Biochem. J. (1977) **165**, 355–365
Printed in Great Britain

Kinetic Properties of a Magnesium Ion- and Calcium Ion-Stimulated Adenosine Triphosphatase from the Outer-Membrane Fraction of Rat Spleen Mitochondria

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(*Received* 6 *January* 1977)

1. Isolated outer membranes from rat spleen mitochondria can be stored in liquid N_2 for several weeks without significant loss of ATPase (adenosine triphosphatase) activity. 2. The ATPase reaction has a broad pH optimum centering on neutral pH, with little significant activity above pH9.0 or below pH5.5. 3. A sigmoidal response of the ATPase activity to temperature is observed between 0 and 55°C, with complete inactivation at 60°C. The Arrhenius plot shows that the activation energy above the transition temperature (22°C) ($E_a = 144$ kJ/mol) is one-third of that calculated for below the transition temperature ($E_a' = 408$ kJ/mol). 4. The outer-membrane ATPase (K_m for MgATP = 50 μM) is inactive unless Mg^{2+} is added, whereas the inner-membrane ATPase (K_m for ATP = 11 μM) is active without added Mg^{2+} unless the mitochondria have been depleted of all endogenous Mg^{2+} (by using ionophore A23187). 5. The substrate for the outer-membrane ATPase is a bivalent metal ion–nucleoside triphosphate complex in which Mg^{2+} ($K_m = 50\,\mu$M) can be replaced effectively by Ca^{2+} ($K_m = 6.7\,\mu$M) or Mn^{2+}, and ATP by ITP. Cu^{2+}, Co^{2+}, Sr^{2+}, Ba^{2+}, Ni^{2+}, Cd^{2+} and Zn^{2+} support very little ATP hydrolysis. 6. Univalent metal ions (Na^+, K^+, Rb^+, Cs^+ and NH_4^+, but not Li^+) stimulate the MgATPase activity (<10%) at low concentrations (50 mM), but, except for K^+, are slightly inhibitory (20–30%) at higher concentrations (500 mM). 7. The Mg^{2+}-stimulated ATPase activity is significantly inhibited by Cu^{2+} ($K_i = 90\,\mu$M), Ni^{2+} ($K_i = 510\,\mu$M), Zn^{2+} ($K_i = 680\,\mu$M) and Co^{2+} ($K_i = 1020\,\mu$M), but not by Mg^{2+}, Ca^{2+}, Ba^{2+} or Sr^{2+}. 8. The outer-membrane ATPase is insensitive to the inhibitors oligomycin, *NN'*-dicyclohexylcarbodiimide, NaN_3, ouabain and thiol-specific reagents. A significant inhibition is observed at high concentrations of $AgNO_3$ (0.5 mM) and NaF (10 mM). 9. The activity towards MgATP is competitively inhibited by the product MgADP ($K_i = 0.7$ mM) but not by the second product P_i or by 5'-AMP.

We have reported previously that rat spleen mitochondria, in common with rat kidney-cortex (Gmaj *et al.*, 1974) and heart mitochondria, have a highly active, but oligomycin-insensitive, Mg^{2+}-stimulated ATPase† activity associated exclusively with the outer-membrane fraction (Vijayakumar & Weidemann, 1976). This enzyme is absent from the outer membranes of rat liver mitochondria, which do, however, contain a bicarbonate-stimulated ATPase activity (Grisolia & Mendelson, 1974) that may be an enzyme of the same general class.

The oligomycin-sensitive ATPases and purified F_1 preparations from mammalian heart and liver mitochondria (Cooper & Lehninger, 1957) and the ATPases of chloroplasts (Vambutas & Racker, 1965) and bacteria (Adolfsen & Moudrianakis, 1973) that participate in oxidative phosphorylation share the common property of catalysing maximum ATP hydrolysis with Mg^{2+} as the sole added metal ion. These ATPases can be distinguished from the transport ATPases, at least in part, by virtue of differences in their metal-ion requirements and in their sensitivities to specific inhibitors. For instance, the sarcoplasmic-reticulum ATPase, which is inhibited by mersalyl (MacLennan, 1970), and the erythrocyte-membrane-bound ATPase, which has been implicated in Ca^{2+} transport (Walter & Hasselbach, 1973; Schartzmann & Rossi, 1971), both have an absolute requirement for Ca^{2+} as well as Mg^{2+} for optimum activity. Similarly, the $Na^+ + K^+$–dependent ATPase of the mammalian plasma membrane requires both Na^+ and K^+ in addition to Mg^{2+} to elicit its maximum activity (Glynn, 1956; Post *et al.*, 1960) and is specifically inhibited by ouabain.

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† Abbreviations: ATPase, adenosine triphosphatase; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

As a first step towards defining the distinctiveness and the possible functional significance of the spleen mitochondrial outer-membrane ATPase, we have studied the influence of metal ions and inhibitors on its kinetic properties both *in situ* and in isolated outer-membrane vesicles. The close resemblance between this enzyme and an ATPase observed in the outer-membrane fraction of rat kidney-cortex mitochondria by Gmaj *et al.* (1974), which is thought to be involved in Ca^{2+} transport, may eventually require a reconsideration of the selective permeability properties of the outer membranes of mitochondria that possess enzymes of this type.

Experimental

Materials

$[^{32}P]P_i$, digitonin, oligomycin, atractyloside and $CaCl_2$ were obtained from the sources described previously (Vijayakumar & Weidemann, 1976). Norit-A (activated charcoal), bovine serum albumin (fraction V), mersalyl {*O*-[(3-hydroxymercuri-2-methoxypropyl)carbamoyl]phenoxyacetic acid} and nitrilotriacetic acid (trisodium salt) were from Sigma Chemical Co., St. Louis, MO, U.S.A. *NN'*-Dicyclohexylcarbodi-imide was from Calbiochem, Carlingford, N.S.W., Australia. Ionophore A23187 was from Lilly Research Laboratories, Indianapolis, IN, U.S.A. $AgNO_3$ was from BDH Chemicals Ltd., Poole, Dorset, U.K. All other chemicals used were of analytical grade.

