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# TIGHTLY BOUND NUCLEOTIDES OF THE ENERGY-TRANSDUCING ATPase, AND THEIR ROLE IN OXIDATIVE PHOSPHORYLATION

## II. THE BEEF HEART MITOCHONDRIAL SYSTEM

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SUMMARY

1. Beef heart mitochondrial ATPase, in both the membrane-bound and isolated form, contains tightly bound ATP and ADP. Each mol of ATPase contains about 2.2 mol ATP and 1.3 mol ADP.

2. In the absence of ATPase activity, these nucleotides exchange only slowly with nucleotides in solution. The exchange rate is increased during coupled ATPase activity, but not when the ATPase is uncoupled.

3. Oligomycin and dicyclohexylcarbodiimide inhibit exchange of the bound nucleotides, as does the ATPase inhibitor protein, although in each case some residual exchange occurs. Aurovertin, although inhibiting phosphorylation, does not inhibit the exchange. This is discussed in terms of the reversibility of these inhibitors.

4. The stimulation of exchange seen during coupled ATPase activity requires energisation of the ATPase molecule. Using the exchange reaction as a probe of energisation, it is deduced that energy can be transferred between different ATPase molecules.

5. It is proposed that coupled ATPase activity and phosphorylation in submitochondrial particles involve the tight nucleotide binding sites and the (weak) ATPase site, while uncoupled ATPase activity involves only the weak site.

## INTRODUCTION

Coupling ATPases isolated from a variety of sources bind ATP and ADP very tightly and are isolated with stoichiometric amounts of nucleotides bound to them [1–8]. These nucleotides are not removed by gel filtration, treatment with charcoal or Dowex, or precipitation by ammonium sulphate. The tightly bound nucleotides are not

Abbreviations: $S_{13}$, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide; FCCP, Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, dicyclohexylcarbodiimide, AdN, adenine nucleotide.

involved directly in the (uncoupled) ATPase activity of the isolated coupling ATPase [2]. The tight binding sites do, however, interact with the ATPase site as is shown by the fact that the ATPase activity is lost if the tightly bound ATP is replaced by ADP [9], or all the bound nucleotides by a non-hydrolysable imido-analogue of ATP [7].

Tightly bound nucleotides are also associated with coupling ATPases in their native, membrane-bound state [4, 5, 8, 10–13]. In photophosphorylating systems, it has been shown that these nucleotides become 'active' (exchangeable with added nucleotides and able to incorporate $^{32}P_i$) when the membranes are energised [4, 5, 10–17]. This is consistent with their being involved in the mechanism of photophosphorylation.

In this and the accompanying paper [8], it is shown that the bound nucleotides of oxidative phosphorylating systems respond to energisation of the ATPase in the same way as do those of chloroplasts. The scheme for a photophosphorylation mechanism involving the bound nucleotides [4] can thus be extended to oxidative phosphorylation systems. The basis of the model is that energy is not required for the synthesis of ATP on the ATPase, but for the release of ATP from this enzyme. In the light of these and other results, an attempt to delineate the roles of the various nucleotide binding sites of the coupling ATPase, within such a model, is made here.

As previously pointed out [4, 8, 9], irrespective of any role in phosphorylation, changes in behaviour of the bound nucleotides can give information on the state of the ATPase itself. Using the nucleotides as a probe, we propose here that coupled ATPase activity in submitochondrial particles involves a different conformation of the ATPase than that of uncoupled ATPase activity, and that energy can be transferred from one coupled ATPase molecule to another. The mode of action of various inhibitors of phosphorylation is also discussed.

## MATERIALS AND METHODS

Heavy beef heart mitochondria were prepared by the methods of van de Stadt et al. [18] (Type I) and Smith [19] (Type II). It was found that submitochondrial particles prepared from Type I mitochondria (isolated largely in a sucrose medium) contained much larger amounts of the ATPase inhibitor protein [20] than those prepared from Type II mitochondria (isolated with succinate present throughout) (Ferguson, S. J., Harris, D. A. and Radda, G. K., in the press).

