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Inhibition of energy-transducing reactions by 8-nitreno-ATP covalently bound to bovine heart submitochondrial particles: direct interaction between ATPase and redox enzymes

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1. The photoaffinity label 8-azido-ATP has been used to study the effect of inhibition of ATP synthase on ATP-driven reverse electron transfer from succinate to NAD^+ ('reversal'), succinate- and NADH-driven ATP synthesis and ATP-P_i exchange. 2. In reversal, where ATPase functions as primary proton pump, inactivation by covalently bound nitreno-ATP results in an inhibition that is proportional to the inactivation of ATP hydrolysis, or, consequently, with the concentration of inactivated ATP synthases. Up to 60% inactivation of the reversal rate does not lead to a decrease in $\Delta\tilde{\mu}_{H^+}$. 3. Inhibition of ATP synthase as secondary proton pump results in case of NADH-driven ATP synthesis in a proportional inhibition, but with succinate as substrate ATP synthesis is less than proportionally inhibited, compared with inactivation of ATP hydrolysis. 4. Inhibition of one of the primary pumps of NADH-driven ATP synthesis, the NADH:Q oxidoreductase, with rotenone also resulted in an inhibition of the rate of ATP synthesis proportional to that of the NADH oxidation. 5. ATP-P_i exchange is much more affected than ATP hydrolysis by photoinactivation with 8-azido-ATP. Contrary to reversal and NADH-driven ATP synthesis the rate of ATP-P_i exchange does not depend linearly, but quadratically on the concentration of active ATP synthases. 6. The observed proportional relationships between inhibition of the primary or secondary pump and the inhibition of the overall energy-transfer reactions do not support the existence of a pool intermediate in energy-transduction reactions. However, the results are consistent with a direct transfer of energy from redox enzymes to ATP synthase and vice versa.

Introduction

According to the theory of Mitchell [1] the phosphorylation of ADP in mitochondria is driven by a delocalized gradient of chemiosmotic protons: $\Delta\tilde{\mu}_{H^+}$. This $\Delta\tilde{\mu}_{H^+}$ is formed by substrate oxidation in the respiratory chain: dependent on the substrate the respiratory-chain enzymes transfer at two or three sites protons over the inner mitochondrial membrane. This mechanism implies that $\Delta\tilde{\mu}_{H^+}$ is the kinetically competent intermediate in ATP synthesis and energy transduction does not involve direct interaction between ATP synthase and the respiratory chain. However, such an interaction has been found by several workers. In rat liver mitochondria [2,3], plant mitochondria

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazinemethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; S13, 2,5-dichloro-3-tertiary butyl-4'-nitrosalicylanilide; SMP, submitochondrial particles; PEP, phospho*enol*pyruvate.

[4], bovine heart submitochondrial particles [5], chromatophores of *Rhodopseudomonas sphaeroides* [6] and of *Rhodopseudomonas capsulata* [7] it was found that the rate of ATP synthesis depends linearly on the rate of electron transfer through the respiratory chain, and that ATP synthesis can be nearly fully inhibited by respiratory-chain inhibitors with only a slight decrease in $\Delta\tilde{\mu}_{H^+}$ [3,4]. Nigericin + valinomycin also abolish oxidative phosphorylation without a drop in $\Delta\tilde{\mu}_{H^+}$ [2]. Double-inhibitor titrations also suggested that there is a direct interaction between ATP synthase and the respiratory chain in chromatophores of *Rps. capsulata* [7]. Venturoli and Melandri [6] have observed that the ATP yield per flash in chromatophores of *Rps. sphaeroides* was decreased by inhibition of electron transfer and ATP synthase without affecting the amplitude of the carotenoid band shift, which is thought to reflect the membrane potential. Earlier, Baum et al. [8] who introduced the double-inhibitor titrations, obtained evidence for direct interaction between ATP synthase and the respiratory chain in the ATP-driven reduction of NAD^+ in bovine heart submitochondrial particles (see also Ref. 9). In view of the importance of this question, we decided to reinvestigate it, using the photoaffinity label 8-azido-ATP irreversibly to inactivate the ATP synthase [10]. Irreversible inhibitors have an advantage over reversible inhibitors in inhibitor titrations in that their binding is not affected by the state of energization of the membrane. Furthermore, 8-azido-ATP specifically binds to the catalytic sites of F_1, labelling three neighbouring amino acids on the β subunit [11,12], whereas oligomycin used in previous experiments binds to an F_0 component, thereby affecting not only ATP synthesis, but also passive H^+ flux through F_0.

