3-phosphate dehydrogenase [4]. In the presence of Ca^{2+} the K_m for L-glycerol 3-phosphate decreases in rat liver mitochondria [10,11]. Ca^{2+} affects the kinetic behavior of the enzyme in liver mitochondria of hyperthyroid rats as well. In Ca^{2+}-free medium, at bulk pH 7.4, two independent L-glycerol 3-phosphate binding sites can be distinguished kinetically, whereas in the presence of the cation only one binding site seems to be effective [11].

Preliminary investigations on the mechanism of action of calcium ions have been performed with purified L-glycerol-3-phosphate dehydrogenase that preserved its sensitivity towards activation by calcium ions [12]. We showed the essential role of SH group(s) in the calcium sensitivity of the enzyme and suggested that calcium ions induced a conformational change of the protein molecule [11]. These results do not exclude the possibility that alterations in the surface charge [13] and/or in

Abbreviations: DPG, diphosphatidylglycerol (cardiolipin); DCIP, dichlorophenol-indophenol; FITC, fluorescein isothiocyanate; GP, DL-glycerol 3-phosphate; PC, phosphatidylcholine; TMA-DPH, trimethylammoniumdiphenylhexatriene.

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the structure of the membrane may also play a role in the effect of Ca^{2+} on the enzyme.

In this work we addressed the above problem by using partially purified L-glycerol-3-phosphate dehydrogenase incorporated into unilamellar phospholipid liposomes as a model system. The steady-state anisotropy of fluorescence labels, either covalently coupled to the enzyme or incorporated into the membrane, was recorded in the presence or absence of sodium oleate and calcium ions. We present evidence that calcium ions induce conformational change of the enzyme in both solubilized and liposome-embedded forms. The mechanism of action of the free fatty acids (modelled by sodium oleate) seems to involve changes induced both in the enzyme itself and in the structural properties of the membrane.

Materials and Methods

Materials

DL-Glycerol-3-phosphate, egg yolk phosphatidylcholine (type II), cardiolipin, FITC, *n*-octyl glycoside were from Sigma (St. Louis, MO). DCIP was obtained from LOBA Chemie, TMA-DPH from Molecular Probes (U.S.A.). All other chemicals (analytical grade) were from Reanal (Hungary).

Methods

Male CFY rats (200–250 g) were used throughout. Hyperthyroid state was induced according to Nelson et al. [14] as described earlier [11].

Partially purified L-glycerol-3-phosphate dehydrogenase from liver mitochondria of hyperthyroid rats was prepared by adopting the procedure described in [12] for normal rats. 8 mg/ml Triton X-100 (0.4 mg detergent/mg protein) was used for the solubilization of the enzyme and 0.2 mg/ml Triton X-100 was applied during the subsequent operations. Partially purified enzyme utilized in this study was obtained by omitting the final purification step (separation by FPLC) of the original procedure, which yields a preparation containing at least 70% L-glycerol-3-phosphate dehydrogenase as determined by SDS-PAGE.

L-Glycerol-3-phosphate dehydrogenase activity with DCIP as artificial electron acceptor was determined as described earlier [12] with DL-glycerol 3-phosphate as substrate. Apparent kinetic parameters were calculated from Lineweaver-Burk plots with the linear least-squares method. In the case of activation by excess of substrate, a kinetic model assuming two independent binding sites [15] was applied.

Ca^{2+} concentration was stabilized by 1 mM EDTA; free cation concentrations were calculated using lg K' = 7.82 for EDTA-Ca^{2+} at pH 7.4 [16].

Protein concentration was determined according to Petterson [17] with bovine serum albumin as standard. Phospholipid content of the enzyme preparation was determined via the phosphor content according to Chen et al. [18].

Incorporation of L-glycerol-3-phosphate dehydrogenase into pure or cardiolipin-containing phosphatidylcholine liposomes was performed with *n*-octyl glycoside as a detergent according to the method of Klingenberg and Winkler [19,20].

