Pathways of Hydrogen Transport in the Oxidation of Extramitochondrial Reduced Diphosphopyridine Nucleotide in Flight Muscle

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(Received for publication, February 23, 1962)

Carbohydrates, principally glycogen, trehalose, and glucose, are the energy reserves for the flight of flies. Glycogen deposits are found in the soluble cytoplasm of the muscle, and the initial reactions of glycogen, trehalose, or glucose catabolism are localized in the cytoplasm (1). During glycolysis, the first oxidative step occurs at the triose level and reduced coenzyme, reduced diphosphopyridine nucleotide (DPNH), is formed by glyceraldehyde phosphate dehydrogenase. The pathways in flight muscle whereby this extramitochondrial DPNH becomes oxidized, or the mechanism whereby hydrogen passes through the cytoplasmic-mitochondrial barrier, are examined in this paper.

The various pathways which have been described (2-7) for oxidizing exogenous DPNH by flight muscle include: Pathway 1, the direct mitochondrial oxidation of exogenous DPNH; Pathway 2, the oxidation of DPNH by pyruvate catalyzed by the soluble cytoplasmic lactic dehydrogenase; Pathway 3, the oxidation of DPNH by dihydroxyacetone phosphate catalyzed by the soluble cytoplasmic \( \alpha \)-glycero-phosphate dehydrogenase; and Pathway 4, the oxidation of DPNH by oxaloacetate catalyzed by the soluble cytoplasmic malic dehydrogenase.

The direct mitochondrial oxidation of extramitochondrial DPNH assumes no barrier for DPNH. Pathway 2 leads to the accumulation of lactic acid in the cytoplasmic compartment. On the other hand, Pathways 3 and 4, coupled with the respective mitochondrial oxidation of \( \alpha \)-glycero-phosphate and malate, enable reducing equivalents to pass the cytoplasmic-mitochondrial barrier. These coupled reactions may be illustrated as

\[
\text{DPNH} + H^+ + \text{dihydroxyacetone-P} \rightarrow \text{DPN}^+ + \alpha\text{-glycero-P (cytoplasmic enzyme)} \quad (3a)
\]

\[
\alpha\text{-Glycero-P} + \frac{3}{2}O_2 \rightarrow \text{dihydroxyacetone-P} + \text{H}_2O \quad (\text{mitochondrial enzyme}) \quad (3b)
\]

Sum: \( \text{DPNH} + H^+ + \frac{3}{2}O_2 \rightarrow \text{DPN}^+ + \text{H}_2O \)

Reactions 3a and 3b represent the \( \alpha \)-glycero-P cycle (8, 9), in which substrates acting as carriers to transport hydrogen may affect the complete oxidation of extramitochondrial DPNH.

The malate-oxaloacetate system may function in a manner similar to the \( \alpha \)-glycero-P-dihydroxyacetone-P system. It was demonstrated previously that flight muscle contained both a mitochondrial and an active cytoplasmic malic dehydrogenase (2). The two malic dehydrogenases were later found to be distinct enzymes (5). These enzymes, coupled, could function in a cycle similar to the \( \alpha \)-glycero-P cycle.

Preliminary observations on the possible participation of each of these pathways of hydrogen transport in flight muscle of the blowfly, Phormia regina, were noted earlier (6, 7). The purpose of this communication is to elaborate on these observations. The data demonstrate that exogenous DPNH is not oxidized directly by teased muscle preparations and suggest that, in flight muscle, oxidation of the extramitochondrial pool of DPNH is mediated for the most part by \( \alpha \)-glycero-P dehydrogenase. This enzyme, in combination with the mitochondrial oxidation of \( \alpha \)-glycero-P, provides the major vehicle for passing hydrogen of DPNH through the cytoplasmic-mitochondrial barrier.

