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AFFINITIES OF ATP FOR THE DINITROPHENOL-INDUCED ATPase

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SUMMARY

1. The effect of Mg^{2+} on ATP-dependent processes catalysed by intact rat-liver mitochondria can be explained quantitatively by the formation of Mg–ATP complexes that cannot act as a substrate for the adenine nucleotide translocator.

2. The dinitrophenol-induced ATPase is characterized by two affinities of ATP: $K_{m(1)} = 6.7\ \mu M$ and $K_{m(2)} = 63\ \mu M$, which contribute to the extent of 70 % and 30 %, respectively, to the total ATPase activity under the standard conditions employed.

3. $K_{m(1)}$ of ATP is competitively increased by atractyloside, and is insensitive to changes in cation concentration or to oligomycin or aurovertin.

4. $K_{m(2)}$ is as sensitive to atractyloside as the $K_{m(1)}$ and is also insensitive to oligomycin. However, it is increased by decreasing the cation concentration, and disappears in the presence of aurovertin.

5. It is proposed that two conformations of the adenine nucleotide translocator exist, characterized by their different affinities for ATP. The distribution of the enzyme over these two conformations appears to be a function of the energy state of the mitochondria (coupled or uncoupled).

INTRODUCTION

Since the discovery of the atractyloside-sensitive barrier in intact mitochondria for exogenous ATP and ADP[1–3], it has been suggested by several authors that the reaction catalysed by the adenine nucleotide translocator is rate-limiting for the uncoupler-induced ATPase[4–6]. The main arguments for such a proposal were obtained from studies that compared the uncoupler-induced hydrolysis of exogenous and endogenous ATP. In view of the fact, however, that the atractyloside-effect curve describing the inhibitory action of atractyloside on the hydrolysis of exogenous ATP in the presence of uncoupler is sigmoidal, Mitchell and Moyle[7] concluded that the hydrolysis is limited by the dephosphorylation reaction itself. If the rate of the uncoupler-induced ATP hydrolysis is limited by the transport of ATP across the mitochondrial inner membrane, one would expect that the K_m of ATP for the uncoupler-induced ATPase would be equal to that for the transport of ATP. For the latter

Abbreviation: PEP, phosphoenol pyruvate.

process K_m values have been reported between 2 5 and 10 μM[8-10] Although no accurate values for the K_m of ATP for the uncoupler-induced ATPase have been reported, all lie 20–100 times higher than that reported for ATP transport[11-13].

It has long been known that Mg^{2+} can inhibit ATP-dependent processes Myers and Slater[14] suggested that the inhibitory action of added Mg^{2+} on the dinitrophenol-induced ATPase may be due to the complex formation of the substrate ATP with Mg^{2+}. The inhibitory effect of high concentrations of Mg^{2+} on the ATP-driven reversal of electron transport from succinate to NAD^+ has been explained similarly[15-17] Pfaff and Klingenberg[18] observed a clear increase in the $K_{m\,obs}$ of ATP for ATP transport in the presence of excess Mg^{2+}, and suggested as explanation the formation of ATP–Mg complexes that cannot enter the mitochondria *via* the adenine nucleotide translocator. Recently, Slater *et al* [19] suggested that the sharpness of the State 3 → State 4 transition in the presence of ATP and Mg^{2+} is due to the presence of Mg–ATP complexes that cannot compete with ADP for the translocation reaction.

In the experiments described in this paper, use has been made of the complex formation of ATP with Mg^{2+} to determine the K_m of ATP for the dinitrophenol-induced ATPase The choice of dinitrophenol as uncoupler was made on basis of the observation that the rate of the dinitrophenol-induced ATPase (nmoles/min per mg protein) is independent of the protein concentration (below 1 mg/ml) at a fixed dinitrophenol concentration, which is not the case for more efficient uncouplers such as the substituted phenylhydrazones

Calculation of concentration of free ATP

Unless indicated otherwise, the ATPase experiments were carried out at pH 7 15, *I* 0 08 and 25 °C. For the calculation of the free ATP concentration (*i e.* ATP^{4-} + $ATPH^{3-}$) the ionization and complex formation constants listed in Table I were used. For most equilibria the p*K* values for zero ionic strength were converted to