Methods

Isolation of rat spleen mitochondria. Mitochondria from rat spleen were isolated in iso-osmotic sucrose solution as described previously (Vijayakumar & Weidemann, 1976), except that the wash medium was free of bovine serum albumin to avoid later interference with mitochondrial protein estimation.

Preparation of spleen mitochondrial outer membrane. The outer-membrane fraction from rat spleen mitochondria was prepared as described previously (Vijayakumar & Weidemann, 1976) by using 2.0mg of digitonin/10mg of mitochondrial protein.

Analytical methods

Depletion of bivalent metal ions from rat spleen mitochondria by treatment with ionophore A23187. Intact rat spleen mitochondria (4.0mg) were depleted of endogenous bivalent metal ions by incubation in a medium containing sucrose (0.25M), Hepes buffer (20.0mM; pH adjusted to 7.4 with KOH), EDTA (2.0mM) and ionophore A23187 (0.5nmol/mg of mitochondrial protein) for 5min at 0°C, in a final volume of 2.0ml, essentially as described by Reed & Lardy (1972). At the end of the incubation period the solution was centrifuged at 8500*g* for 15min to sediment the mitochondrial pellet. The pellet was resuspended in 2.0ml of a solution containing sucrose (0.25M), bovine serum albumin (1%, w/v) and Hepes buffer (20.0mM; pH7.4).

Synthesis of $[\gamma\text{-}^{32}P]ATP$. $[\gamma\text{-}^{32}P]$ATP was synthesized as described previously (Vijayakumar & Weidemann, 1976).

Assay of ATPase activity. (*a*) With intact mitochondria. ATPase activity was assayed at 30°C in Eppendorf centrifuge tubes. The reaction medium contained sucrose (250mM), Hepes (25mM; pH adjusted to 7.4 with KOH), unlabelled ATP (2.0mM), $[\gamma\text{-}^{32}P]$ATP (1.2×10^5–1.5×10^5d.p.m.) and $MgCl_2$ (5.0mM) in a final volume of 0.5ml. The reaction was started by the addition of 80–100μg of mitochondrial protein. (*b*) With the isolated outer-membrane preparation. The reaction medium used was identical with that for (*a*) except that sucrose was replaced by water. The reaction was started by the addition of mitochondrial outer-membrane preparation (40–50μg of protein).

The reaction was stopped at different time intervals (usually every 10min unless shown otherwise in the legends) by adding ice-cold $HClO_4$ (100μl; 1.0M), and the tubes were placed on ice. The mixture was centrifuged (2min at 5000*g*) in an Eppendorf centrifuge to sediment the precipitated protein. The total radioactivity in a sample (100μl) of clear supernatant was determined. Activated charcoal (20mg) was added to the remaining portion of the reaction mixture and mixed for 20s with a Vortex-Genie mixer, to adsorb the unchanged labelled ATP. The mixture was centrifuged again (2min at 5000*g*) to sediment the charcoal. The radioactivity in an equivalent volume (100μl) of clear supernatant was determined, which now contained only $[^{32}P]P_i$. The labelled P_i liberated was always corrected for the zero-time blank value ($HClO_4$ was added to the reaction medium before the addition of enzyme protein). The ATPase activity was linear with time for more than 10min if the protein concentrations indicated above were used. Although the results obtained with this method were quite comparable with those obtained previously (Vijayakumar & Weidemann, 1976), the present method proved to be less time-consuming and equally reproducible.

The ^{32}P radioactivity was determined in a Beckman LS-350 liquid-scintillation counter as described before (Vijayakumar & Weidemann, 1976).

Measurement of protein. Mitochondrial protein was determined by the biuret method of Layne (1957), with bovine serum albumin as the protein standard.

Calculation of free metal ion concentrations. In the experiment described in Table 1, Mg^{2+}/EDTA (1.0mM) and Ca^{2+}/nitrilotriacetate (3.0mM) buffers

were used in the reaction medium to generate the free metal ion concentrations given by the computation method of Perrin & Sayce (1967). The following dissociation constants for $EDTAH_2$ ($K_1 = 10.25$ and $K_2 = 16.47$) and $nitriloacetateH_3$ ($K_1 = 1.89$; $K_2 = 2.49$ and $K_3 = 9.73$) and the following stability constants for Mg^{2+}/EDTA ($K_1 = 8.69$ and $K_2 = 10.97$) and Ca^{2+}/nitrilotriacetate ($K_4 = 6.46$) (Sillén & Martell, 1971) were used in making the calculations.

Results

Stability of the outer-membrane ATPase on storage

Isolated outer membranes from rat spleen mitochondria suspended in Hepes buffer (50mM; adjusted to pH7.4 with KOH) can be stored in liquid N_2 for several weeks without significant loss of the ATPase activity (Fig. 1). The stability of this preparation is quite comparable with that of the purified F_1 factor described by Horstman & Racker (1970). Repeated freezing and thawing caused little change in activity, in contrast with the increase in specific activity of the F_1 enzyme observed by Pullman *et al.* (1960). At higher storage temperature (e.g. −20°C) the enzyme was very unstable and lost its activity progressively at the rate of about 13–15% per day (Fig. 1).

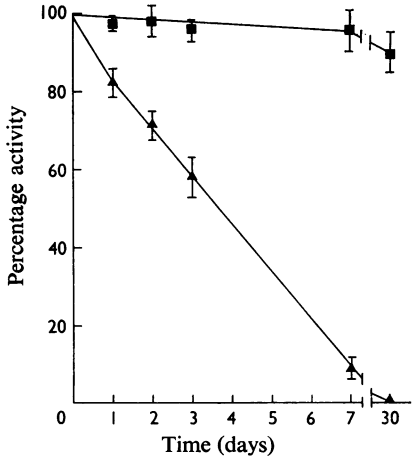

Fig. 1. *Stability of rat spleen mitochondrial outer-membrane ATPase during storage*

The mitochondrial outer-membrane preparation was stored either in liquid N_2 (■) or at −20°C (▲), and a small portion was removed after different storage times. The ATPase activity was assayed as described in the Experimental section, by using 40μg of membrane protein. The results represent means ±S.E.M. (bars) of triplicate determinations.

