# Membrane lateral pressure as a modulator of glycerol-3-phosphate dehydrogenase activity

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Michaelis-Menten kinetics of glycerol-3-phosphate dehydrogenase activity in proteoliposomes from brown adipose tissue mitochondria with exogenously added phospholipids or cholesterol was measured. It was shown that changes in membrane lipid composition affected the membrane lateral pressure and therefore modulated the enzyme activity, namely $V_{max}$ value. Contrarily, changes in surface charge caused by minute amounts of phosphatidylserine or charged organic substances influenced only the apparent $K_m$ value. The role of bulk phospholipids in regulation of glycerol-3-phosphate dehydrogenase is discussed.

Membrane lateral pressure; Förster energy transfer; Surface charge; Model membrane; Glycerol-3-phosphate dehydrogenase; Brown adipose tissue mitochondrion

## 1. INTRODUCTION

FAD-linked glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) is an inner mitochondrial membrane-bound enzyme which shuttles transfer of reducing equivalents from cytosol via respiratory chain to molecular oxygen [1–3]. Earlier studies revealed that this electron (hydrogen) transfer between GPDH and coenzyme Q is subjected by downward regulation of free fatty acids [4,5]. Furthermore, it was found that the inhibitory effect of free fatty acids is due to the changes of both structural and dynamic properties of lipid membrane phase [6]. On the other hand, the membrane surface charge dependent on phospholipid composition has also been shown to have modulatory effect on this enzyme in reconstituted system [7].

The present study was undertaken to obtain more detailed information on the mechanism of modulation of GPDH activity by the lipid phase.

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*Abbreviations:* GPDH, glycerol-3-phosphate dehydrogenase; MLP, membrane lateral pressure; $Cl_2Ind$, 2,6-dichloroindophenol; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; ODA, octadecylamine (1-aminooctadecane); DCP, dicetylphosphate

## 2. MATERIALS AND METHODS

### 2.1. *Phospholipid purification*

Egg yolk phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) were purified on aluminum oxide column according to [8]. Bovine brain phosphatidylserine (PtdSer) was prepared by a combination of the extraction method [9] and column chromatography on CM-cellulose [10]. Each of the purified phospholipids migrated as a single spot on silica gel thin-layer chromatography with the two-dimensional system used [11]. Phospholipid phosphorus was assessed following the method [11].

### 2.2. *Proteoliposome preparation*

Brown adipose tissue mitochondria were isolated from cold-adapted golden hamster [12] and kept frozen at −20°C. Proteoliposomes were obtained as described in [13] with minor modifications. Briefly, mitochondria (1 mg protein) were solubilized with 60 mM *n*-octyl glucoside in the absence or presence of dried phospholipid, cholesterol or charged components. After dialytical removal of the detergent against buffer containing 320 mM sucrose, 10 mM Tris, 3 mM $Ca^{2+}$ and adjusted to pH 6.0, reaggregates were recovered by centrifugation for 60 min at 135000 × *g* with a 40.3 Beckman rotor. The pellet containing reaggregated vesicles, was resuspended in 320 mM sucrose, 10 mM Tris-HCl buffer (pH 7.4). Protein concentration was determined according to Lowry et al. [14].

### 2.3. *Enzyme assay*

GPDH activity was measured with $Cl_2Ind$ as an artificial electron acceptor [15] in the medium containing 250 mM sucrose, 1 mM EDTA, 1 mM KCN, 10 mM Tris-HCl buffer (pH 7.4), 0.06 mM $Cl_2Ind$, mitochondria or proteoliposomes (10–100 μg protein) and 60 mM DL-glycerol 3-phosphate in a final volume of 1 ml. The assay was started by addition of substrate and the progress of reaction was followed by decrease of light absorption at 600 nm on Beckman DU-6 Spectrophotometer or Philips Spectrophotometer PU 8700. The enzyme activity was expressed as $mU \cdot mg^{-1}$ protein.