$Mg^{2+}$-ATP particles were prepared by the method of Low and Vallin [21], and $Mg^{2+}$ particles by a modification of this procedure where ATP was omitted during sonication. $Mg^{2+}$-ATP particles were depleted of ATPase inhibitor by centrifuging the particles down under energised conditions, as described by van de Stadt et al. [22] except that ADP was omitted from the incubation medium. This procedure was only successful with inhibitor-rich particles (from Type I mitochondria). Reconstitution with ATPase inhibitor was carried out as described [22]. The mitochondrial ATPase was prepared by the method of Knowles and Penefsky [23] and the ATPase inhibitor by the method of Nelson et al. [24].

Experiments to study the behaviour of the bound nucleotides under energised conditions were performed according to the general method shown in Scheme I (solid arrows) of the accompanying paper [8].

The protein estimations here are based on bovine serum albumin (or ovalbu-

min) as standard, using an extinction coefficient of $E_{280\,nm} = 6.67\ ml \cdot mg^{-1} \cdot cm^{-1}$ [25]. Previous work used $E_{280\,nm} = 8.95\ ml \cdot mg^{-1} \cdot cm^{-1}$. Using the value of $6.67\ ml \cdot mg^{-1} \cdot cm^{-1}$, the protein content of mitochondrial ATPase determined by the Lowry method calibrated with serum albumin agrees closely with that determined by the Kjeldahl method. (Muller, J. M., Rosing, J. and Slater, E. C., unpublished observations).

As a result, previous values for ATP and ADP bound to the mitochondrial ATPase [2] are too high, and should be decreased to about 2 mol of ATP and 1 of ADP per mol ATPase. This is more in line with the values obtained by other workers [6, 7], and also in the chloroplast system [4] (where calibration was based on chlorophyll concentration).

FCCP was kindly donated by Dr. P. Heytler. DCCD was obtained from British Drug Houses. Other chemicals were obtained as previously [4, 8].

## RESULTS

### *Bound nucleotides on the mitochondrial ATPase*

It has previously been shown that the bound nucleotides of the isolated mitochondrial ATPase are not adventitiously trapped by the protein during its isolation [26]. The same is true for the chloroplast [4] and *Paracoccus denitrificans* [8] ATPases, which are prepared in the absence of ATP.

This is confirmed by work on the membrane-bound ATPase. Table I shows that beef heart mitochondria (Type I, stored frozen, and thawed before assay) contain ATP and ADP which must be shielded from the action of the mitochondrial ATPase and myokinase, although most of the internal nucleotide pool is AMP. This finding is in agreement with the results of Mansurova et al. [27]. The ATP and ADP 'copurifies' with the coupling ATPase, i.e. the ATP/protein ratio increases with the coupling ATPase content of the system.

The results in Table II eliminate the possibility that the nucleotides are trapped inside submitochondrial particles rather than bound to protein. The nucleotide content of the particles is little affected by further sonication of $Mg^{2+}$-ATP particles or

TABLE I

BOUND NUCLEOTIDES ON FREE AND MEMBRANE-BOUND ATPase IN BEEF HEART MITOCHONDRIA

Type I mitochondria were stored frozen and thawed before use. Washed $Mg^{2+}$-ATP particles and washed coupling ATPase were prepared as described (above, [2]). ATPase-deficient particles were prepared by washing $Mg^{2+}$-ATP particles with a medium containing 2 M urea [28]. Nucleotides were assayed on neutralised perchlorate extracts of the protein [2].

| | nmol/mg protein | | | mol/mol |
|---|---|---|---|---|
| | ATP | ADP | AMP | ATP/ATPase |
| Mitochondria | 0.60 | 0.63 | 3.4 | — |
| $Mg^{2+}$-ATP particles | 0.90 | 0.60 | 0.2 | 2.2* |
| ATPase-deficient particles | 0.16 | 0.34 | 0.2 | — |
| Isolated ATPase | 5.9 | 3.7 | 0.0 | 2.2 |

* assuming 0.42 nmol ATPase/mg protein [12].

TABLE II

BOUND NUCLEOTIDES ON SUBMITOCHONDRIAL PARTICLES

$Mg^{2+}$ and $Mg^{2+}$-ATP particles were washed 4 times by centrifugation. Resonicated particles were prepared by sonicating washed $Mg^{2+}$-ATP particles in the presence of $Mg^{2+}$ only, followed by centrifugation. Sephadex-treated particles were prepared from washed $Mg^{2+}$-ATP particles as described in [28].

| | nmol/mg protein | | |
|---|---|---|---|
| | ATP | ADP | AMP |
| $Mg^{2+}$-ATP particles | 0.85 | 0.60 | 0.24 |
| $Mg^{2+}$ particles | 0.82 | 0.53 | 0.06 |
| $Mg^{2+}$-ATP particles resonicated | 0.94 | 0.35 | 0.12 |
| $Mg^{2+}$-ATP particles filtered through Sephadex | 0.66 | 0.41 | 0.12 |

preparation of $Mg^{2+}$ particles (see Methods) in the absence of ATP. Passage of the particles through Sephadex [28], although removing the ATPase inhibitor protein, does not remove the bound nucleotides.

