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ROLE OF THE INTRAMITOCHONDRIAL ADENINE NUCLEOTIDES AS INTERMEDIATES IN THE UNCOUPLER-INDUCED HYDROLYSIS OF EXTRAMITOCHONDRIAL ATP

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SUMMARY

1. A formula is given that describes the appearance of [^{14}C]ADP outside the mitochondria after the addition of [^{14}C]ATP during the steady-state uncoupler-induced hydrolysis of extramitochondrial ATP. If the transported adenine nucleotides equilibrate with the intramitochondrial pool, [^{14}C]ADP_o would be expected to appear with a lag phase that corresponds with the time needed for the radioactive labelling of the intramitochondrial adenine nucleotide pool.

2. The rates of formation of [^{14}C]ADP outside the mitochondria after addition of [^{14}C]ATP during the steady-state uncoupler-induced ATP hydrolysis catalysed by rat-liver mitochondria at 0 °C were measured.

3. In the presence of carbonyl cyanide *m*-chlorophenylhydrazone the time course of the [^{14}C]ADP_o formation was the same as that predicted on the basis of the above assumption.

4. In the presence of the less effective uncoupler, 2,4-dinitrophenol, the time course of [^{14}C]ADP_o formation was not consistent with the theoretical predictions: no lag phase was present and the measured rate was higher than the maximal calculated rate. These results can be explained by assuming a functional interaction between the adenine nucleotide translocator and the mitochondrial ATPase (F_1).

5. It is concluded that under phosphorylating as well as dephosphorylating conditions, the adenine nucleotide translocator and the mitochondrial ATPase can be functionally linked to catalyse phosphorylation or dephosphorylation of extramitochondrial ADP or ATP, without participation of the intramitochondrial adenine nucleotides.

INTRODUCTION

The intramitochondrial adenine nucleotides are thought to be on the main pathway of the reactions involved in the uncoupler-induced hydrolysis of extramito-

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; subscript o and i, outer and inner compartment, respectively.

chondrial ATP. This idea is based essentially on the observations that (1) after the addition of an uncoupler and [^{14}C]ATP to a suspension of mitochondria, the ADP formed initially has a low specific activity, and this increases during the course of the reaction [1, 2] and (2) the hydrolysis of intramitochondrial ATP induced by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) is much faster than that of extramitochondrial ATP [3].

On the other hand, we have given evidence that the intramitochondrial pool of adenine nucleotides is not on the main pathway of the steady-state phosphorylation of extramitochondrial ADP [4, 5]. Vignais et al. [6] concluded from the difference in kinetics of phosphorylation of extra- and intramitochondrial ADP that the two processes occur independently of one another. Recently, they have reported [7] that under the particular conditions used, 20–25 % of the phosphorylation of extramitochondrial ADP proceeded without mixing of the ADP or ATP with the internal pool. Our results and those of Vignais et al. were explained by assuming a functional interaction of the adenine nucleotide translocator and the mitochondrial ATPase (F_1) (see also [8]).

In this paper, experiments are reported concerning the behaviour of the intramitochondrial adenine nucleotides during the uncoupler-induced hydrolysis of extramitochondrial ATP. Assuming that the transport of adenine nucleotides is rate-limiting and that the adenine nucleotide translocator does not differentiate between ADP_i and ATP_i, it is possible to calculate the formation of [^{14}C]ADP outside the mitochondria after the addition of [^{14}C]ATP during the steady state, if the rate of the adenine nucleotide transport and the ATP/ADP ratio inside the mitochondria are measured. The [^{14}C]ADP_o formation calculated is compared with the experimentally measured [^{14}C]ADP_o formation. We have compared the effects of two different uncouplers, a very effective one, CCCP, and the much less effective uncoupler, 2,4-dinitrophenol, since it was shown earlier that the characteristics of the CCCP-induced ATP hydrolysis are different from those of the dinitrophenol-induced reaction [9, 10]. Some of the results have been briefly reported [11].

METHODS

Rat liver mitochondria were prepared by the method of Hoogeboom, as described by Myers and Slater [12]. Protein was measured by the biuret method as described by Cleland and Slater [13].

The adenine nucleotide transport was measured in so-called forward exchange reactions, i.e. the uptake by the mitochondria of added radioactive nucleotide was measured. The reaction was terminated by the atractyloside stop-rapid filtration method, as described by Souverijn et al. [14], and the rate of transport calculated also according to these workers. The solutions of radioactively labelled ATP were treated with phosphoenolpyruvate, pyruvate kinase (EC 2.7.1.40) and Mg^{2+} in order to eliminate most of the ADP [14]. The [^{14}C]ADP_o was corrected for the [^{14}C]ADP left after this treatment (1 % of the [^{14}C]ATP). The adenine nucleotide transport and the formation of [^{14}C]ADP_o were measured at 0 °C in a cold room (0–2 °C). The standard incubation medium contained 100 mM sucrose, 50 mM triethanolamine/HCl buffer, 15 mM KCl and 4 mM EDTA, pH 7.4.

The formation of [^{14}C]ADP outside the mitochondria was measured in the

following way: samples (0.25 ml) of the incubation mixture were added at appropriate time intervals to the standard reaction medium (0.11 ml) containing 1.25 mM atractyloside. After terminating the reaction, the mitochondria were immediately separated by centrifugation for 1.5 min at maximal speed in an Eppendorf microfuge and the supernatant was decanted. ADP, ATP and AMP in the supernatant were separated by thin layer chromatography on polyethyleneimine cellulose plates (Baker Chem. Co.) using 0.85 M LiCl as eluant [15]. After separation, the spots were detected with the aid of ultraviolet light (254 nm) and the radioactivity was measured.