### 2.4. *Fluorescence measurements*

TMA-DPH, a hydrophobic probe with high quantum yield [16,17] was applied in this study. The TMA-DPH probe (final concentration

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was $4 \times 10^{-7}$ M) was incorporated by addition of the ethanolic (less than 1% of total volume) solution to an aqueous suspension of proteoliposomes, and the mixture was incubated for 2 min at room temperature under vigorous stirring.

Steady-state fluorescence anisotropy measurements were performed with a SLM 4800 S spectrofluorometer with radio-frequency electronics switched off or a Perkin-Elmer spectrofluorometer Model LS-5B, equipped with polarizers. The excitation and emission wavelengths were 360 and 450 nm with a bandwidth of 4 and 8 nm, respectively. Emission spectra or anisotropies were measured through a Corning 7-54 band-pass filter. Anisotropies were calculated as described in [18,19].

The phospholipid structural parameters used for the correlation with the enzyme activity were estimated from steady-state fluorescence values ($r_s$) using Eqn. (1) according to van der Meer et al. [20].

$$r_\infty = r_0 r_s^2/(r_0 r_s + (r_0 r_s)^2/m) \quad (1)$$

The parameter, $m$, from Eqn. (1) was obtained from the measurement of the limiting anisotropy $r_\infty$ and rotational relaxation time $\tau_c$ using phase cross-correlation spectrofluorometer SLM 4800 S and Weber's theory of hindered rotations [21]. The order parameter $S_{\delta\infty}$ was calculated from the limiting anisotropy value as $S_{\delta\infty} = (r_\infty/r_0)^{1/2}$, where $r_0 = 0.395$ was the fluorescence anisotropy value observed in the absence of depolarizing factors. Membrane lateral pressure was then calculated from $S_{\delta\infty}$ according to Fulford and Peel [18].

The actual distances between donors (tryptophan residues of protein) and acceptors (fluorescent cholesterol analog) were estimated from the Förster resonance energy transfer measurements as described previously [19]. The actual distances are related to the efficiency of energy transfer, which was determined by steady-state fluorescence donor quenching measured as the extent of quenching of donor quantum.

## 3. RESULTS

Brown adipose tissue mitochondria are known to possess high content of GPDH [3,22,23]. To study the effect of lipid phase on this enzyme, we have adopted a solubilization-reconstitution procedure utilizing the non-ionic detergent, *n*-octyl glucoside. With this procedure we were able to follow the enzyme activity in its natural and modified, i.e. lipid-enriched environment. The method was revealed to be gentle and proper, since specific activity of GPDH in proteoliposomes obtained in the absence of exogenous lipids was nearly the same as in intact mitochondria (data not shown). The proteoliposomes formed without exogenous phospholipids served as a control in all further experiments.

Incorporation of exogenous phospholipids or cholesterol into reconstituted vesicles evoked changes of MLP as estimated from fluorescence anisotropy decay of TMA-DPH. As shown in Table I, PtdCho incorporated at 40–50% into proteoliposomes decreased the MLP in a concentration-dependent manner. The opposite effect, i.e., increase in MLP, was observed upon addition of cholesterol or its fluorescent analog, cholesteryl anthracene-9-carboxylate. To estimate the incorporation of the former, a trace amount of [$^{14}$C]cholesterol was added, whereas the incorporation of the latter was based on fluorescence intensity. Both lipids added simultaneously in a ratio 3:1 were incorporated in approx. 20%, regardless of the concentration. The changes of MLP were again concentration-dependent. The estimated MLP value increased from 49.2 ± 0.3 $mN \cdot m^{-1}$ for proteoliposomes formed in the presence of lowest amounts of cholesterol and its analog (15 and 5 $nmol \cdot mg^{-1}$ protein) to 57.6 ± 0.3 $mN \cdot m^{-1}$ at the highest concentration given, i.e., 776 and 258 $nmol \cdot mg^{-1}$ protein, respectively. However, a linearity in MLP increase was reached at 40 nmol cholesterol incorporated. Raising of MLP was also induced by introducing PtdEtn into proteoliposomes (data not presented).

