The Control of the Oxidation of Proline by Isolated Flight Muscle Mitochondria

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SUMMARY

The oxidation of proline by blowfly (Phormia regina) flight muscle mitochondria is stimulated by ADP in the presence of oligomycin. The stimulation is enhanced by high levels of P:\textsubscript{i}, is still evident with intact mitochondria in the presence of rotenone, arsenate, or arsenite, and is seen with sonically treated particles. The findings indicate that the site of action of the nucleotide is L-proline:cytochrome c oxidoreductase (proline dehydrogenase).

It is suggested that ADP is an allosteric effector of the dehydrogenase and lowers the apparent \( K_m \) for proline. The \( K_m \) in the presence of ADP approximates the concentration of proline found in the flight muscle at rest. Pyruvate also increases the affinity of the proline dehydrogenase for its substrate.

The rate of proline oxidation is dependent on the relative proportions of the adenine nucleotides as well as on the absolute level of ADP. Sensitivity to ADP in the presence of an uncoupling agent or atractyloside suggests that there is an external site for ADP.

The physiological significance of the activation of proline oxidation is considered.

On the initiation of flight, the level of proline in fly flight muscle drops abruptly (1). It has been suggested that the mitochondrial oxidation of proline is facilitated by the rest to flight transition and that this oxidation is crucial in providing the Krebs cycle intermediates necessary for the rapid and complete oxidation of pyruvate formed by glycolysis (2). In view of this role, a study was initiated to explore mechanisms for the regulation of proline oxidation. Previous studies have indicated that mitochondria isolated from flight muscle of flies oxidize proline via \( \Delta^1 \)-pyrroline-5-carboxylate and glutamate to yield intermediates of the Krebs cycle (2-6).

In experiments in which the formation of \( \Delta^1 \)-pyrroline-5-carboxylate was measured, the reaction mixtures contained 50 \( \mu \)moles of L-proline, 50 \( \mu \)moles of potassium phosphate, pH 7.1, 10 \( \mu \)moles of ADP, 400 \( \mu \)moles of potassium chloride, 2.5 mg of bovine serum albumin, and 10 \( \mu \)moles of rotenone in a total volume of 4.8 ml. In those incubations containing pyruvate, 20 \( \mu \)moles of sodium pyruvate were added. After addition of 0.2 ml of mitochondrial suspension (approximately 5 mg of protein) the mixtures were incubated at 25° for 22 min. Then 2 ml of ethanol, followed by 1 ml of saturated aminobenzaldehyde and 10% v/v trichloroacetic acid, also in ethanol, were added to each tube. \( \Delta^1 \)-Pyrroline-5-carboxylate was determined according to the method of Strecker (9).

RESULTS

It was found that addition of ADP subsequent to the addition of oligomycin stimulated the rate of proline oxidation (Table I). This finding suggested that ADP was acting at the dehydrogenase level, or at the level of substrate access. This was confirmed in experiments with phenazine methosulfate and cyanide; under these conditions the respiratory chain was bypassed, yet ADP still stimulated.

Fig. 1 illustrates the dependence of this effect upon ADP concentration. It is seen that approximately 3 \( \mu \)mole nucleotide was required for a maximal stimulation; a half-maximal rate was achieved at about 0.3 \( \mu \)mole ADP.

Table I also shows that the rate of proline oxidation was not enhanced by uncoupling agents; however, a subsequent addition of ADP resulted in a large increase in rate. In parallel experiments in which the same amount of uncoupler, carbonyl cyanide

EXPERIMENTAL PROCEDURE

Analytical grade reagents were used throughout. Solutions were neutralized to pH 7.1. Oligomycin and rotenone were bought from Sigma and made up in ethanol. Atractyloside was a gift from Professor J. B. Chappell of the University of Bristol. Mitochondria were prepared from the flight muscle of the blowfly (Phormia regina) as described (7). Flies were fed milk and sugar and were used from 7 to 21 days after emergence. Sonic particles were prepared by subjecting suspensions of mitochondria in 0.13 M KCl-10 mM triethanolamine, pH 7.1, at about 10 mg of protein per ml, to 2 min of sonic treatment by the Branson Instruments Sonifier tuned at 1.8 amp. This was done in 20-sec bursts, with the mitochondrial suspension cooled in an ice-NH\textsubscript{4}Cl mixture.

