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Mechanisms of Energy Conservation in the Mitochondrial Membrane*

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Introduction

The importance of the inner mitochondrial membrane for respiratory chain-linked energy conservation is well recognized. The precise role of the membrane in the process, however, is not known. A problem of great current interest in this context concerns the mode of interaction of the mitochondrial electron-transport and ATPase systems in catalysing oxidative phosphorylation. According to the chemiosmotic hypothesis[1] this interaction is indirect and involves as an obligatory intermediate a proton gradient across the membrane. In contrast, the chemical hypothesis of oxidative phosphorylation envisages the formation of high-energy intermediates as functional links between the two systems,[2] possibly with the involvement of conformational changes of proteins[3] and localized proton gradients.[4] Since these intermediates most probably are of macromolecular nature, with only limited mobility within the membrane, such a mechanism would be likely to involve a direct interaction between the electron-transport and ATPase systems. Thus, whereas the chemiosmotic hypothesis considers the two systems as separate units, where each ATPase ought to be able to interact with any electron-transport chain within the same membrane (Fig. 1A), the chemical hypothesis is compatible with an assembly-like arrangement of the two systems, where any given ATPase may interact with only one electron-transport chain (Fig. 1B).

Indications for an assembly-like interaction of the electron-transport and ATPase systems were reported by Lee *et al.*[5] who found that addition to submitochondrial particles of oligomycin, which inhibited the ATPase activity of the particles, induced a respiratory control, the extent of which was unaffected by electron-transport inhibitors such as rotenone, antimycin, cyanide or azide. Similar indications were

* The following non-conventional abbreviations are used: ANS, 8-anilino-naphthalene-1-sulfonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PMS, phenazine methosulfate.

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obtained by Baum *et al.*[6] from measurements of the effects of rotenone and oligomycin on the rate of ATP-driven succinate-linked NAD^+ reduction. These authors concluded that their results were quite inconsistent with the chemiosmotic hypothesis. They pointed out, however, that this conclusion may be invalidated if the conserved energy were also dissipated through processes other than the reduction of NAD^+.

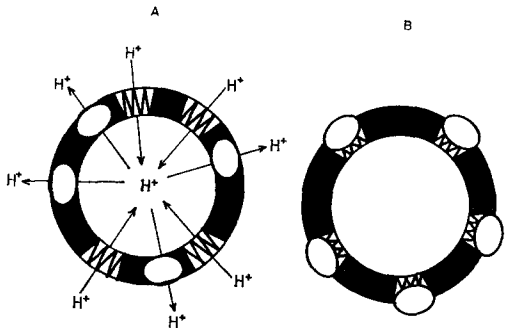

Figure 1. Functional relationship between electron-transport (zigzag line) and ATPase (ellipse) systems in submitochondrial particles. A: interaction via proton gradient; B: interaction via assemblies. (For explanation, see text.)

The purpose of this paper is to summarize the results of experiments carried out in this laboratory which appear to lend strong support to the concept of an assembly-like interaction of the mitochondrial ATPase and electron-transport systems. The evidence is based on comparative studies of the effects of an uncoupler (FCCP), an energy-transfer inhibitor (oligomycin) and the mitochondrial ATPase inhibitor described in 1963 by Pullman and Monroy[7] on various energy-linked reactions catalysed by submitochondrial particles. These results have recently been presented in a symposium lecture,[8] and a detailed account is being published elsewhere.[9]

Experimental Procedure

Submitochondrial particles were prepared from beef-heart mitochondria by sonication and isolated by differential centrifugation as described by Lee and Ernster.[10] Two types of particles were used: "nonphosphorylating" particles, obtained by sonication of mitochondria at alkaline pH in the presence of EDTA ("EDTA particles"), and "phosphorylating" particles obtained by sonication of mitochondria at neutral pH in the presence of Mg^{2+} and ATP ("Mg-ATP particles").