TABLE I

VALUES FOR THE IONIZATION AND COMPLEX-FORMATION CONSTANTS OF ATP, ADP AND P_i

Ref	*Equilibrium*	*Equilibrium constant*	*pK*				
			25 °C				*0 °C*
			I 0 00	0 04	0 05	0 08	0 08
20	$HATP^{3-} \rightleftharpoons ATP^{4-} + H^+$	K_{ATP}	7 68	7 16	7.13	7 06	6 95
20	$MgATP^{2-} \rightleftharpoons ATP^{4-} + Mg^{2+}$	K_{MgATP}	5 83	4 77	4.71	4.59	4.25
20	$MgHATP^{1-} \rightleftharpoons HATP^{3-} + Mg^{2+}$	K_{MgATPH}	3 60	2 84	2 80	2.73	2 58
20	$HADP^{2-} \rightleftharpoons ADP^{3-} + H^+$	K_{ADP}	7 20			6 78	6 69
20	$MgHADP \rightleftharpoons HADP^{2-} + Mg^{2+}$	K_{MgHADP}	2 58			1 92	1 86
20	$MgADP^{1-} \rightleftharpoons ADP^{3-} + Mg^{2+}$	K_{MgADP}	4 20			3 41	3 12
20	$H_2PO_4^{1-} \rightleftharpoons HPO_4^{2-} + H^+$	$K_{H_2PO_4}$	7 18			6.90	
			I	*Temp.* (°C)		*pK*	
21	$KATP^{3-} \rightleftharpoons ATP^{4-} + K^+$	K_{KATP}	0 20	25		1 06	
23	$MgHPO_4 \rightleftharpoons HPO_4^{2-} + Mg^{2+}$	K_{MgHPO_4}	0 20	25		1 88	

values for I 0.08, the ionic strength employed, on the basis of the experimentally established relations between pK and ionic strength[24]. In the kinetic calculations, it is assumed that neither the Mg–ATP nor the K–ATP complex is substrate for the dinitrophenol-induced ATPase. Since initial rates of ATP hydrolysis were measured, no allowance need be made for binding of Mg^{2+} to ADP and P_i. The ATP used contained less than 1 % ADP and P_i. No insoluble Mg–ATP complexes, that would disturb the calculations of the free ATP concentration, were formed

For the calculation of the free ADP and ATP concentrations at 0 °C, the ionization- and complex-formation constants were calculated for 0 °C with the aid of the van 't Hoff isochoor equation.

$$\log \frac{K(25°)}{K(0°)} = -\frac{\Delta H_0}{2.3\,R}\left(\frac{1}{298} - \frac{1}{273}\right)$$

The ΔH_0 values were derived from Phillips *et al*[20], assuming that these values are virtually constant between 25 and 0 °C

RESULTS

Affinity of free ATP for the 2,4-dinitrophenol-induced ATPase

The experiment summarized in Fig. 1A shows that a sharp increase in the rate of hydrolysis of ATP occurs when the amount of ATP added is greater than that of the magnesium (in excess of the EDTA). Two apparent affinities of ATP for the dinitrophenol-induced ATPase can be calculated: $K_{m(1)} = 420\ \mu M$ and $K_{m(2)} = 1320\ \mu M$ When instead of the total ATP concentration the calculated free ATP concentration is used as substrate concentration (see Fig. 1B), there is also a sharp discontinuity in the K_m values, with $K_{m(1)} = 11\ \mu M$ and $K_{m(2)} = 58\ \mu M$.

Fig. 2 shows the results of an experiment where no $MgCl_2$ was present in the reaction medium. After correction for K–ATP complex formation, only one K_m of

