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THE ATPase INHIBITOR PROTEIN IN OXIDATIVE PHOSPHORYLATION

THE RATE-LIMITING FACTOR TO PHOSPHORYLATION IN SUBMITOCHONDRIAL PARTICLES

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Summary

1. Purified luciferase and luciferin were used to study the time course of phosphorylation in submitochondrial particles. The light emitted was detected by a single-photon counter, using a multichannel analyser, and the results were analysed by an 'on-line' digital computer.

2. Using NADH as substrate, phosphorylation showed, in general, four phases. These were (i) a period of increasing rate ('lag'); (ii) a period of constant (positive) rate; (iii) a period of zero net rate (plateau), when the phosphorylation potential was maintained at its equilibrium value, and (iv) a period of negative rate (ATP hydrolysis) after all the oxygen had been consumed.

3. The lag phase, several seconds in length, was a function of the inhibitor protein content of the particles. It was decreased in particles treated to remove the inhibitor protein, either by prior energisation of the particles with NADH, or by addition of aurovertin, which competes with the inhibitor protein for the ATPase. It was concluded that the ATPase inhibitor protein inhibits both ATP synthesis and hydrolysis by the ATPase.

4. The rate constant for the release of the inhibitor protein from the energised membrane was determined from the time course of ATP production during the lag phase. The activation energy of this process was measured from the temperature dependence of the lag, and was shown to be 13.3 kcal/mol, lower than the activation energy of ATP synthesis or NADH oxidation.

5. The rate constant for inhibitor release was dependent on 'energisation' of

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Abbreviations: FCCP, carbonyl dyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

the membrane, being lower in the presence of uncouplers. However, it was possible to decrease the rate constant considerably with agents that collapsed the membrane potential without uncoupling the membrane. It was concluded that the inhibitor protein responded to the membrane potential component of the energisation.

6. A kinetic model for energy-dependent dissociation of the ATPase-inhibitor complex is proposed.

Introduction

The ATPase inhibitor protein, first isolated from mitochondria by Pullman and Monroy [1], is a small, heat-stable protein (molecular weight 10 000) which inhibits ATP hydrolysis by the coupling ATPase. On the membrane-bound ATPase it also inhibits all ATP-driven coupled processes [2,3].

The effect of the inhibitor protein on phosphorylation is less clear. The inhibitor protein is not necessary for phosphorylation, nor does it inhibit phosphorylation when measured over a 5 min period [1] (see below). On thermodynamic grounds it is unlikely that the inhibitor protein can inhibit the back reaction of phosphorylation (ATP hydrolysis) and not the forward reaction. The solution to this paradox presumably lies in the finding that the inhibitor is released from its inhibitory site on the ATPase under energised conditions [4,5], e.g. when phosphorylation is occurring.

We have measured phosphorylation in submitochondrial particles during the first few seconds after the initiation of oxidation. This was made possible by using a stopped-flow mixing system and by continuous monitoring of ATP production using purified luciferin and luciferase. We show that the release of the inhibitor protein is limiting to phosphorylation and that, as has been shown previously in chloroplasts [6], the ATPase inhibitor protein does indeed inhibit phosphorylation.

Using particles containing different amounts of inhibitor protein, we demonstrate the existence of an equilibrium between 'free' and bound inhibitor protein, the position of which depends on the energisation of the membrane and, in particular, the membrane potential component of the 'proton motive force' [7]. A quantitative model is proposed to describe the ATPase-inhibitor interaction during the period of net phosphorylation.

Materials and Methods

Submitochondrial particles with a low inhibitor content were prepared by the method of Low and Vallin [8] from 'type II' mitochondria [3]. ATPase inhibitor was prepared by the method of Nelson et al. [9]. Inhibitor-supplemented submitochondrial particles were prepared according to Horstman and Racker [10]. Inhibitor-depleted particles were prepared by incubating 1—2 mg particles, as prepared, in buffer I (200 mM sucrose, 20 mM Hepes, 5 mM sodium phosphate, 2 mM $MgCl_2$, pH 7.5 with NaOH) with 100 μM NADH for about 10 min, i.e. until all the NADH had been used up and the particles become de-energised again (cf. Ref. 11). Under these conditions, (no ATP,

high pH) the ATPase inhibitor does not recombine with the ATPase after its release in the time intervals used here.

ATP was measured using the luciferin/luciferase system, essentially as described by Lemasters and Hackenbrock [12]. The sensitivity of the method was increased by using purified luciferase [13] and synthetic luciferin (Sigma biochemicals). Under these conditions, the light produced is proportional to ATP concentration over a wide range, and the time-dependent 'decay' of the light produced, seen with unpurified luciferase, is virtually eliminated [14].