Treatment of submitochondrial particles with detergent, prior to deproteinisation with perchloric acid, releases no more nucleotide than acid treatment alone (not shown).

The results in Table I suggest that these nucleotides are not bound to the translocase protein since they purify with the ATPase. Further evidence against this possibility is summarised elsewhere [29].

The bound nucleotides are not only inaccessible to the action of mitochondrial ATPase or myokinase (above), but also to luciferase added to the particle suspension (Table III). No release of ATP is seen in this system, even when oxidisable substrate is added. If release of bound ATP is linked to ADP binding, however, it would not be detected in this system. It is not possible to investigate this hypothesis, since addition of ADP to the assay mixture leads to the production of ATP via myokinase.

*Energised exchange of bound nucleotides in the mitochondrial system*

Table IV shows that bound nucleotides on washed submitochondrial particles exchange with ATP in solution. Maximum exchange occurs when the coupled ATPase

TABLE III

AVAILABILITY OF THE BOUND NUCLEOTIDES TO LUCIFERASE

To a solution containing luciferase were added, first 0.2 mg washed $Mg^{2+}$-ATP particles, followed by 20 $\mu$l of 2 $\mu$M ATP as an internal standard. Experimental conditions were as described in Table IV of the accompanying paper [8].

| Addition | counts in 6 s | free ATP | bound ATP* |
|---|---|---|---|
| None | 147 | — | — |
| Submitochondrial particles (0.2 mg) | 252 | 2 pmol | 240 pmol |
| 40 pmol ATP | 2600 | 40 pmol | 240 pmol |

* Estimated value.

TABLE IV

EXCHANGE OF BOUND NUCLEOTIDES IN SUBMITOCHONDRIAL PARTICLES DURING ATP HYDROLYSIS

About 6 mg of washed $Mg^{2+}$-ATP particles (from Type II mitochondria) were incubated at 30 °C in 3 ml of a solution containing 200 μmol triethanolamine hydrochloride, 120 μmol KCl, 120 μmol sucrose, 9 μmol $MgCl_2$, 3 μmol EDTA, 30 μmol phospho*enol*pyruvate 30 U (μmol/min) pyruvate kinase (ammonia-free) and 0.5 μmol [$^3$H]ATP (3000 cpm/nmol) at pH 7.5. Other additions were made as indicated. After 5 min, the suspension was diluted with cold sucrose solution (250 mM) containing $MgCl_2$ (10 mM), and the particles centrifuged down at 0 °C. The particles were then washed by repeated centrifugation until no counts appeared in the supernatant (routinely 4 times) and the bound nucleotides checked for radioactivity as described.

| Addition | [$^3$H]AdN bound (mol/mol ATPase) | Total ATP bound | Total ADP bound |
|---|---|---|---|
| None | 1.57 | 2.5 | 1.2 |
| $S_{13}$ (6 nmol/mg protein) | 0.59 | 2.0 | 1.6 |
| Oligomycin (40 μg/mg protein) | 0.56 | 2.2 | 1.1 |
| None (Heated particles)* | <0.05 | <0.1 | — |

* For 15 min at 100 °C.

is operating. If the ATPase is inhibited by oligomycin, or energisation prevented (without inhibiting the ATPase) by an uncoupler, the exchange is much reduced without any appreciable effect on the ATP+ADP content of the particles. Oligomycin and uncoupler together have the same effect as oligomycin alone (not shown).

The additional exchange occurring during operation of the coupled ATPase ('energised' exchange) is a measure of the involvement of the bound nucleotides in the operation of the ATPase on a coupled membrane. Unlike in chloroplasts [4], complete exchange of the bound nucleotides does not occur, probably because some of the particles present in the preparation are uncoupled. In Table IV and all further tables, the nucleotide content of membranes is expressed as mol/mol ATPase (cf. Table I), so that the stoichiometry of the processes can be easily seen.