Dependence on pH and temperature

The effect of varying the pH on the hydrolysis of [γ-^{32}P]ATP by the outer-membrane ATPase is shown in Fig. 2. There was a broad activity maximum centring on neutral pH, with no measurable activity either above pH9.5 or below pH5.5, which may reflect denaturation of the enzyme rather than the influence of pH on the activity itself.

The activity of the enzyme responded sigmoidally to increasing temperature between 0 and 55°C (Fig. 3*a*). Complete inactivation was observed at 60°C, indicating that denaturation of the enzyme protein occurred between 55 and 60°C. A typical twofold increase in the activity occurred when the temperature was raised through 10°C from 22 to 32°C. An Arrhenius plot of the temperature-dependent rise in ATPase activity (Fig. 3*b*) shows a clear change in the slope at 22°C, which is consistent with a phase transition in the outer-membrane phospholipids at this temperature (Grisham & Barnett, 1973). A threefold decrease in the activation energy (E_a) of the reaction was observed at temperatures above 22°C ($E_a = 144$kJ/mol) compared with that calculated below the transition temperature ($E_a = 408$kJ/mol).

Kinetic properties

Effective substrate. Both intact mitochondria and an outer-membrane preparation were used to determine the kinetic constants of the outer-membrane

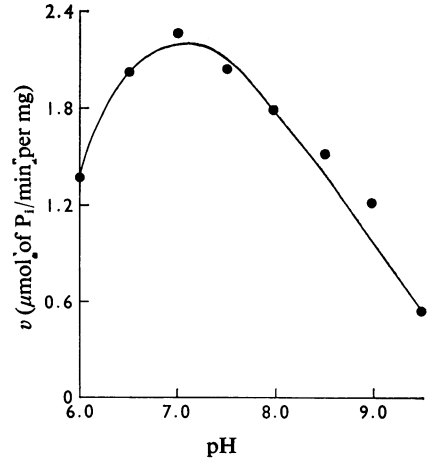

Fig. 2. *pH-dependence of the outer-membrane ATPase of rat spleen mitochondria*

The ATPase activity of the outer membrane fraction (40μg of protein) was assayed essentially as described in the Experimental section, except that Hepes buffer was replaced by Tris (20mM) buffered with maleic acid to give the indicated pH values.

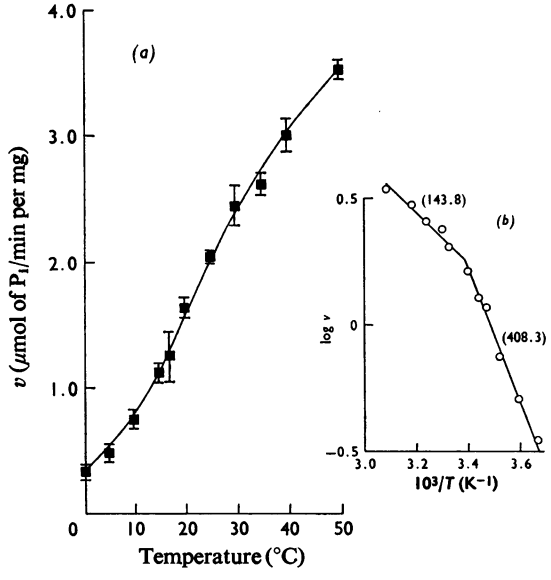

Fig. 3. *Effect of temperature on the outer-membrane ATPase of rat spleen mitochondria*
(*a*) ATPase activity (v) of the outer-membrane fraction (50 μg of protein) was assayed after preincubation at the indicated temperatures for 10 min. The results of triplicate determinations are expressed as means ± S.E.M. (bars). (*b*) Same values as in (*a*) replotted as an Arrhenius plot with E_a values (given in parentheses) in kJ/mol.

Table 1. *K_m and $V_{max.}$ values of rat spleen mitochondrial ATPases*
ATPase activities were assayed by using either intact mitochondria (80 μg of protein) or outer-membrane preparation (50 μg of protein) as described in the text, except that free Mg^{2+} and ATP were varied as indicated in the Table. Free metal ion concentrations were calculated by using metal ion buffers as described in the Experimental section. At fixed ATP concentrations (0.1, 0.3 and 0.5 mM) the free Mg^{2+} was varied over the range 0.01–3.0 mM (Expts. 1 and 4); at 0.5 mM fixed ATP concentration the free Ca^{2+} was varied to give 0.0–0.1 mM (Expt. 5); when the free Mg^{2+} concentration was fixed (at 3.0 mM) the ATP was varied to give 0.0–1.0 mM (Expts. 2 and 6). The oligomycin-sensitive ATPase activity was measured in the absence of oligomycin, atractyloside and Mg^{2+} (Expt. 3) at various ATP concentrations (0.0–0.1 mM). [γ-^{32}P]ATP (3×10^6 d.p.m.) was added uniformly in all incubations. The K_m and $V_{max.}$ values were calculated from an average of three experiments and are given as averages ± S.E.M.