The hydrolysis of intramitochondrial ATP was measured as follows. Procedure 1 : mitochondria were added to a medium containing 15 mM KCl, 1 mM EDTA, 1 mM potassium phosphate, 125 mM sucrose and 50 mM Tris · HCl buffer (pH 7.4). The mixture was aerated for 2 min at 25 °C, brought into a reaction vessel thermostatted at the indicated temperature, and aeration was continued for another 2 min for temperature equilibrium. The reaction was started by addition of 1 mM KCN and the indicated amount of uncoupler. The reaction was terminated after 1 to 20 s by the addition of 4.5 % $HClO_4$ (final concentration). Each reaction rate was based on the measurement after two different time intervals to check that the initial rate was measured. After removal of the protein the samples were neutralized with KOH containing 5 mM Tris. The $KClO_4$ was removed and ATP was measured according to Bergmeyer [16]. The amount of ATP at zero time was measured by addition of $HClO_4$ first and KCN and uncoupler afterwards. Procedure 2 : ATP hydrolysis was measured according to the method described by Heldt and Klingenberg [22]. Mitochondria were incubated in a medium containing 50 mM KCl, 50 mM sucrose, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM α-oxoglutarate, 50 mM Tris · HCl buffer (pH 7.6) and 1 mM $^{32}P_i$ ($10 \cdot 10^6$–$20 \cdot 10^6$ dpm) at the indicated temperature. After 30 min incubation at 0 °C or 15 min at 15 °C rotenone (1–1.5 μg/mg protein) was added and the reaction was started by addition of uncoupler. The reaction was terminated by addition of 5 % trichloroacetic acid (final concentration), after which 0.5 mM ATP was added to minimize loss of ^{32}P from the [^{32}P]ATP. Protein was removed and the total amount of ^{32}P was measured. The amount of ^{32}P in organic phosphate esters was measured after extraction of inorganic phosphate [23].

Radioactivity of ^{32}P-labelled compounds was measured in dried samples with a Nuclear Chicago gas-flow counter. Radioactivity of ^{14}C-labelled compounds was determined with a Nuclear Chicago liquid scintillation counter, type Mark I. A mixture of toluene and alcohol (19 : 6, v/v) containing 4 g 2,5-diphenyloxazole and 50 mg 1,4-bis-(5-phenyloxazolyl-2-)benzene per l was used as scintillation liquid.

Intramitochondrial adenine nucleotides were determined after centrifugation of mitochondria through silicone oil into 15 % $HClO_4$. After neutralization, the adenine nucleotides were determined as described by Bergmeyer [16]. Corrections were made for the adenine nucleotides in the sucrose space.

Assuming that the adenine nucleotide transport is rate-limiting for the hydrolysis of extramitochondrial ATP [3] and that ADP and ATP are transported from the inside to the outside with the same kinetic parameters [17, 18], the appearance of [^{14}C]ADP outside, after the addition of [^{14}C]ATP during the steady-state uncoupler-induced ATP hydrolysis, is given by the equation:

$$\frac{d[^{14}C]ADP_o}{dt} = v \cdot \frac{ADP_i}{ADP_i + ATP_i} \cdot \frac{[^{14}C]ADP_i}{ADP_i} \qquad (1)$$

Substitution of $\frac{[^{14}C]ADP_i}{ADP_i} = \frac{I^*}{I} = \frac{I^*}{E+I}(1-e^{-kt})$

(see Souverijn et al. [14]) and integration yields:

$$[^{14}C]ADP_o = \frac{vT^*}{E+I}\left\{t - \frac{1}{k} + \frac{1}{k}e^{-kt}\right\}\frac{ADP_i}{ADP_i+ATP_i} \quad (2)$$

Substitution of I_t^* and of $I_{t=\infty}^*$ [14] in Eqn. 2 yields

$$[^{14}C]ADP_o = \frac{vT^*}{E+I}\left\{t - \frac{1}{k}\frac{I_t^*}{I_{t=\infty}^*}\right\}\frac{ADP_i}{ADP_i+ATP_i} \quad (3)$$

The abbreviations used in the equations are: v (in nmol · mg protein^{-1} · min^{-1}), rate of adenine nucleotide transport; E and I, the amounts in the medium and matrix, respectively, of the adenine nucleotides participating in the exchange reaction; I_t^* and E_t^*, the amount of radioactivity in the I and E compartment at time t; $T^* = I^*+E^*$; $k = v(E+I)/EI$ (see ref. 14).

By measuring the adenine nucleotide transport, k and $I_t^*/I_{t=\infty}^*$ can be determined (see [14]) and substituted in Eqn 3. When in addition the amounts of the intramitochondrial adenine nucleotides are measured, the complete time course of the appearance of $[^{14}C]ADP_o$ can be calculated.

According to this equation, the formation of $[^{14}C]ADP_o$ is constant only after the intramitochondrial pool of adenine nucleotides has reached a constant specific activity, when the rate of formation becomes equal to $v \cdot ADP_i/(ADP_i+ATP_i)$.

