A Thermodynamic Analysis of the Interaction between the Mitochondrial Coupling Adenosine Triphosphatase and its Naturally Occurring Inhibitor Protein

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1. The naturally occurring ATPase (adenosine triphosphatase)-inhibitor protein, from bovine heart mitochondria, was obtained as a single pure protein. It was not identical with any of the five subunits (α - ϵ) of the isolated ATPase, and appeared to be a single polypeptide chain. 2. The inhibitor combined with the ATPase in a 1:1 molar ratio, producing a completely inhibited ATPase molecule. The affinity of the ATPase for its inhibitor is high; the K_d is of the order of 10^{-8} M. 3. The enthalpy of the ATPase-inhibitor complex-formation is positive, the value of K_d decreasing as the temperature is raised. This suggests that the forces involved are largely hydrophobic in nature. 4. Hydrolysis of a nucleoside triphosphate promoted formation of the ATPase-inhibitor complex, although the equilibrium position was almost unaffected by the rate of hydrolysis. At low salt concentration, less than 200 turnovers of the ATPase suffice for the ATPase to combine with the inhibitor protein. At higher salt concentrations, a larger number of turnovers is required. It is suggested that the inhibitor binds to a form of the ATPase that is produced transiently during hydrolysis. 5. In the presence of 75 mM- K_2SO_4 , the rates of association and dissociation are slow enough to allow their kinetics to be studied. Association is first-order in inhibitor concentration, but fractional order in ATPase concentration. Dissociation is first-order in ATPase-inhibitor complex concentration. The temperature coefficients of the 'on' and 'off' processes were also measured. 6. A simple kinetic model for the ATPase-inhibitor interaction is proposed that can be extended to take into account release of inhibitor protein under energized conditions on the membrane. 7. The isolated ATPase is inhibited by preincubation with Mg^{2+} , reversible by subsequent addition of EDTA, and by ADP, reversible by subsequent addition of ATP. These effects are not found on the membrane-bound ATPase. The mechanism of these effects is discussed.

The ATP synthase/coupling ATPase of coupling membranes from a wide range of organisms has been shown to be associated *in vivo* with a low-molecular-weight (about 10000) protein that inhibits ATP hydrolysis (Pullman & Monroy, 1963; Nelson *et al.*, 1972; Satre *et al.*, 1975; Adolfsen & Moudrianakis, 1976; Chan & Barbour, 1976; Smith & Sternweis, 1977). This protein may be lost from the ATPase to a greater or lesser extent when the ATPase is isolated from the membrane and purified.

It seems likely that the inhibitor protein is involved in regulation of the ATPase protein. It will inhibit all ATP-driven processes of the coupling membrane (Asami *et al.*, 1970; Ferguson *et al.*, 1977) and

phosphorylation (Harris & Crofts, 1978). Its association with the ATPase is regulated by the energy state of the membrane (van de Stadt *et al.*, 1973; Bakker-Grunwald & van Dam, 1974; Harris & Crofts, 1978) and also by the amount of inhibitor protein actually present *in vivo* (Satre *et al.*, 1975), which may be regulated, for example, by proteolysis and/or synthesis *de novo*.

In spite of its potential importance in regulation, the inhibitor protein has never been fully characterized, nor its interaction with the ATPase quantified. In the work described below, it is shown that the mitochondrial ATPase inhibitor is not identical with any of the five subunits (α - ϵ) of the isolated coupling ATPase [for a review, see Senior (1973)], and that it combines with the ATPase in a 1:1 ratio. Association of the ATPase and inhibitor protein has a positive enthalpy and the interaction is largely hydrophobic in nature. Further, the interaction is not a simple bimolecular reaction and must consist of at least two steps.

Abbreviations used: ATPase, adenosine 5'-triphosphatase (EC 3.6.1.3); rro-ATP (ribose-ring-opened ATP), 2,2'-[1-(9-adenyl)-1'-triphosphoryloxymethyl]-dihydroxydiethyl ether; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