Expt. no.	Fixed concentration	K_m value (μM)	$V_{max.}$ (nmol/min per mg)
Intact mitochondria			
(1)	ATP (300 μM)	for Mg^{2+} 45.0 ± 9.4	192.8 ± 12.6
(2)	$MgCl_2$ (3.0 mM)	for ATP 44.3 ± 3.7	204.7 ± 8.2
(3)	No $MgCl_2$	for ATP 11.9 ± 1.4	114.8 ± 6.0
Outer-membrane fraction			
(4)	ATP		
	(100 μM)	for Mg^{2+} 42.6 ± 3.9	2206 ± 91
	(300 μM)	for Mg^{2+} 47.5 ± 2.9	2180 ± 139
	(500 μM)	for Mg^{2+} 49.7 ± 1.8	2251 ± 90
(5)	ATP (500 μM)	for Ca^{2+} 6.7 ± 1.6	1561 ± 118
(6)	$MgCl_2$ (3.0 mM)	for ATP 48.6 ± 2.7	2113 ± 78

ATPase (Table 1). In both cases, oligomycin (10 μg/mg of protein) and atractyloside (50 μM) were included in the incubation medium to eliminate possible interference from the ATPase activity in the matrix space. Since the outer-membrane ATPase is totally inactive with respect to ATP in the absence of an added bivalent metal ion (both *in situ* and in the outer-membrane preparation) its natural substrate

must be a nucleoside triphosphate–bivalent metal ion complex. When intact mitochondria were used, the ATP concentration was fixed at 300 μM, and the enzyme responded to variation in free Mg^{2+} concentration (0–1.0 mM) in a hyperbolic manner to give a K_m value for Mg^{2+} of approx. 50 μM (Expt. 1). Similarly, when the free Mg^{2+} concentration was fixed at 3.0 mM, variation in the ATP concentration (0–1.0 mM) gave a K_m value for ATP that was practically identical (50 μM) (Expt. 2). Thus MgATP rather than free ATP appears to be the effective substrate.

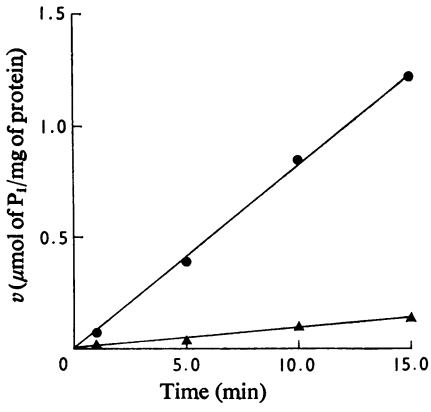

Fig. 4. *Effect of ionophore A23187 on the oligomycin-sensitive ATPase of rat spleen mitochondria*
The ATPase activities were assayed as described in the Experimental section by using 80 μg of protein of either intact mitochondria (●) or mitochondria pretreated with ionophore A23187 (▲). For full details of the depletion of bivalent metal ions from mitochondria with ionophore A23187, see the Experimental section.

In the second case, when the outer-membrane preparation was used, the kinetic constants were determined under identical conditions; in addition, ATP was fixed at three concentrations (100, 300 and 500 μM) to determine the K_m value for Mg^{2+} (Expt. 4). In all cases the K_m values were comparable with those obtained by using intact mitochondria. The 11-fold increase in the $V_{max.}$ is quite compatible with the increase in specific activity observed with outer-membrane preparations (Vijayakumar & Weidemann, 1976). Because the basic kinetic constants of the enzyme measured *in situ* appear to be unaltered in isolated outer-membranes, the kinetic constants for Ca^{2+} were determined with the outer-membrane preparation alone (Expt. 5). When the ATP concentration was fixed at 500 μM, variation in free Ca^{2+} (0–0.5 mM) gave a K_m value of 6.7 μM for Ca^{2+}, which was about one-eighth of that observed with Mg^{2+}. With Ca^{2+} the $V_{max.}$ was only about 80% of the activity stimulated by Mg^{2+}.

The K_m value of the outer-membrane ATPase for ATP (50 μM) is much higher than that observed with the atractyloside- and oligomycin-sensitive ATPase, which does not require added Mg^{2+} (Expt. 3). The K_m for ATP in this case (11 μM) is the same order of magnitude as that reported for the adenine nucleotide translocase of both liver and heart mitochondria (Weidemann *et al.*, 1970), which the substrate must encounter before entering the mitochondrial matrix. The lack of requirement for added Mg^{2+} can be explained by the dependence of the oligomycin-sensitive reaction on endogenous Mg^{2+} present in the

Table 2. *Effect of bivalent metal ions, in the presence and absence of Mg^{2+}, on outer-membrane ATPase activity*
ATPase activity was assayed in a reaction medium as described in the text, by using either the outer-membrane preparation (40 μg) or intact mitochondria (100 μg). $MgCl_2$ was excluded from the reaction medium when the specificity of the enzyme for bivalent metal ions was studied; the calcium concentration of the reaction mixture when reagent-grade salts of Mg^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Sr^{2+}, Ba^{2+} and Ni^{2+} were present was estimated by atomic absorption spectrophotometry (Pybus, 1969). The ATPase activity stimulated by various bivalent metal ions is expressed as a percentage of the Mg^{2+}-stimulated activity. The values were calculated from an average of four experiments and are given as averages ± S.E.M.; n.d., not determined.

Addition (mM)	Calcium concn. of medium (μM)	Intact mitochondria		Outer-membrane fraction	
		No addition	Plus $MgCl_2$ (5.0 mM)	No addition	Plus $MgCl_2$ (5.0 mM)
$MgCl_2$ (5.0)	1.05	100	89.5 ± 7.2	100	93.7 ± 1.6
$CaCl_2$ (3.0)	3000.00	88.0 ± 4.7	96.8 ± 5.4	78.5 ± 1.9	70.2 ± 3.9
$MnCl_2$ (3.0)	0.90	53.9 ± 2.3	59.1 ± 3.1	51.7 ± 1.5	46.7 ± 1.4
$CuCl_2$ (3.0)	0.54	32.0 ± 4.8	29.1 ± 4.1	29.3 ± 4.1	32.1 ± 6.4
$CoCl_2$ (3.0)	0.96	24.9 ± 6.4	28.1 ± 2.8	26.2 ± 1.5	26.6 ± 2.3
$SrCl_2$ (3.0)	1.41	16.1 ± 3.1	101.4 ± 7.8	22.8 ± 1.8	84.8 ± 3.3
$BaCl_2$ (3.0)	0.72	13.5 ± 2.5	102.9 ± 6.9	20.2 ± 0.9	86.3 ± 8.1
$ZnCl_2$ (3.0)	n.d.	11.4 ± 1.7	20.2 ± 1.9	15.6 ± 0.6	23.7 ± 1.4
$CdCl_2$ (3.0)	n.d.	16.6 ± 3.6	18.9 ± 3.1	14.4 ± 2.4	19.1 ± 5.3
$NiCl_2$ (3.0)	1.26	10.1 ± 2.1	17.1 ± 3.2	10.2 ± 1.0	15.7 ± 1.8

matrix, as mitochondria depleted of bivalent metal ions with the ionophore A23187 (Reed & Lardy, 1972) failed to show any ATPase activity in the absence of added Mg^{2+} (Fig. 4).