The reactions studied included ATP-driven succinate-linked NAD^+ reduction,[11] ATP-driven energy-linked transhydrogenase,[12] ATPase,[13]

and respiration-induced ANS-fluorescence enhancement;[14] these were assayed as described in the appropriate references. Details of the assay systems are given in the figure legends.

ATPase inhibitor purified from beef-heart mitochondria was prepared and assayed as described by Horstman and Racker.[15]

Results

Effects of FCCP and Oligomycin

Figure 2 compares the effects of increasing concentrations of FCCP and oligomycin on the ATP-driven succinate-linked NAD^+ reduction and energy-iinked transhydrogenase reaction of Mg-ATP particles. It may be seen that the half-inhibitory concentrations of both compounds

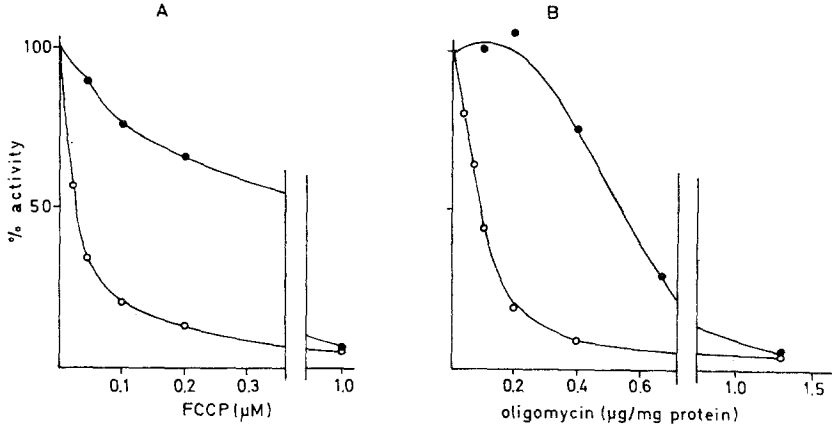

Figure 2. Effects of FCCP and oligomycin on ATP-driven succinate-linked NAD^+ reduction (○) and nicotinamide nucleotide transhydrogenase reaction (●) in Mg-ATP particles. The reaction mixtures contained, in a final volume of 1 ml, the following additions.

Succinate-linked NAD^+ reduction: 170 mM sucrose, 50 mM tris-Ac, pH 7·5, 5 mM succinate, 1 mM NAD^+, 3 mM $MgSO_4$, 3 mM ATP, 2 mM KCN, 0·2 mg particle protein, and FCCP or oligomycin as indicated. The activity in the absence of FCCP and oligomycin was 91 nmoles NADH formed per min and mg protein.

Transhydrogenase: 170 mM sucrose, 50 mM Tris-Ac, pH 7·5, 0·2 mM NADH, 1mM $NADP^+$, 1 mM glutathione disulfide 2 μg glutathione reductase in 1% bovine serum albumin, 1 μM rotenone, 4·5 mM $MgSO_4$, 4·5 mM ATP, 0·2 mg particle protein, and FCCP or oligomycin as indicated. The activity in the absence of FCCP and oligomycin was 80 nmoles NADPH formed per min and mg protein.

The reactions were followed spectrophotometrically at 340 nmoles. Temperature, 30°C.

were considerably lower in the case of the former than the latter reaction. The rates of the two reactions in the absence of FCCP were approximately equal.

Results similar to those described above were obtained with EDTA particles (Fig. 3), except that here, in accordance with earlier findings of Lee and Ernster,[16] low concentrations of oligomycin stimulated the ATP-driven reactions (Fig. 3B). In order to ensure maximal reaction

rates, the measurements with increasing concentrations of FCCP (Fig. 3A) were performed in the presence of a maximally stimulating concentration of oligomycin. As in the case of the Mg-ATP particles, it is evident that both FCCP and oligomycin inhibited the ATP-driven succinate-linked NAD^+ reduction more efficiently than the ATP-driven transhydrogenase reaction.