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Materials and Methods

Bovine heart mitochondria were prepared as previously described (Ferguson *et al.*, 1977) and the ATPase inhibitor protein by the procedure of Nelson *et al.* (1972), except that, to remove a high-molecular-weight impurity, the inhibitor protein was finally chromatographed on a DEAE-Sephadex A50 column (6 cm × 0.8 cm) and eluted with a gradient of 0–100 mM-K₂SO₄. The mitochondrial ATPase was isolated by the method of Knowles & Penefsky (1972a). Sub-mitochondrial particles (A particles) were prepared by sonication of mitochondria in the presence of EDTA and NH₃ (Fessenden & Racker, 1966). Inhibitor-depleted particles (AS particles) were prepared by gel filtration of A particles at high ionic strength (Horstman & Racker, 1970); refiltration of these particles gave no further increase in ATPase activity. The inhibitor protein was assayed by the procedure of Horstman & Racker (1970). ATPase activity was measured in the continuous assay described by Rosing *et al.* (1975), except that (i) 100 mM-KCl was included in the assay medium (to prevent any further ATPase-inhibitor combination after dilution into the assay medium), and (ii) the final pH was 8.0.

The dissociation constant (K_d) of the ATPase (E) with the inhibitor protein (L) was calculated from the following expression (cf. Klein *et al.*, 1977):

initial concentration

$$E_0 \quad L_0 \quad 0$$

equilibrium concentration

$$E_0 - \alpha E_0 \quad L_0 - \alpha E_0 \quad \alpha E_0$$

[where α is the fraction of E in the form EL (ATPase-inhibitor complex) at equilibrium]

$$K_d = \frac{(E_0 - \alpha E_0)(L_0 - \alpha E_0)}{\alpha E_0} \quad (1)$$

$$\frac{L_0}{\alpha} = \frac{K_d}{1 - \alpha} + E_0 \quad (2)$$

and thus, from a plot of L_0/α against $1/(1 - \alpha)$, the slope of the line obtained gives K_d , and the intercept on the ordinate, E_0 , the concentration of binding sites. Polyacrylamide-gel electrophoresis in dodecyl sulphate was carried out on 12% cylindrical gels (0.7 cm × 7 cm) as described by Weber & Osborn (1969) and stained and destained by the method of Fairbanks *et al.* (1971). Particulate protein was determined by the biuret method as modified by Cleland & Slater (1953), and soluble protein by the procedure of Lowry *et al.* (1951). The sources of enzymes and nucleotides were as described elsewhere (Harris *et al.*, 1977).

Results and Discussion

Nature of the inhibitor protein

There are conflicting reports as to whether the ATPase inhibitor protein is equivalent to the smallest (ϵ) subunit of the ATPase (Brooks & Senior, 1971; Knowles & Penefsky, 1972b). Their different preparations also show differences in amino acid composition.

Fig. 1 shows the results of co-electrophoresing a sample of pure inhibitor protein with the isolated ATPase under dissociating conditions (in dodecyl sulphate). Clearly, the inhibitor is not identical with, but intermediate in molecular weight between, the δ and ϵ subunits of the ATPase. A mol.wt. of about 10000 can be calculated for the inhibitor protein.

The gel pattern was unaltered if either the ATPase and inhibitor were pretreated with 2-mercaptoethanol (1% at 80°C for 30 min) or if the ATPase

Fig. 1. Electrophoretic pattern of the inhibitor protein and the ATPase in dodecyl sulphate

Gel electrophoresis and staining the protein bands with Coomassie Blue stain were carried out as described in the Materials and Methods section. The gels were scanned at 600 nm with a Gilson spectrophotometer with scanning attachment. (a) Purified inhibitor protein (I) (5 μ g); (b) purified ATPase (100 μ g); (c) co-electrophoresis of inhibitor (4 μ g) and ATPase (150 μ g). The bands are numbered α – ϵ in order of decreasing molecular weight, using the nomenclature of Brooks & Senior (1971). The ATPase is present in relatively large amounts so that the δ and ϵ bands are clearly visible.

inhibitor were combined to form the inhibited complex before electrophoresis (results not shown). The isolated inhibitor, therefore, seems unlikely to be a dimer associated by disulphide bridges, nor to change in molecular weight on binding to the ATPase.

The ATPase inhibitor protein in heart mitochondria is thus not equivalent to the ϵ subunit of the ATPase (mol.wt. 7500). Whether this is true in other organisms, in particular chloroplasts (Nelson *et al.*, 1972) and *Escherichia coli* (Smith & Sternweis, 1977), remains unclear (but see Yoshida *et al.*, 1977). The possibility of the inhibitor being a sixth subunit very close in molecular weight to the ϵ subunit in these organisms has not been ruled out.