As energy-transducing reactions ATP-driven reduction of NAD^+ by succinate ('reverse electron transfer'), ATP synthesis and ATP-P_i exchange were studied. In the former the ATP synthase functions as the primary, energy-producing proton pump. In ATP synthesis it is the secondary, energy-consuming proton pump, and in ATP-P_i exchange it functions both as primary and secondary pump. The results indicate a direct coupling between the primary and secondary pumps, both in reverse electron transfer and in oxidative phosphorylation. Some of these results have been already published in a preliminary form [13].

Methods and Materials

Preparation of submitochondrial particles

Mn/Mg/succinate/ATP submitochondrial particles (SMP) were prepared from heavy bovine heart mitochondria [14] that had been stored at 80 mg protein/ml in 0.25 M sucrose/1 mM ATP/1 mM $MgCl_2$/1 mM succinate/10 mM Hepes-KOH buffer (pH 7.5) at −70°C. Preparation of SMP was essentially by the method of Hansen and Smith [15] with the exception that Hepes-KOH buffer was used instead of Tris-HCl and that the succinate dehydrogenase of the particles was activated by incubation of the crude particle fraction (supernatant after sedimentation of intact mitochondria by centrifugation) in the presence of 5 mMm potassium malonate for 30 min at 30°C [16]. After one wash with 0.25 M sucrose, 10 mM Hepes-KOH buffer (pH 7.5) succinate oxidation was maximally activated. No further activation could be achieved by preincubation with succinate. The particles were suspended at a concentration of about 60 mg of protein/ml in 0.25 M sucrose, 10 mM Hepes-KOH buffer (pH 7.5), supplemented with 3% (v/v) glycerol, quickly frozen and stored in 0.25 ml portions in liquid N_2. When measured in 180 mM sucrose/10 mM Hepes-KOH (pH 7.5), NADH oxidation in the absence of ADP was stimulated 4–5-times by uncoupler. The State-4 oxidation rate was not further inhibited by oligomycin.

Photolabelling with 8-azido-ATP

Photolabelling of the particles with 8-azido-ATP under ultraviolet light of 360 nm was performed at 0°C, but otherwise as described by Wagenvoord et al. [11]. During illumination EDTA was added to prevent hydrolysis of azido-ATP. Azido-ATP concentrations varied between 0.5 and 3.0 mM, and incubation times from 5 to 45 min. Samples were removed from the incubation mixture at intervals and stored in the dark on ice. Activities in the samples were determined as described below.

ATP hydrolysis assay

ATP hydrolysis was measured at 30°C in a

Zeiss spectrophotometer at 340 nm in 2 ml of a medium containing 83 mM sucrose/6 mM $MgCl_2$/33 mM Tris-HCl buffer/5 mM ATP and 10 mM $NaHCO_3$ as activating anion. As ATP-regenerating system 0.5 mM phospho*enol*pyruvate and 4.0 units pyruvate kinase were added together with 3.0 units lactate dehydrogenase and 0.25 mM NADH to couple the reaction to the consumption of NADH. The final pH was 8.0. Also 1.0 μM S13 and 3.0 μM rotenone were added to the assay mix. The reaction was started by addition of 25–50 μg SMP.

ATP synthesis assay

To measure ATP synthesis SMP were incubated at 30°C in an 1.4 ml oxygraph vessel containing 0.25 M sucrose, 4 mM $MgCl_2$, 10 mM potassium phosphate, 0.1 mM EDTA, 0.33 mM ADP and 1 mM NADH or 10 mM succinate as substrate. 20 mM glucose and 2.6 units of hexokinase per ml medium were added as ADP-regenerating system and 3.3 mM AMP to inhibit the myokinase activity. The final pH was 7.5. The medium was saturated with oxygen, and oxygen consumption was measured with a Clark oxygen electrode. The reaction was started by adding 0.3 mg particles. When all oxygen was consumed, 1.0 ml of the reaction mixture was quenched in perchloric acid (final concentration, 0.54 M), the denatured protein was removed by centrifugation and 1 ml of the supernatant was neutralized with 6 M KOH/0.3 M Mops. Afterwards glucose 6-phosphate was determined in the sample by adding 1 ml of the neutralized sample to 1 ml of a 50 mM Tris-HCl buffer (pH 7.5), containing 5 mM EDTA, 10 mM $MgCl_2$ and supplemented with 0.25 mM $NADP^+$. The reaction was started by adding 0.7 units glucose 6-phosphate dehydrogenase, and NADPH formation was followed at 340 nm in a Zeiss spectrophotometer.

