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ROLE OF CARDIOLIPIN IN THE FUNCTIONING OF MITOCHONDRIAL L-GLYCEROL-3-PHOSPHATE DEHYDROGENASE

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SUMMARY. Adriamycin was used *in situ*, in isolated liver mitochondria of hyperthyroid rats to study the role of cardiolipin in the functioning of FAD-linked L-glycerol-3-phosphate dehydrogenase.

The apparent kinetic parameters of the reaction catalyzed by the enzyme were affected by adriamycin. The effect of adriamycin was dependent on the electron acceptor, suggesting the existence of distinct binding sites for hydrophobic and hydrophilic acceptors.

Assuming a correlation between the two plateaus observed upon binding of adriamycin to the mitochondria and the penetration of the drug into the two leaflets of the inner membrane [Cheneval et al. (1985) J. Biol. Chem. *260*, 13003-13007], we can deduce that cardiolipin in both leaflets influences predominantly the electron acceptor binding site(s).

FAD-linked L-glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) is tightly bound to the inner mitochondrial membrane. This enzyme, as isolated from pig brain (1) and rat liver (2), contains cardiolipin. Enhanced activity was observed when cardiolipin was present among the liposome-forming lipids in reconstitution studies with partially purified enzyme (1,3). Based on these results, L-glycerol-3-phosphate dehydrogenase might belong to the group of proteins for which cardiolipin is essential for functioning. In the present

ABBREVIATIONS: BQ - 2,3-dimethoxy-5-methyl-1,4 benzoquinone; CoQ - coenzyme Q, ubiquinone; DCIP - 2,6-dichloro-phenol indophenol; GP - L-glycerol-3-phosphate; K_3 - menadione (K_3 vitamin, 3-methyl-1,4-naphtoquinone); PMS - phenazinemethosulfate.

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study we addressed this issue using adriamycin (doxorubicin) as a tool. This drug forms a specific complex with cardiolipin (4) in a molar ratio of 2 : 1. As a result of the complex formation phase segregation of cardiolipin occurs, and the hexagonal (non-bilayer) structure of this phospholipid is eliminated (5). These changes lead to the impairment of the functioning of cardiolipin-requiring proteins, such as cytochrome c oxidase (5), the phosphate carrier (6) and creatine phosphokinase (7). In isolated mitochondria adriamycin affects only the inner membrane, due to the exclusive localisation of cardiolipin there (8).

Here we present kinetic analysis of the L-glycerol-3-phosphate dehydrogenase reaction in adriamycin-treated liver mitochondria from hyperthyroid rats, showing deterioration of the functioning of the enzyme by cardiolipin complexation. Our results argue for two distinct binding sites for the hydrophobic and hydrophilic electron acceptors on the enzyme. When functioning with hydrophobic electron acceptors, cardiolipin in both leaflets of the membrane seems to be essential for enzyme action.

MATERIALS AND METHODS

Male CFY rats of 200-250 g were used throughout. Hyperthyroid state was induced according to Nelson et al. (9).

Mitochondria were isolated as described by Johnson & Lardy (10) using a medium containing 0.25 M sucrose, 1 mM EDTA and 10 mM Hepes, pH = 7.4. Freshly isolated mitochondria were used for each experiment.

Adriamycin binding to mitochondria was done according to Cheneval et al. (4); unbound adriamycin was determined in the supernatants; L-glycerol-3-phosphate dehydrogenase activities were measured in the pellets. Enzyme activities with DCIP or DCIP + BQ were measured according to Dawson & Thorne (11). L-glycerol-3-phosphate oxidase as well as L-glycerol-3-phosphate dehydrogenase activities with PMS or K_3 were determined by the method of Estabrook and Sacktor (12), with a Clarke-type oxygen electrode.

Liver mitochondria isolated from hyperthyroid rats were treated as described above at 2 mg/ml protein concentration with 130 and 390 nmol adriamycin/mg protein. Apparent kinetic parameters were determined from the initial velocities. In the case of activation by excess substrate the experimental points were fitted to a kinetic model based on two independent substrate binding sites (13). Experimental data were fitted to the theoretical curves by the linear least squares method.

Protein concentrations were determined as in (14) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Mitochondrial GP oxidation is influenced by adriamycin.

The role of cardiolipin in L-glycerol-3-phosphate dehydrogenase function was examined in liver mitochondria prepared from hyperthyroid rats.