Conditions for formation of the ATPase-inhibitor complex

In agreement with Horstman & Racker (1970) we find that low pH (6.5–7.0) and MgATP are necessary for maximal inhibition of the ATPase by a given amount of inhibitor, although some inhibition occurs in the absence of any MgATP (even if glucose and hexokinase were added to remove traces of ATP).

The role of MgATP is uncertain. It is not related to the ability of ATP to energize the membrane, since AS particles show no coupled behaviour. As shown in Table 1, almost any hydrolysable analogue of ATP will serve to promote inhibition in AS particles; even N^1 -oxidoadenosine triphosphate, which is hydrolysed at about 5% of the rate of ATP under these conditions, yields inhibition identical to ATP. Only N^6 -bromoacetyladenosine triphosphate (which is not hydrolysed detectably) and rro-ATP (hydrolysed at 3% of the rate of ATP) show a significant decrease in inhibition.

These results are in contrast with those reported by Kozlov & Skulachev (1977), who claimed that the equilibrium position for inhibition was related to the rate of hydrolysis of the various nucleotides. That this is not in general the case is shown also by the lack of effect of 2mM-ADP on the final degree of inhibition reached with ATP (Table 1). ADP, however, does slow down the combination of the ATPase and inhibitor induced by MgATP. [ADP alone does not promote combination of the ATPase and its inhibitor (Table 1), as long as glucose and hexokinase are present to nullify ATP production by adenylate kinase.]

An attempt was made to determine the number of ATP molecules needed to be hydrolysed for formation of an inhibited ATPase molecule. AS particles were incubated with a small excess of inhibitor, and a low concentration (40 μ M) of [γ - 32 P]ATP. Inhibition, and release of [32 P] P_i , were measured as a function of time (Fig. 2). From the initial part of this curve an upper limit of about 200 mol of ATP hydrolysed/mol of ATPase inhibited could be estimated.

Table 1. *Ability of nucleoside triphosphates to promote the ATPase-inhibitor interaction*

A 0.2ml portion of 250mM-sucrose/10mM-Tes brought to pH 6.7 with Tris base, containing 160 μ g of AS particles was incubated with 4 μ g of ATPase inhibitor or protein and 500 μ M-MgNTP as indicated. (Decreasing the nucleotide concentration to 200 μ M, which decreased the hydrolysis rate about 2-fold, had no effect on the ultimate degree of inhibition.) Portions were removed for assay of ATPase activity as described in the text, and the degree of inhibition was recorded after 5 min (see Fig. 2). The hydrolysis rate of the various nucleotides was measured, in a separate assay, by measurement of released phosphate as described elsewhere (Ferguson *et al.*, 1977). The error given represents the range of four duplicate readings; 100% hydrolysis rate represents 9.5 μ mol of ATP hydrolysed/min per mg of protein.

Nucleotide added	Inhibition (%)	Hydrolysis rate (relative to ATP)
ATP	95 $\pm$ 4	100 $\pm$ 6
dATP	93	55
Iso-GTP	94	34
ITP	93	22
GTP	94	21
N^1, N^6 -Etheno-ATP	93	13
N^1 -Oxido-ATP	93	10
rro-ATP	67	3
N^6 -Bromoacetyl-ATP	54	<2
No addition	50	—
ADP (2mM) + ATP	93	2
ADP*	21	—

* 500 μ M-ADP, 500 μ M-MgCl₂, 4 units of hexokinase (salt-free) and 40mM-glucose were present during the incubation. One unit of hexokinase activity is the amount of enzyme required to convert 1 μ mol of substrate/min.

It seems likely, therefore, that hydrolysis of a triphosphate results in a transient conformation of the ATPase to which the inhibitor can bind rapidly. This is consistent with the finding that adenosine 5'-[β -imido]triphosphate, a non-hydrolysable analogue of ATP, does not promote inhibitor binding (Klein *et al.*, 1977). In the absence of hydrolysis, although the same ATPase-inhibitor equilibrium is approached, the approach is much slower and inhibition is incomplete even after 20min. It is not known whether the inhibitor in fact binds to the active site of the ATPase or if, on binding, it 'traps' nucleotide bound to the ATPase (cf. Harris & Slater, 1975).

