The Interaction of Energy and Electron Transfer Reactions in Mitochondria

V. THE ENERGY TRANSFER PATHWAY*

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A study of the velocity with which adenosine 5'-triphosphate interacts with the carriers of the respiratory chain presents an unusual opportunity to explore the mechanism of action of the energy transfer pathway (1). Previous studies of this mechanism have been limited to: (a) systems in which a portion of the pathway toward the carriers was measured by the O2 exchange reaction (2), the adenosine 5'-triphosphate-P exchanges exchange reaction (3, 4) (Equations 1 and 2); and (b) adenosine 5'-triphosphatase reactions (5, 6) in which high energy intermediates (X - I (7)) are rapidly hydrolyzed by treatment with uncoupling agents (Equations 1 to 3):

\[
\begin{align*}
\text{ATP} + X & \rightarrow \text{ADP} + X \sim P \quad (1) \\
X \sim P + I & \rightarrow X \sim I + P_1 \quad (2) \\
X \sim I & \rightarrow \text{dinitrophenol} \quad (3)
\end{align*}
\]

In adenosinetriphosphatase activity, the pathway of energy transfer is studied under conditions under which no oxidative phosphorylation can be observed. In neither adenosinetriphosphatase nor exchange reactions does the reaction proceed to the carrier level, despite suggestions to the contrary (3), although indirect effects of the carrier oxidation state are observable (3, 4).

The kinetics of two types of adenosine 5' triphosphate linked reactions are discussed here: (a) those leading to pyridine nucleotide reduction in aerobic mitochondria and (b) those leading to cytochrome oxidation and pyridine nucleotide reduction in anaerobic or terminally inhibited mitochondria. The effects of inhibitors such as dinitrophenol, magnesium, adenosine 5'-diphosphate, phosphate, and oligomycin on the pathways of energy transfer are examined. The inhibitor sensitivities identify the energy transfer pathway for the reversal of electron transfer with that for oxidative phosphorylation. Thus, a new approach to the kinetic properties of reactions coupling electron transfer to the formation and breakdown of adenosine 5'-triphosphate is provided; these data can be compared with those on adenosinetriphosphatase and adenosine 5'-triphosphate-P exchanges.

EXPERIMENTAL PROCEDURE

The mitochondrial preparations, reaction media, and physical techniques are described elsewhere (see Paper I of this series (8)).

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RESULTS

Succinate-linked Pyridine Nucleotide Reduction—As discussed elsewhere (9), small concentrations of ATP cause appreciable reduction of pyridine nucleotide, the rate of the reaction being proportional over a limited range to the concentration of ATP (Fig. 1A). Concentrations below a certain minimum (20 \mu M ATP) cause a scarcely measurable rate of reduction of pyridine nucleotide, whereas a hyperbolic relationship is obtained at larger concentrations. The maximal rate at which ATP can transfer its energy into the reduction of pyridine nucleotide is estimated to be a plateau value of \(~0.44 \mu M\) DPNH per second. This value can be converted to a cytochrome c or protein basis. In the latter case, the value corresponds to 24 mmoles of DPNH per minute per mg of protein; in the former, the "turnover number" of DPNH, referred to the concentration of cytochrome c, gives 0.66 1-electron equivalents in DPNH per second per mole of cytochrome c. These data can be compared with the "turnover number" of cytochrome c in the oxidative reaction which is 1.5 sec\(^{-1}\) for state 4,\(+\) and 5.6 sec\(^{-1}\) for state 3,\(+\) (cf. Table II).

The kinetic curves for such an experiment indicate that an accurate second order velocity constant for the reaction of ATP with the respiratory chain cannot be obtained, although it apparently approaches 0.25 \mu M DPNH per 100 \mu M ATP per second per mg of protein.

Another approach to the kinetics of succinate-linked reduction of pyridine nucleotide is provided by \(\alpha\)-ketoglutarate, low concentrations of which cause a specific and highly efficient (9, 10) reduction (Fig. 1B) attributable to internally generated succinate and ATP. The plateau value is 0.9 \mu M DPNH per second, which when converted as for Fig. 1A gives an over-all activity of 10.8 mmoles of DPNH per minute per mg of protein and a "turnover number" of 0.26 per second per mole of cytochrome c (cf. Table II).