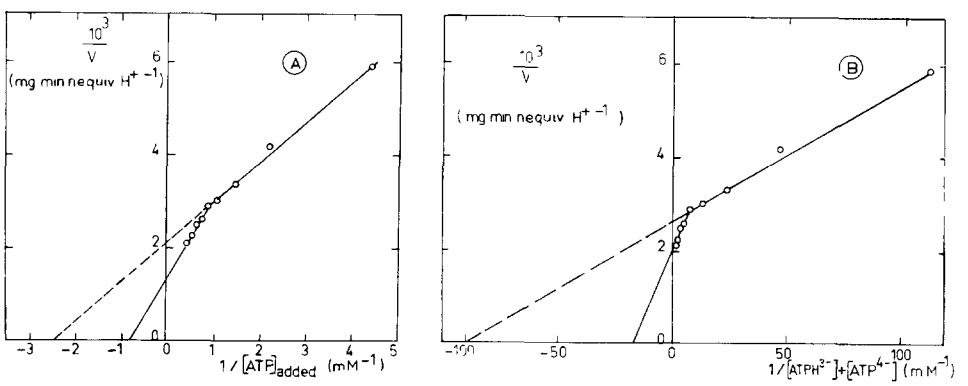

Fig 1 Effect of varying the ATP concentration on the dinitrophenol-induced ATPase in the presence of added Mg^{2+}. $MgCl_2$, 1.8 mM; EDTA, 0 5 mM, 2,4-dinitrophenol, 0 18 mM. Final volume, 1 10 ml, pH, 7 15; protein, 0 30 mg/ml. The results are plotted as a Lineweaver–Burk plot, with A, total ATP concentrations as substrate concentrations yielding $K_{m(1)} = 420\ \mu M$, $K_{m(2)} = 1320\ \mu M$; and B, calculated ($ATP^{4-} + ATPH^{3-}$) concentrations as substrate concentrations yielding $K_{m(1)} = 11\ \mu M$ and $K_{m(2)} = 58\ \mu M$

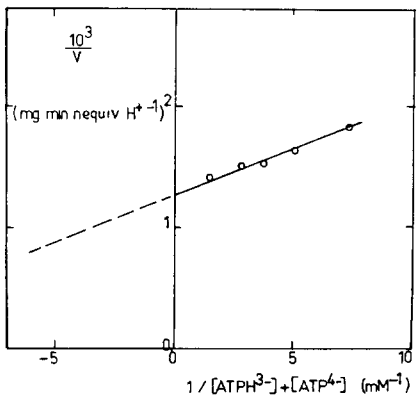

Fig 2 Effect of varying the ATP concentration on the dinitrophenol-induced ATPase in the absence of added $MgCl_2$. 2,4-Dinitrophenol, 0.18 mM. Final volume, 1.10 ml, pH, 7 15, protein, 0.09 mg/ml. The results are plotted as a Lineweaver–Burk plot with calculated ($ATPH^{3-} + ATP^{4-}$) concentrations as substrate concentrations. $K_{m(2)} = 54\ \mu M$

ATP is found (54 μM), but under these conditions it is for technical reasons not possible to obtain reliable initial rates at ATP concentrations below 100 μM. Thus, the presence of the lower K_m, seen at low free ATP concentrations in the presence of $MgCl_2$, cannot be excluded. Table II shows that $K_{m(2)}$ is largely independent of Mg^{2+} concentration up to about 3 mM.

If the assumption is correct that the magnesium and potassium complexes of ATP cannot act as a substrate for the dinitrophenol-induced ATPase, the K_m values obtained in experiments similar to that of Fig. 1A will be independent of the way by which the free ATP concentration is varied. Fig. 3A shows the inhibitory effect of increasing $MgCl_2$ concentrations upon the dinitrophenol-induced ATPase at fixed total ATP concentration. After calculating the free ATP concentration corresponding to each point in Fig. 3A, the Lineweaver–Burk plot of Fig. 3B was obtained. This

TABLE II

AFFINITIES OF ATP FOR THE DINITROPHENOL-INDUCED ATPase BEFORE AND AFTER CORRECTION FOR Mg- AND K-COMPLEXED ATP

Experimental conditions and calculations as described in Fig 1

$[MgCl_2]_{added}$ minus $[EDTA]_{added}$ (mM)	$K_{m(1)}$* (μM)	$K_{m(2)}$* (μM)	$K_{m(1)}$** (μM)	$K_{m(2)}$** (μM)
0	–	86	–	60
0	–	79	–	63
0	–	74	–	54
0 1	–	137	–	68
0 7	354	877	17	76
1.26	420	1320	11	58
2 95	235	1430	3	52

* Total ATP concentration used as substrate concentration

** Calculated free ATP concentration used as substrate concentration.

yields within the experimental error the same K_m values of ATP for the dinitrophenol-induced ATPase as were obtained in Fig 1B, where the concentration of ATP was varied at constant Mg^{2+} concentration, *viz.* $K_{m(1)} = 5.1\ \mu M$, $K_{m(2)} = 69\ \mu M$. Another way of varying the free ATP concentration is the simultaneous variation of

