ATP synthesis and hydrolysis in submitochondrial particles subjected to an acid–base transition

Effects of the ATPase inhibitor protein

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ATP hydrolysis or succinate oxidation by inhibitor-rich submitochondrial particles leads to a 3-fold increase in ATPase activity, with concomitant loss of about 30% of bound inhibitor protein. An acid–base transition causes similar, but smaller, effects (a 30% ATPase increase, and a loss of 8% of the inhibitor). Omitting the electrical component of the gradient completely abolished these effects. The inhibitor protein inhibits ADP phosphorylation induced by an acid–base transition but not by NADH oxidation. This is suggested to reflect the slow movement of the inhibitor protein and the brief period of acid–base jump phosphorylation.

Mitochondria *ATPase inhibitor protein* *Phosphorylation* *ATP hydrolysis* *Acid base transition*

1. INTRODUCTION

The rate of ADP phosphorylation by submitochondrial particles lags behind the oxidation rate, when phosphorylation is driven by NADH oxidation. Thus in particles prepared by sonication with MgATP, a lag of 5–10 s is observed before maximal phosphorylation rates are attained, while maximal oxidation rates are attained without delay [1]. In particles prepared by sonication with EDTA and then recoupled with oligomycin, the lag is somewhat longer [2].

During this lag period, the ATPase inhibitor protein is displaced (presumably electrophoretically) from its inhibitory site on the F_1-ATPase, under the influence of the membrane potential. The lag is abolished if the inhibitor protein is removed from the F_1-ATPase prior to the onset of phosphorylation [1,2]. Furthermore, energisation of submitochondrial particles leads to an activation of the hydrolytic capacity of F_1, correlated with loss of inhibitor protein from the membranes into free solution [3]. It was concluded that, prior to phosphorylation, the ATPase inhibitor protein must be displaced from its inhibitory site on F_1, in an energy dependent process, and that this process takes some 5–10 s in submitochondrial particles. It has been claimed in [9], however, that phosphorylation begins considerably before stimulation of ATPase activity is observed.

Submitochondrial particles will also phosphorylate ADP under the influence of an (artificially-imposed) proton gradient. Although phosphorylation under these conditions occurred only over a short period (5–10 s), rates were comparable with the steady state rates observed when oxidisable substrates are used [4]. Notably, however, phosphorylation induced in this way reached its max-

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid

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imal rate within 1 s; i.e., no lag in phosphorylation was observed [4].

To clarify these points, we studied both the effects of an acid–base transition on the interaction between the inhibitor protein and F_1-ATPase, and the effects of the inhibitor on acid–base jump-induced phosphorylation. We find that, while an artificially-imposed proton gradient can cause inhibitor displacement from the F_1-ATPase, this displacement is too slow to affect phosphorylation. Phosphorylation induced by an acid–base transition is thus inhibited by the ATPase inhibitor protein.

2. MATERIALS AND METHODS

Preparation of inhibitor-depleted and inhibitor-supplemented coupled submitochondrial particles was done as in [1]. The pure inhibitor protein was prepared as in [5], and radio-iodinated at its single tyrosine residue as in [3]. ATP hydrolysis was measured in an ATP regenerating system [5], and phosphorylation measured by incorporation of [^{32}P]phosphate into glucose 6-phosphate [6]. The artificially imposed proton gradient was applied to submitochondrial particles essentially as in [4].

3. RESULTS AND DISCUSSION

3.1. *Effect of acid–base jump on F_1-inhibitor interaction*

Inhibitor-supplemented submitochondrial particles (particles pretreated with inhibitor protein and then washed by centrifugation) have a relatively low ATPase activity, when uncoupled, of 1–1.3 μmol.min^{-1}.mg^{-1} (see table 1). Since the activity expected if all F_1 molecules were active is around 12 μmol.min^{-1}.mg^{-1} (as observed on par-

Table 1

ATPase activity and release of inhibitor protein on energisation of submitochondrial particles [Results are expressed ± SEM (4 readings)]

Additions	ATPase activity (μmol.min^{-1}.mg^{-1})	% Inhibitor released
1 mM MgATP	3.69 (± 0.27)	27.9 (± 3.1)
2.5 mM succinate	2.68 (± 0.29)	24.2 (± 3.5)
1 mM MgATP + 1 μM FCCP	1.32 (± 0.06)	0
pH 5 + valinomycin	1.64 (± 0.12)	
to pH 8 + KCl	2.21 (± 0.20)	8.5 (± 0.8)
pH 5 + valinomycin	1.56 (± 0.16)	
to pH 8 (no KCl)	1.45 (± 0.16)	0

