CONTRIBUTION OF ATP SYNTHESIS FROM ENDOGENOUS SUBSTRATES TO THE OLIGOMYCIN-SENSITIVE ADP-ATP EXCHANGE ACTIVITY OF RAT LIVER MITOPLASTS

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Summary

The oligomycin-sensitive ADP-ATP exchange of intact mitoplasts (inner membrane + matrix particles) of rat liver mitochondria is inhibited less than 22% by KCN when assayed without added phosphate at either high or low nucleotide concentrations. ^{32}P-ATP formation amounts to less than 35% of the ^{14}C-ATP formed and is almost completely insensitive to KCN. Oxidation of endogenous substrates is inhibited nearly 100% by KCN. It is concluded that net ATP synthesis supported by oxidation of endogenous substrates accounts for only a small fraction of the total ^{14}C-ATP formed in the ADP-ATP exchange assay and that the bulk of this activity is catalyzed independently by the terminal reactions of oxidative phosphorylation.

When assayed in the absence of added Mg^{++} or added respiratory substrate intact mitochondria and mitoplasts catalyze an oligomycin-sensitive ADP-ATP exchange reaction whose properties strongly suggest that it is catalyzed by enzymes participating in the terminal enzymatic steps of oxidative phosphorylation (1-3). This exchange is inhibited by aurovertin, an inhibitor of both oxidative phosphorylation (4) and the soluble mitochondrial ATPase (5,6) and by conditions which destroy inner membrane integrity and frequently give rise to ATPase activity (3,7). For this reason we postulated that the ADP-ATP exchange is catalyzed by a highly ordered inner membrane (M) complex containing at the minimum the ATPase (F_1), an oligomycin-sensitivity conferring factor (F_c) and a divalent cation as represented below (3).

$$ATP + (F_c\ M\ Mg^{++}\ F_1) \rightleftharpoons (F_c\ M\ Mg^{++}\ \underset{\underset{ADP}{|}}{F_1}) \sim P \rightleftharpoons (F_c\ M\ Mg^{++}\ F_1) \sim P + ADP$$

Recently Reboul and Vignais (8) have concluded that mitochondria and mitoplasts do not actually catalyze an ADP-ATP exchange reaction but rather that the observed incorporation of ^{14}C-ADP into ATP is exclusively due to net phosphorylation of ADP to ATP coupled to oxidation of endogenous substrates. They based their argument on studies which showed that the respiratory inhibitor KCN completely inhibits ^{14}C-ATP formation from ^{14}C-ADP at low nucleotide concentrations (< 1.0 mM) in the presence of added phosphate. Since their conclusions are in direct contrast to previous studies carried out by Wadkins and Lehninger (9) and Bygrave and Lehninger (7) on intact rat liver mitochondria, and to our own views of the ADP-ATP exchange derived from studies on mitoplasts (3) and depicted above, the following experiments were carried out to reassess the effect of KCN on the ADP-ATP exchange reaction and therefore the contribution of net ATP synthesis to this exchange.

METHODS

Mitoplasts were prepared by the procedure of Schnaitman and Greenawalt (10) and resuspended at a concentration of 35 mg/ml in the mitochondrial isolation medium containing 220 mM D-mannitol, 70 mM sucrose, 2.0 mM HEPES buffer, pH 7.4, and 0.5 mg/ml crystalline bovine serum albumin.

ADP-ATP exchange activity was assayed in a manner similar to that described previously by Pedersen and Schnaitman (3). The preliminary incubation mixture contained in a volume of 0.45 ml, 294 mM D-mannitol, 93 mM sucrose, 0.5 mM EDTA, 2.7 mM HEPES, pH 7.4, 0.875 mg protein and, where indicated, 1.33 mM KCN or 1 μg oligomycin. KCN, which is strongly basic and unstable in aqueous solutions, was always dissolved fresh and adjusted to pH 7.4 with HCl. Incubation was carried out for 4.0 minutes at 30°. When measurements were made at high nucleotide concentrations, 6.0 μmoles ATP, 2.4 μmoles ADP, 0.75μC ^{14}C-ADP and where indicated 0.10 μC $^{32}P_i$ were then added in a volume of 0.150 ml. When measurements were made at low nucleotide concentration, 0.4 μmole ATP, 0.03 μmole ADP, 0.064 μC ^{14}C-ADP, and where indicated 0.2 μC $^{32}P_i$ were added in a volume of 0.150 ml. Incubation was