The results described so far are similar to those reported in 1963 by Danielson and Ernster,[17] who found that the ATP-driven transhydrogenase reaction catalysed by rat-liver submitochondrial particles was

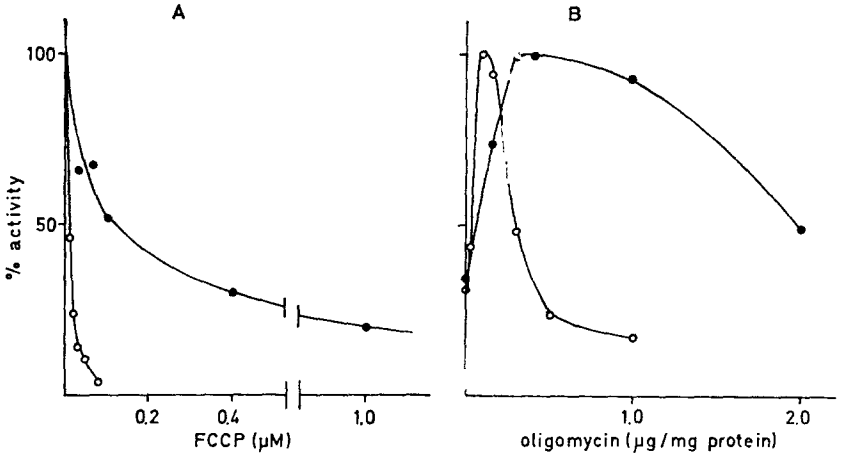

Figure 3. Effects of FCCP and oligomycin on ATP-driven succinate-linked NAD^+ reduction (○) and nicotinamide nucleotide transhydrogenase (●) in EDTA particles. The reaction mixtures were the same as in Fig. 2, except that in the series in Fig. 3A, the samples contained a maximally stimulating amount of oligomycin as deduced from the data in Fig. 3B. The maximal activities were 130 nmoles NADH formed per min and mg protein for the succinate-linked NAD^+ reduction, and 150 nmoles NADPH formed per min and mg protein for the transhydrogenase.

much less sensitive to the uncoupler 2,4-dinitrophenol than was the ATP-driven succinate-linked NAD^+ reduction. They also found that replacement of ATP by another nucleoside triphosphate, e.g. ITP, which is less efficient as substrate for ATPase than is ATP, resulted in a decrease in the rate of the energy-linked transhydrogenase reaction that was proportional to the decrease in rate of the nucleoside triphosphatase, whereas the rate of the succinate-linked NAD^+ reduction decreased much more.

Danielson and Ernster[17] interpreted these findings in terms of a difference in energy requirement between the ATP-driven succinate-linked NAD^+ reduction and transhydrogenase reactions. According to their interpretation, the thermodynamically unfavourable succinate-linked NAD^+ reduction requires a higher level of the high-energy intermediate ~X, i.e., a higher "energy pressure", in order to proceed

at maximal rate, than does the thermodynamically favourable transhydrogenase reaction. Thus, when the steady-state level of ~X is decreased, either by decreasing its rate of generation from ATP (by replacing ATP with a kinetically less efficient substrate of the ATPase, such as ITP), or by increasing its rate of dissipation (by adding an uncoupler), this will affect the succinate-linked NAD^+ reduction more than the energy-linked transhydrogenase reaction. In line with this interpretation was also the finding that addition of ADP and/or P_i in combination with ATP, i.e., lowering of the phosphate potential,