Labeling of L-glycerol-3-phospahte dehydrogenase by FITC was done as described earlier [21] for Na^+/K^+-ATPase. The specific activity of the enzyme did not change upon reaction with FITC under these experimental conditions.

Liposomes were labeled with TMA-DPH as described in Ref. 22.

Steady-state fluorescence anisotropies of both TMA-DPH incorporated in the membrane and FITC covalently bound to the enzyme were determined at room temperature using a Perkin-Elmer MPF-3 spectrofluorimeter equipped with polarizers and a Kiethley 614 electrometer as in Ref. 3. Anisotropy values were corrected for the scattering of the excitation beam, using a liposome suspension of the same absorbance. In addition, to correct for the depolarization due to the reflections both before absorption and before reaching the detector, true anisotropy values (r_s) were calculated from observed ones (r') and the absorbances (A):

$$r_s = \frac{r'}{1 - K \times A}$$

where K is the actual proportionality constant determined according to Ref. 23. The excitation wavelengths were 360 and 494 nm, whereas emission was measured at 430 and 515 nm in the case of TMA-DPH and FITC, respectively.

Results and Discussion

Incorporation of L-glycerol-3-phosphate dehydrogenase into liposomes of different phospholipid composition

Mitochondrial L-glycerol-3-phosphate dehydrogenase, purified from rat liver by our method [12], can be activated by Ca^{2+} and possesses the same affinity towards L-glycerol 3-phosphate as within the mitochondria. This method, adopted for liver mitochondria of hyperthyroid rats, was applied in the present work, which provided a partially purified enzyme preparation similar to that described [12]. The enzyme was incorporated into unilamellar liposomes composed from egg yolk phosphatidylcholine alone or supplemented with cardiolipin. Cardiolipin has been chosen to supplement phosphatidylcholine (egg yolk lecithin), as previous study [24] has shown its advantageous effect on the activity of L-glycerol-3-phosphate dehydrogenase. The apparent kinetic parameters of the enzyme incorporated

TABLE I

Apparent kinetic parameters of the mitochondrial L-glycerol-3-phosphate dehydrogenase incorporated into unilamellar liposomes of different lipid composition

For the determination of the apparent kinetic parameters, the concentration of one of the substrates was varied while keeping the concentration of the other substrate constant (L-glycerol-3-phosphate, 75 mM; DCIP, 71 μM). $V_{max,app}$ values are expressed as mU/mg protein. Apparent kinetic parameters for the two independent L-glycerol-3-phosphate binding sites ($K_{1,app}$, $V_{1,app}$ and $K_{2,app}$, $V_{2,app}$, respectively) were calculated as described in Materials and Methods. Proteoliposomes were prepared as described in Materials and Methods; the protein to phospholipid ratio was 1:14 and in DPG-containing proteoliposomes the phosphatidylcholine to cardiolipin ratio was 20:1.

	L-Glycerol 3-phosphate				2,6-Dichlorophenolindophenol	
	$K_{1,app}$ (mM)	$V_{1,app}$ (mM)	$K_{2,app}$ (mM)	$V_{2,app}$ (mM)	K_m (μM)	$V_{max,app}$ (μM)
Control (soluble GDH)	2.9	18	22.5	26	92	48
GDH in PC liposomes	8.4	21	35.5	41	101	63
GDH in PC/DPG liposomes	5.1	24	40.5	59	106	67

into these liposomes are shown in Table I. The $V_{max,app}$ values probably represent an underestimation of the maximal capacity of the functioning enzyme molecules, since a given fraction of the protein is possibly ineffective due to its inappropriate orientation in the lipid bilayer.

Both kinetic parameters for L-glycerol 3-phosphate are significantly higher in proteoliposomes as compared with the solubilized enzyme. On the other hand, the $K_{m,app}$ for the electron acceptor does not change significantly upon incorporation. The V_{max} value for the electron acceptor, on the other hand, is slightly higher in the presence of lipids, but it is practically unaffected by the phospholipid composition of the liposomes.