Fig. 1 shows that GPDH activity decreased at both, i.e. higher and lower, MLP as compared to control proteoliposomes. At higher MLP the enzyme seemed to be less sensitive than at the lower, as was found for PtdCho-enriched proteoliposomes.

To elucidate whether cholesterol is incorporated into lipid annulus of the enzyme we measured the Förster energy transfer efficiency between tryptophan residues of the proteins and fluorescent cholesterol analog. The MLP of proteoliposomes increased in parallel to amount of the analog, but the average distance between tryptophan residues and cholesterol remained unchanged (energy transfer efficiency corresponded to 11%). Although a decrease of donor (tryptophan residues) intensity in proteoliposomes with relatively low cholesterol added (13 $nmol \cdot mg^{-1}$ protein) was observed, the higher cholesterol concentrations did not further decrease the donor fluorescence intensity, implying that cholesterol in any significant amount was not incorporated into the close vicinity of the proteins with tryptophan residues exposed. The decrease of the enzyme activity under these conditions, nevertheless, was observed (see Fig. 1).

To elucidate the connections between the modulation effects of membrane structure and surface charge, we measured the Michaelis-Menten kinetics of GPDH in

Table I

Changes in MLP upon increasing PtdCho incorporation into proteoliposomes

| PtdCho added ($\mu mol \cdot mg^{-1}$) | PtdCho incorporated (% ± SD) | MLP ($mN \cdot m^{-1}$ ± SD) |
|---|---|---|
| 0.5 | 40 ± 9 | 48.1 ± 0.2 |
| 1.0 | 39 ± 8 | 47.5 ± 0.1 |
| 2.0 | 55 ± 7 | 46.7 ± 0.2 |
| 4.0 | 49 ± 3 | 44.1 ± 0.2 |
| 8.0 | 45 ± 3 | 42.0 ± 0.3 |

Two types of proteoliposome with addition of fluorescent 3-palmitoyl-2-(1-pyrenedecanoyl)-L-phosphatidylcholine or non-fluorescent PtdCho were formed. The former served for calculation of MLP, the latter for estimation of PtdCho incorporation. The average values and standard errors were calculated from four measurements of two independently prepared samples. The steady-state fluorescence values in this case as well as in following measurements in this paper were averaged from 50 readings.

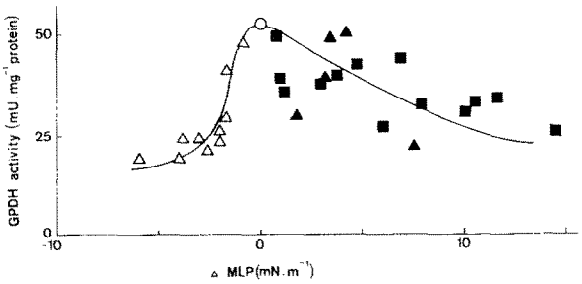


Fig. 1. Dependence of GPDH activity on MLP changes. GPDH activity was measured in proteoliposomes prepared without (○) and with the following lipids: (△) PtdCho (0.2–8.0 μmol·mg$^{-1}$ protein); (▲) PtdEtn (0.5–8.0 μmol·mg$^{-1}$ protein); (■) cholesterol (0.013–1.044 μmol·mg$^{-1}$ protein).

proteoliposomes enriched with uncharged and charged molecules. The latter by contributing to the surface potential of proteoliposomes will affect the affinity and binding of negatively charged substrate to the enzyme [7]. As shown in Table II, PtdCho added at low amount did not change either MLP or kinetic parameters of the enzyme. Introducing a positive charge into proteoliposomes with ODA caused a decrease of the Michaelis-Menten constant but not of the $V_{max}$ or MLP. The apparent $K_m$ value was, however, increased when proteoliposomes were enriched with PtdCho plus DCP or PtdSer. Also PtdSer added alone acted analogically. When the sample contained 0.2 μmol PtdSer together with 1.5 μmol PtdCho the $V_{max}$ value of the enzyme in proteoliposomes formed was decreased whereas the $K_m$ value remained comparable to the estimated for control vesicles. With the highest PtdCho concentration the $K_m$ value could no longer be estimated and the velocity of enzymatic reaction was significantly reduced.