Bivalent-metal-ion specificity. A spectrum of bivalent metal ions was used to test (i) their ability to support ATP hydrolysis independently of Mg^{2+} and (ii) their ability to modify the Mg^{2+}-stimulated ATPase activity (Table 2). Mg^{2+} was the most effective metal-ion activator, although it could be replaced by Ca^{2+} with only 20–30% loss of activity. Mn^{2+} alone was 50% less effective than Mg^{2+}, whereas Co^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, Cd^{2+} and Ni^{2+}, in decreasing order of efficiency, supported very little ATP hydrolysis. The small amount of hydrolysis found with these ions can mostly be accounted for by the presence of Ca^{2+}, for which the enzyme has a very low K_m (Table 1), as a contaminating ion in the analytical-reagent-grade salts used (Table 2). The most striking observation was made with Cu^{2+}, which supported ATP hydrolysis at high concentrations (3.0mM) but was a very effective inhibitor at low concentrations (see Fig. 6). The dependence of the ATPase activity on bivalent metal ions was almost identical whether the experiments were conducted *in situ* or with the outer-membrane preparation. Ca^{2+}, however, caused significantly more inhibition of the Mg^{2+}-stimulated ATPase activity when the outer-membrane preparation was used.

Nucleoside triphosphate specificity. To determine the specificity of the ATPase reaction for nucleoside triphosphates, the liberation of $[^{32}P]P_i$ from [γ-^{32}P]-ATP added at saturating concentration (100μM) was measured in the presence of a tenfold excess (1.0mM) of either unlabelled ATP or one of several unlabelled competing nucleoside triphosphates (Table 3). The specificity of the reaction for ATP was clearly shown by the strong inhibition of $[^{32}P]P_i$ release by unlabelled ATP (a decrease to only 34% of the control rate). Of the other unlabelled nucleoside triphosphates tested, only ITP produced a significant diminution in $[^{32}P]P_i$ release, decreasing the rate to 69% of the control value. Thus ITP is able to replace ATP with approx. 50% efficiency.

Table 3. *Ability of various nucleoside triphosphates to replace ATP as substrate for the rat spleen mitochondrial outer-membrane ATPase*

ATPase activity was assayed by using the mitochondrial outer-membrane preparation (50μg of protein) as described in the text. $MgCl_2$ (4.0mM) and [γ-^{32}P]ATP (0.1mM; 1.5×10^5d.p.m.) were added uniformly in all incubations. All values where unlabelled nucleoside triphosphates (1.0mM) were added are expressed as a percentage of the ^{32}P released at saturating concentration of [γ-^{32}P]ATP (0.1mM). The values are from an average of three experiments and are given as averages ± S.E.M.

Additions	^{32}P liberated (%)
None	100
ATP	33.9 ± 3.1
GTP	89.9 ± 6.4
UTP	86.9 ± 4.7
CTP	84.7 ± 2.9
ITP	69.2 ± 3.2

Modifiers of outer-membrane ATPase activity

Stimulation and inhibition by univalent cations. The effect of Na^+ and K^+ was tested over a wide concentration range (0–500mM) (Fig. 5). Both metal ions stimulated the Mg^{2+}-dependent ATPase activity (about 15%) at concentrations below 50mM, but, at 500mM, Na^+ but not K^+ was inhibitory. When 5mM-K^+ was included in the incubation medium, the inhibitory effect of Na^+ at 500mM was slightly decreased, and 5mM-Na^+ produced a mild inhibition of the K^+-stimulated activity.

When the effect of low (20mM) concentrations of a variety of univalent cations (Li^+, Cs^+, Rb^+ and NH_4^+) was tested on the Mg^{2+}-stimulated ATPase activity (result not shown), all except Li^+ stimulated the enzyme activity (<10%). This stimulation was insensitive to ouabain, which rules out the possibility that it may have been due to the presence of contaminating plasma-membrane or microsomal en-

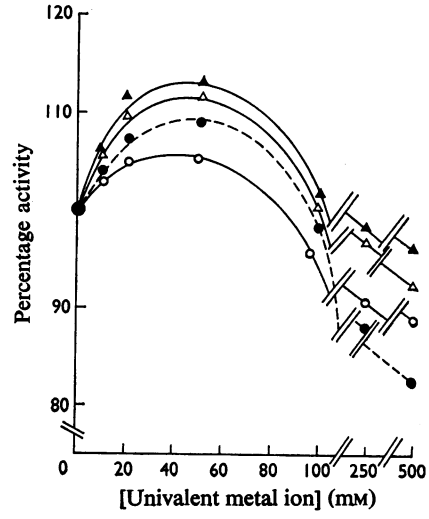

Fig. 5. *Effect of univalent cations on spleen mitochondrial outer-membrane ATPase activity*

ATPase activity was assayed by using mitochondrial outer membranes (50μg of protein) as described in the text, at different concentrations of univalent cations: Na^+ (●); $Na^+ + K^+$ (5.0mM) (○); $K^+ + Na^+$ (5.0mM) (△); K^+ (▲).

zymes. As shown for Na^+ (Fig. 5), all of the univalent metal ions except K^+ inhibited the Mg^{2+}-stimulated activity by 20–30% at 500mM.