A third approach to the kinetics of the reduction is to pretreat the mitochondria with glutamate. Data obtained with this and the two preceding methods are summarized in Table I. When 4 mM glutamate is added before succinate, it is slightly more effective than 0.1 mM ATP. When succinate is added before glutamate, however, the rate of formation of DPNH is comparable to that in the presence of \(\alpha\)-ketoglutarate. Glutamate pretreatment apparently has the advantage of precharg-

1 The abbreviations used are: subscript "g," in the presence of glutamate; subscript "a," in the presence of succinate.
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reduction or cytochrome c oxidation and the concentrations of ATP, ADP, or \( \alpha \)-ketoglutarate added. Mitochondria pretreated with 3.6 mM succinate; 1.4 \( \mu \)M pyridine nucleotide reduction. Aerobic mitochondria pretreated with 4 mM succinate; 1.2 mg of protein per ml (Experiment 140e); B. Effect of concentrations of \( \alpha \)-ketoglutarate on rate of pyridine nucleotide reduction; 2.5 mg of protein per ml (Experiment 149e); C. Relationship between rate of cytochrome c oxidation and concentration of ATP added. Mitochondria pretreated with 300 \( \mu \)M sodium sulfide (no succinate); 1.2 mg of protein per ml (Experiment 198b); D. Effect of various concentrations of ADP on kinetics of cytochrome c oxidation and pyridine nucleotide reduction. Aerobic mitochondria (state 4), pretreated with 4 mM succinate, 140 \( \mu \)M ATP, and 4 mM phosphate; 1.2 mg of protein per ml (Experiment 201b).

**Table I**

Effect of various energy sources on rate of pyridine nucleotide reduction in pigeon heart mitochondria

Measured spectrophotometrically at 340 to 374 m\( \mu \). Mannitol-sucrose-Tris medium (pH 7.4); 2.6 mg of protein per ml; temperature 20°C (Experiment 165a).

<table>
<thead>
<tr>
<th>Energy source</th>
<th>Concentration</th>
<th>Rate of formation of DPNH on adding 4 mM succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>4</td>
<td>0.35</td>
</tr>
<tr>
<td>( \alpha )-Ketoglutarate</td>
<td>0.6</td>
<td>0.16</td>
</tr>
<tr>
<td>ATP</td>
<td>0.1</td>
<td>0.26</td>
</tr>
</tbody>
</table>

ing the system with very low concentrations of succinate and ATP so that, upon addition of excess succinate, the reduction can proceed rapidly. Glutamate alone produces only 15% of the reduction obtained with both glutamate and succinate (8). The kinetics of reduction caused by added succinate and ATP, by \( \alpha \)-ketoglutarate, and by glutamate are sufficiently similar that they may involve the same reaction mechanism for pyridine nucleotide reduction.

**Anaerobic Energy Transfer from ATP to Cytochrome and Pyridine Nucleotide**—By measuring the rates of change of cytochrome and pyridine nucleotide concentrations in records such as those of Paper II (9), plateau values for the maximal rates of energy transfer from ATP to cytochrome oxidation or pyridine nucleotide reduction are obtained; those for several experiments are given in Table II. On the basis of protein content or “turnover number,” the values obtained under anaerobic conditions (Experiment 195b) exceed those obtained under aerobic conditions by factors of 2 to nearly 10. An explanation for this increased rate (9) is that, under anaerobic conditions, oxygen does not compete for reducing equivalents from succinate. An explanation for the greater rate of pyridine nucleotide reduction as compared with that of cytochrome oxidation is that in the anaerobic system succinate can also contribute reducing equivalents to pyridine nucleotide.

At low concentrations of ATP, the relationship between the concentrations and the rate of cytochrome oxidation (Fig. 1C) is linear; when the slope of this trace is converted to units similar to those of Table II (\( \mu \)M cytochrome c per second per mg of protein per 100 \( \mu \)M ATP), values of 1.5 (1-electron) and 0.75 (2-electron) are obtained. The values computed from Figs. 1A and 1B are 0.25 and 0.84, respectively, on a 2-electron basis.