carried out at 30° for 10 minutes before stopping the reaction with 0.10 ml 2.5 M perchloric acid. After allowing the mixture to sit 15 minutes at 0° 0.10 ml 2.5 M KOH was added and the resultant precipitate was sedimented at 2000 x g. Appropriate aliquots (3-25μl) were spotted on thin layer ion exchange sheets, Polygram Cel 300 PEI (Brinkman Instruments, Inc., Westburg, N.Y.). When necessary 0.001 ml of 5 mM ADP and 5 mM AMP were added also to permit detection of these nucleotides. Chromatograms were developed with 1.0 M LiCl in about 15-20 min as described by Randerrath and Randerrath (11). Spots were cut out, added directly to a liquid scintillation solvent system (Toluene-ethanol, 1.67:1 (v/v) containing 2.5 mg/ml PPO and 0.062 mg/ml POPOP) and counted. Exchange rates were calculated by use of the equation of Boyer (12).

Adenosine triphosphatase activity was estimated by determining the amount of phosphate produced during the ADP-ATP exchange assay by the method of Gomori (13).

Respiration studies were conducted in a closed 3.0 ml reaction vessel equipped with a Clark oxygen electrode. Conditions were identical to those of the ADP-ATP exchange assay.

Endogenous phosphate in the mitoplast fraction was determined by the following procedure. To 0.10 ml of frozen-thawed mitoplast (35 mg/ml) was added 0.10 ml of 2.5 M perchloric acid. After standing 15 minutes at 0° centrifugation was carried out for 10 min at 10,000 x g. The supernatant was saved and the pellet reextracted with 0.10 ml of 2.5 M perchloric acid. The content of phosphate in the combined supernatants was then determined by the method of Gomori (13) in a total reaction volume of 0.5 ml.

RESULTS AND DISCUSSION

In order to compare the contribution of net ATP formation supported by oxidation of endogenous substrates to ^{14}C-ATP formation during the ADP-ATP exchange assay as carried out in our laboratory (3) and in the laboratory of Reboul and Vignais (8), assays were conducted both at high nucleotide concen-

TABLE I

Sensitivity of ADP-ATP Exchange And Other Mitoplast Activities To Potassium Cyanide And Oligomycin When Assayed At High Nucleotide Concentration[a]

Species Formed or Utilized	nmoles Formed (+) or Utilized[b] (-) No inhibitor	With KCN	With Oligomycin
^{14}C-ATP	+585.0	+459.0	+9.0
^{32}P-ATP	+ 19.7	+ 17.5	+1.2
^{14}C-AMP	0	+ 10.8	0
P_i[c]	+ 67.0	+151.0	+16.7
Oxygen	- 16.2	0	-16.2

[a] ATP = 10 mM, ADP = 4.0 mM

[b] Assay time = 10 min

[c] Initial amount of P_i in the assay was 18.1 nmoles from endogenous P_i added with the mitoplast.