Bivalent-metal-ion inhibitors. The magnitude of the inhibition of the outer-membrane ATPase by bivalent metal ions (Table 2) was studied at fixed concentrations of ATP (2.0mM) and Mg^{2+} (5.0mM) (Fig. 6). When the concentration of inhibitory metal ions (e.g. Cu^{2+}, Co^{2+}, Zn^{2+} and Ni^{2+}) was increased up to 1.0mM, there was a progressive and strong inhibition of Mg^{2+}-stimulated ATPase activity. Calculation of the K_i value for each metal ion as described by Dixon & Webb (1964) showed that Cu^{2+} was the strongest and Co^{2+} the weakest inhibitor in the order Cu^{2+} ($K_i = 90\mu M$) > Ni^{2+} ($K_i = 510\mu M$) > Zn^{2+} ($K_i = 680\mu M$) > Co^{2+} ($K_i = 1020\mu M$). Cu^{2+} inhibited the activity of the enzyme very strongly up to 100μM, but at higher concentrations (up to 10mM) it also behaved as a substrate and re-stimulated the ATPase activity up to 25% of the uninhibited Mg^{2+}-stimulated rate. Although Mg^{2+} and Ca^{2+} were the most effective bivalent metal ion activators, they were slightly inhibitory at higher concentrations (Fig. 6).

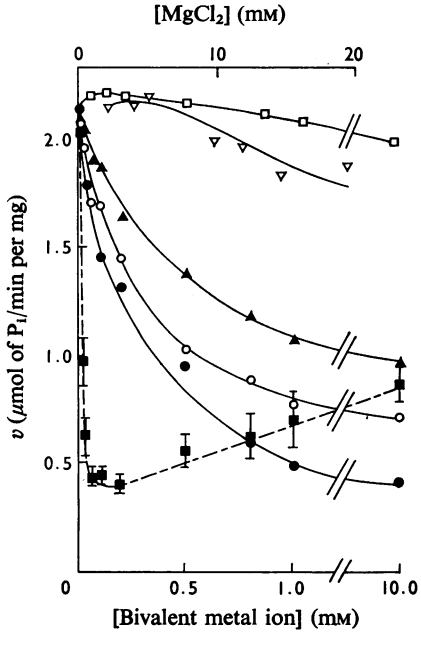

Fig. 6. *Effect of various bivalent metal ions on Mg^{2+}-stimulated ATPase of rat spleen mitochondria*

ATPase activity (v) was assayed by using mitochondrial outer membranes (50μg of protein) as described in the text, at different concentrations of bivalent metal ions: Mg^{2+} (▽), Ca^{2+} (□), Co^{2+} (▲), Zn^{2+} (○), Ni^{2+} (●) and Cu^{2+} (■). Except where variation in the Mg^{2+} concentration itself was tested (▽), $MgCl_2$ was always present at 5.0mM.

'Membrane-bound ATPase' inhibitors. The inhibitors oligomycin, *NN'*-dicyclohexylcarbodi-imide and NaN_3, which inhibit oxidative phosphorylation and the partial reactions catalysed by mitochondrial ATPases (Pedersen, 1975, 1976) had little effect on the outer-membrane ATPase activity (Table 4). Similarly, mersalyl, which is a potent inhibitor of the sarcoplasmic-reticulum ATPase (MacLennan, 1970), produced very little inhibition of the outer-membrane ATPase (Table 4).

Effect of other compounds on ATPase activity. We have been unable to detect any significant inhibition of the outer-membrane ATPase with the thiol-specific reagent *N*-ethylmaleimide or with low concentrations of NaF (1.0mM) and $AgNO_3$ (10μM) (Table 4). However, about 60% inhibition was observed at very high concentrations of NaF (10mM) and $AgNO_3$ (0.5mM) (Table 4), which may be non-specific effects.

End-product inhibition. When the effect of P_i was tested over a wide concentration range (0–20mM), it failed to produce any significant inhibition of the enzyme (Fig. 7).

Table 4. *Effect of various inhibitors on spleen mitochondrial outer-membrane ATPase*

ATPase activity was assayed as described in the Experimental section, by using either intact mitochondria (100μg of protein) or outer-membrane preparation (40μg of protein). Inhibitors were added to give the final concentrations indicated in the Table. Values are given as an average of duplicate experiments.

Addition	Concn. (mM)	Activity (%) Intact mitochondria	Activity (%) Outer membranes
$MgCl_2$	5.0	100	100
NN'-Dicyclohexylcarbodi-imide	0.01	96.7	—
	0.10	92.9	91.7
Oligomycin (μg/mg of protein)	0.5	—	95.6
	5.0	98.1	97.2
	10.0	92.7	91.4
NaN_3	0.01	91.8	91.2
	0.10	89.9	84.0
Mersalyl	0.001	—	89.6
	0.005	93.7	92.4
	0.010	87.3	91.5
N-Ethylmaleimide	0.004	88.8	96.2
	0.010	88.5	95.3
NaF	1.0	87.2	98.7
	10.0	40.8	40.2
$AgNO_3$	0.01	—	87.4
	0.10	55.2	54.5
	0.50	61.7	42.3