ATP hydrolysis was measured as previously [3].

Luminescence was followed by single-photon counting, using an ORTEC multichannel analyser model 6220 to collect and store the data. Counts were corrected for double-photon events, and analysed by a PDP 11/05 computer.

Theoretical treatment

Our model for the ATPase-inhibitor (E-I) interaction is as follows:

1. The interaction is represented by the equilibrium [6]

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \tag{1}$$

2. The value of $K_s = k_1/k_{-1}$ depends on the state of energisation of the membrane [4–6], since k_{-1} is energy dependent.

3. The energisation of the membrane is very rapid compared to k_{-1} (see below).

4. Thus, after energisation, the equilibrium (Eqn. 1) relaxes to a new position where the final concentration of (free) E is $\bar{e}$, and of I is $\bar{i}$. From Eqn. 1 the progress of the dissociation is given by

$$e = \bar{e}[1 - (1 - e_0/\bar{e})\exp(-t/\tau)] \tag{2}$$

where e, the concentration of (free) E at time t;
e_0, the initial concentration of E;
$\bar{e}$, the equilibrium concentration of E;
i_0, the initial concentration of I is zero;
τ, the relaxation time of the process.

In general, $\tau = (k_{-1} + (\bar{e} + \bar{i})k_1)^{-1}$. However, in this sytem, when $t \to 0$, the rate of association will be very low (free [1] low, [ATP] low) and k_1 can be neglected. Thus $\tau(t = 0) = 1/k_{-1}$, as for a simple unimolecular reaction.

5. It is not possible to see the EI dissociation directly. It is monitored by measuring ATP production. Assuming excess P_i to be present, we assume

$$\frac{d[ATP]}{dt} = k_2[ADP]e \tag{3}$$

i.e. only the non-inhibited ATPase makes ATP. Substituting Eqn. (2) in (3)

$$\frac{d[ATP]}{dt} = k_2 ADP\,\bar{e}\,[1 - (1 - e_0/\bar{e})\exp(-t/\tau)]$$

Integrating, we have

$$[\mathrm{ATP}] = \underbrace{([\mathrm{ATP}]_{t=0} + k_2[\mathrm{ADP}]\bar{e}[t - (1 - e_0/\bar{e})\tau])}_{A(t)} + \underbrace{(k_2[\mathrm{ADP}]\bar{e}(1 - e_0/\bar{e})\tau \exp(-t/\tau))}_{B(t)} \quad (4)$$

The linear term in Eqn. (4), $A(t)$ shows the situation when $t > \tau$. The exponential term $B(t)$ then vanishes. ATP is then produced at a constant rate, $k_2[\mathrm{ADP}]\bar{e}$, corresponding to phase II (see below). This rate, extrapolated to [ATP] = 0, does not pass through $t = 0$ but is displaced along the time axis to cross it at a positive value of t. This value is referred to below as the 'lag' and, in our model, is given by 'lag' = $(1 - e_0/\bar{e})\tau$. When $e_0 \approx 0$, as in inhibitor-supplemented particles, the lag is equal to τ, the relaxation time.

The exponential term, $B(t)$ represents that quantity of ATP made in the period before the rate becomes linear, i.e. it corrects for the fact that some ATP is formed during the 'lag' period. τ can be calculated from $B(t)$ for any particles (i.e. e_0 need not necessarily ≈ 0). A plot of $\ln B(t)$ against t yields a line, the initial region of which is linear with a slope $-1/\tau = -k_{-1}$.

As stated above, the amount of free E increases to an equilibrium value $\bar{e}$. This implies that, as t increases, the association reaction becomes significant (free [I] increases, [ATP] increases). As this occurs, the slope of $\ln B(t)$ versus t deviates from $-k_{-1}$. An increase in slope is expected. τ is therefore measured close to $t = 0$, i.e. from the initial slope of $\ln B(t)$.

Results

Characterisation of the submitochondrial particles used

Submitochondrial particles with a relatively high ATPase activity, about 3 μmol · min^{-1} · mg^{-1} protein, can be prepared from mitochondria, themselves prepared in the presence of succinate throughout (type II mitochondria) [3]. Apparently some ATPase inhibitor protein is lost during preparation and/or storage of the mitochondria. An estimate of the amount of inhibitor lost can be made by comparing this ATPase activity with that of 'AS' particles, which are completely stripped of inhibitor protein by Sephadex treatment [10]. This latter ATPase activity is between 9.5 and 11 μmol · min^{-1} · mg^{-1} [15]. Thus the particles used here, designated 'untreated particles' below, probably have about 70% of their ATPase still inhibited.