EXPERIMENTAL PROCEDURE

Blowflies, Phormia regina, 1 to 2 weeks old, were taken from a laboratory culture. The larvae were reared on horsemeat, and the adult flies were fed powdered milk and sugar. The flies were deprived of food for approximately 24 hours before use. Flight muscle was quickly isolated by pinching the posterior portion of the thorax of the decapitated fly and forcing the muscle bundles out of the anterior end. In spectrophotometric experiments with teased flight muscle, 1 to 2 mg, wet weight, of tissue were put directly into a cuvette containing 0.2 ml of a medium of 5% polyvinylpyrrolidone, 0.05 M sodium phosphate buffer (pH 7.4), and 5 mM EDTA. The muscle was gently teased with the end of a glass rod, the experimental reaction mixture was quickly added, and measurements of DPNH oxidation were started immediately. In respiratory experiments with flight muscle homogenates, muscle bundles were pooled and homogenized in a low speed, loose fitting Potter-Elvehjem homogenizer in a medium of 0.21 M mannitol, 0.07 M sucrose, and 1.0 mM Tris buffer, pH 7.2. Oxygen uptake was determined polarographically with a vibrating platinum electrode (10).

DPNH and pyruvic acid were obtained from the Sigma Chemical Company. Pyruvic acid was redistilled. Dihydroxyacetone-P was purchased from the California Corporation for Biochemical Research and treated with Dowex 50 H+ resin to remove cyclohexylammonium. Oxaloacetate was recrystallized as described by Heidelberger (11).

RESULTS

Flight muscle bundles from Phormia were teased apart within the cuvette, so that measurements of DPNH oxidation could be
started within 1 minute from the time the muscle was removed from the fly. Microscopic examinations of these teased preparations revealed that a large number of the mitochondria were still in situ, adjacent to the myofibrils. As shown in Fig. 1, with this mild treatment there was no direct oxidation of DPNH. This demonstrated the presence of a complete barrier to extramitochondrial DPNH. These observations with teased muscle contrast with earlier studies with isolated mitochondria, wherein appreciable DPNH oxidase activity was found (2, 12). When the muscle was teased vigorously, however, a very slow rate of oxidation was seen.

There was no oxidation of DPNH on addition of pyruvate. In contrast to the veritable absence of Pathways 1 and 2, Fig. 1 also shows that the addition of dihydroxyacetone-P to reaction mixtures containing DPNH and flight muscle caused an immediate oxidation of all of the DPNH. The remarkable activity of this enzyme in fly flight muscle was noted previously (3). In the present experiments, as many as 1200 μmoles of DPNH were oxidized per minute per g, wet weight, of flight muscle. This value in fly skeletal muscle may be compared with an activity of 27 found in the mouse gastrocnemius muscle (13).

The stoichiometry of the α-glycero-P dehydrogenase reaction in teased flight muscle is shown in Fig. 2. In this experiment, in contradistinction to the procedure for the one illustrated in Fig. 1, a limited amount of dihydroxyacetone-P relative to DPNH was used. Antimycin A was added to prevent the mitochondrial oxidation of α-glycero-P formed by the reduction of dihydroxyacetone-P. Addition of 0.115 μmole of dihydroxyacetone-P brought about the oxidation of 0.120 μmole of DPNH. In a stepwise manner, a second addition of dihydroxyacetone-P caused another stoichiometric oxidation of the reduced coenzyme.

According to the reasoning behind the α-glycero-P cycle, limited quantities of dihydroxyacetone-P should oxidize all of the extramitochondrial DPNH. Thus, if a small amount of dihydroxyacetone-P were added to a reaction mixture containing excess DPNH and teased muscle, α-glycero-P would be formed by the cytoplasmic dehydrogenase. The α-glycero-P would be oxidized, in turn, by the mitochondrial oxidase, thereby regenerating additional dihydroxyacetone-P. This dihydroxyacetone-P would then be available for further oxidation of the extramitochondrial DPNH. Such an experiment is shown in Fig. 3, in which a relatively small quantity of dihydroxyacetone-P, in the absence of antimycin A, caused the oxidation of all of the excess extramitochondrial DPNH. These data provided demonstration in vitro of the α-glycero-P cycle.