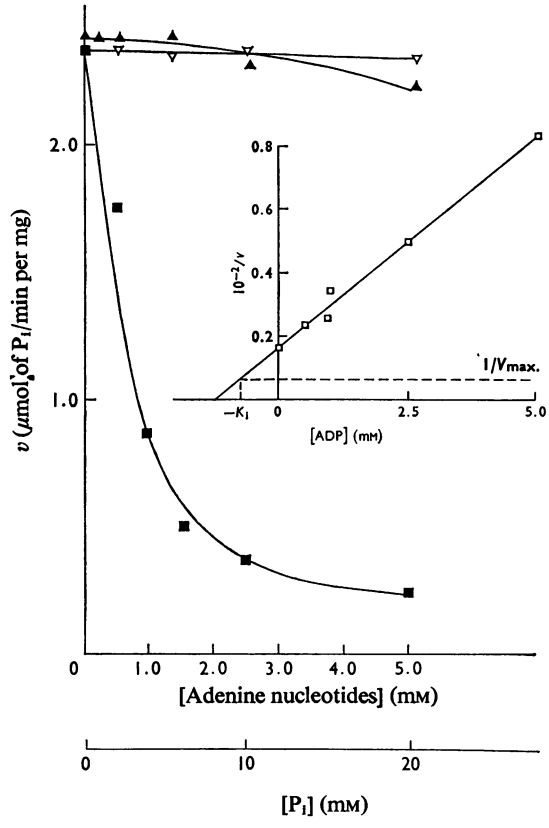

Fig. 7. *Influence of ADP, AMP and P_i on the ATPase activity of rat spleen mitochondrial outer membranes*
ATP activity (v) was assayed in mitochondrial outer membranes (50μg of protein) as described in the text at a fixed, saturating concentration of MgATP (2.0mM), under conditions where the concentrations of ADP (■), AMP (▽) and P_i (▲) were varied. The inset shows the values in the presence of ADP (□) replotted by the method of Dixon & Webb (1964).

At saturating [γ-^{32}P]MgATP concentration, MgADP but not MgAMP caused a large and significant inhibition of ATP hydrolysis, with a K_i value of 0.7mM (inset, Fig. 7). At three concentrations of MgADP, the K_m value of the reaction for MgATP increased with increasing MgADP concentration, whereas the $V_{max.}$ was unchanged (Fig. 8). This observation is consistent with the view that MgADP inhibits the enzyme by competing directly with MgATP for occupation of the catalytic site. In the absence of added MgADP, and also at all three MgADP concentrations tested, titration of the ATPase activity with MgATP gave conventional Michaelis–Menten kinetics (Hill coefficient $h = 1.0$), indicating that there is unlikely to be more than one type of binding site for MgATP.

Discussion

Substrate specificity of the outer-membrane ATPase

The ability of the rat spleen mitochondrial outer-membrane ATPase to catalyse ATP hydrolysis in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+} (Table 2) is similar to that reported for the bacterial ATPase (Hachimori *et al.*, 1970) and shows the same order of specificity. At equimolar concentrations, Co^{2+} is only one-third as potent as Mg^{2+} in activating the outer-membrane ATPase, whereas it supports high rates of ATP hydrolysis by the inner-membrane ATPase and F_1 preparations of heart and liver mitochondria (Pullman *et al.*, 1960; Cooper & Lehninger, 1957). Although Ca^{2+} is essentially without effect on the oligomycin-sensitive ATPase

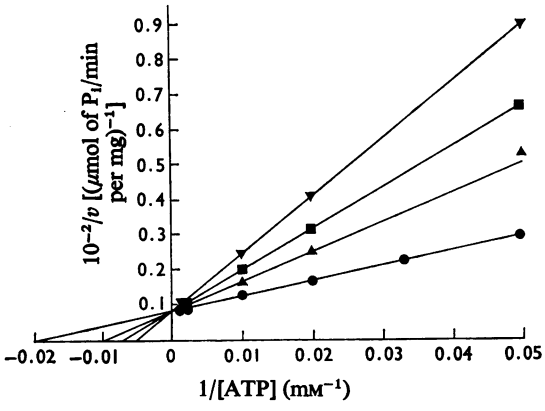

Fig. 8. *Competitive inhibition of the Mg^{2+}-stimulated outer-membrane ATPase of rat spleen mitochondria by MgADP*

ATPase activity was assayed over the [γ-^{32}P]ATP concentration range 0.01–2.0mM, by using mitochondrial outer membranes (60μg of protein). In each experiment the initial ADP concentration was set as follows: none (●), 0.5mM (▲), 1.0mM (■) and 2.0mM (▼). $MgCl_2$ was added in all cases to give a final concentration of 4.0mM. The double-reciprocal plot is expressed as the reciprocal of the reaction rate (v) against 1/[ATP] at each ADP concentration tested.

(Tzagoloff *et al.*, 1968) and is only 50% as effective as Mg^{2+} in activating the ATPase activity of F_1 preparations (Pullman *et al.*, 1960; Pedersen, 1975), its ability to support ATP hydrolysis by the outer-membrane enzyme approaches that of Mg^{2+} (Table 2). However, stimulation of the outer-membrane ATPase activity by Mg^{2+} does not require added Ca^{2+}, as Mg^{2+} alone can produce optimum activity even in the presence of 1.0mM-EGTA (Vijayakumar & Weidemann, 1976). Further, addition of Ca^{2+} does not enhance the Mg^{2+}-stimulated ATPase activity; on the other hand, it behaves as a mild inhibitor of the enzyme when the outer-membrane preparation is used (Table 2 and Fig. 6). These properties are comparable with those of the Ca^{2+}-independent, Mg^{2+}-stimulated ATPase activity of the delipidated sarcoplasmic-reticulum enzyme preparation studied by Walter & Hasselbach (1973), although the native form of that enzyme requires both Ca^{2+} and Mg^{2+} for full activity (MacLennan, 1970).

The difference in effect of the various bivalent metal ions on Mg^{2+}-stimulated ATP hydrolysis can be explained in terms of their affinity for ATP. For instance, Cu^{2+} binds ATP 120 times as strongly as Mg^{2+} (Sillén & Martell, 1971) and effectively displaces Mg^{2+} from the MgATP complex, which explains the strong inhibition by Cu^{2+} at much lower concentration (<100μM) than that of the added Mg^{2+} (5.0mM) (Fig. 6). Similarly, Zn^{2+}, Ni^{2+} and Co^{2+} have 3–6 times the affinity for ATP as Mg^{2+} (Sillén & Martell, 1971), which is consistent with their ability to inhibit Mg^{2+}-stimulated ATP hydrolysis (Fig. 6). The inability of Ca^{2+}, Sr^{2+} and Ba^{2+} to inhibit the Mg^{2+}-stimulated ATPase activity in intact mitochondria (Table 2) is again compatible with their lower affinity for ATP compared with Mg^{2+} (Sillén & Martell, 1971). The inhibition of the outer-membrane ATPase by bivalent metal ions in direct proportion to their relative ability to displace Mg^{2+} gives further support to the view that the effective substrate is a bivalent metal ion–nucleoside triphosphate complex.