RESULTS

Figs. 1A and 1B show the adenine nucleotide exchange during the steady-state CCCP-induced hydrolysis of extramitochondrial ATP. $[^{14}C]ATP$ was added 1 min after initiating the ATP hydrolysis by the addition of CCCP, and it is this time that is referred to as zero time in the figures. From this experiment, v and k can be calculated (5.0 nmol · min^{-1} · mg^{-1} protein and 0.4 min^{-1}, respectively). In Table I, Expt. 1, a parallel incubation, the measured amounts of intramitochondrial ADP and ATP are given. The appearance of $[^{14}C]ADP_o$ was measured in the same incubation. In Fig. 2, the calculated formation of $[^{14}C]ADP_o$ (dashed line) and the measured $[^{14}C]ADP_o$ are shown. The calculated and the experimental curve agreed during the first 8 min, after which the hydrolysis of ATP becomes inhibited; this is not allowed for in the theoretical curve. A lag phase in the formation of $[^{14}C]ADP_o$, corresponding with the calculated lag phase, was clearly present, and the rate of the $[^{14}C]ADP_o$ formation also agreed with the calculated rate (see also Table I, Expt. 1). In the presence of atractyloside, a low rate of ATP hydrolysis was observed. In Fig. 3, the results of an experiment carried out in the presence of a high concentration of CCCP, sufficient to bring about maximal stimulation of the hydrolysis of intramitochondrial ATP [3], are shown. There is again a good correlation between the calculated and the experimentally measured curve, although the inhibition of the ATP hydrolysis occurred somewhat earlier than at the lower CCCP concentration.

The results so far confirm the hypothesis of Heldt et al. [1, 2] that the hy-

TABLE I

THE ADENINE NUCLEOTIDE TRANSPORT AND THE [^{14}C]ADP_o FORMATION DURING THE UNCOUPLER-INDUCED ATP HYDROLYSIS IN RAT-LIVER MITOCHONDRIA AT 0 °C

Expt. 1 is the same experiment as that shown in Figs. 1, 2 and 3. For details, see the legends of those figures. Expt. 2 is the same experiment as that shown in Figs. 4 and 5. For details see the legends of those figures. In Expt. 3, conditions were the same as in Expt. 2, protein 3.42 mg/ml; [^{14}C]ADP_o was measured during 10 min, $k = 0.693/2.6\ min^{-1}$. In Expt. 4, conditions were the same as in Expt. 2, protein 3.33 mg/ml, [^{14}C]-ADP_o was measured during 7 min, $k = 0.693/2.6\ min^{-1}$. The rates are given in nmol/min per mg protein.

Expt.	Additions	Rate of adenine nucleotide transport	Rate of [^{14}C]ADP_o formation*		ADP_i (nmol/mg protein)	ATP_i (nmol/mg protein)	$(ATP/ADP)_i$
			Measured	Calculated			
1	CCCP, 0.1 μM	5.0	3.8	3.8	9.9	2.3	0.23
	CCCP, 4 μM	5.0	3.8	3.8	7.2	1.7	0.24
	CCCP, 0.1 μM+ atractyloside, 1.25 mM	—	0.45				
2	2,4-Dinitrophenol, 0.15 mM	1.3	1.91	0.55	3.8	5.5	1.3
	2,4-Dinitrophenol, 0.15 mM, +atractyloside, 1.25 mM	—	0.73				
3	2,4-Dinitrophenol, 0.15 mM	2.5	2.63	1.79	8.5	3.4	0.4
4	2,4-Dinitrophenol, 0.15 mM	3.1	3.7	1.9	7.0	4.4	0.63
	2,4-Dinitrophenol, 0.15 mM, +atractyloside, 1.25 mM	—	0.73				

* The rates are the maximal rates reached when the intramitochondrial adenine nucleotides have a constant specific activity.

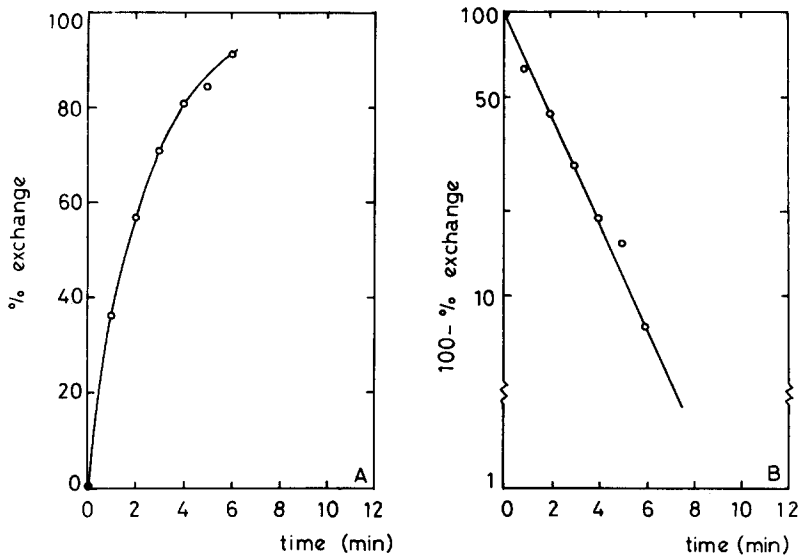

Fig. 1. ATP exchange during CCCP-induced ATP hydrolysis. (A) Mitochondria were added to the incubation medium, containing 1 mM ATP and rotenone (1.1 μg/mg protein) to a concentration of 1.8 mg/ml. After 3 min, 0.1 μM CCCP was added and 1 min later [^{14}C]ATP of high specific activity was added (final specific activity of [^{14}C]ATP, $1.36 \cdot 10^3$ cpm/nmol). The addition of [^{14}C]ATP is referred to as zero time in the figure. Samples were taken and treated as described under Methods. 100 % exchange is measured at $t = 27$ min and corresponds to $1.67 \cdot 10^4$ cpm/mg protein. (B) Semilogarithmic plot of the results shown in A.

