# ACTIVE/INACTIVE STATE TRANSITIONS OF MITOCHONDRIAL ATPase MOLECULES INFLUENCED BY $Mg^{2+}$, ANIONS AND AUROVERTIN

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## 1. Introduction

The complete proton-translocating ATPase of mitochondria is activated by certain anions, such as phosphate, sulphate and chromate, but is inhibited by other anions, such as azide and thiocyanate [1–3]. Similar anion activations and inhibitions have also been observed in preparations of the $F_1$ component of the ATPase [4–8]. These activations and inhibitions have generally been attributed to effects of the anions on the reaction velocity $V$, characteristic of all the ATPase catalytic units in the ATPase preparations. However, the relatively slow time-dependence of some kinetic properties of the ATPase [1,2,7,8,14], and the fact that oligomycin inhibits the ATPase reaction by almost the same factor, independently of the degree of anion activation or inhibition [1,2], suggested to us recently that the enzyme population might consist of a dynamic mixture of active and virtually inactive catalytic units, and that the equilibrium ratio of active to inactive ATPase units or molecules might be influenced by the ionic composition of the medium, and possibly by other agents, such as aurovertin.

The experiments described in this paper show that the complete ATPase of rat liver mitochondria is activated in a KCl medium at low $Mg^{2+}$ concentration, and that 5 mM $MgCl_2$ induces inactivation at a rate that is slow compared with the rate of the ATPase reaction. This inactivation is enhanced by azide. The converse process of anion activation proceeds at a rate that depends on the activating anion and may take as long as ten minutes to reach equilibrium.

* *Abbreviation*: FCCP, carbonylcyanide trifluoromethoxy-phenylhydrazone.

The anion activation of a preparation of $F_1$ from beef heart mitochondria (kindly provided by Professor E. Racker) was found to exhibit kinetic characteristics similar to those of the complete ATPase of our sonic particle preparations from rat liver mitochondria.

We conclude that activation and inhibition by various salts, and inhibition by aurovertin, are not mainly attributable to effects on $V$, but are attributable to sluggish, reversible active/inactive state transitions in the $F_1$ component of the ATPase.

## 2. Materials and methods

Rat liver mitochondria were isolated as described previously [10]. Sonic particles were prepared [11] from mitochondrial suspensions and were stored frozen in 250 mM sucrose at a concentration equivalent to 80 mg of protein/ml. Samples were thawed, diluted tenfold in 250 mM sucrose, and kept at 4°C for each day's experiments.

The $F_1$ preparation supplied by Professor E. Racker was a suspension in 2 M ammonium sulphate, containing 1.4 mg of protein/ml. It had been isolated from beef heart mitochondria by the method of Horstman and Racker [12], and it was stored at 4°C. Stock solutions of $F_1$ were prepared each day by dissolving samples of the suspension in a medium containing 250 mM sucrose, 1 mM EDTA and 10 mM Tris chloride buffer at pH 7.4 at room temperature (about 20°C) to give a final protein concentration of 0.2 mg/ml.

The ATPase activities of the sonic particle and $F_1$ preparations were routinely measured, using the pH method of Nishimura, Ito and Chance [13], as

described before [1], using, in the case of the sonic particle preparations, 1.6 mg of protein in 3.3 ml of a medium containing 150 mM KCl, 3.3 mM glycylglycine, 5 mM $MgCl_2$, 0.1 mg of carbonic anhydrase (Sigma) and 1 $\mu$M FCCP* at pH 7.0 to 7.1 and at 25°C. In the case of the $F_1$ preparation, 10 $\mu$g of protein was used in the 3.3 ml of assay medium, and FCCP was omitted. To measure the activating or inhibitory effects of the anions, they were added to the assay medium as $K^+$ salts, and the KCl concentration was decreased to maintain the total anion concentration at 150 mM. The $MgCl_2$ in the assay medium (but not that added with the ATP) was omitted in certain experiments. In some experiments using oligomycin-treated enzyme preparations, 8.0 mg of protein was used in the assay instead of 1.6 mg of protein. It is noteworthy that the enzyme preparations were incubated at 25°C with the assay medium for a given time before commencement of the ATPase reaction by the addition of ATP (as equimolar ATP and $MgCl_2$).