**Kinetics of Reaction with ADP**—To compare the velocity of the reaction in which energy is transferred from ATP to the respiratory carriers with that with which ADP can interact in the forward direction, we have obtained the maximal values of the latter for pyridine nucleotide and cytochrome c in pigeon heart mitochondria (Fig. 1D). Under the experimental conditions used, 3.6 \( \mu \)M pyridine nucleotide and 0.01 \( \mu \)M cytochrome c are oxidized per second, the “turnover” of the former being rapid and that of the latter being slow. On comparison of these with ATP-activated rates (Table II), it is seen that the rates of pyridine nucleotide oxidation on adding ADP are faster than those of its reduction on adding ATP.

**Effects of Inhibitors: ADP and Phosphate**—According to Equations 1 to 3, either ADP or phosphate should inhibit the transfer of energy from ATP to X \( \sim \) I. The effect of ADP on ATP-induced reduction of pyridine nucleotide in the presence of succinate is illustrated in Fig. 2, where the reduction (downward deflection of the trace) is interrupted and reversed by addition of 100 \( \mu \)M ADP. The reaction of Equation 1 has clearly been affected, and added phosphate was not necessary for the ADP inhibition of energy transfer.

As discussed elsewhere (10, 11), \( \alpha \)-ketoglutarate functions chiefly to provide succinate and ATP in a highly efficient energy-linked reduction of pyridine nucleotide. The effects of ADP and phosphate on the transfer reactions involved can be compared in Fig. 3, which illustrates three titrations of pyridine nucleotide reduction by \( \alpha \)-ketoglutarate: in the absence of added phosphate and ADP (O); in the presence of 0.3 mM ADP (D); and in the presence of 12 mM phosphate (A). Under the two latter conditions, both the rate and extent of the reaction are considerably inhibited; the effects of ADP and phosphate are attributable to their effects upon Equations 1 and 2.

Equations 1 and 2 also suggest that ADP and phosphate inhibit pyridine nucleotide reduction and cytochrome oxidation in terminally inhibited preparations. Such effects are illustrated, in Fig. 4, in pigeon heart mitochondria treated with succinate and inhibited with sodium sulfide. A control experiment (Fig. 4A) demonstrates the kinetics and extent of pyridine nucleotide reduction (measured spectrophotometrically) and cytochrome oxidation (measured fluorometrically), the initial rates being...
indicated on the diagram. As shown previously (9), addition of ATP elicits a cyclic oxidation of cytochrome c and a biphasic reduction of pyridine nucleotide. In the presence of succinate, pyridine nucleotide is reduced faster than cytochrome is oxidized (9). As shown in Fig. 4C, prior addition of ADP inhibits cytochrome oxidation to a large extent, pyridine nucleotide reduction to a lesser extent. Fig. 4D shows that 3.6 m\(\text{M}\) phosphate does not inhibit the initial phase of the reaction (in fact, a slight acceleration occurs) but does affect the extent of cytochrome oxidation and the slow phase of pyridine nucleotide reduction. Fig. 4B shows the combined effects of ADP and phosphate. The initial rate of pyridine nucleotide reduction is again unaffected. However, the slow phase is inhibited 4-fold and the rate and extent of the cytochrome reaction are highly inhibited, suggesting a similar sensitivity of the two components to the joint effect of ADP and phosphate.

The combined effects of ADP and phosphate in inhibiting the anaerobic oxidation of cytochrome are further discussed in Paper VI (10) and these results are considered on a thermodynamic basis.

**Effect of Added Magnesium**—The pathway of energy transfer from succinate to pyridine nucleotide can be studied from specific reactions affecting the high energy intermediates. The effect of magnesium on ATP-induced reduction of pyridine nucleotide in the presence of succinate is illustrated in Fig. 5, under experimental conditions similar to those of Fig. 2. After the reaction has proceeded 20 seconds, 9 m\(\text{M}\) magnesium is added, almost completely inhibiting the reaction but not reversing it, as ADP did.