In addition to studying the α-glycero-P cycle by direct spectrophotometric measurements of DPNH oxidation, we made determinations of oxygen uptake during these reactions. In the latter experiments, whole muscle homogenates were used rather than teased muscle preparations, because the large muscle bundles in the suspensions made from the teased tissue caused considerable extraneous “noise” in the polarographic traces and prevented precise determinations of respiratory rates. Although the teased muscle did not oxidize extramitochondrial DPNH, the homogenates, prepared by limiting homogenization to merely two passes of a loose fitting pestle, had an appreciable direct DPNH oxidase activity. This difference between the teased muscle and the coarse homogenates suggested that even the mild procedure used in preparing the homogenate resulted in partial damage of the mitochondrial membrane. Addition of DPNH to an aerobic medium containing muscle homogenate initiated a respiration of 0.69 μm O2 per second (Fig. 4). About 1 minute later, when approximately 0.32 μmole of DPNH remained, 0.32 μmole of dihydroxyacetone-P was added. An immediate decrease in the oxidation rate to a value of 0.34 μm O2 per second was observed. This rapid fall in oxygen uptake upon addition of dihydroxyacetone-P indicated a sharp decline in DPNH concentration and suggested that the cytoplasmic α-glycero-P dehydrogenase in the homogenate competed successfully with the mitochondrial DPNH oxidase for extramitochondrial DPNH.

**Fig. 1.** Oxidation of exogenous DPNH by teased flight muscle. The reaction mixture contained 33.3 mM triethanolamine-HCl buffer, pH 7.4; 0.165 mM DPNH; 10 mM MgCl2; and flight muscle (1.89 mg, wet weight). Final volume, 3.0 ml. Pyruvate and dihydroxyacetone-P (DHAP) were added as indicated.

**Fig. 2.** The stoichiometry of the α-glycero-P dehydrogenase reaction in teased flight muscle. The reaction mixture contained 33.3 mM triethanolamine-HCl buffer, pH 7.4; 0.165 mM DPNH; 10 mM MgCl2; and flight muscle (4.72 mg, wet weight). Final volume, 3.0 ml. Dihydroxyacetone-P (DHAP) and antimycin A (ANTIM. A), 3 μg, were added as indicated.

**Fig. 3.** Oxidation of extramitochondrial DPNH. Vol. 237, No. 10.
Fig. 3. Oxidation of an excess quantity of DPNH by dihydroxyacetone-P. The reaction mixture contained 33.3 mM sodium phosphate buffer, pH 7.4; 0.14 mM DPNH; 10 mM MgCl₂; 3.3 mM ADP; and flight muscle (0.90 mg, wet weight). Final volume, 3 ml. Dihydroxyacetone-P (DHAP) was added as indicated.

The relatively low respiratory rate found after addition of triose-P was due probably to the fact that the maximal level of α-glycero-P that could be produced in the reaction is 0.16 mM, a value considerably below the \( K_m \) of the mitochondrial α-glycero-P oxidase (8). Fig. 4 also shows that the new respiratory rate was not affected by 1 mM Amytal. This observation indicated that the oxygen uptake occurring after dihydroxyacetone-P had been added was not due to oxidation of DPNH by the mitochondrial DPNH oxidase, for the concentration of Amytal used would have completely blocked this pathway. On the other hand, it has been shown previously (12) that mitochondrial oxidation of α-glycero-P is not inhibited by this concentration of barbiturate. Thus, the lack of inhibition by Amytal in the present experiment provided supplementary evidence that the respiration following addition of dihydroxyacetone-P was due to α-glycero-P oxidation.

It was shown earlier that EDTA competitively inhibited mitochondrial oxidation of α-glycero-P but had no effect on the oxidation of DPNH or succinate (8, 14). This finding was used to provide further evidence that α-glycero-P was the substrate for the oxygen uptake observed after dihydroxyacetone-P had been placed into reaction mixtures containing DPNH and muscle homogenates. Fig. 5 shows that in such an experiment dihydroxyacetone-P caused a decrease in oxygen uptake similar to that seen previously in Fig. 4. Now, 0.5 mM EDTA was added. Oxidation was immediately inhibited by over 90%. Subsequent additions of α-glycero-P reversed this inhibition.

