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Localization of the L-Glycerol 1-Phosphate–Flavoprotein Oxidoreductase on the Outer Surface of the Inner Membrane of Insect Flight-Muscle Mitochondria

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The oxidation of L-glycerol 1-phosphate by insect flight-muscle mitochondria follows Michaelis–Menten kinetics in the presence of 0.1 μM-Ca^{2+} or 10 μM-Sr^{2+} or 1.0 mM-Mg^{2+} (Estabrook & Sacktor, 1958; Hansford & Chappell, 1967; Donnellan & Beechey, 1969). In the presence of ethanedioxybis(ethylamine)tetra-acetate to effectively remove all multivalent metal ions the velocity–substrate concentration curve is sigmoidal. We have examined the effects of the following compounds on the oxidation of L-glycerol 1-phosphate in the presence and absence of 0.1 μM-Ca^{2+}: DL-glyceraldehyde 3-phosphate, 2-deoxyglycerol 1-phosphate (propane-1,3-diol 1-phosphate), dihydroxyacetone phosphate, DL-3-phosphoglycerate, DL-2-methylglycerol 1-phosphate, DL-2-propylglycerol 1-phosphate, propyl phosphate and 2-chloroethyl phosphonate. In the presence of Ca^{2+} DL-glyceraldehyde 3-phosphate, 2-deoxyglycerol 1-phosphate, dihydroxyacetone phosphate and D-3-phosphoglycerate are competitive inhibitors, the K_i values being 70 μM, 12 mM, 7 mM and 12·4 mM respectively. In the absence of Ca^{2+} DL-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate still inhibit the oxidation of L-glycerol 1-phosphate. However, all the other compounds in the concentration range 5–30 mM increase the rate of oxidation of L-glycerol 1-phosphate, and the sigmoidicity of the substrate–velocity curve is decreased and in most cases becomes hyperbolic. None of these substances is oxidized by flight-muscle mitochondria. These results imply different specificity requirements for the enzymic and modifier sites on the oxidoreductase molecule.

Thus many phosphate or phosphonate esters affect the oxidation of L-glycerol 1-phosphate. However, the flight-muscle mitochondrial membrane is peculiarly impermeable (Van den Bergh & Slater, 1962). If the L-glycerol 1-phosphate–flavoprotein oxidoreductase (EC 1.1.99.5) is located on the inside of the inner mitochondrial membrane it would imply that the L-glycerol 1-phosphate porter has a very low specificity. The location of the oxidoreductase on the outside of the inner membrane would more satisfactorily explain the ease with which these substances have access to the enzyme and exert their effect.

The latter location of the L-glycerol 1-phosphate–flavoprotein oxidoreductase is supported by the following observations. Antimycin A inhibits the oxidation of pyruvate but not that of L-glycerol 1-phosphate with cyanoferrate as electron acceptor in cyanide-treated mitochondria. This implies that the electron-transport chain from NADH–flavoprotein oxidoreductase to cytochrome *b* is not available to react with cyanoferrate. The latter is a non-permeant anion in rat liver mitochondria (Mitchell & Moyle, 1969). Thus either the L-glycerol 1-phosphate–flavoprotein oxidoreductase or its associated non-haem-iron protein is available to react with cyanoferrate. Also, flight-muscle mitochondria do not swell in iso-osmotic solutions of the ammonium salt of DL-glycerol 1-phosphate, and swelling is noted in iso-osmotic ammonium pyruvate. From this result we conclude that L-glycerol 1-phosphate is an impenetrant anion. This renders it improbable that the L-glycerol 1-phosphate–flavoprotein oxidoreductase is located on the inside of the inner mitochondrial membrane.

The cyanoferrate technique employed in this work was based on information supplied by Professor M. Klingenberg.

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Effects of Calcium Ion and Electron-Acceptor Concentrations on the Activity of Mitochondrial Glycerol 1-Phosphate Dehydrogenase from Insect Flight Muscle

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Mitochondrial glycerol 1-phosphate dehydrogenase (EC 1.1.2.1) can be considered a regulatory enzyme of the glycerol 1-phosphate cycle, and the work of Hansford & Chappell (1967) has suggested that Ca^{2+} controls its activity. The enzyme activity was measured by following the oxygen uptake of mitochondria provided with glycerol 1-phosphate as substrate. In the present investigation the enzyme was assayed more directly by following the reduction of an electron-acceptor dye, INT.* Activation of the enzyme by raising the Ca^{2+} concentration from 1 nm to 10 μM was confirmed. In these assays the enzyme activity is limited by the concentration of

* Abbreviation: INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

INT, but this is overcome by extrapolation of the experimental results to infinite dye concentration. However, on extrapolation of the above results no effect of Ca^{2+} was observed. At 10μM-Ca^{2+} the slopes of reciprocal plots of activity against concentrations of both INT and glycerol 1-phosphate were independent of the invariant substrate. At 1nM-Ca^{2+}, however, slopes of activity against INT varied with concentrations of glycerol 1-phosphate, and plots of activity against glycerol 1-phosphate concentration were not linear.