Uncoupled ATPase activity on membranes therefore does not involve the bound nucleotides. Similarly, the nucleotides of the isolated ATPase, which is always uncoupled, do not participate in its ATPase activity [2]. With increasingly intact systems, we would expect increasing 'exchange activity' of the bound nucleotides, and indeed, considering $^{32}P_i$ incorporation into the bound nucleotides for example, less than 0.01 mol $^{32}P_i$ can be incorporated on the isolated enzyme, 0.2–0.3 mol $^{32}P_i$ on submitochondrial particles and 1.6–1.9 mol $^{32}P_i$ in mitochondria (in all cases per mol ATPase), even over a long incubation (> 2 h).

*Concentration dependence of the exchange of bound nucleotides*

In these experiments, the rate of bound nucleotide exchange is not measured directly. Exchange of bound for free nucleotides once, or any number of times greater than one, will give the same observable result (see ref. 8). The exchange process would therefore not be expected to show Michaelis-Menton dependence on substrate concentration.

Fig. 1 shows the dependence of 'energised' (oligomycin-sensitive) and 'non-

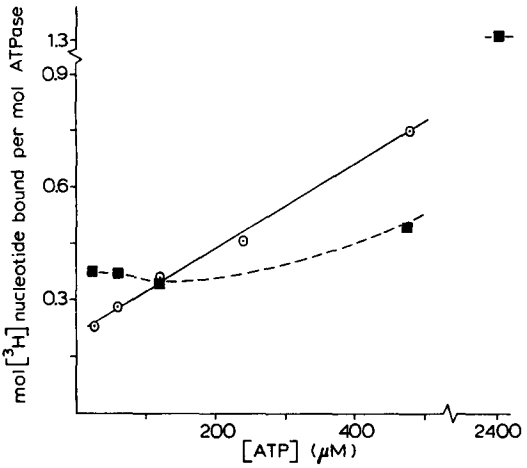


Fig. 1. Concentration dependence of exchange of the bound nucleotides of submitochondrial particles. The exchange was measured against [$^3$H] ATP as in Table IV, except that varying concentrations of ATP were used. Exchange at each concentration was measured in the presence and absence of 40 $\mu$g oligomycin per mg protein. ⊙—⊙, 'non-energised' exchange = exchange in the presence of oligomycin. ■---■, 'energised' exchange = exchange in the absence of oligomycin – exchange in the presence of oligomycin.

energised' (oligomycin-insensitive) exchange on the concentration of added $Mg^{2+}$-ATP. The concentration was varied from 24 $\mu$M to 2.4 mM, but were kept constant in each individual incubation using a regenerating system. The energised exchange is approximately constant over the region 24 to 600 $\mu$M, while the $K_m$ for the ATPase is about 300 $\mu$M [30]. This suggests that even at low concentrations of ATP, 5 min is sufficient time to allow at least one exchange reaction at every energisable ATPase. This is clearly not the case for the oligomycin-insensitive exchange, which increases linearly with concentration over this range. The non-energised exchange, therefore, seems to be much slower than the energised exchange. This is confirmed by experiments to measure the time dependence of this exchange. These are hampered in that no good stopping procedure is available, but we find that the non-energised exchange does increase by about 50 % over a 1.5 h incubation. The mechanism of the non-energised exchange, and why it is higher on the membrane-bound than the isolated ATPase, is unknown.

The increase in energised exchange at high $Mg^{2+}$-ATP concentrations ($\geqslant$ 1 mM) is probably due to the release of ATPase inhibitor protein from these particles at high substrate concentrations (see below). This explanation is consistent with kinetic studies on the ATPase [30].

*Effect of the ATPase inhibitor protein*

Although the exchange was performed in fairly high salt concentrations (150 mM) it was still affected by the ATPase inhibitor protein [20, 28]. Higher exchanges were routinely seen on particles prepared from Type II mitochondria as compared to Type I. A more direct comparison can be made between Type I particles prepared normally, and Type I particles from the same batch depleted of ATPase inhibitor