### 3. Results and discussion

Table 1 shows the coefficients $K_M$ (ATP), $K_i$ (ADP) and $V'$ for the ATPase activity of the complete ATPase of the rat liver mitochondrial sonic particles and for the beef heart mitochondrial $F_1$ preparation at 25°C and at pH 7.0 to 7.1 after preincubation in the presence of various salts. These coefficients were obtained in the orthodox way from Lineweaver-Burk plots, which gave straight lines, but the apparent maximum velocity is described by the symbol $V'$, which may be related to $V$ by the relationship $V' = V\alpha$, where $\alpha$ means the proportion of enzyme catalytic units in the active state, as explained below. As observed previously [1,2], ADP inhibition was found to be strictly competitive with

Table 1
Kinetic coefficients for the complete ATPase of sonic particle preparations from rat liver mitochondria and for the $F_1$ ATPase from beef heart mitochondria

| Assay medium (containing 5 mM $MgCl_2$) | Complete ATPase | | | | $F_1$ ATPase | | | |
|---|---|---|---|---|---|---|---|---|
| | $K_M$ (ATP) ($\mu$M) | $K_i$ (ADP) ($\mu$M) | $V'$ | $\alpha$ | $K_M$ (ATP) ($\mu$M) | $K_i$ (ADP) ($\mu$M) | $V'$ | $\alpha$ |
| $MgCl_2$ omitted | 500 | 66 | 44 | 0.75 | 440 | 44 | 6000 | 1.00 |
| + 20 mM sulphate, $MgCl_2$ omitted | 500 | 35 | 59 | 1.00 | | | | |
| – | 106 | 9 | 4.3 | 0.07 | 210 | 15 | 1340 | 0.22 |
| + 2 mM phosphate | 106 | 4.5 | 14 | 0.24 | 200 | 8.3 | 1610 | 0.27 |
| + 20 mM phosphate | 106 | 4.0 | 29 | 0.49 | | | | |
| + 20 mM sulphate | 105 | 3.0 | 18 | 0.30 | 200 | 6.5 | 1680 | 0.28 |
| + 150 mM sulphate | | | 25 | 0.42 | 210 | 6.7 | 2680 | 0.45 |
| + 4 mM sulphite | 210 | 202 | 56 | 0.94 | 216 | 93 | 5500 | 0.92 |
| + 0.5 mM 2,4-dinitrophenate | 250 | 107 | 12 | 0.20 | 190 | 47 | 3350 | 0.56 |
| + 0.5 mM picrate | 240 | 100 | 18 | 0.30 | 205 | 34 | 3220 | 0.54 |
| + 15 $\mu$M azide | 106 | 12 | 1.3 | 0.02 | | | | |
| + 0.2 mM azide | | | 0.8 | 0.01 | | | | |
| + aurovertin D, $MgCl_2$ omitted | 75 | 35 | 6.9 | 0.12 | | | | |
| + aurovertin D | 56 | 180 | 4.4 | 0.07 | | | | |
| + aurovertin D + 20 mM sulphate | 50 | 45 | 4.4 | 0.07 | | | | |

The assay medium contained 5 mM $MgCl_2$, unless stated otherwise, and the routine method allowed a 7.5-min preincubation of the sonic particle preparations, and a 1-min preincubation of the $F_1$ ATPase preparations, in the assay medium at pH 7.0 to 7.1 and at 25°C before addition of MgATP or MgADP. Where indicated, aurovertin D was present at 12.5 mg/g of protein. Calculations of $K_M$ (ATP) and $K_i$ (ADP) values are based on total ATP and ADP concentrations. Values of $V'$ are expressed as $\mu$mol of ATP hydrolysed/sec per g of protein.