Submitochondrial particles (200 μg) pretreated with radiolabelled inhibitor protein (10^4 cpm/μg protein) (see section 2) were incubated at 30°C: (a) in 100 μl 250 mM sucrose, 10 mM Tris, pH 8.0 (H_3PO_4) (buffer A); or (b) in 100 μl 200 mM malonic acid, 10 mM NaCN, 0.5 μg valinomycin (pH 5.0) (NaOH) with FCCP and other additions as indicated. After 1 min aliquots were taken for measurement of uncoupled ATPase activity. MgATP (1 mM) or succinate (2.5 mM) was added to samples of type (a) and 400 μl buffer A containing, in addition, 100 mM KCl (buffer A*) to type (b). The samples were incubated for a further 2 min and aliquots again removed for ATPase assay. Alternatively, duplicate samples were made up to 1 ml with 500 μl 250 mM sucrose, 25 mM HEPES, 5 mM sodium phosphate, 2 mM $MgCl_2$ (pH 7.4) (NaOH), containing 0.1 mg cytochrome *c*/ml, plus 400 μl 'buffer A*' in the case of the type (a) incubations. The membranes were pelleted by centrifugation, and the radioactivity in the supernatant (below) and the pellet (not shown) measured [3]. The radioactivity in the supernatant is expressed in relation to that washed off the membranes in the absence of energisation (FCCP present), which differed slightly between the two types of incubation

ticles from which all inhibitor protein has been removed see [3]) it appears that only about 10% of their F_1 molecules are able to hydrolyse ATP, the remainder being bound to inhibitor protein. This estimate is confirmed if the inhibitor content of these particles is measured directly using an isotope dilution technique [3], when 0.92 mol inhibitor is found to be bound per mole F_1.

Energisation of the particles, by MgATP hydrolysis or by succinate oxidation, leads to an increase in ATPase activity of 2–3-fold. If the inhibitor used to supplement the particles is radioactively labelled, we can see that this increase in ATPase activity is accompanied by appearance of inhibitor protein in solution (table 1) and, indeed, by loss of inhibitor protein from the membrane (not shown). Typically, some 25% of bound inhibitor is released into solution.

As demonstrated in [3], loss of inhibitor from the membrane correlates well with an increase in ATPase activity. Here, for example, we expect to activate some 25% of the 90% of inactive molecules, leading to an expected increase in ATPase activity of $0.25 \times 0.9 \times 12 = 2.7$ μmol. $min^{-1}.mg^{-1}$. This is very close to the observed value (table 1).

The results obtained when submitochondrial particles are energised by an artificial proton gradient are also given in table 1. Although the overall changes are smaller, it can clearly be seen that imposition of a proton gradient (acid inside) plus a charge gradient (positive inside) causes displacement of some 8% of the bound inhibitor protein with a proportionate increase in ATPase activity. It should be noted that simply exposing the particles to pH 5 does not destroy the F_1-inhibitor interaction – in fact slightly more inhibitor is found bound after exposure to pH 5.

In table 2, the effects on inhibitor release of altering various components of the electrochemical gradient are investigated. It can be seen that the potential gradient plays a particularly important role in inducing inhibitor protein release. If this component is abolished by either omitting KCl in the base (outside) medium, or by including KCl in the acid (inside) medium, stimulation of ATPase activity is completely abolished even though a proton gradient is still present (see also table 1). Nigericin, which also abolishes the electrical component of the gradient, also prevents ATPase stimulation (not shown). Conversely, omitting the proton gradient abolishes only about 50% of the response if an electrical gradient is still present, and FCCP will not abolish the response completely unless nigericin is also included in the mixture (table 2). The finding that the electrical gradient is the major factor responsible for inhibitor displacement accords with earlier conclusions based on studies on oxidation-induced phosphorylation [1].

Table 2

Effects of altering components of the electrochemical gradient on the release of the inhibitor protein

Conditions	Stimulation of ATPase activity (%)
Complete system	31.5
KCl omitted from base stage	3.3
KCl included in acid stage	4.0
Both stages at pH 8.0	17.0
Both stages at pH 8.0	
2 μg nigericin present	4.3
1 μM FCCP present	10.4
1 mM MgATP at pH 8.0	201.5

An acid–base jump was imposed on (inhibitor-supplemented) submitochondrial particles essentially as in table 1, except that the reaction mixtures were varied as indicated. The ATPase stimulation is calculated thus:

Stimulation (%) = (ATPase activity after transition − ATPase activity before transition)/ activity before transition × 100

The range of duplicate readings was ± 3.5%. The result obtained after energisation by MgATP is included for comparison

3.2. *Effect of inhibitor protein on acid–base jump phosphorylation*

As we have shown (above), an electrochemical gradient of protons (and in particular, its electrical component) can displace the inhibitor from its inhibitory site on the ATPase, whether this gradient is set up by oxidation, ATP hydrolysis, or imposed artificially. Simply incubating the particles at high or low pH, or high salt, does not cause inhibitor release over the period of our experiments (see also [5]).