In adriamycin titration experiments, with different hydrophilic electron acceptors either no effect (in case of DCIP or DCIP + BQ) or a slight decrease of activity (in the case of PMS) can be detected (Fig. 1). On the contrary, adriamycin significantly inhibits GP oxidation by the hydrophobic electron acceptors, CoQ or K_3 (Fig. 2.). The inhibition measured with K_3 correlates well with the two plateaus observed during the binding of adriamycin. In the case of CoQ (state 4 respiration) the situation is more complex, due to the existence of an adriamycin-sensitive site in the respiratory chain, between Complex III and IV (15). The drug was shown to compete with cytochrome c for the same binding site on the cytoplasmic surface of the inner membrane.

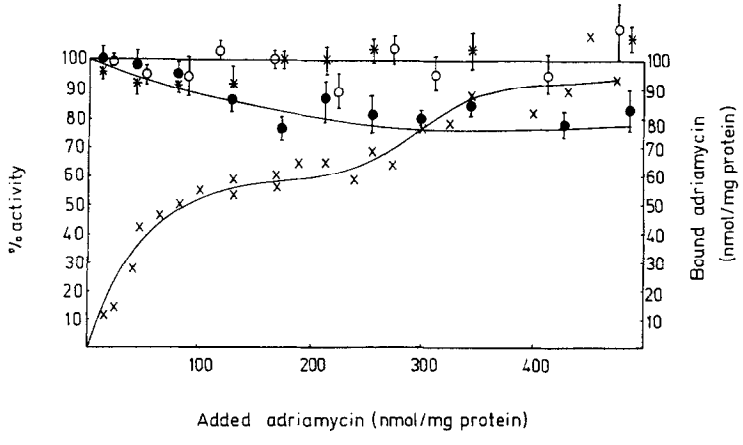

Figure 1. Effect of adriamycin on L-glycerol-3-phosphate dehydrogenase activity with hydrophilic electron acceptors in liver mitochondria of hyperthyroid rats.

75 mM GP was used throughout. 78 µM DCIP (*), 78 µM DCIP + 20 µM BQ (o) or 333µM PMS (•) served as electron acceptor. 100 % activities were 14.3, 28.3 and 107.2 natom O_2/ min x mg protein, respectively. Adriamycin binding: x - x.

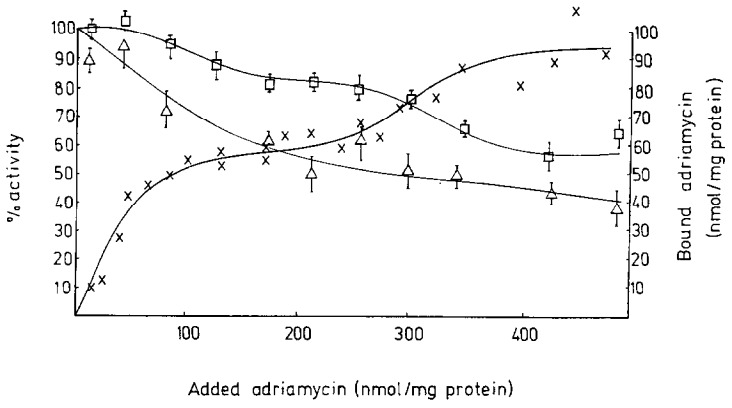

Figure 2. Effect of adriamycin on L-glycerol-3-phosphate dehydrogenase activity with hydrophobic electron acceptors in liver mitochondria of hyperthyroid rats.

GP concentration was 75 mM. CoQ (Δ; state 4 respiration) or 33 μM K_3 (□) were applied. 100 % activities : 38.2 and 57.4 natom O_2/ min x mg protein, respectively. Adriamycin binding: x - x.

This is superimposed on the inhibition of L-glycerol-3-phosphate dehydrogenase.

Kinetic analysis of the effect of adriamycin in the case of different electron acceptors.

Apparent kinetic parameters were determined in adriamycin treated liver mitochondria from hyperthyroid rats. Two adriamycin concentrations were chosen, corresponding to the first and second plateaus observed in the binding of adriamycin to the mitochondria (Figs. 1. and 2., see also (4)); either K_3 or PMS served as electron acceptor.

With PMS as electron acceptor, the apparent kinetic parameters for GP were not influenced significantly by adriamycin (results not shown); parameters for PMS were slightly modified by adriamycin treatment (Table I.). Both $K_{m,app,PMS}$ values were practically unchanged, whereas both $V_{max,app,PMS}$ decreased slightly (non-competitive inhibition).