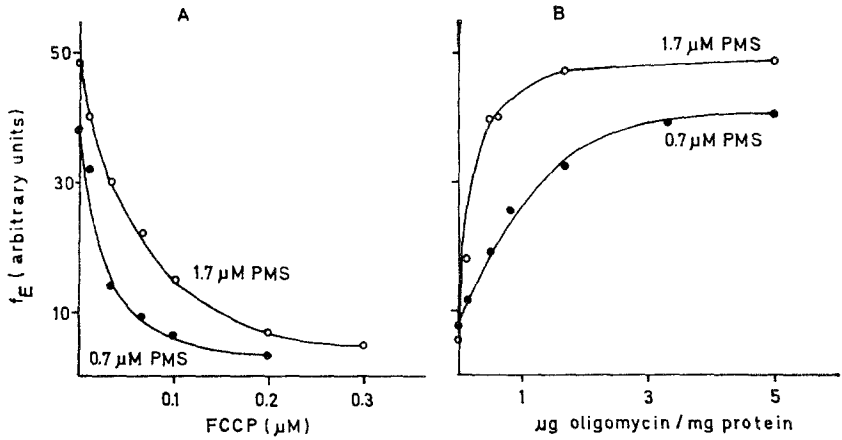

Figure 4. Effects of FCCP and oligomycin on the energy-dependent ANS-fluorescence enhancement (f_E) of EDTA particles. The reaction mixture contained, in a final volume of 3 ml, 10 mM Tris-Ac, pH 7·5, 170 mM sucrose, 1·7 μM rotenone, 17 μM ANS, 5 mM ascorbate, 0·6 mg particle protein, and 0·7 or 1·7 μM PMS. FCCP (Fig. 4A) and oligomycin (Fig. 4B) were added as indicated. In Fig. 4A, 3 μg oligomycin was added to all samples. Temperature, 24°C. (Data quoted from ref. 18.)

greatly suppressed the succinate-linked NAD^+ reduction but had virtually no effect on the energy-linked transhydrogenase reaction.

Evidently, a similar interpretation may be valid for the present data, and may thus apply also for the case of oligomycin, which, like FCCP, lowers the "energy pressure" generated from ATP. In fact, independent evidence supporting this interpretation comes from data recently published from this laboratory,[18] quoted in Fig. 4, concerning the effects of increasing concentrations of FCCP and oligomycin on the respiration-induced ANS-fluorescence enhancement in EDTA particles. Here, just as in the case of the ATP-driven reactions, uncouplers act as inhibitors, by promoting energy dissipation. Oligomycin, on the other hand, promotes energy accumulation from the respiratory chain, by tightening an "energy leak" present in these particles, and thereby enhancing respiration-supported energy-linked reactions. Using ascorbate + PMS as substrate, one can regulate the rate of

respiration, and thereby the respiration-generated "energy pressure", by varying the concentration of PMS. As illustrated by the data in Fig. 4, the higher the concentration of PMS, i.e., the higher the "energy pressure", the higher is the FCCP concentration required to inhibit, and the lower is the oligomycin concentration required to stimulate the respiration-induced ANS-fluorescence enhancement.

These data seem to eliminate the possibility that the differences in FCCP and oligomycin sensitivity between the ATP-driven succinate-linked NAD^+ reduction and transhydrogenase reaction would be due to intrinsic features of the two reactions other than those concerning energy requirement.

It may thus be concluded that the ATP-driven succinate-linked NAD^+ reduction and nicotinamide nucleotide transhydrogenase reaction show different sensitivities to both uncouplers and oligomycin, and that this difference is a reflection of the different energy requirements of the two reactions. As will be shown in the following, a different situation is found when the two reactions are compared with respect to their sensitivities to the mitochondrial ATPase inhibitor.

Effects of Mitochondrial ATPase Inhibitor

In 1963 Pullman and Monroy[7] reported the purification of an ATPase inhibitor from beef-heart mitochondria. The inhibitor, a protein of an estimated molecular weight of 10,000–15,000, was shown to inhibit the ATPase activity of both submitochondrial particles and coupling factor 1 (F_1), while it had no effect on oxidative phosphorylation. The inhibitor was shown to form a complex with F_1, a finding that was subsequently confirmed by several investigators.[19–20]

In 1970, it was reported from this laboratory[21] that the ATPase inhibitor also inhibited ATP-driven energy-utilizing reactions of submitochondrial particles such as succinate-linked NAD^+ reduction, energy-linked transhydrogenase, and ATP-induced ANS-fluorescence enhancement. The same reactions when driven by the respiratory chain were unaffected.