Measurement of inhibitor binding to the isolated ATPase

In the above experiments on AS particles it was assumed that inhibition of the ATPase activity represents combination of ATPase and inhibitor

Fig. 2. Hydrolysis of ATP during combination of the ATPase and its inhibitor

A series of tubes containing 0.45 ml of 250 mM-sucrose, 10 mM-Tes brought to pH 6.7 with Tris base, 4 μ g of inhibitor protein, and 40 μ M-Mg[γ - 32 P]ATP (sp. radioactivity 100 c.p.m./nmol) where indicated, were incubated at 23°C and at $t = 0$; 400 μ g of AS particles in 50 μ l of 250 mM-sucrose was added. From one tube, portions were removed at the times indicated and the ATPase activity assayed. At the same times, hydrolysis was stopped in the duplicate tubes sequentially by the addition of 4% (v/v) perchloric acid, and the amount of [32 P]P_i released measured as previously (Harris & Slater, 1975). The arrows indicate the calculated value of ATP hydrolysed per ATPase molecule inhibited (corrected for inhibition in the absence of ATP), assuming 0.35 nmol of ATPase per mg of AS-particle protein (Vadineau *et al.*, 1977). Symbols: $\square$, [32 P]P_i released; $\circ$, ATPase activity in the absence of added MgATP; $\bullet$, ATPase activity in the presence of added MgATP.

protein. This is consistent with the non-competitive nature of the inhibition (van de Stadt *et al.*, 1973) and is justified further below. This cannot be assumed in the case of the association of the inhibitor with the isolated ATPase, since inhibition (stable to subsequent dilution) can occur in addition by interaction of the ATPase with ADP (Harris, 1977) and Mg²⁺ (Moyle & Mitchell, 1975).

Fig. 3 shows the effect of preincubation with Mg²⁺ alone, or in combination with ATP and/or inhibitor protein, on the subsequent activity of the ATPase. Mg²⁺ (Fig. 3) or MgATP (Fig. 4) (rapidly hydrolysed to Mg²⁺ + ADP) have little effect on the activity of the membrane-bound ATPase in the absence of

inhibitor protein. Mg²⁺, however, causes a slow inactivation of the isolated ATPase to about 60% of the original activity. This inactivation was reversed by EDTA (Fig. 3). In contrast to Moyle & Mitchell (1975), we found that phosphate or sulphite ions had little effect on the enzyme inactivated by Mg²⁺ (results not shown).

If MgATP is added to the preincubation, a 40–45% inactivation also results. However, reactivation by EDTA is small, and ATP is required in addition for maximal reactivation (Fig. 3). Since ADP inactivates the isolated ATPase, and this inactivation is reversed by ATP (Harris, 1977), it would appear that both Mg²⁺ and ADP are contributing to the inactivation observed in this incubation, and that both must be removed from the enzyme for full reactivation. It is interesting that the two types of inhibition are not additive, and that the maximal inactivation attained with Mg²⁺ + ATP is only about 50%.

A mechanism for the inactivation due to ADP is proposed elsewhere (Harris *et al.*, 1977). The nature of the inhibition induced by Mg²⁺ is uncertain, but is not due to interaction of Mg²⁺ at the active site of the ATPase, since it persists during several turnovers of the enzyme. Although not additive with ADP-induced inhibition, Mg²⁺-induced inhibition was not due to an identical process (e.g. conversion of bound ATP into bound ADP) since EDTA reversed only the latter (Fig. 3). A conformational change is known to occur when Mg²⁺ binds to the isolated ATPase, as shown by change in fluorescence of bound aurovertin (Chang & Penefsky, 1973) or formycin bisphosphate (D. A. Harris, unpublished work).

The lowest curve in Fig. 3 shows the inactivation induced in the isolated ATPase when preincubation occurs with MgATP and a 2-fold molar excess of inhibitor protein. The inhibition proceeds to far greater than 50% and is not reversed by EDTA or ATP, and thus is taken to represent the combination of ATPase with its inhibitor protein.

At low concentrations, therefore, inactivation due to Mg²⁺ or ADP will interfere with measurement of ATPase-inhibitor combination on the isolated ATPase by the hydrolytic assay. Even in the absence of inhibitor protein, about 50% inhibition is observed (Fig. 3, insert, upper curve). To measure the ATPase-inhibitor combination, it was necessary to reverse the Mg²⁺- and ADP-induced inactivation, and the experiments were thus carried out as in Scheme 1. At time B, the only remaining inhibition should represent the ATPase-inhibitor combination (Fig. 3, insert, lower curve). After correction for the small denaturation due to the long incubation, this curve could be used for the calculation of K_d (see below). ATP and EDTA did not appear to affect the position of the ATPase-inhibitor equilibrium (Fig. 3, main Figure, lowest curve).