TABLE V

EXCHANGE ON INHIBITOR-RICH AND INHIBITOR-DEPLETED SUBMITOCHONDRIAL PARTICLES

60 mg of once-washed Mg-ATP particles were prepared from Type I mitochondria. 30 mg were washed by two further spins through sucrose (250 mM), $MgCl_2$ (15 mM). 30 mg were depleted of inhibitor protein by centrifuging down under energised conditions [22], followed by a single wash in sucrose (250 mM), $MgCl_2$ (15 mM). The uncoupler-stimulated ATPase of these latter particles was about twice that of the inhibitor-rich particles, showing that some of the inhibitor had been removed. The total nucleotide content of both types of particle was the same, in agreement with ref. 29. The bound nucleotide exchange of both sets of particles was measured as in Table IV, with additions as indicated. Results are expressed in mol [$^3$H]AdN exchanged per mol ATPase.

| Addition | Inhibitor-rich particles | | Inhibitor-depleted particles | |
|---|---|---|---|---|
| | Total | Energised | Total | Energised |
| None | 0.88 | 0.54 | 1.12 | 0.80 |
| NADH (1 mM) | 0.91 | 0.57 | 0.93 | 0.61 |
| Oligomycin (40 $\mu$g/mg protein) | 0.34 | 0 | 0.32 | 0 |

protein by the method of van de Stadt et al. [22]. Table V shows that removal of the inhibitor protein increases the 'energised' exchange while leaving the oligomycin-insensitive exchange unchanged.

In the presence of NADH, inhibitor-depleted and inhibitor-rich particles give the same level of exchange (Table V), in keeping with the view that energisation by oxidisable substrate can release the ATPase from its inhibition by the inhibitor

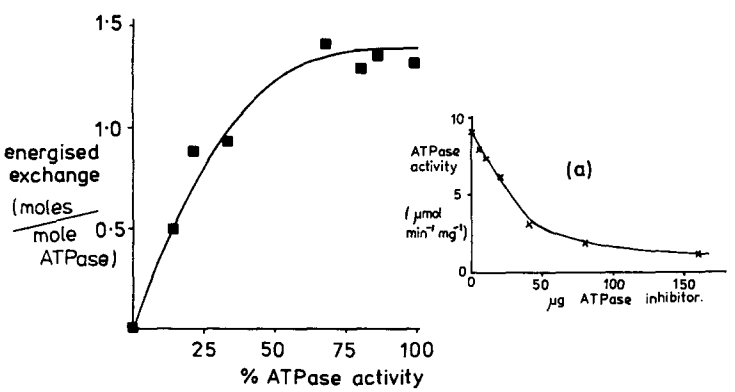


Fig. 2. Dependence of exchange on the amount of ATPase inhibitor present on submitochondrial particles. Submitochondrial particles prepared from Type II mitochondria were combined with different amounts of ATPase inhibitor as in ref. 22. The uncoupled ATPase activity was measured in the presence of an ATP regenerating system [2], in a buffer containing 250 mM sucrose, 20 mM Tris-TES, 3 mM $MgCl_2$, 1 mM EDTA at pH 7.1 (so as to avoid dissociation of the inhibitor from the particles). 1 $\mu$M FCCP was also present. The bound nucleotide exchange was measured, in the same buffer (without FCCP), essentially as described in Table IV. The 'energised' exchange was calculated as in Fig. 1. The point at zero ATPase activity was obtained in the presence of 20 nmol DCCD per mg membrane protein. The insert (a) shows the decrease in ATPase of the particles with the addition of inhibitor protein. 8 mg submitochondrial particles were used in each incubation.

protein [22]. It is found, surprisingly, that the exchange level in inhibitor-depleted particles falls to that of inhibitor-rich particles, rather than the latter increasing. The reason for this is uncertain, but it may be due to an inhibitor of energised exchange present in the NADH preparation.

The effects of addition of the ATPase inhibitor protein to inhibitor-depleted (Type II) particles are shown in Fig. 2. The exchange was measured at low ionic strength to avoid any salt-induced removal of inhibitor. Similar results were obtained with Type I particles stripped of the inhibitor protein as in Table V. The exchange is not directly proportional to ATPase activity but shows a hyperbolic dependence on it. Indeed, 30 % of the ATPase activity can be removed without any effect on the exchange reaction. This might suggest that energy can be transferred between ATPase molecules, as discussed later.

*Effect of other inhibitors on energised exchange*

Table VI shows the effect of other inhibitors of oxidative phosphorylation on the exchange of bound nucleotides with added ATP. Like $S_{13}$, other uncouplers such as FCCP reduce the exchange. An inhibitor of electron transfer, antimycin, as expected, has no effect.