The phospholipid composition of the proteoliposomes affects the kinetic parameters for L-glycerol 3-phosphate; the most significant change occurs in the apparent maximal velocity of the looser L-glycerol 3-phosphate binding site (denoted as $V_{2,app}$ in Table I). The $V_{max,app}$ value for the electron acceptor is barely affected by the presence of cardiolipin. The possibility cannot be excluded that cardiolipin does not affect the binding site of the electron acceptor because this site changes irreversibly upon removal of the enzyme from the mitochondrial membrane. An alternative, more probable, explanation is provided by the mechanism of the enzyme reaction. Our kinetic analysis, performed in intact mitochondria (unpublished data), shows that the kinetic mechanism of the L-glycerol-3-phosphate dehydrogenase reaction with DCIP as electron acceptor is ping-pong bi-bi. Thus, GP binding and oxidation to dihydroxyacetone phosphate occurs before the binding of the electron acceptor. Consequently, the binding site for the hydrophylic electron acceptor might be constituted upon the reduction of the enzyme, from those amino acid side chains (or other groups) which became enriched in electrons. This is in line with the above finding, indicating the necessity of cardiolipin for the optimal functioning of (at least one of) the GP binding sites.

Comparison of the effects of sodium oleate and Ca^{2+} on L-glycerol-3-phosphate dehydrogenase

The mechanism of action of calcium ions and free fatty acids, two possible physiological effectors of L-glycerol-3-phosphate dehydrogenase, was studied in the following model system.

Partially purified L-glycerol-3-phosphate dehydrogenase was allowed to react with the fluorescent label FITC and the (fully active) enzyme derivative was incorporated into liposomes. The FITC-labelled enzyme preparation and the proteoliposomes were labelled with TMA-DPH as well, and the steady-state fluorescence anisotropy of both labels was monitored separately.

The binding of TMA-DPH to the solubilized enzyme, as well as the high anisotropies observed (first lines in Table II and III) indicate either the existence of an ordered lipid annulus or the binding of the label to a hydrophobic pocket (accessible for the label) on the enzyme itself. We could show the presence of only seven molecules of phospholipid per enzyme molecule (M_r = 78000) in our preparations (cf. Ref. 24 as well). This argues against a lipid annulus, and makes it more probable that TMA-DPH binds to a hydrophobic pocket on the protein molecule.

A significant enhancement of the steady-state fluorescence anisotropy of FITC by calcium ions has been observed both with the solubilized and the liposome-incorporated enzymes (Table II). Presence of cardiolipin in the liposomes does not influence this effect, in good agreement with our result [25] showing that the enzyme can be activated with calcium ions even after the selective removal of cardiolipin by adriamycin from the mitochondrial membrane. Maximal activation of the enzyme occurs at 10^{-4}–10^{-5} M Ca^{2+} [11]. The calcium concentration dependence of the enhancement of the FITC anisotropy correlates well with the concentration dependence of the enzyme activation. Influence of calcium ions on the anisotropy of TMA-DPH (Table II) is manifested at significantly higher (1–2 mM) calcium concentrations. This argues against the possibility that

TABLE II

Effect of Ca^{2+} on the steady-state fluorescence anisotropy of FITC-labeled L-glycerol-3-phosphate dehydrogenase and TMA-DPH in proteoliposomes