Fig. 3. Effect of preincubation with Mg^{2+} or $MgATP$ on the activity of the isolated ATPase

About 1 unit ($\mu\text{mol}/\text{min}$) of ATPase (isolated or membrane-bound) was incubated in 0.2ml of 250mM-sucrose/10mM-Tes brought to pH6.7 with Tris base, at 23°C . At $t = 0$, 1mM- $MgCl_2$ or 500 μM - $MgATP$ was added and the activity measured at various times as shown in Fig. 2. Subsequent additions of 2mM-EDTA ('solid' arrow) and 2mM-ATP ('open' arrow) were made as indicated; 100% activity represents 88 μmol of ATP hydrolysed/min per mg of protein for the isolated ATPase and 10.2 $\mu\text{mol}/\text{min}$ per mg for the membrane-bound ATPase. Main Figure: ●, AS particles, initial addition $MgCl_2$ (1mM); ○, isolated ATPase, initial addition $MgCl_2$ (1mM); △, isolated ATPase, initial addition $MgATP$ (500 μM); □, isolated ATPase, initial additions $MgATP$ (500 μM) and inhibitor protein (1 μg). Insert: inhibition measured as in □ above, except that differing concentrations of inhibitor protein were added; △, inhibition at time A (no further additions); ○, inhibition at time B (EDTA, ATP added).

Scheme 1. Sequence of additions for measuring combination of inhibitor with the isolated ATPase

The ATPase was incubated at pH6.7 with the inhibitor protein and 500 μM - $MgATP$, and after 4min a steady-state activity was reached (A, see Fig. 3). EDTA (2mM) and ATP (2mM) were then added to reverse inactivation due to Mg^{2+} and ADP respectively, and the final level of inhibition, at B, taken to represent the combination of the ATPase with its inhibitor protein. (It is essential to add $MgATP$ after the inhibitor and ATP after EDTA to avoid hydrolysis.)

Binding parameters of the ATPase-inhibitor complex

The inhibitor protein binds very tightly to AS particles, and in fact an estimate of the stoichiometry of binding (about 0.8mol of inhibitor/mol of ATPase) can be read from a plot of inhibition against amount of inhibitor added (Fig. 4, insert). These data also justify the assumption that the inhibitor can completely inhibit the ATPase. If this is the case, and we

assume that every molecule of bound inhibitor inhibits the ATPase equally, α of eqn. (1) is given by $1 - \alpha = V/V_0$, where V_0 = rate of hydrolysis in the absence of inhibitor and V = rate of hydrolysis after equilibration with inhibitor.

A plot of L_0/α against $1/(1 - \alpha)$ is linear (Fig. 4), confirming that these assumptions are correct, i.e. that only one class of inhibitory binding site is present in these particles, with a K_d of $1.4 \times 10^{-8}\text{M}$. The

Fig. 4. Inhibition of the membrane-bound ATPase: titration with inhibitor protein

Inhibition of the membrane-bound ATPase was measured as in Table 1, except that MgATP (500 μM) was present throughout and the inhibitor and particle concentrations were varied as indicated. The results are plotted either directly (insert) or as in eqn. (2) (see the Materials and Methods section). Symbols: Δ, 1.5 mg of particle protein/ml; ○, 0.75 mg of particle protein/ml; □, 0.38 mg of particle protein/ml.

intercepts indicate a concentration of binding sites of 3 ± 0.2 μg of inhibitor/mg of particle protein, namely 0.86 mol/mol of ATPase, assuming 0.35 nmol of ATPase/mg of particle protein in AS particles (Vadineau *et al.*, 1976). In yeast, a value of 0.56 μg of inhibitor/mg of particle protein has been estimated (Klein *et al.*, 1977) by using radioactively labelled inhibitor, but the concentration of ATPase on these particles was unknown.

The binding of inhibitor protein to particle-bound ATPase at various temperatures is shown in Fig. 5. The number of inhibitor-binding sites (E_0) remains constant, whereas the K_d changes. The binding becomes stronger as the temperature rises, indicating a positive enthalpy of formation of the complex.