ATP-driven electron transfer from succinate to NAD (reversal)

Reversal was measured according to Ernster and Lee [17] at 340 nm in a Zeiss spectrophotometer at 30°C in a buffer containing 0.18 M sucrose/50 mM Tris-HCl/5 mM succinate/6 mM $MgCl_2$/1.6 mM KCN/1.0 mM NAD^+. The final pH was 7.8. 0.1–0.3 mg particles were incubated for 1 min in 2 ml of this medium and the reaction was started with 3.0 mM ATP.

ATP-P_i exchange assay

To measure ATP-P_i exchange 0.1 mg particles were incubated at 30°C in a medium containing 25 mM potassium phosphate, 10 mM ATP, 0.1 M sucrose, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mg/ml bovine serum albumin, 20 mM Hepes-KOH, final pH 7.5. To allow for the lag phase encountered in ATP-P_i exchange measurements with submitochondrial particles (Cornelissen, J. and Berden, J.A., unpublished observations, and Ref. 18), $^{32}P_i$ (specific activity, about 100 cpm/nmol P_i) was added after a preincubation for 10 min. After a further 10 min the reaction was quenched with ice-cold trichloroacetic acid (5% final concentration). Inorganic phosphate was extracted as a phosphate-molybdate complex [19] with isobutanol-toluene (1 : 1) and [$^{32}P_i$]ATP in the water layer was counted after one wash with isobutanol, and one with ether. When $^{32}P_i$ was added 10 min after the start of the incubation, the formation of [γ^{32}P]ATP was always linear with time for at least 20 min.

Determination of $\Delta\tilde{\mu}_H$ under reversal conditions

ΔpH and $\Delta\psi$ were determined with flow dialysis, essentially as described by de Jonge and Westerhoff [20]. $\Delta\psi$ was calculated from the disappearance of [^{14}C]thiocyanate from the medium, and ΔpH from the disappearance of amino[^{3}H]methane. To 1.4 ml of a medium containing 0.15 M sucrose,10 mM Hepes-KOH (pH 7.4)/50 mM KCl/5 mM potassium phoshate/10 mM $MgCl_2$/1 mM EGTA/100 mM phosphocreatine/1.5 mM KCN/10 mM succinate/1.5 mg per ml bovine serum albumin, 20 mg particles were added. After 1 min 20 μM [^{14}C]SCN^- (60 Ci/mol) was added, and after 2.5 min 1 μM amino[^{3}H]methane (11.4 Ci/mmol). At 4.5 min 200 units creatine kinase were added and 30 s later 2 mM ATP. At 6 min 1 mM NAD was added, at 13 min 5 μg FCCP and at 18 min 10 μl Triton-X-100. The final pH of the medium was 7.5. The efflux of label through the dialysis membrane was monitored by taking samples at 30-s intervals from the 'flow mix' flowing through the lower compartment of the flow dialysis vessel at about 2 ml per

min. 4 ml scintillation liquid was added to the samples and the samples were counted in a two-channel scintillation counter. The results were corrected for the effect of additions, so that the correct reference line could be obtained through interpolation between the deenergized parts of the plot. By using this line, the membrane potential or the pH gradient was calculated. The internal volume of the particles, determined as described by De Jonge and Westerhoff [20], was 1 μl/mg protein.

Phosphate determination

Inorganic phosphate was determined according to a modification of the Fiske-Subbarow procedure by Sumner [19].

Protein determination

Protein concentrations were measured according to Lowry et al. [21] with bovine serum albumin ($A^{1\%}_{279\ nm} = 6.67$) as standard.

Materials

8-Azido-ATP was synthesized in our laboratory according to the method of Schäfer [22]. $^{32}P_i$ was purchased from New England Nuclear, and [^{14}C]thiocyanate and amino[^{3}H]methane from Amersham. S13 was a gift from Dr. P. Hamm, Monsanto Company, St. Louis, MO, U.S.A. and ACMA from Professor R. Kraayenhof. Piericidin A was isolated from *Streptoverticilium mobaraensis* (obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, F.R.G.) as described by Singer [23].