	Free Ca^{2+} (mM)	Steady-state anisotropy * of	
		FITC	TMA-DPH
Control (soluble GDH)	–	0.052 ± 0.003	0.269 ± 0.024
	0.10	0.084 ± 0.007	0.270 ± 0.003
	0.32	0.092 ± 0.009	0.273 ± 0.003
	1.0	0.112 ± 0.002	0.280 ± 0.018
	2.0	0.118 ± 0.003	0.281 ± 0.021
	3.0	0.107 ± 0.004	0.288 ± 0.012
GDH in PC liposomes **	–	0.053 ± 0.006	0.290 ± 0.003
	0.004	0.063 ± 0.005	0.291 ± 0.003
	0.040	0.080 ± 0.010	0.290 ± 0.002
	0.080	0.116 ± 0.008	0.290 ± 0.002
	0.320	0.118 ± 0.010	0.294 ± 0.004
	1.0	0.128 ± 0.010	0.300 ± 0.003
	2.0	0.122 ± 0.008	0.305 ± 0.002
	3.0	0.125 ± 0.005	0.307 ± 0.005
GDH in PC/DPG liposomes **	–	0.050 ± 0.010	0.295 ± 0.003
	0.10	0.094 ± 0.011	0.294 ± 0.005
	0.32	0.116 ± 0.007	0.298 ± 0.004
	1.0	0.129 ± 0.009	0.303 ± 0.002
	2.0	0.120 ± 0.011	0.304 ± 0.005
	3.0	0.125 ± 0.010	0.310 ± 0.003

* Average values and standard errors were calculated from ten independent measurements. Fluorescence intensities were barely influenced by the addition of calcium ions; thus, the observed anisotropy changes cannot be attributed to a quenching effect.

** For the preparation and composition of the proteoliposomes, cf. legend to Table I.

in the case of the membrane-embedded enzyme calcium exerts its effect indirectly, via the membrane surrounding the enzyme. We conclude that calcium ions act in the same way on the soluble and proteoliposome-embedded enzyme, by inducing a FITC detectable conformational change of the protein.

Sodium oleate, a free fatty acid (Table III), caused an enhancement of the steady-state fluorescence anisotropy of FITC-labelled L-glycerol-3-phosphate dehydrogenase in the solubilized form, but it did not affect this parameter of the liposomes. Apparently, sodium oleate acts differently on the solubilized and on the liposome-incorporated enzyme. This is further supported by the observation (Table III) that the fluorescence anisotropy of the TMA-DPH label is affected differently by sodium oleate in the case of the solubilized and the lipsoome-incorporated enzymes.

In the case of the solubilized L-glycerol-3-posphate dehydrogenase, sodium oleate enhanced the anisotropy of both TMA-DPH and FITC. The specific activity of the enzyme increased in a parallel way. Apparently, the increase of the order in the hydrophobic pocket of the enzyme responsible for the binding of TMA-DPH (see above) and/or in the microenvironment of L-glycerol-3-phosphate dehydrogenase in the detergent-protein micelles, caused by the free fatty acid, leads to higher specific activity.

In the proteoliposomes, on the other hand, the steady-state fluorescence anisotropy of TMA-DPH is lowered by the free fatty acid. This reflects predominantly the decreasing microviscosity of the membrane. The specific activity of the enzyme decreased as well. This result suggests that sodium oleate influences L-

TABLE III

Effect of sodium oleate on the steady-state fluorescence anisotropy of FITC-labeled L-glycerol-3-phosphate dehydrogenase and TMA-DPH in proteoliposomes

Experiments and calculations were performed as described in legends to Table I and II; addition of sodium oleate did not affect fluorescence intensities. n.d., not determined.

	Sodium oleate (μM)	Specific activity (mU/mg protein)	Steady-state anisotropy of	
			FITC	TMA-DPH
Control (soluble GDH)	–	4.9 ± 0.6	0.051 ± 0.003	0.267 ± 0.024
	10	n.d.	0.071 ± 0.002	0.319 ± 0.018
	20	n.d.	0.076 ± 0.003	0.353 ± 0.021
	100	11.6 ± 2.5	0.080 ± 0.004	0.342 ± 0.012
GDH in PC liposomes	–	16.5 ± 0.8	0.054 ± 0.004	0.291 ± 0.002
	10	n.d.	0.051 ± 0.003	0.285 ± 0.002
	20	n.d.	0.050 ± 0.004	0.279 ± 0.003
	100	10.1 ± 1.5	0.049 ± 0.004	0.278 ± 0.003
GDH in PC/DPG liposomes	–	20.6 ± 3.0	0.052 ± 0.005	0.294 ± 0.002
	10	n.d.	0.053 ± 0.004	0.290 ± 0.003
	20	n.d.	0.053 ± 0.004	0.282 ± 0.002
	100	7.1 ± 1.8	0.053 ± 0.004	0.281 ± 0.003

glycerol-3-phospahte dehydrogenase activity indirectly, by changing the physical properties of the membrane.