The same year Horstman and Racker[15] described a new procedure for the purification of the ATPase inhibitor. The inhibitor so obtained was shown to require preincubation with ATP and Mg^{2+} in order to inhibit the ATPase activity of submitochondrial particles and purified F_1. Evidence was presented indicating that ATP and Mg^{2+} promote the binding of the inhibitor to F_1.

The findings of Horstman and Racker[15] were recently confirmed in this laboratory, and it was shown[22] that the inhibitor prepared according to these authors has a molecular weight 4–6 times higher than that reported for the preparation of Pullman and Monroy,[7] thus probably representing a polymer of the latter. It was also found[22] that when the inhibitor is preincubated with EDTA particles in the presence of ATP

and Mg^{2+}, so as to inhibit the ATPase activity, the inhibition persisted even after dilution of the ATP and Mg^{2+} in the preincubating medium. However, the inhibition was relieved, if the diluted preincubation mixture was reincubated in the presence of succinate and a low concentration of oligomycin, i.e., under conditions which give rise to an accumulation of energy from the respiratory chain. These findings were interpreted to indicate that energy derived from ATP promotes the binding of the inhibitor to F_1, whereas energy derived from the respiratory chain promotes its release from F_1. Studies of the effects of P_i and aurovertin indicated, furthermore, that a phosphorylated high-energy intermediate probably is responsible for the respiration-induced release of the inhibitor.

Kinetic studies of the effect of the inhibitor on the ATPase reaction of EDTA particles revealed[9,23] that the inhibition is noncompetitive with respect to the substrate, leaving unaltered the K_m for ATP and ITP, and likewise the K_i for ADP. Oligomycin and aurovertin did not affect the titer of the ATPase with respect to the inhibitor, nor did the ATPase inhibitor alter the inhibition titer of the ATPase with respect to oligomycin. These data are consistent with the conclusion that the ATPase inhibitor firmly binds to a site of the ATPase which is essential for catalytic activity but is not identical with the binding site for either ATP, ADP, oligomycin, or aurovertin.

Next, the kinetics of the effect of ATPase inhibitor on the ATP-driven succinate-linked NAD^+ reduction and energy-linked transhydrogenase reaction were investigated. Mg-ATP particles, which catalyze a maximal activity of these reactions without need for the presence of a low concentration of oligomycin,[24] proved to be unsuitable for this purpose, since it was found that added ATPase inhibitor had little or no effect on their ATPase activity, in contrast to that obtained with EDTA particles (Fig. 5). Also the ATP-driven electron-transport reactions of the Mg-ATP particles showed little or no sensitivity to the ATPase inhibitor. Treatment with Sephadex G-50 resulted, in accordance with earlier findings of Racker and Horstman,[25] in a several-fold increase of the ATPase activity of Mg-ATP particles, with a simultaneous release of ATPase inhibitor. However, this treatment also caused a decrease of the ATP-driven electron-transport activities, which now required the addition of low concentrations of oligomycin to reach maximal levels, similar to EDTA particles. In view of these findings it was decided to use EDTA particles for investigating the kinetics of the effect of ATPase inhibitor on the ATP-driven electron-transport reactions.