Results

As shown before by Wagenvoord et al. [10] photolabelling with 8-azido-ATP inhibits ATP hydrolysis not only in soluble F_1, but also in bovine heart submitochondrial particles. Coupled ATP hydrolysis was inhibited to the same extent as ATP hydrolysis measured in the presence of the uncoupler S13 (not shown). This means that the percentage inhibition of ATP hydrolysis can be seen as a measure for the saturation of ATP binding sites. In F_1 it was already shown that photolabelling is non-cooperative and that 100% inactivation of ATP hydrolysis is reached after 2 mol of

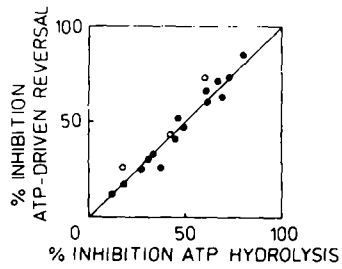

Fig. 1. Inhibition of ATP hydrolysis and ATP-driven reversed electron transfer by photoinactivation with 8-azido-ATP. Submitochondrial particles were incubated on ice water at a protein concentration of 18–19 mg/ml with 1.0 or 3.0 mM azido-ATP and 7 mM EDTA for 0–36 min under ultraviolet light of ($\lambda_{max} \approx 360$ nm). Samples were removed at intervals and stored in the dark on ice. Afterwards ATP-driven reversal was measured as described in methods. Closed symbols: ATP hydrolysis was measured in a separate assay with a coupled enzyme system containing PEP, pyruvate kinase, NADH and lactate dehydrogenase. The assay also contained 10 mM HCO_3^-, 1.0 μM S13 and 3.0 μM rotenone. 100% activity of ATP hydrolysis was 6.3 (±0.2 S.D.) μmol·min^{-1}·mg^{-1}. 100% activity of ATP-driven reversal was 246 (±45 S.D.) nmol·min^{-1}·mg^{-1}. Data were taken from four separate experiments. Open symbols: in this experiment reversal and ATP hydrolysis were measured simultaneously. From the cuvette in which the reversal rate was followed samples were taken in which colorimetrically P_i was determined. The reversal rate was 169 nmol·min^{-1}·mg^{-1} and the ATP hydrolysis rate was 0.82 μmol·min^{-1}·mg^{-1}.

nitreno-ATP are bound. This relationship also holds in submitochondrial particles [35].

Reverse electron transfer from succinate to NAD^+ can be driven by ATP hydrolysis. Inactiva-

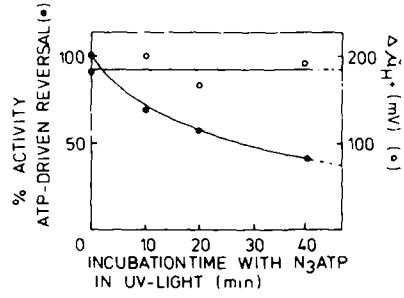

Fig. 2. Effect of photoinactivation with 8-azido-ATP on reversed electron transfer and on $\Delta\tilde{\mu}_{H^+}$. $\Delta\tilde{\mu}_{H^+}$ was determined by flow dialysis, measuring in a single incubation $\Delta\psi$ as accumulation of [^{14}C]thiocyanate and ΔpH as accumulation of [^{3}H]methylamine. Reversal was measured as described in Materials and Methods (see also legend to Fig. 1, open symbols). 100% activity of reversal was in this experiment 263 nmol·min^{-1}·mg^{-1}. In the control particles ΔpH was 77 mV and $\Delta\psi$ 115 mV.

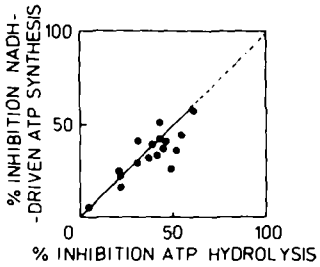

Fig. 3. Inhibition of ATP hydrolysis and NADH-driven ATP synthesis by photoinactivation with 8-azido-ATP. Submitochondrial particles were incubated on icewater with 0.5 mM 8-azido-ATP and 5 mM EDTA for 0–40 min under light of 360 nm. Samples were taken at intervals and stored in the dark on ice. Afterwards ATP synthesis and ATP hydrolysis were measured as described in methods. 100% activity of ATP hydrolysis was 5.8 (±0.4 S.D.) $\mu mol \cdot min^{-1} \cdot mg^{-1}$, measured with HCO_3^- and uncoupler (S13). 100% activity of NADH-driven ATP synthesis was 508 (±85 S.D.) $nmol \cdot min^{-1} \cdot mg^{-1}$. Data were taken from seven separate experiments. The average P/O of NADH-driven ATP synthesis was 0.53.

tion of the submitochondrial particles with azido-ATP caused an inhibition of the NAD^+ reduction. As can be seen in Fig. 1 ATP-driven reversal is inhibited to the same extent as ATP hydrolysis. The closed dots in Fig. 1 represent experiments in which the two activities were measured in separate assays, as described in methods. When ATP hydrolysis was measured in the same assay as ATP-driven reversal, by colorimetric P_i determination, the same relation was found. The results of this experiment are shown by the open symbols in Fig. 1. The proportionality between ATP hydrolysis and ATP-driven reversal indicates a direct rate limitation by ATP hydrolysis. Thus if the pool $\Delta\tilde{\mu}_{H^+}$ were the intermediate between ATP hydrolysis and NAD^+ reduction, an inhibition of hydrolysis would cause a decrease in $\Delta\tilde{\mu}_{H^+}$. Fig. 2 shows, however, that, when the reversal rate is inhibited by more than 50%, there is not a corresponding decrease in $\Delta\tilde{\mu}_{H^+}$.