The results presented above convincingly show that the structure of the whole membrane (the nature of the bulk phospholipids) plays a role in maintaining the structural organization of L-glycerol-3-phosphate dehydrogenase.

We conclude that calcium ions and a free fatty acid, sodium oleate, which were shown to influence the activity of the mitochondrial L-glycerol-3-phosphate dehydrogenase, act in two different ways. Calcium ions predominantly act directly, by causing conformational change of both the soluble and membrane-embedded enzyme. The free fatty acid, besides its direct effect, presumably acts indirectly as well, by altering the physical properties of the membrane.

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References

1 Houstek, J. and Drahota, Z. (1976) Mol. Cell Biochem. 17, 45–50.
2 Rauchová, H. and Drahota, Z. (1984) Int. J. Biochem. 16, 243–245.
3 Amler, E., Rauchová, H., Svobodová, J. and Drahota, Z. (1986) FEBS Lett. 206, 1–3.
4 Wohlrab, H. (1977) Biochim. Biophys. Acta 462, 102–112.
5 Estabrook, R.W. and Sacktor, B. (1958) J. Biol. Chem. 233, 1014–1019.
6 Hansford, R.G. and Chappel, J.B. (1967) Biochim. Biophys. Res. Commun. 27, 686–692.
7 Bukowiecki, L.J. and Lindberg, O. (1974) Biochim. Biophys. Acta 348, 115–125.
8 Carafoli, E. and Sacktor, B. (1972) Biochim. Biophys. Res. Commun. 49, 1498–1503.
9 Fisher, A.B., Scarpa, A., LaNoue, K.F., Bassett, D. and Williamson, J.R. (1973) Biochemistry 12, 1438–1445.
10 Wernette, M.E., Ochs, R.S. and Lardy, H.A. (1981) J. Biol. Chem. 256, 12767–12771.
11 Beleznai, Zs., Szalay, L. and Jancsik, V. (1988) Eur. J. Biochem. 170, 631–636.
12 Beleznai, Zs. and Jancsik, V. (1987) Biochem. Int. 15, 55–63.
13 Wojtzak, L. and Nalecz, M.J. (1979) Eur. J. Biochem. 94, 99–107.
14 Nelson, B.D., Joste, V., Wielburski, A. and Rosenqvist, U. (1980) Biochim. Biophys. Acta 608, 422–426.
15 Batke, J. and Keleti, T. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. 3, 385–395.
16 Portzell, H., Coldwell, P.C. and Rüegg, J.C. (1964) Biochem. Biophys. Acta 79, 581–591.
17 Petterson, G.L. (1977) Anal. Biochem. 83, 346–356.
18 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Biochem. 28, 1756–1759.
19 Klingenberg, M. and Winkler, E. (1986) Methods Enzymol. 127, 772–779.
20 Klingenberg, M. and Winkler, E. (1987) EMBO J. 4, 3087–3092.
21 Amler, E., Teisinger, J. and Svoboda, P. (1987) Biochim. Biophys. Acta 905, 376–382.
22 Kuhry, J.G., Duportail, G., Bronner, C. and Laustriat, G. (1985) Biochim. Biophys. Acta 845, 60–67.
23 Caratorta, P., Casali, E. and Sartor, G. (1986) in Membrane Proteins (Azzi, A., Massoti, L. and Veechi, A., eds.), pp. 24–31, Springer, Berlin.
24 Cottingham, J.R. and Ragan, C.J. (1980) Biochem. J. 192, 9–18.
25 Beleznai, Zs. and Jancsik, V. (1989) Biochim. Biophys. Res. Commun. 159, 132–139.