trations (10 mM ATP, 4 mM ADP) as in our earlier studies (3) and at low nucleotide concentrations (0.6 mM ATP, 0.05 mM ADP) as employed by Reboul and Vignais (8). Experiments summarized in Table I show that at high nucleotide concentrations, conditions optimal for ADP-ATP exchange activity in the mitoplast fraction (3), 1.0 mM KCN inhibits oligomycin-sensitive ^{14}C-ATP formation less than 22 percent, but completely inhibits oxidation of endogenous substrates, the imputed energy source for ^{14}C-ATP formation via net phosphorylation. Moreover, the oxidation of endogenous substrates (-16.2 nmoles O_2) could in any case account for formation of a maximum of only 97.2 nmoles ATP, or 17 percent of the 585 nmoles of ATP-ADP exchange observed, based on a P:O=3.0. When the inorganic phosphate is labeled with ^{32}P the amount of ^{32}P-ATP formed under these conditions amounts to only 3.4 percent of the ^{14}C-ATP formed and it is not significantly inhibited by the presence of KCN. The insensitivity of ^{32}P-ATP formation to KCN shows that this activity reflects predominantly the well-known P_i-ATP exchange and not net

oxidative phosphorylation of ADP. Taken together results obtained at high nucleotide concentrations which are optimal for the ADP-ATP exchange strongly suggest that there is essentially no net synthesis of ATP energized by oxidation of endogenous substrates under conditions of the exchange assay, and that the small inhibition of ^{14}C-ATP formation by KCN is probably due to some other property of the inhibitor. In this latter regard it is important to note that 1 mM KCN causes a significant stimulation of ATPase activity (Table 1) which is known to be inversely related to ADP-ATP exchange activity (7).

These experiments were repeated using the low nucleotide concentrations employed by Reboul and Vignais (8). The results summarized in Table 2 are not notably different from those obtained at high nucleotide concentrations although the rate of ^{14}C-ATP formation was of course lower under these sub-optimal conditions. In these experiments, as in those discussed above, no phosphate was added so that any phosphorylation of ADP fueled by oxidation

TABLE 2

Sensitivity of ADP-ATP Exchange And Other Mitoplast Activities To Potassium Cyanide And Oligomycin When Assayed At Low Nucleotide Concentration[a]

Species Formed or Utilized	nmoles Formed (+) or Utilized[b] (-)		
	No inhibitor	With KCN	With Oligomycin
^{14}C-ATP	+42.8	+37.0	+10.6
^{32}P-ATP	+14.0	+12.7	+ 2.6
^{14}C-AMP	+ 0.4	+ 0.5	0
P_i[c]	+88.0	+ 134	+33.0
Oxygen	- 9.1	0	-11.3

[a] ATP = 0.6 mM, ADP = 0.05 mM

[b] Assay time = 10 min

[c] Initial amount of P_i in assay was 18.1 nmoles from endogenous P_i added with the mitoplast.

of endogenous substrates would be totally dependent on endogenous phosphate (19-21 nmoles/mg mitoplast protein, see Methods). Again it is seen that KCN completely inhibits oxidation of endogenous substrates, has only a small inhibitory effect on oligomycin-sensitive ^{14}C-ATP formation ($\sim$ 18 percent), stimulates ATPase activity, and is essentially without effect on ^{32}P-ATP formation. Moreover, as in the experiments described above at high nucleotide concentration, there was essentially no interference by adenylate kinase activity. Conclusions derived from these experiments therefore remain the same as those derived from studies at high nucleotide concentration; i.e., formation of ^{14}C-ATP under conditions of the ADP-ATP exchange assay cannot be accounted for by net synthesis of ATP supported by oxidation of endogenous substrates.

Although it is difficult to reconcile these results with those obtained by Reboul and Vignais (8), it should be noted that they are consistent with findings of Wadkins and Lehninger (9) and Bygrave and Lehninger (7) on intact rat liver and beef heart mitochondria respectively. Wadkins and Lehninger (9) showed that KCN only moderately inhibits the oligomycin-sensitive ADP-ATP exchange catalyzed by rat liver mitochondria, whereas Bygrave and Lehninger (7) showed that the rate of oxidation of endogenous substrates in beef heart mitochondria is not sufficient to support the formation of ^{14}C-ATP at the rate observed in the ADP-ATP exchange assay. Thus, the oligomycin-sensitive ADP-ATP exchange reaction appears to be catalyzed by the enzyme system catalyzing the terminal steps of oxidative phosphorylation.

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