ATP, allowing for the fact that $K_i$ (ADP) of the complete ATPase is a function of time after ADP addition in the presence of $Mg^{2+}$ and phosphate or sulphate. Accordingly, equilibrium $K_i$ (ADP) values, obtained after preincubating the ATPase with ADP for 1 min, are given in table 1. In a medium containing 5 mM $MgCl_2$, and in the presence of sulphite, 2,4-dinitrophenate or picrate, $K_i$ (ADP) reached its equilibrium value relatively quickly (i.e. in 1 sec or less). Low values of $V'$ were induced by the presence of $Mg^{2+}$ in excess of that added as MgATP. Thus, high $V'$ was obtained in the absence of activating anions, provided that the $Mg^{2+}$ concentration was low; and it appears that anion activation might alternatively be regarded as the release of $Mg^{2+}$ inhibition. Moreover, in other experiments, inhibition by 20 $\mu$M azide was increased from 10% to 55% by preincubation in the presence of 5 mM $MgCl_2$, showing that azide inhibition is $Mg^{2+}$ dependent. It is significant for the interpretation of this result that azide inhibition was time dependent, taking some 10 min to reach virtual equilibrium.

Fig.1A shows the time-course of induction of the inhibited state by $Mg^{2+}$ in the complete ATPase. The inhibitory effect of $Mg^{2+}$ develops much too slowly to be attributable to direct interference by $Mg^{2+}$ in the catalytic turnover of the ATPase. Presumably it must depend on a relatively slow change of the population of ATPase molecules, in which the individual molecules undergo stepwise transitions from one or more states of higher $V$ to one or more states of lower $V$. Likewise, the release of the $Mg^{2+}$ inhibition by phosphate or sulphate, shown in fig.1B, indicates a slow reversion of the ATPase population to one or more molecular states of higher $V$. The reactivation of the complete ATPase by sulphite, at a concentration of 1.5 mM, was found in other experiments to be somewhat faster than that shown for phosphate or sulphate in fig.1, being virtually complete within 1 min.

It was desirable to discover whether the relatively slow reversible $Mg^{2+}$ inactivation and anion reactivation were characteristic only of the complete ATPase, or whether they could be attributed to the $F_1$ component, as might be inferred from the time-dependent $Mg^{2+}$ inactivation of the ATPase activity of $F_1$, observed by Catterall and Pedersen [7]. Fig.2 shows that the ATPase activity of $F_1$ undergoes a time-dependent inactivation by $Mg^{2+}$, and that this is reversible by

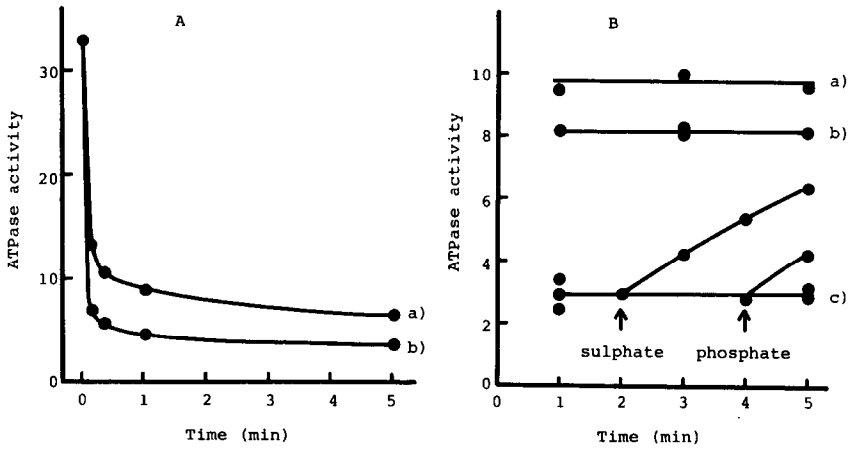


Fig.1. Time-course of inactivation of the complete ATPase of sonic particle preparations of rat liver mitochondria by $MgCl_2$, and reactivation by phosphate and sulphate. In (A) sonic particles were preincubated in an assay medium initially containing no $MgCl_2$ for a total of 7.5 min before ATP hydrolysis was initiated. In (a), 0.2 mM $MgCl_2$, and in (b), 5 mM $MgCl_2$, was added at various time intervals before adding the 150 $\mu$M MgATP. In (B) the assay medium contained: (a) 5 mM $MgCl_2$, 2 mM phosphate; (b) 5 mM $MgCl_2$, 20 mM sulphate; and (c) 5 mM $MgCl_2$. In (c), sulphate (20 mM) and phosphate (2 mM) were added to some samples, at the arrows. The sonic particles were preincubated in the assay medium for the time indicated before ATP hydrolysis was initiated by adding 150 $\mu$M MgATP. ATPase activity is expressed as ng ions of $H^+$ produced/sec per 1.6 mg of sonic particle protein.