In ATP synthesis it is the secondary proton

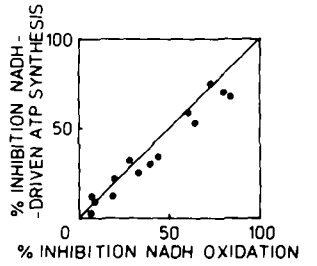

Fig. 4. Inhibition of NADH oxidation and NADH-driven ATP synthesis by roterone. Particles were incubated for 45 min at room temperature with 16–64 ng roterone per mg particle protein. NADH oxidation was measured polarographically with a Clarck oxygen electrode or spectrophotometrically at 340 nm, in medium given in Materials and Methods for the ATP synthesis. Assay temperature was 30°C. To determine simultaneously the ATP synthesis rate, samples were taken at intervals and quenched in perchloric acid. Glucose 6-phosphate in the samples was measured as described in Materials and Methods. 100% activity of NADH-driven ATP synthesis was 434 (±92 S.D.) $nmol \cdot min^{-1} \cdot mg^{-1}$ and 100% activity of NADH oxidation was 854 (±183 S.D.) $nmol \cdot min^{-1} \cdot mg^{-1}$. Data were taken from three separate experiments.

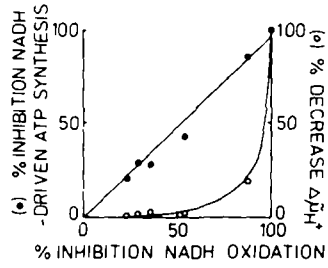

Fig. 5. Effect of inhibition by piericidin on NADH-driven ATP synthesis, NADH oxidation and $\Delta\tilde{\mu}_{H^+}$ as measured by ACMA quenching. Particles were incubated for 30 min on ice with 60–150 pmol piericidin per mg particle protein. NADH oxidation was measured spectrophotometrically at 340 nm, in the medium given in Methods for the ATP synthesis. In one experiment the medium was supplemented with 1 μM ACMA and 0.24 μg valinomycin per mg particle protein. Samples were taken at intervals and quenched in $HClO_4$, and glucose 6-phosphate was determined as described in Methods. ACMA quenching was measured in a separate incubation with the same medium in a Perkin-Elmer MPF-2A fluorescence spectrophotometer, with emission wavelength 478 nm and excitation wavelength 412 nm. Both assays were performed at 30°C and the protein concentration was 0.1 mg/ml. Data were taken from two separate experiments. In the experiment in which ATP synthesis was measured in the absence of ACMA and valinomycin, 100% activity of NADH oxidation was 892 $nmol \cdot min^{-1} \cdot mg^{-1}$ and 100% activity of ATP synthesis was 539 $nmol \cdot min^{-1} \cdot mg^{-1}$. In the experiment in which ACMA and valinomycin were included in the medium during the ATP synthesis assay, the 100% values were 988 $nmol \cdot min^{-1} \cdot mg^{-1}$ for NADH oxidation and 437 $nmol \cdot min^{-1} \cdot mg$ for ATP synthesis. 100% $\Delta\tilde{\mu}_{H^+}$, calculated according to the Schuldiner equation [34], was equivalent to 3.75 ΔpH unit in the first experiment and 3.77 ΔpH unit in the second experiment. In this calculation 1 μl/mg particle protein was used as internal volume.

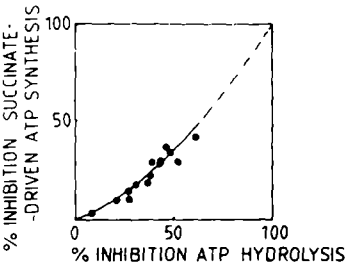

Fig. 6. Inhibition of ATP hydrolysis and succinate-driven ATP synthesis by photoinactivation with 8-azido-ATP. Submitochondrial particles were incubated on ice water with 0.3–0.6 mM 8-azido-ATP and 50 mM EDTA for 0–30 min under light of 360 nm. Samples were taken at intervals and stored in the dark on ice. Afterwards ATP synthesis and ATP hydrolysis were measured as described in methods. 100% activity of ATP hydrolysis was 6.4 (±0.8 S.D.) $\mu mol \cdot min^{-1} \cdot mg^{-1}$ measured with HCO_3^- and 1.0 μM S13. 100% activity of succinate-driven ATP synthesis was 385 (±87 S.D.) $nmol \cdot min^{-1} \cdot mg^{-1}$. Data were taken from five separate experiments. The average P/O of succinate-driven ATP synthesis was 0.45.