In another experiment, designated as 61-1-3 in Table I, dihydroxyacetone-P was added first to the aerobic medium containing muscle homogenate. A slow rate of oxygen uptake, 0.14 \( \mu \)M O₂ per second, was found. Now, 0.52 \( \mu \)mole of DPNH was added. Respiration increased immediately to 1.11 \( \mu \)M O₂ per second. This rapid rate of oxygen uptake lasted for less than 30 seconds, however, and then decreased to 0.20 \( \mu \)M O₂ per second. Calculation of the total oxygen uptake during this transitory phase of enhanced respiration showed that only 0.16 \( \mu \)atom of oxygen was used. This quantity was not enough to deplete the 0.52 \( \mu \)mole of DPNH added and indicated that DPNH was oxidized mostly via the α-glycero-P dehydrogenase reaction. That the lowered level of respiration resulted truly from a decreased titer of DPNH was indicated by the increased respiration subsequent to a second addition of the coenzyme. Again, dihydroxyacetone-P immediately caused the decreased oxygen uptake characteristic of oxidation in the presence of low levels of α-glycero-P. Finally, the addition of a sufficient titer of α-glycero-P initiated a very rapid oxygen uptake until oxygen was exhausted. In a control experiment, 61-1-4, 0.52 \( \mu \)mole of DPNH was added to the medium. This caused an initial rapid O₂ uptake, but after approximately 2 minutes, respiration ceased. It was calculated that 0.52 \( \mu \)atom of oxygen was utilized during the reaction. The addition of α-glycero-P restored oxygen consumption, demonstrating that the mito-
Oxidation of Extramitochondrial DPNH

A comparison of the activities of the cytoplasmic α-glycero-P and malic dehydrogenases in *Phormia* flight muscle homogenates is shown in Fig. 6. Oxaloacetate, at optimal concentration, caused the oxidation of exogenous DPNH at a rate one-fourth of that obtained with dihydroxyacetone-P. However, the rate of DPNH oxidation by malic dehydrogenase fell sharply after several minutes. Attempts to show the oxidation of an excess of exogenous DPNH with a limited quantity of oxaloacetate, thereby demonstrating a malate-oxaloacetate cycle, were unsuccessful. Efforts to stimulate the cycle through increasing the mitochondrial oxidation of malate by addition of 50 μM pyruvate were without effect.

**DISCUSSION**

“Intact” mitochondria from mammalian tissues do not oxidize added DPNH because of the impermeability of the mitochondria for reduced coenzyme (15-17). However, isolated flight muscle mitochondria oxidized exogenous DPNH at appreciable rates (2, 4, 12). In the present experiments, in which teased muscle was used, extramitochondrial DPNH was not oxidized directly. In agreement with the findings with mammalian mitochondria, the mitochondrial barrier was found to be complete in this “more physiological” preparation. In contrast, Zebe, Delbruck, and Bücher (4) reported that teased muscle preparations of *Locusta* oxidized added DPNH and calculated that 40% of the extramitochondrial DPNH was oxidized directly by the mitochondria. They suggested that their preparations were probably damaged and that this value was too high.

In the absence of direct mitochondrial oxidation of extramitochondrial DPNH, reoxidation of the reduced pyridine nucleotide must be catalyzed by other routes. The data in Fig. 1 clearly showed that in flight muscle the lactate dehydrogenase reaction, Pathway 2, was not used. Instead, the cytoplasmic α-glycero-P dehydrogenase brought about the rapid oxidation of the added DPNH. From earlier observations on the activities of this enzyme as well as the mitochondrial α-glycero-P oxidase, an α-glycero-P cycle was proposed which could shuttle reducing equivalents from extramitochondrial DPNH to the intramitochondrial electron transport system (8, 9, 18). The experiments, exemplified by Fig. 3, which show that the addition of a small quantity of dihydroxyacetone-P results in the oxidation of excess exogenous DPNH, demonstrate the operation of the cycle in *vitro*.

The presence of cytoplasmic and mitochondrial malic dehydrogenases in flight muscle suggested that a coupling of these reactions could participate in a transfer system analogous to that of the α-glycero-P cycle. As shown in Fig. 6, the cytoplasmic enzyme oxidized added DPNH at a rapid initial rate. Efforts to demonstrate the shuttle were unsuccessful, however. It should be pointed out that the oxidation of malate by fly