With K_3 as electron acceptor more significant effects of adriamycin were observed (Table II.). $K_{m,app,GP}$ increased approximately two-fold at

Table I. Effect of adriamycin on the apparent kinetic parameters of L-glycerol-3-phosphate dehydrogenase for PMS

	$K_{m,app,PMS}$ (μM)		$V_{max,app,PMS}$ natom O_2/min x mg protein	
	K_1	K_2	V_1	V_2
control	50 ± 1	450 ± 18	124 ± 2	269 ± 11
1st plateau	50 ± 1	418 ± 15	115 ± 3	208 ± 6
2nd plateau	52 ± 2	424 ± 6	89 ± 5	212 ± 20

Initial velocities were determined in untreated (control) and in adriamycin treated hyperthyroid rat liver mitochondria (130 nmol adriamycin/ mg protein : 1st plateau and 390 nmol adriamycin/ mg protein: 2nd plateau). Measurements were carried out using 125 μg/ml mitochondrial protein, 50 mM GP; PMS concentration was varied from 25 to 833 μM.

K_1, V_1 and K_2, V_2 are the apparent kinetic parameters corresponding to the tight and weak electron acceptor binding sites, respectively, as calculated according to (13).

Table II. Effect of adryamycin on the apparent kinetic parameters of the reaction catalyzed by L-glycerol-3-phosphate dehydrogenase with K_3 as electron acceptor

	$K_{m,app,GP}$ (mM)	$V_{max,app,GP}$ natom O_2 / min x mg protein	$K_{m,app,K3}$ (μM)	$V_{max,app,K3}$ natom O_2 / min x mg protein
control	18 ± 2	231 ± 25	36 ± 2	210 ± 13
1st plateau	23 ± 3	249 ± 30	44 ± 2	155 ± 6
2nd plateau	39 ± 8	261 ± 55	66 ± 7	141 ± 10

For the determination of the apparent kinetic parameters for GP, K_3 concentration was held at 250 μM, and GP concentration was varied between 5 and 167 mM. To determine those for K_3, 100 mM GP was applied throughout, and K_3 concentration was varied between 33 and 333 μM. For further conditions, cf. legend to Table I.

the higher adriamycin concentration (second plateau), while $V_{max, app,GP}$ was not affected (competitive inhibition).

Adriamycin influenced most significantly the kinetic parameters of K_3 (Table II). Increase of $K_{m,app,K3}$ and decrease of $V_{max,app,K3}$ occurred at both levels of adriamycin (mixed type inhibition).

The above differences clearly indicate that the binding sites for the hydrophobic and hydrophilic electron acceptors are different. It is tempting to assume (4) that the two plateaus obtained upon adriamycin titration reflect the complex formation by the drug with cardiolipin in the cytoplasmic and matrix leaflets of the inner mitochondrial membrane, respectively. With the hydrophobic K_3 as electron acceptor, significant increase in the $K_{m,app,GP}$ value occurs at high adriamycin concentration, corresponding to the penetration of adriamycin to the matrix leaflet of the membrane. Thus, the membrane-protruding part of the enzyme seems to play a role in the reaction mechanism. Finally, the binding site of the electron acceptor is influenced by cardiolipin in both membrane leaflets (cf. changes of $K_{m,app,K3}$ and $V_{max,app,K3}$ values in Table II). It appears that the binding site of the hydrophobic electron acceptors is positioned close to the core of the lipid bilayer. This suggestion is in good correlation with the localization of the physiological electron acceptor, CoQ, in the central region of the membrane.

According to Brdiczka and Reith (16) L-glycerol-3-phosphate dehydrogenase resides in, or close to, the contact regions between the inner and outer mitochondrial membranes. Cardiolipin might participate in the formation of these contact regions; a correlation can be assumed between the regulation of the dynamic organization (or the number) of contact points by Ca^{2+} (17) and the induction of non-bilayer formation by cardiolipin upon Ca^{2+}-binding. Thus, the question arises, whether or not cardiolipin is involved in the activation of the enzyme by Ca^{2+} (18). Initial velocity

measurements in adriamycin treated liver mitochondria of hyperthyroid rats (not shown) revealed that the Ca^{2+} sensitivity of the enzyme reaction was not affected up to 400 nmol adriamycin/mg protein. Thus, neither cardiolipin itself nor the possible reorganization of the membrane around the enzyme induced by the binding of Ca^{2+} to cardiolipin plays a role in the activation of L-glycerol-3- phosphate dehydrogenase by this cation..

In this study we showed data in favor of the existence of distinct binding sites on the mitochondrial L-glycerol-3-phosphate dehydrogenase for hydrophobic and hydrophilic electron acceptors *in vitro*. Under physiological conditions evidently the hydrophobic site is of primary importance. However, under specific conditions the hydrophilic binding site might be effective in the cell as well, acting on reducing agents from the cytosol (e.g.: glutathione, cysteine). Furthermore, we conclude that cardiolipin is involved in maintaining the active conformation of L-glycerol-3-phosphate dehydrogenase. It seems to be essential for the quinone binding site of the enzyme, localised probably on a hydrophobic surface of the protein molecule close to the center of the lipid bilayer.

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