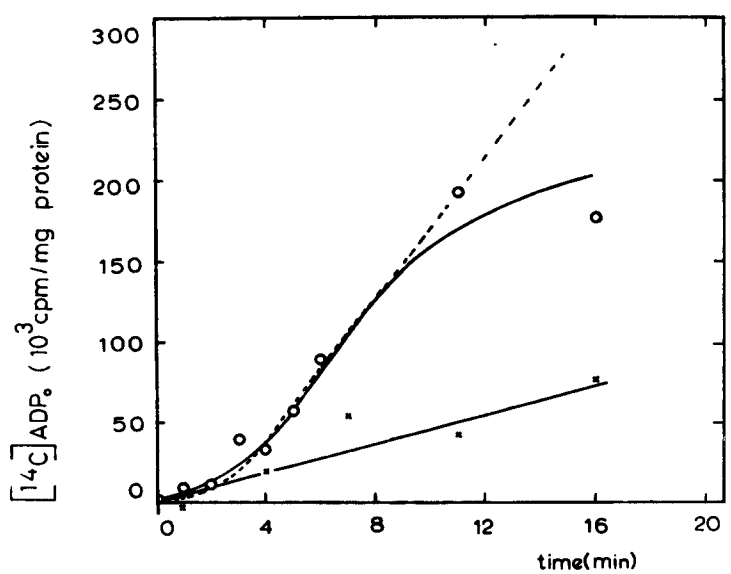

Fig. 2. [^{14}C]ADP_o formation during CCCP-induced ATP hydrolysis. Conditions were the same as in Fig. 1, except for the protein concentration which was 2.7 mg/ml, and the amount of [^{14}C]ATP (final specific activity of [^{14}C]ATP was $5.52 \cdot 10^3$ cpm/nmol). ○—○, measured [^{14}C]ADP_o formation; – – –, calculated [^{14}C]ADP_o formation; ×—×, [^{14}C]ADP_o formation in the presence of 1.25 mM atractyloside.

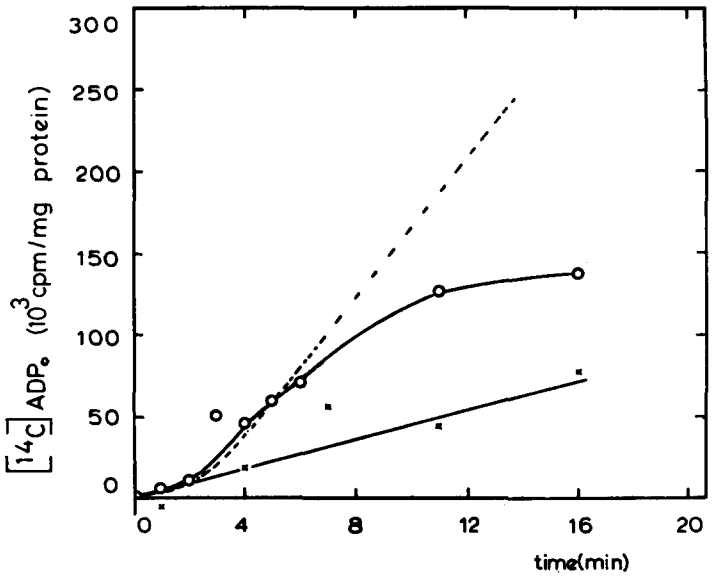

Fig. 3. [^{14}C]ADP$_o$ formation during CCCP-induced ATP hydrolysis, with a high concentration (4 μM) of CCCP. Conditions are the same as in Fig. 2, except for the concentration of CCCP. O—O, measured [^{14}C]ADP$_o$ formation; – – –, calculated [^{14}C]ADP$_o$ formation; ×—×, [^{14}C]-ADP$_o$ formation measured in the presence of 1.25 mM atractyloside.

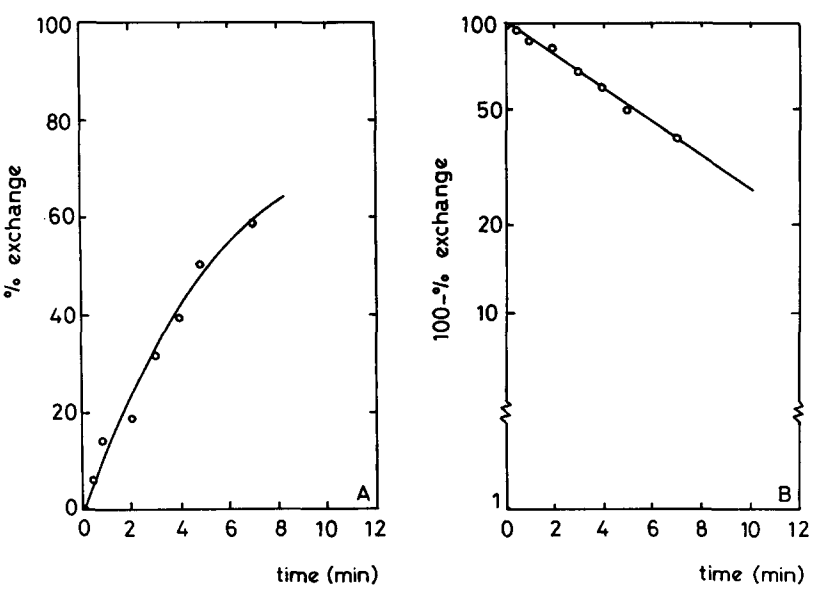

Fig. 4. ATP exchange during 2,4-dinitrophenol-induced ATP hydrolysis. (A) Mitochondria were added to the incubation medium containing 1 mM ATP and rotenone (0.74 μg/mg protein) to a concentration of 2.72 mg protein/ml. After 3 min, 150 μM 2,4-dinitrophenol was added and 1 min later [^{14}C]ATP of very high specific activity was added (final specific activity of [^{14}C]ATP was $1.46 \cdot 10^3$ cpm/nmol). The addition of [^{14}C]ATP is referred to as zero time in the figure. 100 % exchange is measured at $t = 25$ min and corresponds to $1.44 \cdot 10^4$ cpm/mg protein. (B) Semilogarithmic plot of data given in A.