pump that is inhibited by azido-ATP. If $\Delta\tilde{\mu}_H$ is sufficiently high to drive the ATP synthase at maximal velocity, we may expect a proportional relation between occupation of binding sites and inactivation of ATP synthesis, in which case the inhibition of synthesis would be proportional to that of hydrolysis assuming that the same binding sites are involved in ATP hydrolysis and ATP synthesis.

With NADH as a substrate for oxidative phosphorylation this proportional relationship is indeed found (Fig. 3). However, also when the oxidation of NADH is inhibited by rotenone or piericidin proportionality is found between inhibition of NADH oxidation and inhibition of ATP synthesis (Figs. 4 and 5). Apparently, both primary and secondary pumps are rate-limiting. This is rather difficult to explain in the chemiosmotic theory without additional assumptions about regulatory interactions between the components involved (see Discussion). Inhibition of NADH oxidation by piericidin by up to 50% had a very small effect on $\Delta\tilde{\mu}_H$ measured by fluorescence quenching of the probe ACMA in the presence of valinomycin (Fig. 5). We are aware of the difficulties involved in the use of ACMA as ΔpH probe [24], but under conditions of oxidative phosphorylation it was not possible adequately to set up a flow-dialysis experiment, analogous to the experiment described for reverse electron transfer. Due to the high particle concentration (about 10 mg/ml), necessary to have a sufficient ratio of internal to external volume, the rate of oxygen consumption was so high, that even careful additions of H_2O_2 in the presence of added catalase to keep the oxygen supply non-limiting resulted in irreversible inactivation of the particles.

TABLE I

EFFECT OF ILLUMINATION IN PRESENCE OF 8-AZIDO-ATP ON UNCOUPLED NADH AND SUCCINATE OXIDATION

Submitochondrial particles were incubated in ice water with 0.5 mM 8-azido-ATP and 5 mM EDTA for 0–30 min under ultraviolet light of 360 nm. Samples were taken at intervals and substrate oxidation was measured at 30°C in an oxygraph vessel in medium given in Methods for the ATP synthesis assay (State 3). For full uncoupling 0.5 μM S13 was added.

Exposure time (min)	−S13		+S13	
	($\mu mol \cdot min^{-1} \cdot min^{-1}$)	(%)	($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	(%)
Succinate oxidation				
0	1.00	100	1.48	100
5	1.02	102	1.57	106
10	0.98	98	1.50	101
20	0.92	92	1.48	100
30	0.92	92	1.40	94
NADH oxidation				
0	0.92	100	1.16	100
5	0.92	100	1.17	101
10	0.87	95	1.11	95
15	0.84	91	1.11	95
20	0.82	89	1.08	93
30	0.77	84	1.04	90

When succinate was used as substrate for oxidative phosphorylation the relation between inhibition of ATP hydrolysis and ATP synthesis by nitreno-ATP is non-linear (Fig. 6), ATP synthesis being less affected by nitreno-ATP binding than ATP hydrolysis. Clearly the ATP synthase activity is in overcapacity with respect to the energy delivered by the primary pumps. As can be seen in Table I the primary pumps are scarcely affected by the treatment with azido-ATP: uncoupled succinate and NADH oxidation are not inhibited.

In ATP-P_i exchange, also a measure for ATP synthesis capacity, energy for ATP synthesis is provided by ATP hydrolysis. Both processes are inactivated by azido-ATP and this results in a

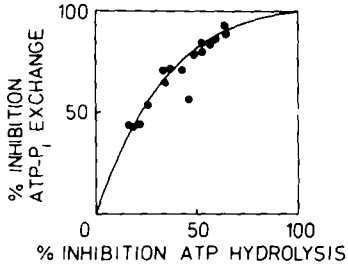

Fig. 7. Inhibition of ATP hydrolysis and ATP-P_i exchange by photo-inactivation with 8-azido-ATP. Submitochondrial particles wree incubated on ice water with 0.5–3 mM 8-azido-ATP and 5.0 mM EDTA for 0–40 min under an ultraviolet lamp (λ_{max} = 360 nm). Protein concentration was 11–15 mg/ml. Samples were taken at intervals and stored in the dark on ice. Afterwards ATP hydrolysis and ATP-P_i exchange were measured as described in methods. 100% activity of ATP hydrolysis is 6.5 (± 1.3 S.D.) $\mu mol \cdot min^{-1} \cdot mg^{-1}$. 100% activity of ATP-$P_i$ exchange is 70 and 76 $nmol \cdot min^{-1} \cdot mg^{-1}$ in one batch of particles, and 23 and 239 $nmol \cdot min^{-1} \cdot mg^{-1}$ in another batch. Data were taken from four separate experiments.