![Fig. 3. Effects of ADP and phosphate on the extent of reduction of pyridine nucleotide on addition of \(\alpha\)-ketoglutarate to aerobic pigeon heart mitochondria; 1.2 mg of protein per ml (Experiment 149e).](image1)

![Fig. 4. Effects of ATP and phosphate on the kinetics of cytochrome c oxidation and pyridine nucleotide reduction in succinate- and sodium sulfide-treated pigeon heart mitochondria. The rates of oxidation and reduction are given in micromoles per liter per second; 1.9 mg of protein per ml (Experiment 294d).](image2)
The effect of magnesium on the sodium sulfide-inhibited system is illustrated by Fig. 6. In a control experiment (left), the mitochondria are treated successively with succinate, sodium sulfide, and 32 μM ATP, the last causing cytochrome oxidation and pyridine nucleotide reduction. If 0.7 mM magnesium is added before ATP (center), pyridine nucleotide reduction is inhibited ~50%, and cytochrome oxidation even more so. Other data show that with an ATP/Mg++ ratio of ~1, the rate of oxidation of cytochrome c by ATP is inhibited ~22%.

**Effect of Uncoupling Agents**—Uncoupling agents are extremely active inhibitors of the reduction of pyridine nucleotide and the oxidation of cytochrome c. If the experiment of Fig. 6, left, is repeated with 4.5 μM dinitrophenol instead of magnesium (right), there is only a brief cycle of oxidation of cytochrome c in which the maximal amplitude is less than 50% of the control value. The fluorescence trace is recorded immediately after addition of dinitrophenol, and the ensuing cycle of pyridine nucleotide reduction is smaller and shorter than in the control experiment, the maximal amplitude being less than 25% of that observed in Fig. 6, left.

In aerobic mitochondria, uncoupling agents have a generalized effect on the succinate-linked reduction of pyridine nucleotide for which a sensitive assay has been developed. In brief, the reduction of succinate-linked pyridine nucleotide is more sensitive to these agents than is the activation of respiration, the former being specifically inhibited in rate and extent.

Effects of another uncoupling agent, dibromophenol, on the kinetics of pyridine nucleotide, flavoprotein, and cytochrome b on adding succinate to glutamate-treated mitochondria are summarized in Table III. In the absence of dibromophenol, pyridine nucleotide is more rapidly reduced than cytochrome b and flavoprotein. With 24 μM dibromophenol, which gives approximately half maximal uncoupling, the rate of pyridine nucleotide reduction falls to ~36% of the control value, whereas the rates of the other components differ only slightly. With 48 μM dibromophenol, the rate of pyridine nucleotide reduction is ~6% of the control value, and the changes in the steady state levels of the other carriers are so small as to be immesurable.

**Effect of Mitochondrial Aging**—Because aging of mitochondria activates ATPase, we compare in Fig. 7 the effects of 2- and 24-hour aging on ATP-induced reduction of pyridine nucleotide and oxidation of cytochrome c in the presence of succinate and sodium sulfide. In the latter preparation, oxidation of cytochrome c is considerably inhibited in rate and extent, and also in duration, indicating a higher rate of ATP turnover. Pyridine nucleotide reduction is somewhat decreased in extent and, more significantly, in duration. It is unlikely that any pyridine nucleotide was lost during the aging process, inasmuch as the mitochondria were maintained in a mannitol-sucrose-Tris medium at 0° for the 24-hour period. In any case, no cytochrome was lost, and the general nature of the conclusions would not be altered by a small loss of pyridine nucleotide.

**Oligomycin**—A number of substances that interfere specifi-
cally with the transfer reactions of Equations 1 and 2 have been tested. Such substances may be identified in several ways, one being by their inhibition of the effect of ADP on the rate of respiration and the steady state of the carriers. Two so identified are guanidine (12) and oligomycin (13). Another inhibitor, azide, blocks an intermediate step in Equations 1 and 2 required for the ATP-P32 exchange reaction (14, 15).