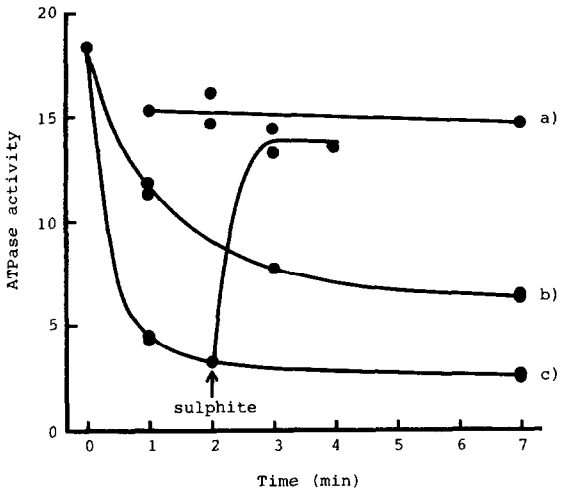


Fig.2. Time-course of inactivation of the $F_1$ ATPase from beef heart mitochondria by $MgCl_2$, and reactivation by sulphite. Samples of the $F_1$ ATPase preparation were preincubated in the assay medium for the time indicated before ATP hydrolysis was initiated by adding 150 $\mu$M MgATP. The assay medium contained: (a) 5 mM $MgCl_2$, 4 mM sulphite; (b) 0.15 mM $MgCl_2$; and (c) 5 mM $MgCl_2$. In (c), 4 mM sulphite was added to some samples at the arrow. ATPase activity is expressed as ng ions of $H^+$ produced/sec per 10 $\mu$g of $F_1$ protein.

sulphite, as in the case of the complete ATPase. We conclude, therefore, that it is the $F_1$ component of the complete ATPase that undergoes the sluggish changes of state responsible for the dependence of $V'$ on the salt composition of the medium.

In the simplest case of the type of activation/inactivation behaviour described here, the ATPase would presumably consist of an equilibrium mixture of molecules in only two states, one being inactive ($V = 0$) and the other exhibiting a fixed velocity $V$. Under these circumstances, the apparent velocity $V'$ would be given by the product of the velocity $V$ of active enzyme molecules and the proportion $\alpha$ of the molecules in the active state. This type of behaviour should be recognisable experimentally, because treatments that would vary $V$ and $\alpha$ independently should be seen to have mutually-independent effects on $V'$. For example, as oligomycin decreases $V$ specifically in the complete ATPase by imposing a rate-limiting step in $F_0$ [2,14], if the activating or inhibiting anions were to act by changing $\alpha$, they should change $V'$ by the same factor independently of the presence or absence of oligomycin. But this should not be so if the anions were to act by changing $V$ through a rate-limiting step in all the $F_1$ molecules.

Table 2
Effect of oligomycin on the factor by which various anion species and aurovertin activate or inhibit the complete ATPase

| Additions to assay medium | Normal ATPase | | Oligomycin-treated ATPase | |
|---|---|---|---|---|
| | Relative activity | Activation factor | Relative activity | Activation factor |
| | 100 | 1.0 | 8 | 1.0 |
| 2 mM phosphate | 350 | 3.5 | 29 | 3.6 |
| 20 mM phosphate | 670 | 6.7 | 55 | 6.9 |
| 20 mM sulphate | 510 | 5.1 | 41 | 5.1 |
| 4 mM sulphite | 820 | 8.2 | 37 | 5.0 |
| 0.2 mM azide | 9 | 0.09 | 2.3 | 0.29 |
| aurovertin D | 124 | 1.2 | 10 | 1.3 |

The assay medium contained 5 mM $MgCl_2$. Where indicated, oligomycin (6.8 mg/g of sonic particle protein) and aurovertin D (2.5 mg/g of sonic particle protein) were present. The sonic particle preparations were preincubated for 15 min in the assay medium (with or without oligomycin) before ATP hydrolysis was initiated by adding 150 $\mu$M MgATP. All activities are expressed relative to the activity in the assay medium with no additions in the absence of oligomycin, given as 100. Activation factors represent the factor by which the relative activity of the normal ATPase or the oligomycin-treated ATPase (i.e. 100 or 8 respectively) is multiplied when the given anion or aurovertin is present.