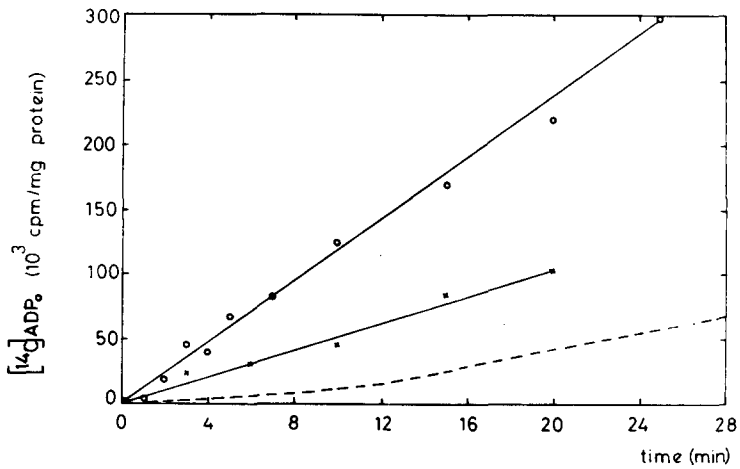

Fig. 5. [^{14}C]ADP_o formation during 2,4-dinitrophenol-induced ATP hydrolysis. The conditions are the same as in Fig. 4, except for the amount of [^{14}C]ATP (final specific activity of [^{14}C]ATP was $6.65 \cdot 10^3$ cpm/nmol). ○—○, measured [^{14}C]ADP_o formation; – – –, calculated [^{14}C]ADP_o formation; ×—×, [^{14}C]ADP_o formation in the presence of 1.25 mM atractyloside.

drolysis of extramitochondrial ATP proceeds via the intramitochondrial pool of adenine nucleotides.

The experiments in the presence of 2,4-dinitrophenol were performed in the same way as those in the presence of CCCP. The ATP_i/ADP_i ratio was found to be higher in the presence of 2,4-dinitrophenol than in the presence of CCCP (see Table I), and this high ratio was almost constant for at least 5 min. In Fig. 4, the adenine nucleotide exchange in the presence of 2,4-dinitrophenol is shown. The theoretical curve describing the formation of [^{14}C]ADP_o, calculated from the data of Fig. 4 and the amount of nucleotides, is shown in Fig. 5, together with the actual [^{14}C]ADP_o measured. The rate of [^{14}C]ADP_o formation was constant for at least 24 min; it was higher than the maximal calculated rate (1.91 nmol/min per mg protein compared with 0.51 nmol/min per mg protein), and any lag phase, if present, was much shorter than would be expected from the theoretical curve. The [^{14}C]ADP_o formation was inhibited by atractyloside.

In Table I, the results of this experiment as well as those of additional experiments of the same type are summarized. Essentially the same results were found as those presented in Figs. 4 and 5. The rate of [^{14}C]ADP_o formation measured was always higher than the maximal calculated rate, and no lag phase was present. To exclude the possibility that the absence of a lag phase in the formation of [^{14}C]-ADP_o is caused by some isotopic exchange reaction (e.g. a reaction catalysed by adenylate kinase), we have added unlabelled AMP or unlabelled ADP to the complete reaction medium containing oligomycin, atractyloside and [^{14}C]ATP. No extra [^{14}C]ADP_o or [^{14}C]AMP_o was formed compared with the amounts formed in the absence of added ADP or AMP. Moreover the presence of a lag phase in the appearance of [^{14}C]ADP_o during the CCCP-induced ATP hydrolysis also indicates that disturbing isotopic exchange reactions do not occur under our experimental conditions.

The results obtained in the presence of 2,4-dinitrophenol cannot be explained by the assumption that the hydrolysis of extramitochondrial ATP proceeds via the pool of intramitochondrial adenine nucleotides. The absence of a lag phase in the appearance of [^{14}C]ADP$_o$ indicates that extramitochondrial ATP is transported inwards directly into the F_1 region, is hydrolysed and that the ADP formed is directly transported outwards, without mixing of ATP and ADP with the intramitochondrial adenine nucleotides.

A difference between results obtained in the presence of 2,4-dinitrophenol and those obtained in the presence of CCCP is in itself not surprising, since it is known that CCCP is a much more effective uncoupler than 2,4-dinitrophenol, even at saturating concentrations of uncoupler. The following experiments demonstrate the differences in the behaviour of the two types of uncouplers towards hydrolysis of intramitochondrial ATP and ATP exchange. In Fig. 6 the effects of 2,4-dinitrophenol and carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP) on the hydrolysis of intramitochondrial ATP at 0 °C are compared. In the presence of 5 μM FCCP, the rate of ATP hydrolysis was 180 nmcl/min · mg protein whereas in the presence of 65 μM 2,4-dinitrophenol, that rate was only 4 nmol/min · mg protein (see also [3] and [24]). Table II shows results concerning the hydrolysis of intramitochondrial ATP in the presence of 2,4-dinitrophenol, CCCP and FCCP at various temperatures. In the presence of 2,4-dinitrophenol, the rate of ATP hydrolysis was considerably lower than that in the presence of CCCP or FCCP (5, 85 and 130 nmol/min · mg protein, respectively, at 0 °C), the difference becoming less the higher the temperature. Even at 15 °C, however, there is a large difference between the maximal rates of ATP hydrolysis in the presence of the different types of uncouplers.