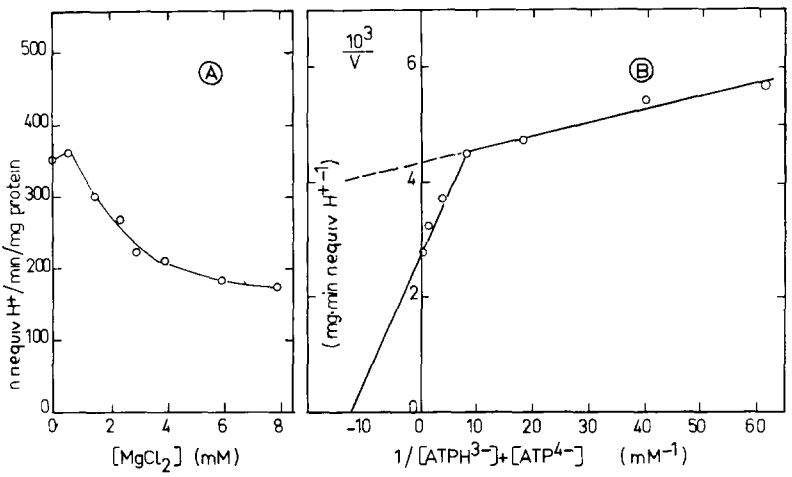

Fig 3 Effect of varying the $MgCl_2$ concentration on the dinitrophenol-induced ATPase. 2,4-Dinitrophenol, 0 22 mM; EDTA, 0 45 mM, ATP, 2 mM. Volume, 2 0 ml; pH, 7.15; protein, 0.40 mg/ml A, the ATPase activity is plotted as function of the added $MgCl_2$ concentration, B, for each added $MgCl_2$ concentration, the $ATPH^{3-}+ATP^{4-}$ concentration is calculated, and the results are plotted as a Lineweaver–Burk plot, yielding $K_{m(1)} = 5\ 1\ \mu M$, $K_m(_2) = 69\ \mu M$

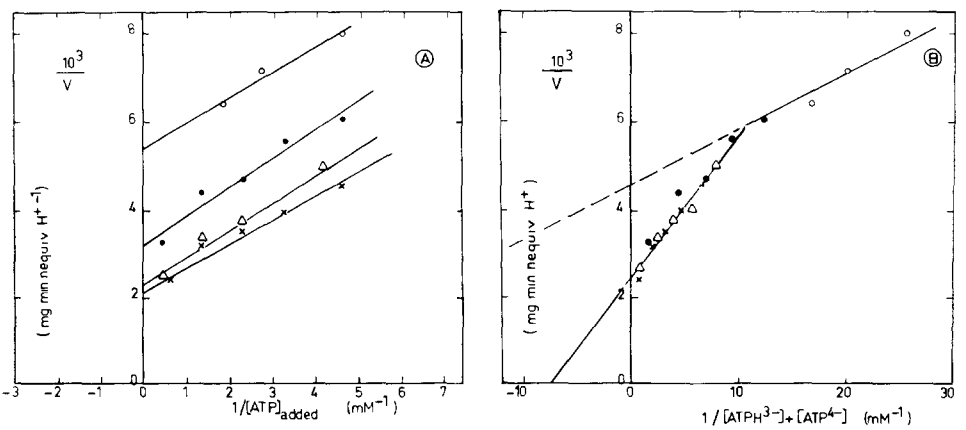

Fig. 4. Effect of varying both the $MgCl_2$ and ATP concentration on the dinitrophenol-induced ATPase The ATPase reaction was started by the addition of $MgCl_2$ and ATP in the ratios 0 1 (–), 1 : 1 6 (●–●), 1 · 4 8 (△–△) and 1 25 . 1 (○–○) The Mg–ATP solutions had the same pH as the reaction medium (pH 8.00±0 01). Final volume, 1.8 ml, 2,4-dinitrophenol, 0 18 mM, no EDTA was present, protein, 0.34 mg/ml. A, Lineweaver–Burk plot of the results, when the total ATP concentration is considered as the substrate concentration, B, Lineweaver–Burk plot of the results when the concentration of $ATP^{4-}+ATPH^{3-}$ is considered as the substrate concentration, yielding $K_{m(1)} = 29\ \mu M$ and $K_{m(2)} = 119\ \mu M$.

both the ATP and $MgCl_2$. Fig 4A shows the results of an experiment where the ATPase activity was measured at different concentrations of Mg–ATP (in the ratios 0 . 1, 1 : 1.6, 1.25 : 1 and 1 : 4.8). Separate lines are obtained for each Mg–ATP ratio, when the total ATP concentration is used in the Lineweaver–Burk plot When, however, each point is recalculated to give the free ATP concentration, the points at different Mg–ATP ratios fall together on two lines (see Fig. 4B), with a discontinuity at about 100 μM free ATP (*cf.* Figs 1B and 3B). The observation that both $K_{m(1)}$ and $K_{m(2)}$ of ATP for the dinitrophenol-induced ATPase are somewhat higher when compared with those obtained in the experiments shown in Figs 1 and 3 may be a consequence of the higher pH (8 00) employed in this experiment.