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The inhibitory effect of oligomycin (15) is illustrated by Fig. 9. In a control experiment (left), the response of succinate- and sulfide-treated pigeon heart mitochondria to 400 μM ATP is observed, cytochrome being abruptly oxidized and pyridine nucleotide slowly reduced. The current results are repeated in the presence also of 0.6 μg (γ) per ml of oligomycin (right), 4,5 both the rate and extent of the responses of cytochrome and pyridine nucleotide are inhibited, the initial rates being about one-third of those in the control experiment. The duration of the cycles, however, is scarcely altered, suggesting that oligomycin has a specific effect on the rate at which the intermediate of Equation 2, X - I, can be used in activating cytochrome oxidation and pyridine nucleotide reduction.

In view of the tight binding of oligomycin to the intermediates of the transfer reaction, a titration would aid in determining the completeness of the inhibition obtained with this substance. In Fig. 10 (left) a titration of the inhibition of cytochrome c oxidation under the conditions of Fig. 9 is graphed. Oligomycin, 1 μg (γ) per ml, almost completely inhibits oxidation of cytochrome c in anaerobic pigeon heart mitochondria at a protein concentration of 0.8 mg per ml, under the conditions indicated.

The generality of the inhibitory effect of oligomycin on the reduction of pyridine nucleotide is seen from experiments under anaerobic (terminally inhibited) conditions (Fig. 10, right) and under aerobic conditions (Fig. 10, center). In the sulfide-treated system, pyridine nucleotide reduction is inhibited at the same concentration of oligomycin as cytochrome c oxidation (Fig. 10, left). In the aerobic system, pyridine nucleotide reduction is extensively inhibited, but more oligomycin is required and the inhibition is not complete.

Chlorpromazine (100 μm) has been observed to give 50% inhibition of the aerobic reduction of pyridine nucleotide in the presence of succinate.6

4 Material generously donated by F. M. Strong.

5 The oligomycin concentration was erroneously given in grams per milliliter instead of micrograms per milliliter in a preliminary report (1).

6 B. Chance, unpublished experiments.
FIG. 11. Nucleotide specificity of pyridine nucleotide reduction and cytochrome c oxidation in succinate- and sulfide-treated pigeon heart mitochondria; 1.3 mg of protein per ml (Experiment 180e).

FIG. 12. Effect of ITP on ATP-activated cytochrome c oxidation and pyridine nucleotide reduction in succinate- and sulfide-treated pigeon heart mitochondria; 0.8 mg of protein per ml (Experiment 209f).

FIG. 13. Effect of concentration of ITP on extent of pyridine nucleotide reduction and cytochrome c oxidation in succinate- and sulfide-treated pigeon heart mitochondria; 0.63 mg of protein per ml (Experiment 210a).

were observed in pigeon heart with a variety of nucleotides or in rat liver with GTP (71 μm). The experiment with GTP (Fig. 11) was repeated in pigeon heart mitochondria in the presence of added ADP, and no reduction of pyridine nucleotide was observed. It is probable that the transfer enzyme has a fairly low activity in these mitochondria. The experiments that follow show that the mitochondria are permeable to ITP and presumably to the other nucleotides.

Effect of Inosine Triphosphate—In investigating whether phosphopyruvate is formed from oxalacetate on addition of ITP to pigeon heart mitochondria, it is observed that pyridine nucleotide reduction is slowed, not accelerated, on subsequent addition of ATP. In fact, ITP causes a rapid inhibition of the ATP-electron transfer reaction. This effect is demonstrated by the experiment of Fig. 12 in which ATP addition gives a typical oxidative response of cytochrome c and reductive response of pyridine nucleotide in succinate- and sulfide-treated pigeon heart mitochondria. As soon as a steady state is reached, 400 μM ITP is added, whereupon cytochrome c is abruptly reduced and pyridine nucleotide is somewhat oxidized.

The graph of Fig. 13 represents a titration of the inhibition of the responses of cytochrome c and pyridine nucleotide by ITP under the conditions of Fig. 12, (succinate and sodium sulfide); in this case, varying concentrations of ITP are now added, and then 80 μM ATP. The inhibition of cytochrome oxidation is much more complete than the inhibition of the initial fast phase of pyridine nucleotide reduction; kinetic studies show that it is the slow or succinate-linked phase of the latter reaction that parallels the inhibition of cytochrome c in its sensitivity to ITP. Fig. 4 illustrates the fast and slow portions of the kinetics of reduction of mitochondrial pyridine nucleotide.