more than proportional inhibition of the exchange reaction (Fig. 7). The results fit quite well with a quadratic dependence of the ATP-P_i exchange activity on the concentration of active ATP synthases: when the concentration of active synthases is decreased by 50%, the ATP-P_i exchange is inhibited by 75%.

Discussion

According to the Mitchell chemiosmotic theory, $\Delta\tilde{\mu}_H$· is built up by the operation of a primary proton pump and is utilized by a secondary pump. The ATP-driven reduction of NAD^+ by succinate can, for example, be written as:

$$ATP + H_2O \rightleftharpoons ADP + P_i + \Delta\tilde{\mu}_H\cdot$$

$$\Delta\tilde{\mu}_H\cdot + \text{succinate} + NAD^+ \rightleftharpoons \text{fumarate} + NADH + H^+$$

In this formulation, $\Delta\tilde{\mu}_H$· acts as a 'pool' between the two pumps (cf. Ref. 25) and its steady-state concentration will be dependent upon their relative capacities.

In this case, the ATPase is the primary pump and a redox enzyme (NADH:Q oxidoreductase) is the secondary pump. These roles are reversed in ATP synthesis driven by substrate oxidation which may be written as:

$$NADH \text{ (or succinate)} + \tfrac{1}{2}O_2 \rightarrow NAD^+ \text{ (fumarate)} + H_2O + \Delta\tilde{\mu}_H$$

$$\Delta\tilde{\mu}_H\cdot + ADP + P_i \rightleftharpoons ATP + H_2O$$

In the ATP-P_i exchange reaction, the ATPase (ATP synthase) acts both as primary and secondary proton pump:

$$ATP + H_2O \rightleftharpoons ADP + P_i + \Delta\tilde{\mu}_H\cdot$$

$$\Delta\tilde{\mu}_H\cdot + ADP + P_i \rightleftharpoons ATP + H_2O$$

All these reactions would be expected to follow typical pool kinetics, so that the rate of the reaction $v = V_1V_2/(V_1 + V_2)$, where V_1 and V_2 are the maximal rates of the primary and secondary pumps, respectively.

In the case of the ATP-driven reversal, when the primary pump was inacativated by covalent binding of nitreno-ATP to the ATP-binding site, the inhibition of the overall reaction was found to be proportional to the inactivation of the ATPase (Fig. 1). Thus, v is proportional to V_1, which, according to the pool model based on chemiosmotic theory is the case only when $V_2 \gg V_1$, in other words when the ATPase is rate-limiting. Under these conditions, one would expect the steady-state value of $\Delta\tilde{\mu}_H$· to be low (compared with that found during substrate-driven ATP synthesis) and to decline further with increasing inactivation of the ATPase. In fact, the value of $\Delta\tilde{\mu}_H$· found was quite high (about 200 mV) and did not decline when the ATPase was inhibited (Fig. 2). Thus, the experiments reported in Figs. 1 and 2 are not consistent with the chemiosmotic theory.

The effect of inactivation of the ATP synthase on the synthesis of ATP driven by NADH oxidation is, in itself, not inconsistent with this theory. The proportional inhibition of ATP synthesis and inactivation of the ATPase with NADH as substrate (Fig. 3) is consistent with the pool model if the ATP synthase is rate-limiting, i.e., $V_2 \ll V_1$. However, the experiment given in Fig. 4 shows that inhibition of NADH oxidation by rotenone results in a proportional inhibition of ATP synthesis, which, on the basis of the pool model, could be explained only if $V_2 \gg V_1$. That, with succinate as substrate, the ATP synthesis is inhibited less than

the ATPase (Fig. 5) could also be consistent with pool function kinetics, with $\Delta\tilde{\mu}_{H^+}$ as pool, provided that, in this case, V_1 is not much greater than V_2. However, this would mean that v would be much lower with succinate than with NADH which was not the case. Thus, it seems that some other intermediate is acting as a pool in the case of succinate as substrate.

The quadratic relationship between inhibition of the ATP-P_i exchange reaction and inactivation of the ATPase is consistent with the pool model only if $V_1 = V_2$, which is not impossible, but seems a rather unlikely coincidence.