From a number of experiments, carried out as described in Fig. 1, the mean values of $K_{m(1)}$ and $K_{m(2)}$ were found to be 6.7 ± 1.5 μM and 63 ± 7 μM, respectively. It was found that the high-affinity system contributes 70 % of the overall rate of ATP hydrolysis at infinite ATP concentration. In 5 experiments where the ATPase activity was measured in the presence of an ATP-regenerating system, a mean value of $K_{m(1)}$ of 20 μM was obtained This value is inflated due to the fact that besides corrections for ATP–Mg and PEP–Mg, no correction for K–ATP complex formation was applied. Moreover, it can not be excluded that the presence of 2 5 mM PEP in the medium (mostly in the form of Mg–PEP) causes a slight increase in the K_m of ATP due to competition of PEP with ATP for ATP transport[25].

Effect of dinitrophenol and cation concentration

In disagreement with the report of Kraayenhof and Van Dam[12] that dinitrophenol competes with ATP for the dinitrophenol-induced ATPase, no systematic effect of varying the dinitrophenol concentration between 50 and 180 μM was found on either $K_{m(1)}$ or $K_{m(2)}$ A relatively high dinitrophenol concentrations (> 200 μM) a non-competitive inhibition of the ATPase reaction (up to 10–25 %) was observed, but the degree of inhibition varied considerably from preparation to preparation The ATPase activity at infinite ATP concentration also varied rather widely

In agreement with the finding that dinitrophenol does not affect the K_m for ATP, ATP was found to have no effect on the K_m for dinitrophenol

TABLE III

THE INFLUENCE OF THE KCl CONCENTRATION ON THE KINETIC PARAMETERS OF THE DINITROPHENOL-INDUCED ATPase

ATPase activity was measured in a medium containing 3.3 mM glycylglycine buffer, 200 mM sucrose, 0 5 mM EDTA and 3 mM $MgCl_2$. The reaction was started by the addition of 0 1 mM 2,4-dinitrophenol Initial rates of H^+ formation were measured. ATP and EDTA were neutralized with Tris The final K^+ concentration was measured by flame photometry The H^+/P_i ratios were calculated from experiments where the H^+ production and the amount of liberated P_i were measured simultaneously Final volume, 2 00 ml, pH, 7 10, protein, 0 65 mg. The values for $K_{m(1)}$, $K_{m(2)}$, V_1 and V_{1+2} presented were calculated from the Lineweaver–Burk plots, using least square analysis

H^+/P_i	KCl (mM)	I (M)	$K_{m(1)}$ATP (μM)	$K_{m(2)}$ATP (μM)	V_1 (nequiv H^+/min)	V_{1+2} (nequiv H^+/min)
0 60	0	0.04	5	1000	115	400
0.61	10	0 05	3	600	140	390
0 60	50	0 08	7 5	250	200	380

A number of authors have reported that the dinitrophenol-induced ATPase requires added cations[14,26-30]. Meisner[13], on the other hand, found that the transport of ATP at 0 °C is competitively inhibited by decreasing the KCl concentration. From results summarized in Table III it appears that V_{1+2} (rate at infinite ATP concentration) is independent of added KCl. This is in good agreement with the result of Meisner[13], when it is assumed that KCl affects the ATPase activity only *via* its action on ATP transport. $K_{m(1)}$ is independent of the cation concentration, whereas $K_{m(2)}$ is increased by decreasing the KCl concentration At the same time the contribution of the high-affinity system to V_{1+2} increases from about 25 % at zero KCl to 55 % at 50 mM KCl. Under the standard conditions (*cf* Fig. 1) where the K^+ concentration exceeds 50 mM, this ratio is 70 %.

Inhibitors of the 2,4-dinitrophenol-induced ATPase

Table IV summarizes some experiments in which the effect of atractyloside, oligomycin and aurovertin on the affinities of ATP for the dinitrophenol-induced ATPase were studied. Bruni *et al*[11] have already demonstrated that the inhibition of the dinitrophenol-induced ATPase by atractyloside is competitive with respect to the substrate ATP (however, they report a $K_{m\,obs}$ ATP of 1.6 mM). Table IV shows that both $K_{m(1)}$ and $K_{m(2)}$ are increased by atractyloside The inhibition was found to be almost completely competitive with a K_i for atractyloside of 0.1 μM