DISCUSSION

Activity Values—The ATP-electron transfer reaction affords a novel approach to the activity of the energy transfer pathway of intact mitochondria, and we have therefore performed a variety of experiments to determine some standard values for the activity of this pathway at 26° and in pigeon heart mitochondria. Inasmuch as it is most useful to refer ATP-electron transfer to the maximal electron transfer activity in oxidative phosphorylation, we make the comparison on the basis of turnover number, e.g. micromoles of pyridine nucleotide reduced per second per micromole of cytochrome c present. Under aerobic conditions, the highest turnover number obtained was 0.66 sec⁻¹, whereas in the anaerobic or terminally inhibited system, a value of 2.4 sec⁻¹ was obtained. Typical turnover numbers for aerobic respiration in pigeon heart mitochondria³ (state 3, succinate) are 5 to 6 sec⁻¹. Although the anaerobic transfer rate is faster than the aerobic rate, it is still only a small fraction of the maximal rate of oxidation. The rate of ATP-electron transfer at the level of cytochrome c is 7-fold less than that of pyridine nucleotide, provided succinate is present; otherwise the rates approach equality (see Paper II (9)). The rates for cytochrome c, however, represent the difference between the actual rate of its oxidation by ATP and the rate of its reduction by adjacent cytochrome components. These values may be compared with dinitrophenol-stimulated ATPase activities on which numerous values are available for rat liver mitochondria at various concentrations of dinitrophenol and ATP (16, 17). At 200 μM ATP and 10⁻⁴ M dinitrophenol,
values of 8.5 pmoles per mg of protein per hour are obtained at 26° (16). With a cytochrome c concentration of 1 x 10^{-10} moles per mg of protein, the turnover number corresponds to 12 sec^{-1}. The maximal ATPase activity corresponds to a turnover number of 32 sec^{-1}. These values are somewhat greater than respiratory turnover data and much greater than the ATP-electron transfer rates. A similar conclusion is reached on the basis of the ATP-P_{32} exchange reaction in rat liver mitochondria which has been found to be much more rapid than the maximal rate of oxidation (18). Two explanations are possible. First, the rate of the reaction into the level of X ~ I (Equation 2) may be much more rapid than that of the reaction of X ~ I with the carrier. Second, there may be a variety of exchange reactions in the mitochondria, only a fraction of which lead to interaction with the carriers. Some support for the second possibility is afforded by the data of Figs. 2C and 20 of Paper II (9) which show that the rates of ATP-induced cytochrome oxidation and pyridine nucleotide reduction are not remarkably slower in “digitonin” particles (19) than in the intact rat liver mitochondria from which they were derived. However, assays of the exchange enzymes show that these rates are considerably less in “digitonin” particles (18) than in intact mitochondria.

Reversibility of the ATP-Electron Transfer Reaction—A variety of experiments show the separability and joint effects of ADP and phosphate on the rate and extent of the ATP-electron transfer reaction and clearly indicate its reversibility. As expected from the higher affinity of oxidative phosphorylation for ADP than for phosphate, the former is a more effective inhibitor of the ATP-electron transfer. In one experiment (see Fig. 4), a phosphate to ADP ratio of 20:1 was required for equal diminution of the extent of cytochrome c oxidation by 300 μM ATP. In an attempt to determine whether the effects of phosphate and ADP on cytochrome oxidation are merely summed, it is found that concentrations that separately give equal inhibition (30%), jointly give 75% inhibition.

It was anticipated that magnesium would act by causing hydrolysis of high energy intermediates, and indeed it can do so. However, the records of Fig. 6 show a prolonged, inhibited reaction with ATP. It is possible that the added magnesium combines with the added ATP or with the transfer site to inhibit the reaction. This appears to be a rather different mechanism from activation of ATPase by magnesium.