Aurovertin, at concentrations that inhibit ATP-driven reversal of electron transport by more than 95 % (not shown), does not inhibit the exchange but, unexpectedly stimulates it. One might not expect aurovertin to inhibit exchange since it is a reversible inhibitor of the ATPase. Since only one ATPase turnover is necessary for exchange, it could be that aurovertin spends sufficient time off the ATPase to allow each molecule to turn over at least once and thus does not inhibit the exchange.

TABLE VI

EFFECT OF PHOSPHORYLATION INHIBITORS ON 'ENERGISED' EXCHANGE OF THE BOUND NUCLEOTIDES

Exchange was measured on washed submitochondrial particles from Type II mitochondria as in Table IV. Additions were made to the exchange medium as indicated. Percent energised exchange = [$^3$H]AdN bound $-$[$^3$H]AdN bound during incubation with oligomycin*/[$^3$H]AdN bound in control without inhibitor $-$ [$^3$H]AdN bound during incubation with oligomycin* $\times 100$

Oligomycin concentration: = 40 $\mu$g oligomycin/mg protein. The inhibition of the exchange by oligomycin has a $K_{\frac{1}{2}} \approx 0.4$ $\mu$g/mg protein according to this table.

| Addition | Percent energised exchange | |
|---|---|---|
| | No substrate | +1 mM NADH |
| None | 100 | 85 |
| $S_{13}$ (6 nmol/mg) | 6 | — |
| Aurovertin (80 $\mu$M) | 188 | — |
| Antimycin A (20 $\mu$g/mg) | 107 | — |
| DCCD (20 nmol/mg) | 0 | 9 |
| Venturicidin (2 $\mu$g/mg) | 15 | 49 |
| Oligomycin (40 $\mu$g/mg) | 0 | −5 |
| Oligomycin (10 $\mu$g/mg) | 17 | 5 |
| Oligomycin (1 $\mu$g/mg) | 33 | 29 |
| Oligomycin (0.1 $\mu$g/mg) | 78 | 77 |

The stimulation is more difficult to interpret but may be because aurovertin can displace the ATPase inhibitor from its inhibitory site [31], replacing a slowly reversible inhibitor of the ATPase (which does inhibit the exchange reaction) with a rapidly reversible one (ineffective on the exchange). Aurovertin does not affect the exchange of bound nucleotides on the isolated ATPase [29]. The stimulatory effect of aurovertin is also found in *P. denitrificans* membranes.

DCCD and oligomycin inhibit exchange both in the presence and absence of NADH, and appear to have a different mode of action from venturicidin, which is much less effective in the presence of NADH (Table VI). This is not simply due to the relative effectiveness of the two inhibitors at a single concentration, since oligomycin inhibits ATP and NADH-driven exchanges equally at all concentrations tested.

## DISCUSSION

### *Bound nucleotides as probes of the coupling ATPase*

Membrane-bound mitochondrial ATPase, like the isolated ATPase [2], has very tight binding sites for ATP and ADP. It is the only major protein of these membranes that binds nucleotides so tightly. Thus virtually all nucleotides associated with washed submitochondrial particles are bound to the ATPase.

These nucleotides exchange only slowly with added nucleotides when the ATPase is not energised, but on energisation the exchange rate is increased. This is directly comparable to the situation in chloroplasts [4] except that whereas untreated chloroplasts can be energised only by electron transfer, submitochondrial particles can be energised in addition by the hydrolysis of ATP.

Exchange of bound nucleotides is inhibited by preventing turnover of the ATPase, by addition of oligomycin or DCCD, even if NADH ($+O_2$) is present, showing that ATPase turnover is necessary for exchange and not simply energisation of the membrane. Moreover, preventing energisation without inhibiting ATPase turnover, by uncouplers, also inhibits exchange of the bound nucleotides. Thus exchange requires both energisation and ATPase turnover, indicating that during coupled ATPase activity, the coupling ATPase takes up (a) different conformation(s) from that present during uncoupled ATPase activity. We would suggest that, during coupled ATPase activity, both the 'tight' and 'ATPase' nucleotide binding sites come into play, while during uncoupled activity, only the 'ATPase' site is involved [2, 8, 9]. Further, care should be taken when comparing parameters measured on the uncoupled (or isolated) ATPase with those obtained during phosphorylation or coupled ATPase activity [cf. 32, 33].