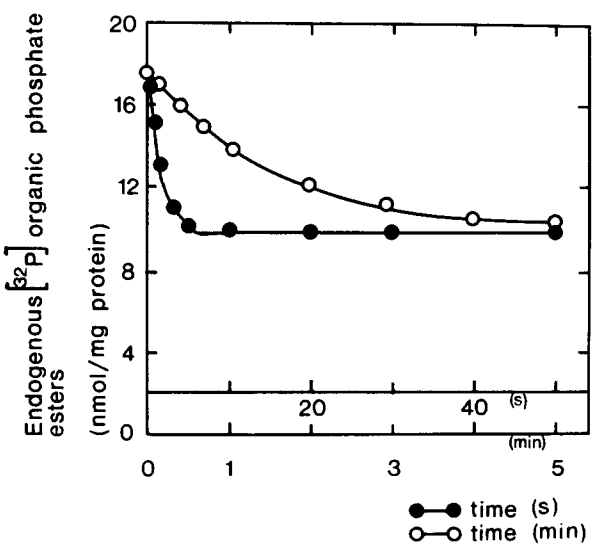

Fig. 6. The effects of 2,4-dinitrophenol and FCCP on the hydrolysis of intramitochondrial ATP in rat-liver mitochondria. The experiment was performed at 0 °C according to procedure 2 as described in the methods section. ○—○, 1.5 mg protein/ml, 1.1 μg rotenone/mg protein and 65 μM 2,4-dinitrophenol; ●—●, 2.0 mg protein/ml, 1.0 μg rotenone/mg protein and 5 μM FCCP. The specific activity of [^{32}P]phosphate was 134 cpm/nmol. The experiment was done by J. Schrijver.

TABLE II

THE HYDROLYSIS OF INTRAMITOCHONDRIAL ATP IN THE PRESENCE OF VARIOUS UNCOUPLERS

(A) The experiments were performed according to procedure 1 as described in the methods section. Protein concentrations (rat-liver mitochondria) varied between 3.1 and 6.4 mg/ml. The experiments were done by C. Heijting. (B) The experiments were performed at 15 °C according to procedure 2, as described in the Methods. Protein concentrations (rat liver mitochondria) varied between 1.6 and 1.7 mg/ml. The experiments were done by J. Schrijver.

Uncoupler	Concentration (μM)	A				B	
		Rate of ATP hydrolysis (nmol/min per mg protein)				V (nmol/min per mg protein)	$K_{m_{obs}}$** (μM)
		0 °C	5 °C	10 °C	15 °C		
Dinitrophenol	100	5	15	32	65	150	8.5
FCCP*	5	130	145	210	230	approx. 350	0.5
CCCP	5	85	140	220	260	approx. 350	0.7

* FCCP, carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone

** $K_{m_{obs}}$, concentration of uncoupler by which half maximal rate is induced.

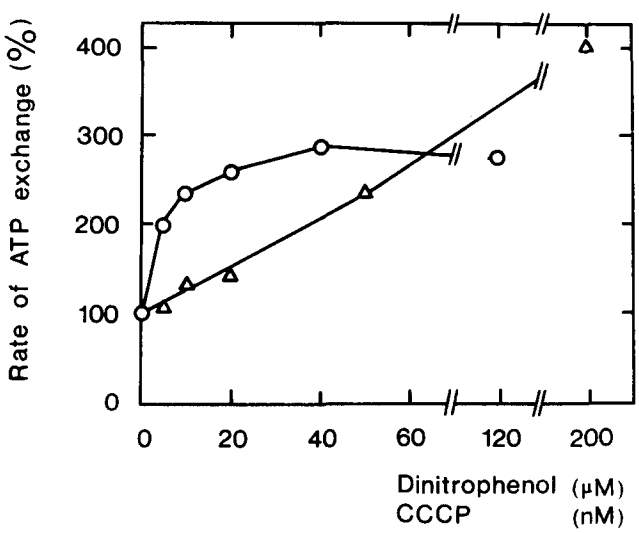

Fig. 7. The effect of 2,4-dinitrophenol and CCCP on ATP exchange. Rat-liver mitochondria (4.1 mg protein/ml) were added to the standard incubation medium, containing 0.49 μg rotenone/mg protein and 1 mM ATP. Uncoupler was added after 0.5 min, and after 1 min the reaction was started by adding [^{14}C]ATP of high specific activity. Samples were taken after 1 and 2.25 min. At the higher uncoupler concentrations, samples were also taken after an additional 20 min, in order to measure the incorporated radioactivity at isotopic equilibrium. Temperature, 0 °C, final vol. 0.8 ml. 100 % represents a rate of 0.95 nmol/min · mg protein. ○—○, 2,4-dinitrophenol; △—△, CCCP.

Moreover, the concentration of 2,4-dinitrophenol that induced half maximal stimulation of ATP hydrolysis was about 10 times higher than that of CCCP or FCCP. A similar difference between 2,4-dinitrophenol and CCCP was observed when the effects of those uncouplers on ATP exchange in the absence of oligomycin was studied. Fig. 7 shows that under conditions that are similar to those of the experiments reported in Figs. 1–5 the stimulation of ATP exchange by 2,4-dinitrophenol is much less than that by CCCP. Also, in this case a much higher concentration of 2,4-dinitrophenol than of CCCP is needed to induce half maximal stimulation (cf. results in Table I). If the stimulation of ATP exchange by uncouplers is taken as a measure for the deenergization of mitochondria it can be concluded that the deenergization by 2,4-dinitrophenol under the experimental conditions is much less than that by CCCP.