The standard free energy (ΔG°), enthalpy (ΔH°) and entropy (ΔS°) for the combination of the ATPase with its inhibitor protein, calculated from these results, are given in Table 2. The large positive entropy of formation of the complex is consistent with the view that the protein-protein interactions

Fig. 5. Temperature-dependence of the ATPase-inhibitor interaction

Inhibition of the membrane-bound ATPase was measured and plotted as in Fig. 4 (main Figure). Particle concentration was 0.38 mg of protein/ml, and the incubation temperature was varied as indicated. Symbols: □, 0°C; ○, 23°C; Δ, 32°C.

Table 2. *Thermodynamic parameters of the ATPase-inhibitor interaction*

The dissociation constant, K_d , for the combination of either particle-bound or isolated ATPase with the purified inhibitor protein was measured as described in Figs. 3 and 4. It should be noted that K_d for this interaction at 0°C could be measured only on the membrane-bound enzyme, at low salt concentrations; ATPase activity was rapidly lost at 0°C in the absence of inhibitor protein both with the isolated enzyme (even at low salt) and on the membrane-bound enzyme in the presence of 75 mM- K_2SO_4 . $\Delta G^{0'}$ (reference temperature 23°C), $\Delta H^{0'}$ (mean value between 23 and 32°C) and $\Delta S^{0'}$ (reference temperature 23°C) refer to the association of ATPase and inhibitor protein at pH 6.7, and are calculated as described in standard texts. Abbreviation used: N.D., not determined.

System	Temperature (°C)	...	K_d (M)				$\Delta G^{0'}$ (kJ/mol)	$\Delta H^{0'}$ (kJ/mol)	$\Delta S^{0'}$ (kJ/mol per degree K)
			0	23	32	42			
AS particles, low salt			6.0×10^{-8}	1.4×10^{-8}	0.65×10^{-8}	N.D.	-45	+48	+0.31
AS particles, +75 mM- K_2SO_4			—	2.0×10^{-8}	1.4×10^{-8}	N.D.	-44	+38	+0.27
Isolated ATPase, low salt			—	5.9×10^{-8}	N.D.	1.6×10^{-8}	-41	+54	+0.32

are largely hydrophobic, in spite of the opposite charges of the two proteins (Senior, 1973). The value of $\Delta H^{0'}$ is not constant over the range 0–37°C. This may reflect changes in the binding site due to phase transitions in addition to the equilibrium studied. The only known transition in the bovine heart ATPase occurs at about 16°C (Bertoli *et al.*, 1973), and thus the value of $\Delta H^{0'}$ between 23 and 37°C may be closer to the true value for the reaction under consideration.

Similar results were obtained with the isolated ATPase, although the affinity of the enzyme for the inhibitor was some five times less when isolated. Values are included in Table 2. A value of K_d at 0°C could not be measured as the isolated ATPase is unstable at this temperature. Clearly, $\Delta H^{0'}$ and $\Delta S^{0'}$ are similar in sign and magnitude in both the isolated and membrane-bound ATPases, suggesting that the inhibitor-binding site is little altered when the ATPase is removed from the membrane.

Kinetics of the ATPase-inhibitor interaction

The combination of the membrane-bound ATPase with its inhibitor was measured by incubating both proteins together, in the presence of MgATP, and withdrawing portions at various times for assay of ATPase activity. As such, the rate of the combination in the standard buffer (250 mM-sucrose/10 mM-Tes/Tris, pH 6.7) was too fast to be measured accurately ($t_{1/2} < 5$ s).

However, as shown in Fig. 6, the rate of association decreased with increasing ionic strength. This occurred apparently because the ATPase had to turn over a larger number of times on average before combining with an inhibitor molecule; i.e. the chance of combination per turnover fell from about 1/200 to <1/2000. This was reflected in the higher MgATP requirement for combination in the presence of salt.

Fig. 6. *Effect of salt on the ATPase-inhibitor interaction*
Inhibition of the membrane-bound ATPase, in the presence of 5 mM-MgATP, was measured as in Table 1, with the addition of K_2SO_4 as indicated. Symbols: Δ , no salt added; $\circ$, 38 mM- K_2SO_4 ; $\square$, 75 mM- K_2SO_4 (150 mM- K_2SO_4 had an effect comparable in magnitude with 75 mM- K_2SO_4).

In a low salt medium, 50 μ M-ATP was sufficient to produce >90% maximal inhibition (Fig. 2), whereas in 75 mM- K_2SO_4 more than 1 mM-ATP was required (results not shown). The curves shown in Fig. 6 confirm also that dilution of the ATPase-inhibitor

reaction medium into buffer at pH 8.0 and high ionic strength 'quenches' the combination reaction.