Treatment of these particles with NADH at pH 7.5 gives particles with a stimulated ATPase activity, 6—7 μmol · min^{-1} · mg^{-1} protein [11]. This increase (which persists even after all the NADH is used up) results from the loss or displacement of the inhibitor protein from its inhibitory site on the ATPase. The NADH-pretreated particles are referred to as 'inhibitor-depleted particles'.

Conversely, particles with a very low ATPase activity can be prepared by incubating the untreated particles with MgATP (500 μM) and excess inhibitor protein in low-salt buffer at pH 6.5 [10]. After excess free inhibitor, and ATP, had been removed by centrifugation, these particles showed an ATPase activity of 0.3 μmol · min^{-1} · mg^{-1}. Nearly all the ATPase (probably greater than 95%) is thus complexed with the inhibitor protein. These particles are referred to as 'inhibitor-supplemented' particles.

Table I shows that, in spite of the large differences in ATPase activity, the

TABLE I

INHIBITOR CONTENT AND COUPLING IN SUBMITOCHONDRIAL PARTICLES

ATP hydrolysis was measured by continuous monitoring of P_i release as previously [3]. 2 μM FCCP was present throughout. Similar results were obtained when hydrolysis was followed using a recording pH meter [4] (not shown). P/O ratios and respiratory control (= rate of NADH oxidation + ADP / rate — ADP) were measured in buffer I as previously [16] using NADH as substrate. The respiratory control ratio with FCCP was 3.8 in both sets of particles. The equilibrium phosphate potential, ΔG_p was calculated from the plateau level (Fig. 1) in the luciferase reaction, assuming AMP production to be negligible under these conditions (low ADP concentration) and thus $ADP_{final} = ADP_{added} - ATP_{final}$. Control experiments in which adenylate kinase activity was measured directly show that such activity would lead to an error (underestimate) in G_p of 0.15 kcal/mol. μ_{H^+} was measured by the flow dialysis technique [17]. Values represent the average of two or three duplicates on the same preparation of particles. n.d., not determined.

Type of submitochondrial particles	Hydrolysis rate (μmol/min per mg)	P/O ratio	Respiratory control	ΔG_p (kcal/mol)	$\Delta\mu_{H^+}$ * (mV)
Untreated	3.93 (±5%)	2.3 (±0.2)	1.45 (±0.05)	11.82 (±0.15)	n.d.
Inhibitor supplemented	0.37	1.9	1.45	11.61	195 (±30)
Inhibitor depleted	6.41	2.0	n.d.	11.77	189

* Kindly measured by Dr. M.C. Sorgato.

three types of particles are equally 'coupled' in the accepted sense of the term. They all show the same P/O ratio, respiratory control ratio with ADP (and with FCCP, not shown), equilibrium phosphate potential ΔG_p, and equilibrium proton motive force, $\Delta\mu_{H^+}$, within experimental error. It was concluded that over the periods of measurement (3—15 min) the inhibitor was not necessary for, nor did it interfere with phosphorylation. Addition of free inhibitor, in five-fold excess over the particles, similarly did not affect the coupling as measured by these parameters (not shown).

Time course of phosphorylation

Phosphorylation over the period 0.5—50 s after the addition of NADH was measured using a rapid mixing device in a stopped-flow arrangement. In the cell used here (volume 5 ml), mixing was complete after 0.5 s. The time constant of the luciferin-luciferase reaction is about 0.2 s [12], and so data were collected over consecutive periods of 0.2 s each.

Phosphorylation, in general, comprised four phases (Fig. 1); phase I of low but increasing rate, or lag phase, of 5—10 s; phase II, characterised by a constant rate of ATP production, of 20—30 s at 30°C at the protein concentration used here; a plateau region, III, where no net ATP production occurs, of 2—3 min; and a phase of net ATP hydrolysis, IV, which occurs when all the oxygen has been consumed.

Fig. 2 shows that the lag phase in ATP production does not result from a lag in NADH oxidation. Oxidation is linear over the period 0.1—15 s. The lag demonstrated in Fig. 1 in 'inhibitor-supplemented' particles disappears in 'inhibitor-depleted' particles (Fig. 2) (and is less in untreated particles, Fig. 4). It was concluded that the lag phase was due to the slow release of the inhibitor protein from the ATPase.