Rates of oxidation were obtained with an oxygen electrode (8). Mitochondrial protein was determined by the biuret method, after solubilization with Triton X-100 (final concentration 1%, v/v).

In experiments in which the formation of \( \Delta^1 \)-pyrroline-5-carboxylate was measured, the reaction mixtures contained 50 \( \mu \)moles of L-proline, 50 \( \mu \)moles of potassium phosphate, pH 7.1, 10 \( \mu \)moles of ADP, 400 \( \mu \)moles of potassium chloride, 2.5 mg of bovine serum albumin, and 10 \( \mu \)moles of rotenone in a total volume of 4.8 ml. In those incubations containing pyruvate, 20 \( \mu \)moles of sodium pyruvate were added. After addition of 0.2 ml of mitochondrial suspension (approximately 5 mg of protein) the mixtures were incubated at 25° for 22 min. Then 2 ml of ethanol, followed by 1 ml of saturated aminobenzaldehyde and 10% v/v trichloroacetic acid, also in ethanol, were added to each tube. \( \Delta^1 \)-Pyrroline-5-carboxylate was determined according to the method of Strecker (9).
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TABLE I

Stimulation of proline oxidation by ADP

Mitochondria were suspended in 2 ml of 0.12 M KCl-10 mM triethanolamine, pH 7.1. In Experiment 1, the reaction mixture contained 10 mM L-proline and 6.3 mM potassium phosphate, pH 7.1. Oligomycin was added to 1 μg per ml and ADP to 1.5 mM, as indicated. In Experiment 2, the reaction contained 12.5 mM L-proline and 6.3 mM potassium phosphate buffer. Potassium cyanide, pH 7.1, was added to 3 mM, phenazine methosulfate to 0.5 mg per ml, and ADP to 1.5 mM, as indicated. Experiments 3 and 4 contained 20 mM L-proline and 10 mM potassium phosphate buffer. Where indicated, carbonyl cyanide p-trifluoromethoxyphenylhydrazone was added to 1 μM, ADP to 1 mM, and atractyloside to 0.5 mg per ml. The temperature was 25°.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Rate (μg atom O2/min/mg mitochondrial protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>Proline + oligomycin</td>
<td>0.016</td>
</tr>
<tr>
<td>ADP</td>
<td>0.051</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>Proline + KCN</td>
<td>Nil</td>
</tr>
<tr>
<td>Phenazine methosulfate</td>
<td>0.013</td>
</tr>
<tr>
<td>ADP</td>
<td>0.050</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>0.019</td>
</tr>
<tr>
<td>Carbonyl cyanide p-trifluoromethoxyphenylhydrazone</td>
<td>0.014</td>
</tr>
<tr>
<td>ADP</td>
<td>0.053</td>
</tr>
<tr>
<td>Experiment 4</td>
<td></td>
</tr>
<tr>
<td>Proline + atractyloside</td>
<td>0.032</td>
</tr>
<tr>
<td>ADP</td>
<td>0.057</td>
</tr>
</tbody>
</table>

FIG. 1. The dependence upon ADP of proline oxidation in the presence of an uncoupler. Mitochondria were suspended in a medium comprising 0.1 M potassium chloride, 12.5 mM potassium phosphate, pH 7.1, 25 mM L-proline, and 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone. ADP was present at the concentrations indicated.