When sufficient ATP was present, K_d and the thermodynamic parameters of the ATPase-inhibitor association in the presence and absence of 75 mM- K_2SO_4 were very similar (Table 2). The 'prevention' of the ATPase-inhibitor interaction by salts reported by others (Horstman & Racker, 1970) probably resulted from using a constant low ATP concentration in all assays. The number of inhibitor-binding sites per ATPase is also unaltered by salts (0.93 mol of inhibitor/mol of ATPase).

By thus slowing down the association, the 'on-rate' of inhibitor could be decreased to a value measurable by the repeated sampling technique. The results are shown in Fig. 7. The combination is

Fig. 7. The rate of combination of ATPase with inhibitor: effect of protein concentration

The time course of inhibition of the membrane-bound ATPase, in the presence of 75 mM- K_2SO_4 , was measured essentially as in Fig. 6 except that four assays were made within the first minute, and the protein concentrations were varied as indicated. The initial rate of inhibition was obtained from the tangent to the inhibition progress curve at the origin, which was estimated mathematically by the method of Noble (1968). This procedure gives much more reproducible results than drawing the tangent by eye. Symbols: □, 0.38 mg of particle protein/ml; ○, 0.75 mg of particle protein/ml; Δ, 1.5 mg of particle protein/ml. [It should be noted that, in the presence of 75 mM- K_2SO_4 , combination of the ATPase with its inhibitor in the absence of MgATP is very small (less than 5% inhibition). This can be contrasted with the significant inhibition obtained at low salt concentrations (Fig. 2).]

approximately first-order in inhibitor concentration, as expected, but of much lower order in ATPase concentration. This suggests that the ATPase-inhibitor association is not a simple bimolecular process. The on-rate showed a small temperature-dependence, increasing by only 30% over the range 23–32°C (results not shown).

The 'off-rate' of the inhibitor was measured as described previously (Ferguson *et al.*, 1977), except that AS particles inhibited by more than 90% were used to start the incubation. In this case, 75 mM- K_2SO_4 increases the rate, confirming that the ATPase-inhibitor interaction cannot be a simple bimolecular process (salt increases k_{off} , decreases k_{on} and yet leaves K_d almost unaltered). The off-rate was first-order in particle protein (results not shown) and very strongly stimulated by temperature (Ferguson *et al.*, 1977), having a Q_{10} value of about 5.

From the values of Q_{10} for the on and off reactions, the activation energy E_a ($\approx \Delta H^\ddagger$) can be calculated for these processes by using the Arrhenius equation. In this case, we have:

$$E_a^{off} = 120 \text{ kJ/mol} \quad E_a^{on} = 20 \text{ kJ/mol}$$

For a simple bimolecular reaction,

$$\Delta H_{r-1}^\ddagger = \Delta H_{r-1}^\ddagger - \Delta H_{1-r}^\ddagger$$

$r-1$ indicates the reaction direction right to left. In the case analysed here, $\Delta H^{0'} = +35 \text{ kJ/mol}$ (Table 2), whereas $\Delta H_{r-1}^\ddagger - \Delta H_{1-r}^\ddagger$ (i.e. for association) = -100 kJ/mol . This confirms that the association cannot be treated as a simple bimolecular one, and the values of E_a obtained represent a complex function of the $\Delta H^\ddagger$ for the various steps in the association.

On the basis of the kinetic data given here, we propose the following mechanism for association (EI representing the inhibited form of the ATPase:

This could account for the fractional order of the reaction with regard to ATPase and the first-order reaction with respect to inhibitor. This cannot be explained by a previously proposed mechanism (van de Stadt *et al.*, 1973)

which must be of the same order in both E and I, since it is symmetrical. (Our proposed mechanism cannot, however, rule out an uninhibited complex $E^1 I$; it would simply be kinetically unimportant.)

Combining eqn. (3) with that for the activation of the ATPase in ATP synthesis (Harris & Crofts, 1978), we simply have to add an energy-dependent reversal to the second step:

Again, it is difficult to draw an equivalent scheme for the mechanism shown in eqn. (4), and thus this self-consistent scheme seems a likely possibility for the situation *in vivo*. It should be noted that E^1 does not represent a classical 'high-energy' state of the ATPase; release of the inhibitor protein and re-binding do not occur each time one molecule of ATP is synthesized (Harris & Crofts, 1978).

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