Table II

Determination of kinetic parameters of GPDH reconstituted in proteoliposomes

| Additions | $K_m$ (mM) | $V_{max}$ (mU·mg$^{-1}$ protein) | Δ MLP (mN·m$^{-1}$) |
|---|---|---|---|
| None | 22 ± 2 | 32 ± 3 (4) | 0 |
| PtdCho (0.5 μmol) | 24 ± 3 | 29 ± 2 (4) | 0.2 |
| PtdCho (0.3 μmol) | | | |
| + ODA (0.2 μmol) | 15 ± 2 | 31 ± 3 (7) | 0.3 |
| + DCP (0.2 μmol) | 32 ± 2 | 35 ± 5 (4) | 0.2 |
| + PtdSer (0.2 μmol) | 35 ± 2 | 28 ± 5 (3) | 0.6 |
| PtdSer (0.2 μmol) | 31 ± 1 | 28 ± 7 (2) | 0.3 |
| PtdCho (1.5 μmol) | | | |
| + PtdSer (0.2 μmol) | 22 | 10 (1) | 4.2 |
| PtdCho (5 μmol) | NM | 5 ± 1 (2) | 7.6 |

Proteoliposomes of different composition were prepared as described in section 2. In each case, 1.0 mg solubilized mitochondrial protein was added to dried lipid. Kinetic parameters data are given as mean ± SD for the number of separate determinations in brackets. NM, not measurable.

## 4. DISCUSSION

Activity of the membrane-bound enzyme can be controlled directly by surfactants, cations etc. or indirectly via bulk phospholipids. There are a number of membrane enzymes described to be sensitive to its lipid environment [19,24,25]. In the present paper, GPDH activity was found to be dependent on MLP, the term we used rather than the often criticized microviscosity or fluidity. As shown in Fig. 1, the activity of GPDH decreased when the MLP was higher or lower than that found for proteoliposomes formed without exogenous phospholipids. Since the same GPDH activity can be observed at different values of MLP it may be suggested that MLP is not the only factor controlling the enzyme activity. An important role in its modulation can also be ascribed to the alterations in cholesterol/phospholipid ratio and phospholipid composition of the membrane. It may be speculated that GPDH in brown adipose tissue mitochondria is located in the environment most suitable for its activity. However, the requirement of GPDH for a special type of phospholipid cannot be excluded.

Borochov and Shinitzky [26,27] have suggested that membrane proteins may be vertically displaced upon increase in membrane viscosity (MLP) caused by cholesterol enrichment. Such changes of GPDH conformation can occur in proteoliposomes used in this study.

Furthermore, the properties of the bulk phospholipids seem to be responsible for the enzyme inhibition rather than phospholipid annulus. Evidence for this was provided from the experiments with fluorescently labeled cholesterol, which influenced the enzyme activity and was found to be incorporated into bulk phase. Another factor known to affect the membrane-bound enzymes and among them the GPDH is a surface charge [7,28]. The question consequently arose as to which of these factors has a primary effect. Our work suggests that the ambiguity mentioned above is only apparent. We measured the influence of surface charge on the apparent $K_m$ value towards DL-glycerol 3-phosphate and compared it simultaneously with the effect of MLP on the enzyme activity. In agreement with the previous data [7], we found that only $K_m$ value, not the $V_{max}$ value, of the enzyme was affected by variation of the surface charge. With membrane that was made more negative the $K_m$ value increased, whereas with a more positive membrane it decreased (Table II). These changes could, however, be observed only under conditions in which the MLP was not affected.

In conclusion, GPDH is an example of membrane-bound enzyme which activity is modulated by phospholipid phase. Membrane surface charge can influence the accessibility of negatively charged substrate through an unstirred layer. As a consequence, the ap-

parent $K_m$ value is changed but not the $V_{max}$ value. On the other hand, changes in MLP result in the decrease of the $V_{max}$ value, probably by modifying the enzyme conformation.

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