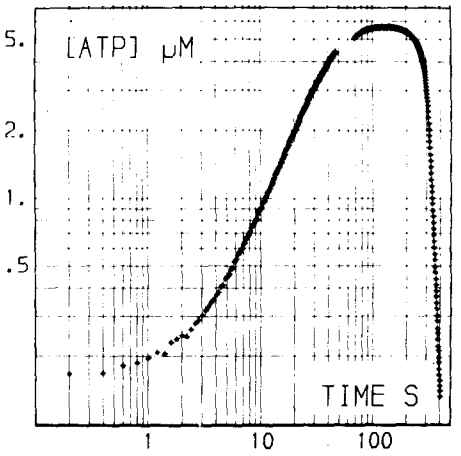


Fig. 1. Time course of ATP production in inhibitor-supplemented particles. The two syringes of the stopped-flow arrangement were each filled with 7 ml 200 mM sucrose, 20 mM Hepes, 5 mM sodium phosphate, 2 mM $MgCl_2$, brought to pH 7.5 with NaOH. To one was added 1—2 mg inhibitor-supplemented submitochondrial particles (see Materials and Methods), 30 μg luciferin, and an aliquot (less than 50 μl) of luciferase, sufficient to yield 10 000 counts in 0.2 s with 1 μM ATP. To the other was added 20 μM ADP and 1 mM NADH. After equilibration at 30°C, the reaction was initiated by manually driving the reagents in to the measuring cell, where they mixed in a 1 : 1 ratio. The time of mixing was 500 ms, and the cell volume 5 ml, the remaining fluid flushing out the cell or remaining in the tubes leading to the cell. The reaction was followed by measurement of luminescence, as described in Materials and Methods. 255 data points were collected on each time scale.

This conclusion was supported by the effect of aurovertin on phosphorylation. In inhibitor-supplemented particles, pretreatment with aurovertin decreases the lag before phosphorylation (Fig. 3). Aurovertin is known to displace the inhibitor protein from the ATPase [18]. The decrease in lag was accompanied by a decrease in steady-state rate of phosphorylation due to the inhibitory action of aurovertin itself (not shown).

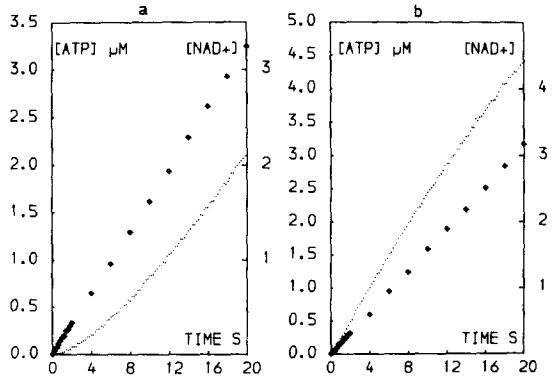
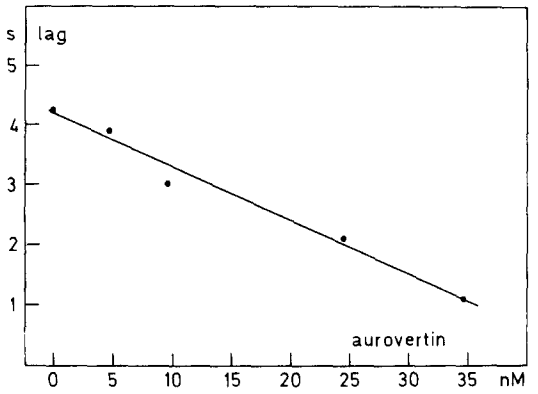


Fig. 2. Phosphorylation and NADH oxidation in submitochondrial particles. Phosphorylation was measured as in Fig. 1, except that inhibitor-depleted particles (see Materials and Methods) (b) replaced inhibitor-supplemented particles (a) as indicated. NADH oxidation was measured spectrophotometrically in an air-driven stopped-flow apparatus (modified from Gibson and Milnes [26]). The path length of the cell was 3 mm. The mixing time was less than 10 ms. Conditions were as in Fig. 1 except that luciferase and luciferin were omitted, the initial NADH concentration was 0.37 mM and the particle concentration was 0.6 mg protein/ml. •, ATP; ▪, NAD^+.