The mechanism of displacement seems similar in all three cases, for example in being highly

Table 3

ADP phosphorylation induced by oxidation of NADH and an electrochemical gradient

Conditions	ATP synthesised (nmol/mg)	
	Inhibitor depleted	Inhibitor supplemented
(a) 400 μM NADH	227 nmol.min^{-1}.mg^{-1}	196 nmol.min^{-1}.mg^{-1}
(b) pH jump	3.74 (± 0.4)	1.05 (± 0.1)
1 μM FCCP included	1.04 (± 0.2)	0.76 (± 0.1)
KCl present in both stages	0.83 (± 0.1)	0.53 (± 0.1)

(a) 200 μg or (b) 1.2 mg of each of inhibitor-depleted and inhibitor-supplemented submitochondrial particles were incubated at 30°C in: (a) 750 μl 166 mM HEPES, 166 mM KCl, 3.3 mM $MgCl_2$, 8.3 mM sodium phosphate, 66 mM glucose (pH 8.0) (NaOH) containing 3.3 mM ADP, 40 units (μmol/min) hexokinase and 10^3 cpm/nmol [^{32}P]phosphate (buffer B); or (b) 300 μl 200 mM malonic acid, 10 mM NaCN, 0.5 μg valinomycin (pH 5.0) (NaOH). Reaction (a) was started by addition of NADH (final conc. 400 μM), and stopped after 1 min by addition of trichloroacetic acid (final conc. 5% (w/v)). Reaction (b) was started by addition of 450 μl buffer B and terminated after 30 s by addition of trichloroacetic acid. ATP formed was measured as in section 2. The range of duplicate readings is given in parentheses

temperature dependent ([1], unpublished). If so, we would expect the time constant to be similar in all cases – in other words, the time constant for release of inhibitor in an acid–base jump is likely to be about 5–10 s. Since acid–base-induced phosphorylation is complete in about 6 s, and rapid for only 1–2 s [4], it seems likely that the inhibitor protein would have insufficient time to adjust its position during acid–base-induced phosphorylation.

This postulate is tested in table 3. Particles supplemented with, and depleted of inhibitor were prepared, and allowed to phosphorylate ADP using either oxidation of NADH or an acid–base jump as a source of energy. We see that phosphorylation rates are equal with both sets of particles (around 200 nmol.min^{-1}.mg^{-1}) when NADH is used as substrate (reaction time 1 min). When phosphorylation is driven by an acid–base jump, however, phosphorylation is about 4-times higher in the inhibitor-depleted particles. Since ATP hydrolysis by these particles is also some 3–4-times faster (2.15 as opposed to 0.6 μmol.min^{-1}.mg^{-1}), we conclude that acid–base jump-induced phosphorylation takes place only on pre-activated F_1 molecules. Release of inhibitor, induced by the acid–base jump, appears too slow to have any effect on phosphorylation rates in these experiments. These findings suggest that ATPase activity, ability to phosphorylate, and inhibitor binding are directly correlated in submitochondrial particles (cf. [9]). As expected, acid–base jump-induced phosphorylation is abolished by omitting either the H^+ or K^+ gradient (table 3). The extent of ATP synthesis during an acid–base transition in inhibitor-depleted particles is similar to that in [4].

4. CONCLUSIONS

The ATPase inhibitor protein inhibits phosphorylation in submitochondrial particles, where phosphorylation is driven by an artificially imposed electrochemical gradient. Removal of the inhibitor protein allows phosphorylation to occur. In this respect, acid–base phosphorylation in submitochondrial particles resembles the initial phase of oxidative phosphorylation in these particles [1]. Induction of ATPase activity in chloroplasts (by light and dithiothreitol) similarly results in stimulation of the initial phases of light-induced [7] and acid–base-induced phosphorylation [8], although the role of a proteinaceous inhibitor in this process is not yet established.

The ATPase inhibitor protein is displaced from

its inhibitory site on F_1 into solution by an artificially imposed electrochemical proton gradient. Although the amount of displacement is only 25–30% of that observed during energisation by Mg^{2+}-ATP or succinate, the displacement of inhibitor protein can be correlated with an increase in ATPase activity by these particles. However, this process is too slow to affect phosphorylation induced by an acid–base jump. This indicates that no lag is observed in the onset of acid–base jump-induced phosphorylation because only those F_1-ATPase molecules already free of inhibitor are involved in this process.

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