**Table I**

Oxidation of exogenous DPNH by muscle homogenates

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions in order</th>
<th>Respiration (μM O₂/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>61-1-3</td>
<td>Dihydroxyacetone-P (0.16 mM)</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>DPNH (0.26 mM)</td>
<td>1.11 → 0.20</td>
</tr>
<tr>
<td></td>
<td>DPNH (0.26 mM)</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>Dihydroxyacetone-P (0.16 mM)</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>α-Glycerol-P (2 mM)</td>
<td>2.48</td>
</tr>
<tr>
<td>61-1-4</td>
<td>DPNH (0.26 mM)</td>
<td>1.33 → 0</td>
</tr>
<tr>
<td></td>
<td>α-Glycerol-P (2 mM)</td>
<td>2.47</td>
</tr>
</tbody>
</table>

**Fig. 6.** Comparison of the activities of the cytoplasmic malic and α-glycero-P dehydrogenases in flight muscle homogenates. The reaction medium was the same as described in Fig. 1, except that 0.01 ml of homogenate was used instead of the teased muscle. Flight muscle was homogenized in 5 ml of teased muscle medium (0.29 mg of protein per ml). The concentrations of dihydroxyacetone P (DHAP) and oxaloacetate (OXALOAC) were as indicated.
muscle mitochondria was only 2% that of \( \alpha \)-glycero-P (12), indicating that this muscle need not be the preferred tissue to establish such a cycle. The absence of \( \beta \)-hydroxybutyrate dehydrogenase from flight muscle mitochondria (1) excludes the oxidation-reduction system involving acetocetate and \( \beta \)-hydroxybutyrate (19, 20), and no evidence has yet been obtained in flight muscle for a cytoplasmic pyridine nucleotide diaphorase linked with a quinone (21) or an extramitochondrial DPNH-intramitochondrial DPNH transhydrogenase (22).

Previous observations that the \( \alpha \)-glycero-P cycle enzymes had sufficient activity to account for the high respiratory rate during flight, whereas the rates with citric acid cycle intermediates were much less, suggested that the \( \alpha \)-glycero-P cycle was of physiological importance in flight muscle (7, 12). Oxygen uptake rates with \( \alpha \)-glycero-P exceeding those with succinate by a factor of 10 and with other citric acid cycle substrates by factors as high as 50 were confirmed (4, 24-26). The low respiratory rates with many of the citric acid cycle substrates were much less, suggested that the \( \alpha \)-glycero-P cycle was of physiological importance in flight muscle (7, 12), whereas the rates with citric acid cycle intermediates were insufficient activity to account for the high respiratory rate during flight, whereas the rates with succinate by a factor of 10 and with other citric acid cycle substrates by factors as high as 50 were confirmed (4, 24-26). The low respiratory rates with many of the citric acid cycle substrates were much less, suggested that the \( \alpha \)-glycero-P cycle was of physiological importance in flight muscle (7, 12), whereas the rates with citric acid cycle intermediates were insufficient activity to account for the high respiratory rate during flight, whereas the rates with succinate by a factor of 10 and with other citric acid cycle substrates by factors as high as 50 were confirmed (4, 24-26).

**SUMMARY**

Teased flight muscle preparations of the blowfly do not oxidize added reduced diphosphopyridine nucleotide (DPNH) directly. This indicates a complete cytoplasmic-mitochondrial barrier to extramitochondrial DPNH. Alternative pathways whereby the reduced pyridine nucleotide becomes oxidized have been examined. DPNH is not oxidized by lactic dehydrogenase, which is essentially absent from flight muscle. The cytoplasmic \( \alpha \)-glycero-phosphate dehydrogenase is 4 times as active as the cytoplasmic malic dehydrogenase. An \( \alpha \)-glycero-phosphate cycle has been demonstrated whereby an excess of exogenous DPNH is oxidized by the alternate reduction of dihydroxyacetone phosphate, mediated by the cytoplasmic \( \alpha \)-glycero-phosphate dehydrogenase, and oxidation of \( \alpha \)-glycero-phosphate, mediated by the mitochondrial \( \alpha \)-glycero-phosphate oxidase. A malate-oxaloacetate shuttle system analogous to the \( \alpha \)-glycero-phosphate cycle has not been demonstrated in flight muscle. The significance of the \( \alpha \)-glycero-phosphate cycle is discussed.

**REFERENCES**

Pathways of Hydrogen Transport in the Oxidation of Extramitochondrial Reduced Diphosphopyridine Nucleotide in Flight Muscle
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