As the bound nucleotides are bound specifically to the coupling ATPase, their exchangeability provides a measure of the energisation of the individual ATPase molecules rather than the membrane as a whole. The results obtained with the ATPase inhibitor protein are particularly interesting. This inhibitor acts effectively irreversibly on the ATPase (i.e. it does not exchange significantly between ATPase molecules over the 5 min incubation used here) as is shown by its inhibitory effect on the exchange of the bound nucleotides (Table V, Fig. 2). Other studies suggest that the ATPase inhibitor does not exchange between ATPases during ATP-driven reactions (e.g. the extensive studies of Ernster et al. reviewed in ref. 34) and this has been investigated further in our laboratory (Ferguson, S. J., Harris, D. A. and Radda, G. K., in

the press). The effect of the inhibitor protein can be contrasted with that of aurovertin, a reversible inhibitor of the ATPase, which does not inhibit the exchange reaction (Table VI) while it reduces coupled ATPase activity by more than 95 % at the concentration used here.

The ATPase inhibitor seems to bind equally to coupled and uncoupled ATPase molecules. This has been demonstrated by Ernster et al. [34] who show that coupled ATP-utilising reactions titrate with the inhibitor protein identically to the uncoupled ATPase activity. In this case, it is somewhat surprising that the ATPase activity of submitochondrial particles can be considerably inhibited by this protein without much change in the exchange of the bound nucleotides (Fig. 2). If the exchange were prevented at a given ATPase molecule by binding the inhibitor, we would expect it to fall linearly with drop in ATPase activity.

This is explained if energy can be transferred between one ATPase molecule and another. In this case, an ATPase molecule with an inhibitor bound to it could be energised by ATPase activity at an (uninhibited) ATPase molecule on the same particle. This energisation could lead to an exchange of bound nucleotide on the 'inhibited' ATPase molecule, and at high levels of energisation over the particle, inhibition of exchange by the inhibitor protein would lag behind inhibition of ATPase activity.

This argument is valid independently of the exact mechanism of the exchange. It is probable that exchange occurs without ATP passing through the 'ATPase site' on the inhibited molecules, since a given inhibitor seems to remain bound to a given ATPase during ATP-driven reactions (above). Even if exchange occurs via the ATPase site, however, with some shift of inhibitor occurring on one ATPase molecule at high levels of energisation, the results of Fig. 2 require that energy can be transferred from one ATPase molecule to another on the membrane. These results thus demonstrate that a mobile 'high energy intermediate' can be generated by ATPase activity and can affect ATPase conformation on submitochondrial particles, at least at high levels of energisation.

*The mode of action of inhibitors of phosphorylation*

We have seen that, to inhibit the exchange reactions, inhibitors must act effectively irreversibly, i.e. over the time the exchange is measured, that they must not allow the ATPase molecule to turn over (in a coupled way) even once.

This is apparently true for uncouplers (once an ATPase is uncoupled, it remains so) oligomycin and DCCD, and for the ATPase inhibitor protein, although the sites of inhibition by these inhibitors varies. Aurovertin (above) and dio-9 in chloroplasts [4] both act directly on the ATPase [35, 36] but do not inhibit the exchange reactions. It is probable that these inhibitors are reversible enough to allow the ATPase to turn over occasionally during their application.

Venturicidin (mitochondria and *P. denitrificans*) and phlorizin (chloroplasts, [13, 17]) are two other 'energy transfer' inhibitors which do not bind to the ATPase but inhibit phosphorylation. Neither inhibit the exchange of the bound nucleotides induced by electron transfer very effectively while venturicidin does inhibit exchange induced by ATP hydrolysis. It is possible that their poor inhibition is due to their mode of action being identical to that of oligomycin and DCCD but their reversibility being greater (and differing on membranes energised by ATP or by electron transfer in

the case of venturicidin). Alternatively, their action may be irreversible and intrinsically different from oligomycin. It has been suggested by Griffiths et al. [37] that venturicidin and oligomycin have different sites of action.

*Bound nucleotides and phosphorylation*

A mechanism of phosphorylation has previously been proposed where energy is used, not in ATP synthesis but in ATP release from the membrane (see ref. 8). These results show that, in oxidative as well as in photophosphorylation, the bound nucleotides become less tightly bound on energisation of the membrane, consistent with this view.