Suboptimal concentrations of oligomycin have no effect on either $K_{m(1)}$ or $K_{m(2)}$ of ATP, whereas the rate at infinite ATP concentration is decreased

In the presence of partially inhibitory concentrations of aurovertin only one K_m (15 μM) was found (see Table IV) This may be because the system defined by $K_{m(2)}$ of ATP is preferentially inhibited by aurovertin, so that at the aurovertin concentration used only the system defined by $K_{m(1)}$ can be measured. An alternative hypothesis would be that aurovertin effects an alteration in System 2 such that $K_{m(2)}$ becomes nearly equal to $K_{m(1)}$. The decrease in the K_m of ATP concomitant with a decrease in the maximal velocity provides a simple explanation for the earlier observation that aurovertin is a more effective inhibitor of ATPase activity at higher

TABLE IV

DEPENDENCE OF $K_{m(1)}$ AND $K_{m(2)}$ ON DIFFERENT INHIBITORS

Conditions as described in the Figs 1 and 2, unless indicated otherwise The effects of inhibitors on $K_{m(1)}$ of ATP were measured in the presence of $MgCl_2$, the effects on $K_{m(2)}$ in the absence of $MgCl_2$. pH, 7.10–7.15

Inhibitor	$K_{m(1)}$		$K_{m(2)}$	
	– Inhibitor	*+ Inhibitor*	*– Inhibitor*	*+ Inhibitor*
Atractyloside*	26	77	120	360
Oligomycin**	9.5	9.0	83	59
Aurovertin***	—	—	116	15.3

* 0.8 μM in the experiment for $K_{m(1)}$ (ATP-regenerating system) mixed competitive inhibition with K_i = 0.1 μM; 0.2 μM in the experiment for $K_{m(2)}$ (sensitive pH measurement) K_i = 0.1 μM

** 0.15 μg oligomycin/mg protein.

*** 2.7 μg aurovertin/mg protein, no correction for K–ATP complex formation was made.

(free) ATP concentrations[9]. However, for the moment it remains unexplained that the $K_{m(2)}$ of ATP is dependent both on atractyloside and aurovertin, the latter being a proven ligand of the mitochondrial ATPase[31,32], but not the former.

Effect of $MgCl_2$ on ATP transport and P_i–ATP exchange

To test if the addition of $MgCl_2$ indeed decreases the effective ATP concentration for adenine nucleotide transport, conditions were chosen so that the exchange against ATP at 0 °C would be inhibited by binding with Mg^{2+} by about 50 % while that against ADP would be hardly affected. The results of such an experiment (see Fig 5) show indeed that the rate of ATP exchange is greatly decreased by addition of $MgCl_2$ (free ATP concentration is 6 μM), while the rate of ADP exchange remains almost the same (free ADP concentration is 46 μM).

Since Groot[33] has reported that the P_i–ATP exchange reaction is hardly affected by the addition of $MgCl_2$, some experiments were done under conditions in which the addition of $MgCl_2$ would be expected to have an appreciable effect on the concentration of free ATP. Under the conditions used by Groot (6 mM ATP, 25 mM P_i) very large concentrations of $MgCl_2$ would be necessary. The experiment given in Fig. 6A shows that 50 % inhibition of the exchange activity was obtained when sufficient Mg^{2+} was added to lower the free ATP concentration from 1800 to 74 μM

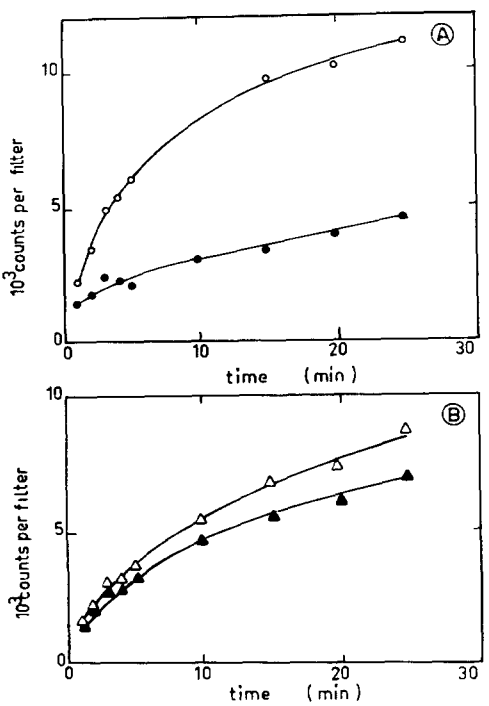

Fig 5 Effect of $MgCl_2$ on the exchange of ATP and ADP at 0 °C. The experiment was carried out in collaboration with J. H. M. Souverijn. Forward exchange was measured with the rapid filtration technique (*cf* ref. 43). Final pH, 7 40, Temp., 0 °C. ATP and ADP were ^{14}C labelled A, ○–○, 0.4 mM ATP; ●–●, 0 4 mM ATP + 5 mM $MgCl_2$ (ATP^{4-} + $ATPH^{3-}$ = 6 μM). B, △–△, 0 4 mM ADP; ▲–▲, 0.4 mM ADP + 5 mM $MgCl_2$ (ADP^{3-} + $ADPH^{2-}$ = 46 μM)