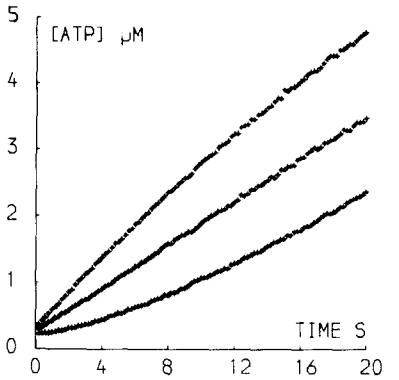


Fig. 3. Effect of aurovertin on release of inhibitor protein. Phosphorylation, in inhibitor-supplemented particles, was measured as in Fig. 1. The lag, as defined in Theoretical treatment, was measured as the displacement of the constant slope region (phase II) from the time of mixing. Varying amounts of aurovertin were added as indicated, to the syringe containing the particles.

Fig. 4. Comparison of phosphorylation rates in particles of different inhibitor content. Phosphorylation was measured as in Fig. 1, except that inhibitor-depleted particles (uppermost curve) or untreated particles (middle curve) replaced inhibitor-supplemented particles (lowest curve) as indicated.

These experiments were carried out at 10 μM ADP (final concentration) where the rate of adenylate kinase activity was negligible (10%) compared with oxidative phosphorylation. Using ADP concentrations between 0 and 20 μM the K_m^{ADP} was found to be 28 μM for phosphorylation under these conditions.

Kinetic parameters of release of the inhibitor protein

During the lag phase of phosphorylation, we consider inhibitor release to be rate limiting, rather than a slow build-up of 'energisation of the membrane' *. This conclusion is based on our experimental finding that inhibitor-deficient particles start to phosphorylate, at maximal rate, within the mixing time (less than 500 ms) (Fig. 2). Thus their membrane is maximally energised within the 500 ms period. Since inhibitor-supplemented particles are equally well 'coupled' by several criteria (Table I), and start to oxidise NADH at their maximal rate immediately (Fig. 2), it is concluded that their membrane also is fully energised within the mixing time. Thus it must be inhibitor release which limits phosphorylation in the lag phase.

As a working model, we consider the lag phase as the relaxation of the ATPase-inhibitor equilibrium to a new equilibrium position under the influence of an (instantaneously) energised membrane. This equilibrium position, indeed, governs the steady-state rate of phosphorylation, phase II. In the steady state, untreated particles phosphorylate faster than inhibitor-supplemented particles (Fig. 4). Inhibitor-depleted particles initially phosphorylate very rapidly, but the rate is quickly reduced to approach the steady-state rate of the parent particles (Fig. 4). This can be explained by a net recombination of displaced

* We use the terms 'energisation of the membrane' or 'energised membrane' in a general sense, meaning any free energy that is available to drive phosphorylation of ADP, irrespective of the mechanism by which the energy is conserved.

inhibitor with the ATPase, as compared to a net dissociation in the supplemented particles (see Discussion).

In its initial stages, the relaxation approximates to a first-order process. Fig. 5 shows a logarithmic plot of $B(t) = [ATP]_t - A(t)$ (see Eqn. 4) versus time. Over the initial region, this plot is linear and the rate constant for the dissociation of the ATPase-inhibitor complex k_{-1} can be measured from the slope (see Theoretical treatment). Its value, at 30°C, is 0.2 s^{-1}.

The inhibitor protein is released more quickly as the temperature is raised. An Arrhenius plot for k_{-1}, determined as above, between 20°C and 40°C is linear (Fig. 6, lower curve), with E_a = 13.3 kcal/mol, as compared to 29 kcal/mol under non-energised conditions [15]. This activation energy is rather lower than for ATP production (14.7 kcal/mol) (Fig. 6, upper curve) or NADH oxidation (17.5 kcal/mol) measured spectrophotometrically (not shown). Both the latter two values obtained on inhibitor-depleted particles where the plots are linear. An Arrhenius plot for phosphorylation by untreated or inhibitor-supplemented particles is curved, apparently because the rate-limiting process