Table 2 shows that, for the anions phosphate and sulphate, and for aurovertin, the activation factor is independent of the action of oligomycin, within experimental error. It seems probable, therefore, that the inhibition by $Mg^{2+}$, and the reactivation by phosphate and sulphate, occur without significant change of the value of $V$ of the active enzyme catalytic units, but reflect active/inactive state transitions of the $F_1$ component of the ATPase. Likewise, aurovertin, which antagonises anion activation (table 1), probably does not change $V$ (table 2), but presumably influences the slow active/inactive state transitions of $F_1$ in the complete ATPase, so that $\alpha$ becomes only around 0.1 under the conditions of our experiments (table 1). This is consistent with observations on slow time-dependent changes of fluorescence exhibited by aurovertin-ATPase complexes under certain conditions [15].

The interaction of aurovertin with the ATPase molecules probably has a dual effect, on the one hand, changing $K_M$ (ATP) and $K_i$ (ADP) of the ATPase molecules that are enzymically active (table 1), and on the other hand, inducing a relatively slow change of $\alpha$. Thus, the enzymically-active aurovertin-ATPase complex would represent a (conformational?) state that is significantly different from that of the normal active ATPase state (or states, see below).

The activation and inhibition effects of sulphite and azide, shown in table 2, do not appear to be completely independent of the action of oligomycin. In the case of azide, this could well be attributable to the presence of only about 1% of a contaminating oligomycin-insensitive and azide-insensitive ATPase activity in our sonic particle preparations. The fact that sulphite activates significantly less in the presence than in the absence of oligomycin suggests that the predominant effect of sulphite is on $\alpha$, but that there may also be a weak effect of sulphite on $V$, under certain conditions. As shown in table 1, it may be relevant in this context that sulphite, unlike phosphate and sulphate, increases the observed $K_M$ (ATP). Incidentally, 2,4-dinitrophenate and picrate also increase the observed $K_M$ (ATP). Other supporting experiments showed that, when azide and sulphite or sulphate were present together, their effects were antagonistic; and the equilibrium ATPase activity could be adjusted to any value over the complete range between virtually zero and maximum activation by appropriately increasing the sulphite or sulphate to azide concentration ratio. This behaviour is reminiscent of the observation by Pullman et al. [16], that 0.5 mM 2,4-dinitrophenate causes activation of the $F_1$ ATPase by a greater factor in the presence of azide than in its absence. It is noteworthy that although sulphite, at a given concentration, was more effective than sulphate at reversing inhibition by 5 mM $MgCl_2$ alone, it was less effective than sulphate at reversing inhibition by 5 mM $MgCl_2$ in the presence of 0.2 mM azide. Thus, the specific $Mg^{2+}$ and anion effects on the relative energy levels of the active and inactive molecular states of the ATPase, that presumably determine the equilibrium value of $\alpha$, appear to be complex.

In accordance with the above conception of the part played by $Mg^{2+}$, various anions and aurovertin in influencing the ratio of active to total $F_1$ molecules in the ATPase preparations, table 1 shows computed values of the fraction $\alpha$ of the ATPase in the active state, on the assumption that all the catalytic units of the ATPase are active under the conditions found to give maximum activity in this research.

The nature of the active/inactive state transitions of the $F_1$ component of the ATPase is a matter of conjecture at present. It is conceivable that they could be attributable to a specific inhibitor subunit in the ATPase preparations, but this seems unlikely because the known inhibitor subunit of the ATPase of beef heart mitochondria, present in the $F_1$ preparation used in this work [12,17], has been shown not to inhibit inhibitor-free ATPase preparations at the high KCl concentration [12] of our assay procedure, and it is doubtful whether any of the subunits of the ATPase of rat liver mitochondria (which lacks the inhibitory subunit of the beef heart enzyme) has a specific inhibitory function [8,17]. However, we have eliminated the possibility that a soluble dissociable factor could be involved either in activation or in inactivation, by centrifuging down sonic particle preparations in the active and inactive states, discarding the supernatant, and observing that the pellets are still susceptible to quantitatively normal inactivation and reactivation respectively. It seems probable that the active/inactive state transition involves some change of conformation of the individual subunits of $F_1$ or some change of packing of the subunits, or both; but it should be stressed that any changes of conformation that may be involved in the relatively sluggish

process of activity change discussed in this paper are not fast enough to be directly involved in the reversible proton-translocating hydrolytic reaction catalysed by the complete ATPase.