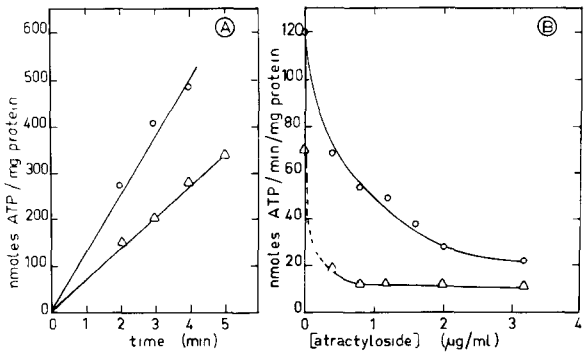

Fig 6 A Effect of $MgCl_2$ on the P_i–ATP exchange. Mitochondria were pre-incubated for 2 min in the complete reaction medium before the reaction was started with carrier-free $^{32}P_i$. ATP, 1 8 mM; EDTA, 0 5 mM; P_i, 5 mM pH, 7 15; final volume, 1.03 ml; protein, 0 36 mg/ml. ○–○, without additions, △–△, +5 mM $MgCl_2$, the concentration of $ATP^{4-}+ATPH^{3-}$ was 74 μM B Effect of atractyloside on the rate of P_i–ATP exchange in the presence or absence of added $MgCl_2$ Atractyloside was present before the mitochondria were added. Conditions as described in A.

That it is really a decrease in effective ATP concentration that is responsible for the decrease in the rate of P_i–ATP exchange is supported by the finding (Fig 6B) that atractyloside is a much more potent inhibitor in the presence of $MgCl_2$ than in its absence.

DISCUSSION

The interpretation of the experiments shown is consistent with the earlier proposal of Myers and Slater[14] that the Mg–ATP complexes cannot act as a substrate for the dinitrophenol-induced ATPase in intact rat-liver mitochondria When the added ATP concentrations are corrected for the presence of $MgATPH^-$, $MgATP^{2-}$ and $KATP^{3-}$, two affinities of free ATP for the dinitrophenol-induced ATPase are obtained· $K_{m(1)} = 7$ μM and $K_{m(2)} = 63$ μM. Moreover, complex formation of ADP and ATP with Mg^{2+}, resulting in lower concentrations of free ADP and ATP, can explain the effect of $MgCl_2$ in increasing the atractyloside sensitivity of various ADP- and ATP-dependent processes.

No conclusions may be drawn concerning which reaction is rate-limiting in the overall process of ATP hydrolysis. The reason for this is the complex nature of the multi-enzyme system catalysing the dinitrophenol-induced hydrolysis of ATP. In fact the nature of this system is such — regeneration of intermediates — that all enzymes involved will contribute to the final steady-state rate of ATP hydrolysis. The affinity of the substrate for a multi-enzyme catalysed reaction in general may deviate appreciably from that for the first enzyme, when examined separately. However, any deviations that do (or do not) occur may provide valuable information on the multi-enzyme character of the particular reaction studied

The value of $K_{m(1)}$ is in reasonable agreement with the values reported for the K_m of ATP for ATP transport at 0 °C[8-10,34]. The existence of $K_{m(2)}$ is rather surprising, since until now no low-affinity site for the transport of ATP has been reported. The observation that this low-affinity site is as sensitive to atractyloside as the high-

affinity site suggests that $K_{m(2)}$ of ATP is also associated with the adenine nucleotide translocator. The contribution of this second system to the total ATPase activity (at infinite ATP concentration) is about 30 % under standard conditions and 70 % at very low cation concentrations (*cf.* Table III).