These results, together with the effect of Ca^{2+}, could be explained if the catalytic reaction was of the single-displacement random-order type (Dalziel, 1957). Thus one pathway would predominate in the presence of excess of INT, and its rate would not be affected by Ca^{2+}; at lower concentrations of INT two pathways would be available, and Ca^{2+} would favour the alternative pathway by increasing the affinity of the enzyme for glycerol 1-phosphate.

If the Ca^{2+} activation of glycerol 1-phosphate dehydrogenase is of physiological importance, then it must be reconciled with the Ca^{2+} inhibition of mitochondrial NAD-linked isocitrate dehydrogenase. The latter is located in the mitochondrial matrix and should respond to Ca^{2+} changes in this compartment whereas glycerol 1-phosphate dehydrogenase is bound to the inner membrane. It is suggested that the latter enzyme may be positioned on the outer face of the inner membrane so that it can respond to Ca^{2+} concentration changes in the outer mitochondrial compartment, which is probably in equilibrium with the sarcoplasm. Thus the increased sarcoplasmic Ca^{2+} concentration caused by nervous stimulation of muscle would lead to stimulation of muscular contraction and the glycerol 1-phosphate cycle, whereas the isocitrate dehydrogenase would respond to changes of Ca^{2+} concentration in the mitochondrial matrix that will be dependent on the transport mechanism of the inner mitochondrial membrane.

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Two Types of Mitochondria in Heart Muscle from Euthyroid and Hyperthyroid Rats

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Previous reports (Hülsmann, De Jong & Van Tol, 1968; Hülsmann, Meijer, Bethlem & Van Wijngaarden, 1969) indicated that skeletal muscle of rat and man contains two types of mitochondria: one with loosely coupled oxidative phosphorylation (M_1) and one with tightly coupled oxidative phosphorylation (M_2).

The present paper extends these findings to rat heart. M_1 mitochondria are released with a Potter–Elvehjem homogenizer (clearance 0.003in) in the following isolation medium: KCl (0.1M), tris–HCl buffer, pH7.4 (0.05M), EDTA (1mM), $MgCl_2$ (5mM), ATP (1mM) and bovine serum albumin (0.8mg/ml). The M_1 mitochondria, isolated by differential centrifugation between 600 and 5000*g*, have a relatively high rate of respiration in the presence of added ATP and Mg^{2+}, due to spontaneous adenosine triphosphatase activity, which is largely oligomycin-resistant. This indicates that M_1 mitochondria are not uncoupled. Indeed, in nine manometric experiments with glutamate as hydrogen donor, the average P/O ratio was 2.7, although the average stimulation of respiration (respiratory control index) by the addition of hexokinase and glucose was only 1.9-fold. M_2 mitochondria, released by Nagarse (EC 3.4.4.16) treatment (Chance & Hagihara, 1960) of the 600*g* sediments, from which the M_1 mitochondria had been extracted, were also isolated by differential centrifugation. They had typical properties of tightly coupled mitochondria: no spontaneous Mg^{2+}-stimulated adenosine triphosphatase, high respiratory control indices and normal P/O ratios when oxidizing glutamate (in nine experiments the average values were 5.1 and 2.8 respectively).

We concluded that M_1 mitochondria had the properties of moderately aged M_2 mitochondria. Phospholipase action has been proposed to contribute to the aging of liver mitochondria, resulting in the unmasking of latent adenosine triphosphatase and loss of respiratory control (Hülsmann, 1958; Hülsmann, Elliott & Slater, 1960; Rossi, Sartorelli, Tato, Baretta & Siliprandi, 1967). Indeed, phospholipase A (EC 3.1.1.4) treatment of M_2 heart mitochondria results in the formation of lipophilic substances, which when added to M_2 mitochondria cause lowering of the respiratory control index.

In thyrotoxicosis the yields of M_1 mitochondria from the hypertrophic hearts are increased. The average yield of M_1 mitochondrial protein from the ventricles of an euthyroid rat heart is 5.6mg (ten animals) and that from a hyperthyroid rat heart 12.0mg (eight animals, with the same average body weight as the controls, namely 240g). If in thyrotoxicosis the amount of M_1 mitochondria in other tissue also increases a simple explanation for the increased basal metabolic rate can be given.

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