Further support for this hypothesis comes from the findings in chloroplasts that unlabelled bound ATP is released from the membrane before newly phosphorylated ATP [38], and that the response of the bound nucleotides to energisation are faster than is phosphorylation [16]. The simple model of ref. 4, however, must be modified to take into account a further finding, that ADP is necessary for ATP release from the membrane (above, refs. 17, 38). This suggests that ATP release from the membrane may be linked to ADP binding (cf. also ref. 16), which in turn implies a multi-site model for phosphorylation.

Other workers have suggested a two site mechanism for phosphorylation [11, 14, 39] in which the two sites remain distinct and each has a different role. We

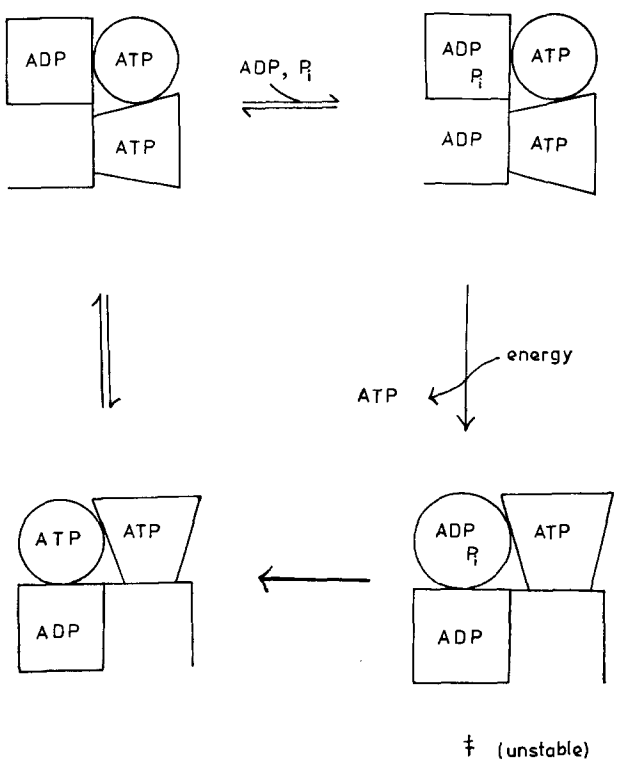


Fig. 3. Four-site model for ATP synthesis on a coupling ATPase (for explanation, see text).

find no evidence for a separation of roles between the nucleotide binding sites during phosphorylation and suggest that all four sites are equivalent and pass through the same conformations during phosphorylation.

A model in which all the bound nucleotides and the 'ATPase' site are involved in phosphorylation is given in Fig. 3. This is similar in concept but not in sequence, to one of the models proposed by Boyer et al. [16]. ADP binds to one site, ATP is lost from a second and ADP is phosphorylated at a third. All four sites are equivalent and pass through each of four states in turn. The thermodynamics of such a system, which may be termed a rotating wheel model, have been discussed by Wyman [40]. This model is one of several which accounts for these results (cf. ref. 29). It does, however set limits on the involvement of the various nucleotide binding sites. First, it is not compatible with the view that the (uncoupled) ATPase site alone is responsible for both phosphorylation and ATPase activity. Second, it is not compatible with the view that the ATPase and phosphorylation sites are completely distinct [32].

An alternative, but similar model involves only two of the nucleotide binding sites in phosphorylation with only these two, equivalent, sites alternating between conformations. (Smith, T. D. and Boyer, P. D., personal communication.) The data above cannot rule out this model but we would suggest that all four nucleotide sites are involved in phosphorylation because

(i) in chloroplasts all the bound nucleotides are exchangeable [4].

(ii) although in oxidative systems not all the bound nucleotides do exchange in vitro, better coupled membranes show a higher amount of exchange (above, [8]). It is postulated that in completely coupled membranes all the nucleotides would be exchangeable (see also accompanying paper, ref. 8).

In summary, therefore, we find that the coupling ATPases of oxidative phosphorylating systems bear bound ATP and ADP, and these nucleotides respond to energisation, either by ATP or by oxidisable substrate, in a way consistent with their involvement in phosphorylation. A similar mechanism operates in chloroplasts [4]. The exact sequence of events on the ATPase awaits accurate rate studies, but a mechanism in which ADP binding and ATP release are linked seems likely. It is probable that the bound nucleotides and the 'ATPase' site of the enzyme become equivalent during phosphorylation.

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