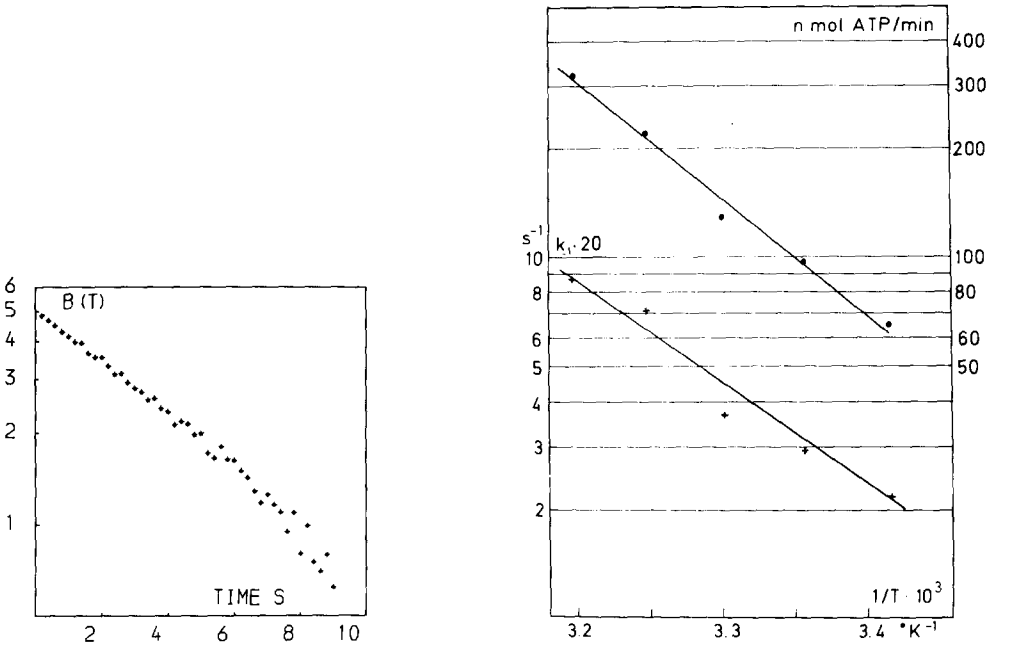


Fig. 5. Progress of relaxation towards the energised ATPase-inhibitor equilibrium. Phosphorylation in inhibitor-supplemented particles, was measured as in Fig. 4. ATP produced during that period when the rate was lower - $A(t)$ above - was subtracted from the total ATP produced, and the resultant 'extra ATP' production plotted logarithmically against t. The values represent $B(t)$ (in relative units) as described in Theoretical treatment, and the slope is $-\tau^{-1}$, the inverse relaxation time of the approach to the steady state. Extrapolation to $t \approx 0$ yields $-\tau^{-1}$ ($t = 0$), which equals $-k_{-1}$.

Fig. 6. Temperature dependence of release of the inhibitor protein. k_{-1}, the rate constant for the inhibitor release was measured between 20°C and 40°C as in Fig. 5 ($\tau = 1/k_{-1}$). Phosphorylation rate, in inhibitor-depleted particles, refers to the initial slope of the time course of ATP synthesis by these particles (Fig. 4). E_a is calculated from the slope of these lines, using the Arrhenius equation; $\ln k = E_a/R(-1/T)$. •———•, phosporylation rate; +———+, inhibitor release.

changes from an unknown process, at low temperature (possibly oxidation), to inhibitor release at high temperature (not shown).

Energisation and the ATPase-inhibitor interaction

The position of the ATPase-inhibitor equilibrium is known to be affected by the 'energisation of the membrane' in submitochondrial particles [4,5]. This is seen, for example, from the increase in the relaxation time, τ, induced by uncoupling the membrane by FCCP, or by freezing and thawing (Table II).

The effect of other reagents on τ is also shown in Table II. While all the reagents used decreased ΔG_p to some extent, it can be seen that at comparable values of ΔG_p, SCN^- and K^+/valinomycin caused a particularly large increase in τ (i.e. decrease in rate of inhibitor release). Under these conditions the membrane potential is decreased. Other reagents, such as methylamine (decreases ΔpH), phenethyl biguanide (blankets the surface charge) and rotenone (decreases the oxidation rate, by 50—70% at this concentration) had much less effect on τ. All these reagents also decreased the rate of phosphorylation (Table II) and, except for rotenone, the P/O ratio (not shown). They can therefore be considered 'uncouplers' to submitochondrial particles, but the mechanism of uncoupling is indirect and different from that of FCCP. This is discussed further below.

None of these reagents, including FCCP, induced a lag in phosphorylation in inhibitor-depleted particles (not shown). Such particles, even when uncoupled by 70—80%, always begin to phosphorylate within the mixing time (less than 500 ms).

TABLE II

EFFECT OF ENERGY STATE ON DISPLACEMENT OF INHIBITOR PROTEIN

The relaxation time, τ, of the ATPase inhibitor release, under energised conditions, was measured in inhibitor-supplemented particles, as in Fig. 6. Various additions were made as indicated. In the case of valinomycin and nigericin, 20 mM KCl was also present and the particles were incubated for 30 min at room temperature with the ionophores + KCl before measurement. (Decreasing KSCN concentration twice, and valinomycin four times, or increasing protein concentration twice, did not affect the results given here). The errors represent a range of three duplicates. n.d., not determined.