FIG. 2. The dependence of the rate of proline oxidation upon the composition of a mixture of ADP and ATP. Mitochondria were added to a medium comprising 0.1 M potassium chloride, 10 mM triethanolamine, pH 7.1, 16.4 mM L-proline, and 1 μg per ml of oligomycin. In addition, each incubation contained mixtures of ADP and ATP of the composition shown, and totaling 4.1 mM. O---O, experiments containing 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone, and the same amount of mitochondrial protein were used, pyruvate oxidation was maximally stimulated, in accord with previous results (10). Since, as shown in Fig. 1, the concentration of ADP needed to activate proline oxidation approximates that needed to activate pyruvate oxidation (10), these observations suggest that the ADP produced from endogenous ATP on uncoupling is not accessible for activation of proline dehydrogenase, whereas it is accessible to the NAD-linked isocitrate dehydrogenase, the rate-limiting enzyme of pyruvate oxidation (10, 11).

ADP also increased the rate of proline oxidation in the presence of atractyloside (Table I), but stimulated pyruvate oxidation under identical conditions to a much lesser extent. In view of the proposal that atractyloside achieves a separation of internal and external adenine nucleotide by inhibiting the translocase (12, 13), it is suggested that in the case of proline oxidation ADP is acting external to the atractyloside barrier.

It should be noted from Fig. 1 that the requirement for 3 mM ADP for maximal proline oxidation was obtained in the absence of ATP and in the presence of uncoupler. On the other hand, as described in Fig. 2, when mixtures of ADP and ATP were added to respiring mitochondria such that the proportions of nucleotides in the mixture varied while the total adenine nucleotide concentration remained the same, it was found that a maximal rate was not achieved until 100% of the nucleotide was ADP. It should be noted that these experiments were performed in the presence of oligomycin to prevent interaction of the ADP with the respiratory chain. Adenylate kinase activity within the mitochondria might have caused some change in the composition of these mixtures, although rates of oxidation were essentially linear with time. The external adenylate kinase activity, i.e. external to the atractyloside barrier, of these preparations is very low in the absence of added Mg++. Under these conditions the internal mitochondrial adenine nucleotide composition should be manipulated through changes in the composition of the external mixture, the total level of the internal...
Fig. 3. The kinetics of the oxidation of proline by intact mitochondria. The medium comprised 0.1 M potassium chloride, 10 mM triethanolamine, pH 7.1, 12.5 mM potassium phosphate, pH 7.1, and L-proline as indicated. C—-C, 2.3 mM ADP added.

Fig. 4. The effect of phosphate concentration on the response to the composition of mixtures of ADP and ATP. Conditions were as described for Fig. 2 except that arsenate was replaced by phosphate at the concentrations indicated. ○, O, M, and □, 41 mM, 90 mM, 11 mM, and 3 mM phosphate, respectively.

nucleotide remaining constant being fixed by that present initially within the mitochondrion (13).

Fig. 2 also illustrates the marked differences obtained with coupled and uncoupled mitochondria. The displacement is most easily explained on the basis that coupled mitochondria effectively take a biased sample of adenine nucleotide, discriminating in favor of ADP (14), although an accumulation of P_i (also an activator, see below) in the coupled state (15) may contribute. When the experiments were examined further with a total of 15 mM adenine nucleotide, this being approximately the level estimated in flight muscle assuming the muscle represents half the wet weight of the fly thorax (16), identical curves were obtained. The steep slope of the curve with coupled mitochondria in the region corresponding to a high percentage of ATP suggests that the relatively small changes in ATP and ADP levels which were found when blowflies began to fly (19) would lead to an appreciable increase in the rate of proline oxidation.

Two explanations of the apparent inhibition by ATP are evident. One is that ATP directly inhibited the proline dehydrogenase. This is less likely since ATP did not inhibit the activation by ADP of proline oxidation by submitochondrial particles, prepared by sonic treatment. The other is that ATP displaced ADP from the inside of the mitochondrion. This alternative explanation is consistent with the difference between the curves for coupled and uncoupled mitochondria, as shown in Fig. 2. It thus appears that there are two requirements for a maximal rate of proline oxidation. One is that there be a certain absolute concentration of ADP in the medium (Fig. 1). The other is that the intramitochondrial nucleotide be largely ADP.