Figure 6 compares the effects of increasing amounts of ATPase inhibitor on the ATP-driven succinate-linked NAD^+ reduction, nicotinamide transhydrogenase and ATPase activities of EDTA particles. The ATP-driven electron-transport reactions were measured

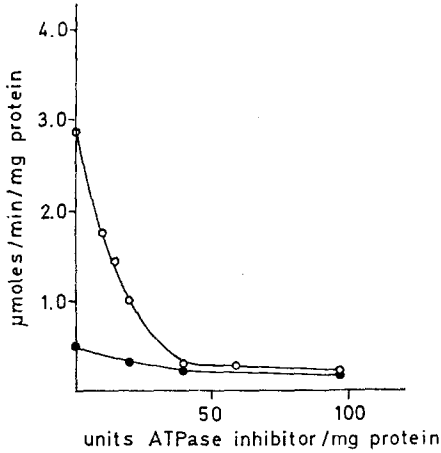

Figure 5. Effect of ATPase inhibitor on the ATPase activity of EDTA particles (○) and Mg-ATP particles (●). ATPase inhibitor (AI), prepared according to Horstman and Racker,[15] was preincubated in the particles at 30°C for 10 min in 0·5 ml of a mixture containing 0·25 M sucrose, 10 mM tris-TES, pH 6·5, 0·45 mM and 0·45 mM $MgSO_4$.

ATPase activity was assayed with an aliquot of the preincubation mixture in 1 ml of a medium containing 5 mM tris-Ac, pH 7·5, 30 mM KCl, 3 mM $MgCl_2$, 0·2 mM NADH, 0·75 mM phosphoenolpyruvate, 15 μg lactate dehydrogenase, 0·1 mg pyruvate kinase, 1·5 μM rotenone, 1 μM FCCP, and 0·02 mg particle protein.

The reaction was followed spectrophotometrically at 340 nmoles. Temperature, 30°C.

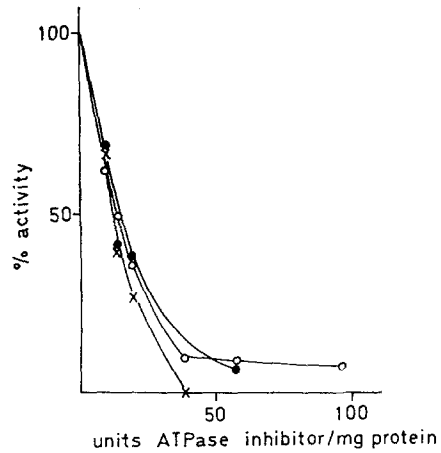

Figure 6. Effects of ATPase inhibitor on the ATPase (○) and ATP-driven succinate-linked NAD^+ reduction (×) and nicotinamide nucleotide transhydrogenase reaction (●) of EDTA particles. Preincubation with ATPase inhibitor (AI) was done as described in Fig. 5.

ATPase activity was assayed as in Fig. 5, and ATP-driven succinate-linked NAD^+ reduction and transhydrogenase were assayed as in Fig. 3A.

Activities in the absence of ATPase inhibitor were: 2·9 μmoles ADP formed per min and mg protein for ATPase; 73 nmoles NADH formed per min and mg protein for succinate-linked NAD^+ reduction; and 128 nmoles per min and mg protein for transhydrogenase.

in the presence of a maximally stimulating concentration of oligomycin (cf. Fig. 3B). It may be seen in Fig. 6 that the ATPase inhibitor inhibited with equal efficiencies the two ATP-driven reactions, in

sharp contrast to FCCP and oligomycin, which inhibited the succinate-linked NAD^+ reduction much more efficiently than the transhydrogenase reaction (cf. Figs. 2 and 3). Furthermore, the ATPase inhibitor titers of the two ATP-driven reactions were the same as that of the ATPase. The ATPase activity of the non-inhibited system was about one order higher, in terms of estimated molar amounts of ATP expended, than those of the ATP-driven reactions (cf. legend of Fig. 6). These findings, which confirm preliminary observations reported from this laboratory,[21] strongly suggest that the ATPase inhibitor acts on a common rate-limiting step of the ATPase and ATP-driven electron-transport reactions.