DISCUSSION

The equation used to calculate the formation of $[^{14}C]ADP_o$ leads to two criteria to test whether the intramitochondrial pool participates in the dephosphorylating reaction of extramitochondrial ATP: (1) a lag phase in the formation of $[^{14}C]ADP_o$ should be observed corresponding with the time needed for maximal radioactive labelling of the matrix pool; (2) after the lag phase, the rate of $[^{14}C]ADP_o$ formation should equal $v \cdot ADP_i/(ADP_i+ATP_i)$. A similar equation was used for the calculation of the appearance of $[^{14}C]ATP_o$ during the State-3 phosphorylation, after the addition of $[^{14}C]ADP_o$ [4, 5].

The presence of a lag phase in the appearance of $[^{14}C]ADP_o$ after the addition of $[^{14}C]ATP_o$ during the steady-state CCCP-induced hydrolysis of extramitochondrial ATP demonstrates that under these conditions the extramitochondrial adenine nucleotides equilibrate with the intramitochondrial adenine nucleotides before they react with the mitochondrial ATPase. Moreover, there is also a good correlation between the measured and the calculated rates. The experiments performed in the presence of CCCP therefore support the hypothesis of Heldt et al. [1, 2] that the hydrolysis of extramitochondrial ATP proceeds via the intramitochondrial adenine nucleotides.

The observed results in the presence of 2,4-dinitrophenol, however, cannot be explained by the assumption that the reaction proceeds via the matrix pool of adenine nucleotides. The results indicate that there is a functional interaction between the adenine nucleotide translocator and the ATPase under those conditions.

With respect to the phosphorylation of ADP_o, it is shown in Fig. 8 that after addition of $[^{14}C]ADP_o$ during the steady-state State-3 respiration, $[^{14}C]ATP$ appears outside without the lag phase to be expected from a labelling of the matrix pool of adenine nucleotides, and at a rate much higher than that expected if the phosphorylation reaction proceeds via the matrix pool of adenine nucleotides. This result, which was already reported earlier [4, 5], was the basis for our conclusion that there is a functional interaction between the adenine nucleotide translocator and the mitochondrial ATPase when the mitochondria are energized.

The results concerning the effects of 2,4-dinitrophenol and CCCP on the hydrolysis of intramitochondrial ATP, as reported in this paper, show that 2,4-dinitrophenol does not effectively induce ATP hydrolysis. The difference between the

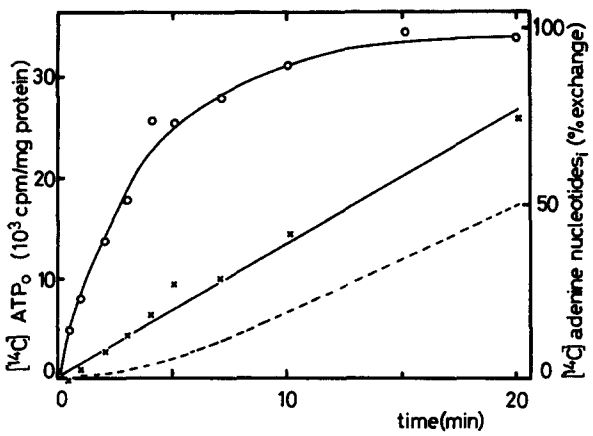

Fig. 8. Appearance of [^{14}C]ATP_o during State-3 phosphorylation. Mitochondria (3.15 mg protein/ml) were added to a medium containing 50 mM sucrose, 10 mM potassium phosphate, 12 mM succinate, 15 mM KCl, 5 mM EDTA, 50 mM triethanolamine/HCl buffer, 1.07 mM ADP and rotenone (0.32 μg/mg protein), pH 7.2. After 1 min, [^{14}C]ADP of high specific activity was added (final spec. act. of [^{14}C]ADP was 330 cpm/nmol). Final vol. 3.0 ml. Temperature, 0 °C. The reaction was terminated as described for the ATP hydrolysis experiments. [^{14}C]adenine nucleotides were separated as described in the methods. $\times-\times$, [^{14}C]ATP_o; $\circ-\circ$, [^{14}C]adenine nucleotides$_i$; – – –, calculated [^{14}C]ATP_o (the calculation was based on the exchange data and on an assumed value for $ATP_i/(ADP_i+ATP_i) = 1$). Adenine nucleotide transport was measured as described in the Methods, in a parallel experiment. The conditions were the same as described above except for the specific activity of [^{14}C]ADP (480 cpm/nmol) and the final volume (1.5 ml). 100 % exchange corresponds to 6950 cpm/mg protein. The experiment was performed in cooperation with R. M. Bertina.

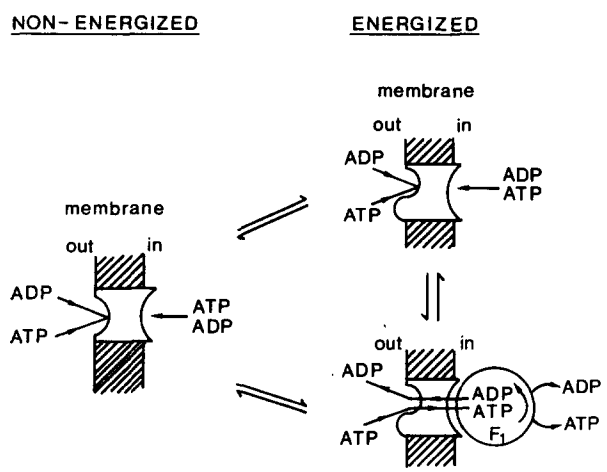

Fig. 9. Schematic representation of the functional interaction of the adenine nucleotide translocator and the mitochondrial ATPase during the uncoupler-induced ATP hydrolysis.