The data in Fig. 3 indicate that the mode of action of ADP on proline oxidation was to increase the affinity of the mitochondria for proline. ADP decreased the apparent K_m for proline (17). Significantly, the K_m in the presence of ADP approximates the concentration of proline found in the flight muscle at rest (1). The possibility that ADP was acting at the level of penetration of the mitochondrion by proline was ruled out by experiments which showed that submitochondrial particles, obtained by sonic disruption of the mitochondria, in which no permeability barriers would be expected to remain, were still sensitive to ADP. In these preparations, as in intact mitochondria, ADP increased the rate of proline oxidation and lowered the K_m for proline. These results indicate that ADP is an allosteric effector of proline dehydrogenase.

Since blowfly flight muscle mitochondria metabolized proline to oxalacetate (2, 17), the possibility that the ADP effect on the oxidation of proline was caused by a requirement for ADP by some component of the Krebs cycle or by the oxidation of Δ1-pyrroline-5-carboxylate or glutamate was also examined. The ADP requirement persisted in the presence of the following: rotenone, which blocked the conversion of Δ1-pyrroline-5-carboxylate to glutamate; arsenite, which prevented the further metabolism of α-ketoglutarate; and arsenate, which uncoupled substrate level phosphorylation. These observations suggest that proline dehydrogenase itself is activated by ADP and is rate-limiting in the oxidation of proline.

As shown in Fig. 4, the rate of oxidation of proline, in the presence of ADP, was enhanced additionally by a high level of phosphate. In fact, a maximal rate was not achieved below 40 mM. Interestingly, it was previously found that these mitochondria accumulated phosphate when coupled (19).
the absence of ADP, the level of phosphate was of little significance.

Addition of pyruvate to mitochondria oxidizing proline in the presence of rotenone stimulated respiration. Control experiments usually indicated that pyruvate oxidation was inhibited 99.9% by rotenone. However, if a perceptible rate with pyruvate alone was obtained, the value was subtracted from the rate with proline plus pyruvate. Nevertheless, the effect was also investigated directly, by estimating the amount of Δ¹-pyrroline-5-carboxylate formed in the presence and absence of pyruvate. The results confirmed the stimulation; in two experiments the Δ¹-pyrroline-5-carboxylate formed in the presence and absence of pyruvate averaged 415 and 175 μmoles, respectively. Fig. 5 shows that pyruvate decreased the apparent Kᵹ for proline. Since there is a severalfold increase in the concentration of pyruvate in flight muscle upon initiation of flight (1), this would also make an attractive way of stimulating proline oxidation. However, it was found that the enhancement of the rate of proline oxidation was still maximal at 0.2 mM pyruvate, the concentration found in resting muscle. The significance of this effect is therefore uncertain.

**DISCUSSION**

Blowfly flight muscle mitochondria oxidize extremely rapidly the two products of glycolysis in this tissue, glycerophosphate and pyruvate. In both cases a key dehydrogenase is sensitive to a signal of the metabolic state of the muscle. Thus, glycerophosphate dehydrogenase is stimulated by Ca²⁺ (7); isocitrate dehydrogenase is sensitive to a multitude of factors, but primarily to adenine nucleotide (10). It now seems that the oxidation of proline, suggested as important to the operation of the Krebs cycle in this tissue, is also affected by ADP at the dehydrogenase level. The stimulation by ADP of proline oxidation in sonically treated particles indicates that the effect is not related to the penetration of proline into the mitochondrion.

The oxidations of glycerophosphate, pyruvate, and proline by flight muscle mitochondria are also subject to control by ADP in the classical manner in the respiratory chain. The regulation at two levels focuses attention as to the control of respiration in isolated mitochondria and in the muscle in situ. This problem has been discussed in some depth in recent reviews (18, 19).

**REFERENCES**

The Control of the Oxidation of Proline by Isolated Flight Muscle Mitochondria
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