Addition	Phosphorylation rate (nmol/min per mg protein)	τ (s)	ΔG_p (kcal/mol)
None	63 ± 4	5.1 ± 0.2	11.4 ± 0.2
FCCP (7 pM)	23	6.6	9.9
Nigericin (2 μg/mg)	35	6.5	10.3
None *	48	7.5	n.d.
KSCN (4 mM)	32	8.5	10.5 ± 0.1
Valinomycin (20 μg/mg)	28	8.7	10.4
Aurovertin (24 nM)	32	2.6	10.3
Methylamine (12 mM)	59	6.1	10.7
Phenethyl biguanide (100 μM)	49	4.9	11.0
Rotenone (0.2 ng/mg) **	46	5.7	11.3

* Particles frozen at −20°C for 24 h, then thawed.

** Respiration inhibited by 50—70% in oxygraph trace.

Discussion

The significance of a 'lag' period in phosphorylation

In agreement with other workers [12,19] we have demonstrated a lag in phosphorylation, of the order of seconds, when phosphorylation is initiated in submitochondrial particles with NADH. These workers showed that the lag disappeared if phosphorylation were initiated with either an acid-base transition, or by addition of ADP to particles already oxidising NADH [12,19]. The significance of this lag was, however, not discussed.

Our results show that the occurrence of this lag is correlated with the presence of the ATPase inhibitor at its inhibitory site (Figs. 2 and 3), and not with either a lag in the oxidation of NADH nor a slow build up of energisation of the membrane (Fig. 2). If the inhibitor is displaced from its inhibitory site (and the particles subsequently de-energised) the lag disappears. This is analogous to the situation in chloroplasts, where a lag observed in phosphorylation [18,19] was also shown to be due to release of the ATPase inhibitor protein [6]. One interesting point is the large difference in rates of inhibitor release in the two systems. τ for submitochondrial particles is about 5 s, while in chloroplasts the process is 2—3 orders of magnitude faster (τ = 5—20 ms) [20].

In two systems at least, therefore, the ATPase inhibitor protein inhibits both phosphorylation and ATP hydrolysis. A corollary of this statement is that the rate of phosphorylation is dependent on the number of activated ATPase molecules in the steady state, and thus not directly on the degree of energisation of the membrane (cf. Refs. 22 and 23). We, and others [6,21], have estimated the number of active ATPase molecules in stead-state-illuminated chloroplasts as about 10%. Starting from 97% inhibited submitochondrial particles, a figure of about 30—40% activated ATPases in the steady state can be estimated from Fig. 4. With only 70% inhibited particles (Table I), the lag preceeding phosphorylation is undetectable, which suggests that these particles start out close to the equilibrium value, with $e_0 \approx \bar{e}$. About the same equilibrium position is reached with 97% inhibited particles, as shown by the rates of the steady-state phosphorylation. Thus these latter must also reach a level of activation of 30—40% of the ATPase molecules.

Kinetics of inhibitor release

For a quantitative analysis, a mathematical treatment of the ATPase inhibitor interaction is proposed. In submitochondrial particles, the interaction is considered to be an equilibrium with an energy-dependent k_{-1} (for inhibitor release) [6] and ATP-dependent k_1 (for combination) [10,15]. Thus, on energisation, the position of the ATPase-inhibitor equilibrium is shifted, and the new equilibrium position is approached with a relaxation time τ.

This model is supported by the experiment of Fig. 4. In inhibitor-supplemented particles, inhibitor release (k_1) is initially dominant and an equilibrium position ($K = k_{-1} = f(\Delta\mu)/k_1 \doteq f([ATP])$, i.e. a constant phosphorylation rate is eventually reached. As untreated particles start off close to this position, the relaxation is hardly observed. More dramatic is the time course in inhibitor-depleted particles. These have been energised in the absence of ADP, and thus

more inhibitor has been displaced than can occur during phosphorylation ($e_0 > \bar{e}$), both because the energy level is somewhat higher in the absence of ADP (state IV) and because $k_1 = f([ATP])$ remains low. Placed under phosphorylation conditions, since the inhibitor is still present in the system, these particles must reach the same equilibrium position as the particles from which they were derived. Thus k_1 becomes dominant and the rate of phosphorylation falls with time (Fig. 4, upper curve) towards this equilibrium value. It should be possible to calculate k_1 from the shape of this curve, but since k_1 is a complex function of [ATP] [15], and [ATP] is changing in time, this calculation was not attempted *.