The nucleoside triphosphate specificity of the enzyme (Table 3) is similar to that of the oligomycin-sensitive ATPase of heart and liver mitochondria (Tzagoloff *et al.*, 1968; Cooper & Lehninger, 1957), chloroplasts (Vambutas & Racker, 1965) and bacteria (Hanson & Kennedy, 1973) in that it catalyses the preferential hydrolysis of purine nucleoside triphosphates, with ATP being hydrolysed at about twice the rate of ITP. ITP can also partly displace the atractyloside-insensitive [^{14}C]ADP binding to ox heart mitochondria (Weidemann *et al.*, 1969), which is compatible with the presence of a purine-nucleoside-binding protein located outside the atractyloside barrier in mitochondria of heart (Chao & Davis, 1972) and spleen (Vijayakumar & Weidemann, 1976). It is suggested that the atractyloside-insensitive binding site for ITP and ADP in these mitochondria could be the outer-membrane ATPase.

When the concentration of MgATP is increased, the double-reciprocal plot obtained with the outer-membrane ATPase of rat spleen mitochondria differs markedly from that observed with the rat liver mitochondrial ATPase. Since the oligomycin-sensitive ATPase of liver mitochondria has two interdependent nucleoside-binding sites (Koshland, 1970; Kitiakowsky & Rosenberg, 1952) similar to ox heart F_1 preparations (Penefsky, 1974), and a separate modifying site for anions (Alberty *et al.*, 1954; Frieden, 1964; Pedersen, 1976), it gives a curvature in the double-reciprocal plot when MgATP is the sole substrate that becomes linear only on the addition of anions (e.g. P_i) (Ebel & Lardy, 1975). In contrast, the outer-membrane ATPase gives a linear double-reciprocal plot even in the absence of added anions (Fig. 8), which may suggest the absence of a modifying site in this case. Lack of stimulation of the outer-membrane ATPase by P_i (Fig. 7) provides additional evidence for the absence of such a modifying site.

Inhibition of the outer-membrane ATPase

Competitive inhibition of the outer-membrane ATPase by ADP (Fig. 7) is similar to that reported

for F_1 preparations from chloroplasts (Vambutas & Racker, 1965) and bacteria (Schnebli & Abrams, 1970). Further, the addition of ADP increased the K_m value of the enzyme for MgATP without changing the $V_{max.}$ or the linearity of the double-reciprocal plot (Fig. 8), which may suggest that this enzyme has only one nucleotide-binding site, for which both ADP and ATP compete. AMP does not inhibit the activity of either the outer-membrane ATPase (Fig. 7) or the oligomycin-sensitive ATPase of rat liver mitochondria (Catterall & Pedersen, 1972). This observation is in agreement with the inability of F_1 preparations from rat liver (Catterall & Pedersen, 1972) and ox heart (Harris *et al.*, 1973) to bind AMP.

Specific inhibition of mitochondrial respiration and the partial reactions of oxidative phosphorylation by oligomycin and *NN'*-dicyclohexylcarbodi-imide is well established (Bulos & Racker, 1968; Lardy *et al.*, 1958). Since the mechanism of inhibition by these agents is dependent on an oligomycin-sensitivity-conferring protein (Beechey *et al.*, 1967), the insensitivity of the outer-membrane ATPase to these inhibitors (Table 4) infers the lack of such a protein subunit associated with it. In addition, the outer-membrane ATPase is insensitive to NaN_3 (Table 4), which is a direct specific inhibitor of the F_1 enzyme (Slater, 1955). These findings distinguish the outer-membrane ATPase from the oligomycin-sensitive enzyme in both its native and F_1 forms. Further, the insensitivity of the outer-membrane enzyme to thiol-specific reagents (Table 4) rules out the possibility that accessible thiol groups are involved at the active site.

Function of the ATPase in relation to outer-membrane permeability

Spleen mitochondria resemble kidney-cortex and heart mitochondria in many respects, especially in their response to the addition of bivalent metal ions. Addition of Mg^{2+} to all of these mitochondria prolongs State-3 respiration (Chance & Williams, 1956) and apparently uncouples them (Chao & Davis, 1972; Gmaj *et al.*, 1974; Vijayakumar & Weidemann, 1976) and also alters their Ca^{2+}-handling capacity (Gmaj *et al.*, 1974; Sordahl, 1974; Jacobus *et al.*, 1975). It has been suggested, at least for rat kidney-cortex mitochondria, that a decrease in the transport of Ca^{2+} in the presence of Mg^{2+} may be due to the involvement of the Mg^{2+}-stimulated outer-membrane ATPase in this process (Gmaj *et al.*, 1974). Unlike liver mitochondria, spleen, heart and kidney-cortex mitochondria have very low ability (<10%) to exchange externally added [^{14}C]ADP with the endogenous adenine nucleotide pool (I. A. Munro & M. J. Weidemann, unpublished work). Finally, all of these mitochondria have a Mg^{2+}-stimulated ATPase in their outer membranes (Vijayakumar & Weidemann, 1976; Gmaj *et al.*, 1974). These observations may argue for the existence of fundamental differences in the permeability properties of the outer membranes of different types of mitochondria, although it is generally accepted that the liver mitochondrial outer membrane is freely permeable to low-molecular-weight substances (Ernster & Kuylenstierna, 1970). Although this hypothesis obviously needs more experimental support, it should provide a useful starting point for further work aimed at defining the role played by the mitochondrial outer-membrane ATPase in regulating the intracellular distribution of bivalent metal ions in the three tissues that contain it.

We acknowledge the award of an Australian National University Research Scholarship to E. K. V. in connexion with this project. The work was supported by Australian Research Grants Committee Grant No. D2 70/17436 to M. J. W.

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