The conclusion is, then, that the effects of irreversibly inactivating the ATPase on the reactions in which this enzyme functions either as primary or secondary proton pump are not consistent with the Mitchell chemiosmotic theory, in which $\Delta\tilde{\mu}_{H^+}$ is a kinetically competent intermediate between the two pumps. This is in agreement with much evidence that has recently been reviewed by Kell [25], Ferguson and Sorgato [26], Westerhoff and coworkers [27–29]. In 1982, Sorgato et al. [30] found that $\Delta\tilde{\mu}_{H^+}$ built up by the hydrolysis of ATP in submitochondrial particles decreased only slightly when the rate of hydrolysis of ATP was varied by a factor of 20 by incubating the particles with mitochondria, respiring in State 3, at varying ratios. Yagi et al. [31] found practically no decrease in $\Delta\psi$ (under conditions in which $\Delta pH \approx 0$) when ATP-driven reversal was inhibited by oligomycin to the extent of 70%. Zoratti et al. [3] and Mandolino et al. [4] found that the degree of inhibition of ATP synthesis driven by substrate oxidation in respectively rat-liver and Jerusalem artichoke mitochondria is proportional to inhibition of the oxidation reaction by malonate or antimycin, with little effect on $\Delta\tilde{\mu}_{H^+}$ (cf. Ref. 5). Bar-Zvi and Shavit [32] found that inactivation of chloroplast ATPase by glutaraldehyde resulted in a proportional inhibition of ATP synthesis, but a greater inhibition of the ATP-P_i exchange reaction. They also found no effect of glutaraldehyde on $\Delta\tilde{\mu}_{H^+}$.

In the above discussion of the results to be expected on the basis of the chemiosmotic theory, it is assumed that the rate of ATP synthesis or of NAD^+ reduction is, as a first approximation, proportional to the $\Delta\tilde{\mu}_{H^+}$. Clark et al. [33], however, have concluded not only that the rate of ATP synthesis depends on the sixth power of $\Delta\tilde{\mu}_{H^+}$, but also that the main proton leak, occurring via the ATPase, has a high-power dependency (but less than the sixth power) on the $\Delta\tilde{\mu}_{H^+}$. On this basis a large decrease in the rate of ATP synthesis could be accompanied by a very slight decrease of $\Delta\tilde{\mu}_{H^+}$. However, a proportional relationship between inhibition of the primary pump and decrease of activity of the secondary pump is not to be expected, unless further assumptions are introduced. Furthermore, what is more important, inhibition of the reduction of NAD^+ by succinate, induced by inhibition of the ATPase, cannot be explained in this way, since the ATPase cannot now act as a proton leak. The possibility of a specific leak through the NADH dehydrogenase under these conditions, with a high-power dependency on $\Delta\tilde{\mu}_{H^+}$, is not supported by any experimental data.

Our results and those cited above are, however, consistent with an alternative view, namely that energy conservation involves direct collision in the mitochondrial membrane between redox enzymes and the ATP synthase. If the rate-limiting step in the ATP-driven reversal and ATP synthesis coupled to NADH oxidation is the frequency of collision between the ATPase (ATP synthase) and a redox enzyme, the proportionality between inhibition of the overall reaction and inactivation of the ATPase (ATP synthase) is to be expected. The quadratic relationship in the case of the ATP-P_i exchange reaction is also to be expected if the rate-limiting step is the collision between two ATP synthase molecules. The hyperbolic relation between inhibition of ATP synthesis linked to succinate oxidation and inactivation of the ATP synthesis is to be expected if, in this case, the collision frequency between the redox enzyme and the ATP synthase is not rate-limiting. That it is so in the case of the NADH oxidation could be because both the concentration of NADH dehydrogenase in the membrane is lower than that of the redox enzymes involved in succinate oxidation and/or the diffusion coefficient of the larger NADH:Q oxidoreductase is smaller than that of succinate:Q oxidoreductase.

Westerhoff et al. [27] have recently proposed an alternative model ('mosaic proton coupling') which retains all the four basic postulates of the chem-

iosmotic theory, but adds a fifth, namely that in the native organelle single primary proton pumps do not share with other primary proton pumps the space (or domain) into which they pump protons, but they do share these domains with other secondary ATP-driven proton pumps (or a few of them). Our finding of a quadratic relationship between inhibition of the exchange reaction, which involves interaction between ATPase (ATP synthase) molecules, and the inactivation of the ATPase shows, however, that primary pumps do share the same domain. We suggest that this is the membrane continuum of energy-transducing membranes.

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