The observation that $K_{m(1)}$ of ATP is independent of the cation concentration appears to be in contrast with the results of Meisner[13], who demonstrated a clear competitive increase in the K_m of ATP for ATP transport from 15 to 256 μM at decreasing KCl concentration (below 20 mM). His experiments, however, do not exclude that the high-affinity system is still present under those conditions. In fact in the range of ATP concentrations used by Meisner[13] only the low-affinity site of ATP would be operating. The apparent competitive character is completely in agreement with our finding that KCl does not affect the rate of the dinitrophenol-induced ATPase at infinite ATP concentration

It would be of interest to compare the relative contributions of the systems defined by $K_{m(1)}$ and $K_{m(2)}$ to the overall rate of different ATP-dependent processes At the moment there is evidence that the system defined by $K_{m(2)}$ is involved in P_i–ATP exchange (*cf.* Fig. 6A), ATP-induced high- and low-amplitude swelling (refs 35, 36) and the ATP-dependent reduction of NAD^+ (ref. 15). Although the range of free ATP concentrations used in these experiments does not allow any conclusion about the presence or absence of the high-affinity system, it can be concluded that at least for the P_i–ATP exchange and the ATP-induced low-amplitude swelling, the contribution of the high-affinity system to the overall rate is much less than in the case of the dinitrophenol-induced ATPase. This suggests that under relatively energized conditions the affinity of ATP for ATP-dependent reactions in intact rat-liver mitochondria is mainly determined by the low-affinity system ($K_{m(2)}$). This appears to be in good agreement with some recent results of Souverijn *et al.*[34] on the nature of the adenine nucleotide translocator. They found that under energized conditions (*i.e.* after pre-incubation with succinate and P_i) the K_m of ATP for ATP transport (0 °C) is 140 μM, while under de-energized conditions (*i e.* in the presence of uncoupler) this K_m is lower than 10 μM.

The existence of two affinities of ATP for the adenine nucleotide translocator (as revealed by direct transport measurements[34] and the study of ATP-dependent processes) suggests that two conformations of this translocator are present. Vignais *et al.*[37] came to the same conclusion on the basis of gummiferin-binding experiments. Their conclusion that atractyloside has the same affinity for both conformations is in good agreement with our observation that the K_i of atractyloside for both conformational states (defined by $K_{m(1)}$ and $K_{m(2)}$ of ATP) is essentially the same

METHODS AND MATERIALS

Rat-liver mitochondria were isolated according to the method of Hogeboom[38] as described by Myers and Slater[14]

Protein was determined by the biuret method as described by Cleland and Slater[39]

Inorganic phosphate was determined according to the method of Fiske and SubbaRow, as described by Sumner[40].

Mg and K concentrations were measured by flame spectrophotometry

The ATPase activity was measured by determination of H^+ formation with sensitive pH recording. Unless indicated otherwise, the medium contained 100 mM sucrose, 5 mM KCl, 0.5 mM EDTA and 10 mM Tris–HCl buffer at 25 °C. Mitochondria were preincubated for 2 min with ATP, before the ATPase reaction was started by adding uncoupler. Initial rates of H^+ formation were calculated after determination of the buffer capacity with standard oxalic acid. Extra additions to the ATPase system were made before the mitochondria were added (except oligomycin). This procedure is based upon that described by Nishimura *et al.*[41] for the sensitive measurement of P/O ratios Experimentally a $\Delta H^+/\Delta P_i$ of 0 61 was obtained at pH 7.15. This value was found to be independent of concentrations of dinitrophenol, protein, Mg^{2+} and K^+ Rates are reported in nequiv H^+/min per mg protein

In some experiments an ATP-regenerating system (with phosphoenolpyruvate and pyruvate kinase) was used. Since, however, Mg^{2+} is necessary for the activity of pyruvate kinase, high concentrations of ATP, that bind with the Mg^{2+}, cannot be used This method is, therefore, not suitable for detecting the second K_m (at higher ATP concentrations).

The P_i–ATP exchange reaction was measured at 25 C in 1 ml of a medium containing 100 mM sucrose, 25 mM Tris–HCl buffer, 1 μg rotenone/mg protein, and EDTA, P_i and ATP in concentrations mentioned in the legends. After 2 min pre-incubation of the mitochondria in the complete reaction medium, the exchange activity was started by the addition of carrier-free $^{32}P_i$ (Philips-Duphar). The reaction was stopped by addition of 1 0 ml 10 % (w/v) trichloroacetic acid and the incorporation of ^{32}P in the organic phosphate esters, present in the protein-free supernatant, was measured after extraction of the inorganic phosphate according to Nielson and Lehninger[42]. The amount of radioactivity in the aqueous phase was determined by counting a dried sample in a Nuclear-Chicago gas-flow counter

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