effects of 2,4-dinitrophenol and CCCP on ATP exchange in the absence of oligomycin indicates that 2,4-dinitrophenol, although inducing ATP hydrolysis, causes de-energization of the mitochondria only to a minor extent. With respect to this it should be mentioned that it was shown by Verdouw and Bertina [9] that during 2,4-dinitrophenol-induced hydrolysis of extramitochondrial ATP, a high K_m of ATP for the adenine nucleotide translocator was observed at high ATP concentrations. Since it was shown by Souverijn et al. [14] that the K_m of ATP for the translocator is 150 μM in energized mitochondria and 1 μM in non-energized mitochondria, Verdouw and Bertina's observations also indicate that the adenine nucleotide translocator occurs in some kind of energized state during 2,4-dinitrophenol-induced hydrolysis of extramitochondrial ATP. Thus, our results concerning the appearance of $[^{14}C]ADP_o$ after addition of $[^{14}C]ATP_o$ during 2,4-dinitrophenol-induced hydrolysis of extramitochondrial ATP are consistent with the hypothesis that there is a functional interaction between the translocator and the ATPase when the mitochondria are in the energized state [5, 20].

From the results presented in this paper and from earlier reported experiments [4, 5], we conclude that the dephosphorylation of extramitochondrial ATP as well as the phosphorylation of extramitochondrial ADP can be catalysed by a functional complex of the adenine nucleotide translocator and the mitochondrial ATPase, in such a way that the intramitochondrial adenine nucleotides do not participate in the reactions. In the non-energized state, the interaction is lost and the reactions proceed via the matrix pool. A schematic picture of the interaction is given in Fig. 9. In the energized state, some of the translocator molecules become functionally linked with F_1, thereby acting as a kind of binding protein for extramitochondrial ADP and ATP. To account for the radioactive labelling of the matrix pool that occurs at the same time, we assume that there are also free adenine nucleotide translocator molecules. A similar interaction has been proposed by Bertagnolli and Hansen [8] and by Vignais et al. [7]. It is not clear to us which particular exchange reactions (ATP against ADP or ATP, ADP against ATP or ADP) are responsible for the labelling of the intramitochondrial adenine nucleotides. With respect to the scheme, it is interesting that the rate of transport towards the intramitochondrial pool is higher in the presence of CCCP, when there is no functional linkage between F_1 and translocator, than in the presence of 2,4-dinitrophenol, when there is a link. The rate is also increased when CCCP is added in State 3 [5, 20].

The functional interaction between the adenine nucleotide translocator and the mitochondrial ATPase supports the idea that the enzymes involved in oxidative phosphorylation occur in separate complexes and that the intermediates between the different enzymes of such a complex do not equilibrate with those of the other complexes [4, 21].

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REFERENCES

1 Heldt, H. W., Jacobs, H. and Klingenberg, M. (1965) Biochem. Biophys. Res. Commun. 18, 174–179
2 Heldt, H. W. (1966) in Regulation of Metabolic Processes in Mitochondria (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds.), BBA Library, Vol. 7, pp. 51–64, Elsevier, Amsterdam
3 Heldt, H. W. and Klingenberg, M. (1968) Eur. J. Biochem. 4, 1–8
4 Bertina, R. M. and Out, T. A. (1973) Abstr. 9th Intern. Congr. Biochem., Stockholm, Aktiebolaget Egnellska, Boktryckeriet, Stockholm, p. 236
5 Kemp, Jr., A. and Out, T. A. (1975) Proc. Koninkl. Ned. Akad. Wetensch., Series C, 78, 143–166
6 Duée, E. D. and Vignais, P. V. (1969) J. Biol. Chem. 244, 3932–3940
7 Vignais, P. V., Vignais, P. M. and Doussiere, J. (1975) Biochim. Biophys. Acta 376, 219–230
8 Bertagnolli, B. L. and Hanson, J. B. (1973) Plant Physiol. 52, 431–435
9 Verdouw, H. and Bertina, R. M. (1973) Biochim. Biophys. Acta 325, 385–396
10 Bertina, R. M. and Slater, E. C. (1975) Biochim. Biophys. Acta 376, 492–504
11 Out, T. A., Valeton, E. and Kemp, Jr., A. (1975) Abstr. 10th Meeting Fed. Europ. Biochem. Socs., Paris, Soc. de Chimie Biol., nr. 1158
12 Myers, D. K. and Slater, E. C. (1957) Biochem. J. 67, 558–572
13 Cleland, K. W. and Slater, E. C. (1953) Biochem. J. 53, 547–556
14 Souverijn, J. H. M., Huisman, L. A., Rosing, J. and Kemp, Jr., A. (1973) Biochim. Biophys. Acta 305, 185–198
15 Randerath, K. and Randerath, E. (1967) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.), Vol. 12, pp. 323–347, Academic Press, New York
16 Bergmeyer, H. U. (1970) Methoden der Enzymatischen Analyse, Band I and Band II, Verlag-Chemie, Weinheim/Bergstrasse
17 Pfaff, E. and Klingenberg, M. (1968) Eur. J. Biochem. 6, 66–79
18 Klingenberg, M. (1970) in Essays in Biochemistry (Campbell, P. N. and Dickens, F., eds.), Vol. 6, pp. 119–159, Academic Press, New York
19 Hopfer, U., Lehninger, A. L. and Thompson, T. E. (1968) Proc. Natl. Acad. Sci. U.S. 59, 484–490
20 Out, T. A. and Kemp, Jr., A. (1974) Biochem. Soc. Trans. 2, 516–517
21 Groot, G. S. P. (1969) Biochim. Biophys. Acta 180, 439–444
22 Heldt, H. W. and Klingenberg, M. (1968) Eur. J. Biochem. 4, 1–8
23 Nielsen, S. O. and Lehninger, A. L. (1955) J. Biol. Chem. 215, 555–570
24 Kemp, Jr., A. (1970) Biochem. J. 116, 10P