The fact, established previously [1,2], that a slow change of $K_i$ (ADP) to a relatively low value is triggered by ADP in a medium containing 5 mM $MgCl_2$ and phosphate or sulphate without significant change of $V'$ (and probably without significant change of $V$), suggests that the normal ATPase molecules can exist in at least two catalytically active states (with different $K_i$ (ADP) values but with the same value of $V$) as well as in the catalytically-inactive state. The absolute dependence of the lowering of $K_i$ (ADP) on $Mg^{2+}$ and phosphate or sulphate as well as ADP, indicates that these ligands are involved in the stabilisation of the molecular state of the ATPase with low $K_i$ (ADP). It is not known whether the actions of ADP, $Mg^{2+}$ and phosphate or sulphate in this $K_i$ (ADP) state transition of the ATPase molecules are effected through the site or sites involved in the active/inactive state transition, or whether the active centre of the enzyme may be involved in either case. Preparations of $F_1$ have been shown to contain tightly-bound nucleotide [7,17], and the slow rate of exchange suggests that this nucleotide may be bound at a 'regulatory' site rather than at the active centre. Such a regulatory site might be involved in the state transitions affecting $\alpha$ and $K_i$ (ADP). At all events, it is possible that the active centre might play some part as an effector site in the slow state-transition processes discussed here, because the effectors $MgPO_4^-$ and ADP are substrates, and many of the magnesium salts that favour the active state of the enzyme appear to be structural analogues of $MgPO_4^-$.

Assuming that all the inorganic activating anions that have been studied [2,4,9,18,19] act by increasing the proportion $\alpha$ of the ATPase in the active state, as shown here for phosphate, sulphate and sulphite, we can list the following in the category of phosphate analogues: chromate, pyrophosphate, arsenate, sulphate, molybdate, sulphite, selenite, phosphite and carbonate. The following activating anions appear to belong to a different category: 4-nitrophenate, 2,4-dinitrophenate, picrate and pentachlorophenate; but we have confirmed by measurements of the slow reactivation of the ATPase in experiments similar to those of fig.1A, that, like the inorganic activating anions, these organic anions activate by increasing $\alpha$ and not by increasing $V$. Although the view of Stockdale and Selwyn [19] that this category of organic anion activates the ATPase by increasing $V$ is probably incorrect, their suggestion that the activation by these anions depends on structural resemblance to an adenine residue may be right. All the magnesium salts so far known to favour the inactive state of the ATPase appear to fall in a single univalent category, as follows: chloride, nitrate, chlorate, perchlorate, thiocyanate and azide.

Our results and conclusions on the effects of changes of $\alpha$ on the apparent value of the velocity of the reaction catalysed by the proton-translocating ATPase of rat liver mitochondria indicate that a much wider exploration of the phenomenon of active/inactive state transition is needed in order to interpret the kinetics of proton-translocating ATPases generally. For example, it will be necessary to consider the effects of the slow changes of $\alpha$ and of the related heterogeneity of the population of the ATPase molecules in interpreting pH-activity curves, the actions of metals analogous to $Mg^{2+}$ [20], the actions of inhibitors like guanidine [16] or the specific inhibitory subunit of $F_1$ [17]. In the light of our studies, it seems especially important that there should be a critical re-evaluation of the function (if any) of the tightly-bound nucleotide, found in certain ATPase preparations, that some workers have assumed to be directly involved in the catalytic mechanism of reversible ATP hydrolysis [21–24]. As pointed out by Senior [17], the tightly-bound nucleotide might well be present in 'regulatory' sites rather than in the active centre of the ATPase. There is actually no evidence that any conformational changes that may be associated with this tight nucleotide binding would occur any faster than, or would indeed be distinct from, the type of slow change of state of the ATPase molecules, influenced by $MgPO_4^-$, ADP and other effectors including aurovertin, which we have described in this paper.

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