Conclusions and Comments

The results described in this paper reveal a striking difference between the effects of the ATPase inhibitor, on one hand, and of FCCP and oligomycin, on the other, on ATP-driven electron-transport reactions of submitochondrial particles. The ATPase inhibitor inhibits the ATP-driven succinate-linked NAD^+ reduction and nicotinamide nucleotide transhydrogenase reaction with equal efficiencies, whereas FCCP and oligomycin inhibit much more efficiently the succinate-linked NAD^+ reduction than the transhydrogenase reaction. Furthermore, the inhibition of the ATP-driven electron-transport reactions by the ATPase inhibitor parallels the inhibition of the ATPase reaction, indicating a common rate-limiting step in the three reactions.

There is strong evidence in the literature[7,15] that the ATPase inhibitor acts on the terminal enzyme of the phosphorylating system, F_1. It might seem conceivable, therefore, that F_1 is the common rate-limiting step for the ATPase and ATP-driven electron-transport reactions, and that this is the reason why the ATPase inhibitor, but not FCCP and oligomycin (which act on steps intermediate between F_1 and the electron-transport system), inhibits the ATP-driven succinate-linked NAD^+ reduction and transhydrogenase reaction with equal efficiencies. This explanation, however, appears unlikely, since replacement of ATP by ITP, which most probably also limits the ATP-driven reactions at the level of F_1, has been shown to lower the rate of the succinate-linked NAD^+ reduction more than that of the transhydrogenase reaction. In fact, it is quite obvious from the data summarized in Table I that half-inhibition of the ATPase activity by either oligomycin or replacement of ATP by ITP results in a substantial decrease in the ratio of the relative activities of the two ATP-driven electron-transport reactions, whereas that by ATPase inhibitor does not.

A more probable explanation of the above findings is that the ATPase inhibitor forms, at least under the conditions here employed, a stable,

TABLE I. Relative ratio of rates of ATP-driven succinate-linked NAD^+ reduction and nicotinamide nucleotide transhydrogenase at 50% inhibition of ATPase of submitochondrial particles

Preparation	ATPase inhibited by	Relative ratio of rates of ATP-driven succ.-linked NAD^+ red. and transhydrogenase
Beef-heart Mg-ATP particles	Oligomycin	0·12
Beef-heart EDTA particles	Oligomycin	0·25
Rat-liver particles	Replacement of ATP by ITP	0·33*
Beef-heart EDTA particles	ATPase inhibitor	0·95

* Calculated from data in ref. 17.

practically undissociable complex with F_1. Thus, addition of increasing amounts of ATPase inhibitor to the particles will inactivate an equivalent amount of F_1 in an all-or-none fashion. If, as predicted by the chemiosmotic hypothesis (1), ATPases and electron-transport chains within the same membrane would interact in a random manner, by way of a common proton gradient (cf. Fig. 1A), then a partial inhibition of the ATPase activity by ATPase inhibitor would result in a greater inhibition of the thermodynamically unfavourable succinate-linked NAD^+ reduction than the thermodynamically favourable transhydrogenase reaction. The fact that the inhibition of the two ATP-driven electron-transport reactions by the ATPase inhibitor closely parallels that of the ATPase strongly suggests, therefore, that the ATPase and electron-transport systems interact in a direct, assembly-like fashion as indicated in Fig. 1B.

It should be pointed out that the above interpretation does not necessitate the postulation of the occurrence of one ATPase per electron-transport system or even a strict stoichiometry within each electron-transport chain, and it is quite possible that there is an excess of F_1 relative to the number of electron-transport systems[26] and of cytochromes relative to flavoproteins,[27] as indicated by estimates of the mitochondrial contents of these components; regarding transhydrogenase, there are no estimates available so far. What the present interpretation does imply is that those ATPases and electron-transport chains which do interact, do this in a direct, assembly-like fashion within the membrane, i.e., in a fashion incompatible with the involvement of a bulk proton gradient or membrane potential as an obligatory intermediate. The role of the membrane, though not yet fully understood, would be to promote this interaction, rather than merely to serve as an inert insulator as envisioned by the chemiosmotic hypothesis.

Acknowledgement

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