Effects of Uncoupling Agents and Aging—It is observed that a transient oxidation of cytochrome c can be obtained in the presence of 4.5 μM dinitrophenol (Fig. 6C). According to Equation 3, a suitable concentration of this uncoupler would completely eliminate the response to ATP; independent experiments show that 9 μM dinitrophenol has this effect. The reaction of X ~ I with the carriers is sufficiently rapid that their response to ATP can be observed in the presence of sufficient dinitrophenol to accelerate the rate of ATP breakdown (~7-fold in Fig. 6), suggesting a new method of measuring the competition between dinitrophenol and the carriers for the high energy intermediate. Similarly, in Fig. 7 where aging has increased the rate of expenditure of ATP 6-fold, the response of cytochrome c is still observable.

The reaction of ATP with the respiratory carriers may be a more sensitive method of detecting the possibilities for coupled phosphorylation than the reverse reaction. ATP-electron transfer depends upon the rate of reaction of high energy intermediates with the carriers:

\[ X + \text{DPNH} + H^+ + \text{flavoprotein} \]

whereas in oxidative phosphorylation, the reverse of Equation 4 occurs. For reasons which we do not fully understand at present, ADP and phosphate do not seem able to compete as effectively for the high energy intermediate, X ~ I, in the forward reaction, as does the carrier couple (DPN-reduced flavoprotein) in the reverse reaction of Equation 4. This disparity suggests that reversal of the ATP-induced reaction be studied in loosely uncoupled systems, particularly to determine which couple ceases to respond first.

Effect of Oligomycin- The ATP-electron transfer reaction is of particular interest as a method for identifying differences in the responses of the various couples of the respiratory chain in the reactions with the transfer system. Titrations of cytochrome c and pyridine nucleotide reactions by oligomycin show identical sensitivity (Figs. 9 and 10), suggesting not only that oligomycin acts at the common intermediate, X ~ I, but also that it acts upon the moiety of this intermediate which is identical for the three carrier sites rather than upon that which is specific for the individual carriers. For example, it has already been postulated that three forms of the intermediate exist, X ~ I₈, X ~ I₁, and X ~ I₉, each representing a ligand suitable for a different site of electron transfer. We must postulate either that oligomycin interacts with X or that the I substances are sufficiently similar that they react with the same concentrations of oligomycin. Neither of these alternatives can be eliminated at present.

The ATP-electron transfer reaction under anaerobic conditions is completely sensitive to oligomycin and is thus completely dependent upon energy transfer through the mechanism of oxidative phosphorylation and completely independent of any other transfer reactions. It is significant, however, that a small portion of the aerobic reduction of pyridine nucleotide in the presence of succinate is resistant to oligomycin and this leads us to consider the possibility that a small portion of the reaction is not energy-linked or that it may proceed through a pathway of energy transfer insensitive to oligomycin.

The effect of ITP is very similar to the effects of ADP + phosphate in that cytochrome c oxidation and the slow phase of pyridine nucleotide reduction are inhibited. It is possible that ITP binds the intermediate X so that the reaction with ATP is impeded.

SUMMARY

1. The transfer of energy from adenosine 5'-triphosphate to the respiratory carriers causing oxidation of cytochrome c reduction of pyridine nucleotide under anaerobic conditions is completely oligomycin-sensitive. Thus, the pathway of energy transfer is identified with that of oxidative phosphorylation.

2. The responses of the energy transfer pathway to dinitrophenol and to aging of the mitochondria are characteristic of an activated hydrolysis of the high energy intermediate. That the carrier couples can successfully compete with 4.5 μM dinitrophenol for the high energy intermediate is clearly demonstrated; higher concentrations of the uncoupling agent (9 μM) almost completely eliminate the response of the carriers to adenosine 5'-triphosphate under these experimental conditions.

3. The adenosine 5'-triphosphate to electron transfer reaction appears to be inhibited by magnesium at concentrations equal to that of adenosine 5'-triphosphate.
4. The adenosine 5'-triphosphate-electron transfer reaction is sensitive to both adenosine 5'-diphosphate and phosphate, the former being 20-fold more effective on a molar basis. These effects confirm the reversibility of the reaction.

5. Preliminary values for the activity of the adenosine 5'-triphosphate-electron transfer reaction have been obtained under various conditions, the maximum being still considerably less than the maximal rate of oxidative phosphorylation. These values are used to evaluate the significance of adenosine 5'-triphosphate-P\textsubscript{32} exchange and adenosine 5'-triphosphatase reactions in relation to the transfer system.

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