It is possible to calculate k_{-1}, the rate constant for the dissociation of the inhibitor protein. In general, τ as defined above = $k_{-1} + (\bar{e} + \bar{i})k_{+1}$ for the relaxation. In the initial stages of the dissociation, however, [I] = 0 and [ATP] $\approx$ 0 and thus the ATPase inhibitor combination can be ignored ($k_1 = 0$). Under these conditions $\tau = 1/k_{-1}$ and k_{-1} can be calculated as 0.2 s^{-1} at 30°C. This value is remarkably constant between different particle preparations (not shown).

As t increases, the combination rate becomes more significant. This leads to the 'lag', measured by extrapolation of the equilibrium rate of ATP synthesis to (ATP) = 0 (Fig. 2), being lower than the relaxation time, calculated from the logarithmic plot (Fig. 5). The former naturally includes a component due to reversal of the inhibitor dissociation, while the latter, measured by extrapolation to $t = 0$, does not. Thus, even on inhibitor-supplemented particles (about 97% inhibited), τ is some 10--20% higher than the 'lag' (Fig. 3, Table II). The ATPase-inhibitor recombination is also reflected in the change in slope of the log plot in Fig. 5 as t increases, the recombination rate increases (as ATP rises) and the curve changes slope (more negative).

The model therefore, seems consistent with the data, and the assumptions used in its derivation are justified (see Theoretical treatment). The rate of release of inhibitor protein under energised conditions ($k_{-1} = 0.2$ s^{-1}) is very much faster than the rate of release under non-energised conditons ($k_{-1} = 10^{-4}$ s^{-1}) [16]. This is, in fact, the increase in rate expected for a decrease in activation energy from 29 kcal/mol under non-energised conditions [15] to 13.3 kcal/mol (Fig. 6) under energised conditions. This in turn suggests that the mechanism of inhibitor release is the same in both cases (i.e. A in the equation $k = A \exp(-E_a/RT)$ is a constant).

Agents which alter the time course of phosphorylation

The rates of phosphorylation and inhibitor release can be changed independently. The best example of this occurs with aurovertin, which decreases the affinity of the ATPase for the inhibitor while also inhibiting phosphorylation [18]. This is seen, in our system, by a decrease in the lag (raised k_{-1}) and a decrease in phosphorylation rate (Fig. 3). The biguanides, which may bind to the inhibitor binding site on the ATPase [24] are somewhat similar. They decrease the rate of phosphorylation, but have little effect on τ because they

* We should note that as [ATP] $\rightarrow \infty$, $k_1 \rightarrow$ constant [15]. This justifies our use of $\bar{e}$ as a constant when $t >> \tau$.

can bind only after the inhibitor is released (Table II).

In contrast, reagents that affect membrane energisation would be expected to change the rates of inhibitor release and phosphorylation in the same direction. If k_{-1} is a function of 'energy state', then decreasing energy state will decrease k_{-1} which will simultaneously increase the lag and decrease $\bar{e}/e_{tot}$ (and thus the phosphorylation rate) in the steady state. This is seen both with true uncouplers, such as FCCP, and with agents which affect specifically the membrane potential (Table II) but do not cycle, and thus should not uncouple. In terms of our model, we would say that true uncouplers decrease k_{-1} and k_2, while other compounds affect only k_{-1}, but both affect the steady state of phosphorylation, which is governed both by k_2 and $\bar{e}$.

It is thus confusing to say, with Ort [25] that all these reagents 'uncouple more strongly in the initial stages of phosphorylation than in the steady state'. The lag in phosphorylation, as we have seen, is due to the inhibitor protein and is not observed if this protein is removed by pretreatment. In the presence of inhibitor, some of the ATPase molecules are initially shut down and, in consequence, more energy is dissipated via leaks during this period. 'Uncoupling', during this 'pre-steady-state' period can be carried out by any direct or indirect inhibitor of the ATP synthase, and does not require that the agent is a proton carrier, or that it decreases $\Delta\mu_{H^+}$.

Various reagents were used in order to determine the component of energisatin most involved in inhibitor release. When ΔG_p is titrated to a fairly constant level (10.5 ± 0.2 kcal/mol) we find that SCN^- and K^+/valinomycin, which collapse the membrane potential, have by far the largest effect on τ. We conclude that, as in chloroplasts [6] the displacement of the inhibitor protein is largely dependent on the membrane potential. Under its influence, the inhibitor is considered to move from its site on the ATPase to another site, presumably on the membrane.

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