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INTERACTION OF F_1-ATPase, FROM OX HEART MITOCHONDRIA WITH ITS NATURALLY OCCURRING INHIBITOR PROTEIN

STUDIES USING RADIO-IODINATED INHIBITOR PROTEIN

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(1) The ox heart mitochondrial inhibitor protein may be iodinated with up to 0.8 mol ^{125}I per mol inhibitor with no loss of inhibitory activity, with no change in binding affinity to submitochondrial particles, and without alteration in the response of membrane-bound inhibitor to energisation. Tryptic peptide maps reveal a single labelled peptide, consistent with modification of the single tyrosine residue of the protein. (2) A single type of high-affinity binding site ($K_d = 96 \cdot 10^{-9}$ M) for the inhibitor protein has been measured in submitochondrial particles. The concentration of this site is proportional to the amount of membrane-bound F_1, and there appears to be one such site per F_1 molecule. (3) The ATP hydrolytic activity of submitochondrial particles is inversely proportional to the occupancy of the high-affinity binding site for the inhibitor protein. No evidence is found for a non-inhibitory binding site on the membrane or on other mitochondrial proteins. (4) In intact mitochondria from bovine heart, the inhibitor protein is present in an approx. 1:1 ratio with F_1. Submitochondrial particles prepared by sonication of these mitochondria with MgATP contain about 0.75 mol inhibitor protein per mol F_1, and show about 25% of the ATPase activity of inhibitor-free submitochondrial particles. Additional inhibitor protein can be bound to these particles to a level of 0.2 mol/mol F_1, with consequent loss of ATPase activity. (5) If MgATP is omitted from the medium, or inhibitors of ATP hydrolysis are present, the rate of combination between F_1 and its inhibitor protein is very much reduced. The equilibrium level of binding is, however, unaltered. (6) These results suggest the presence of a single, high-affinity, inhibitory binding site for inhibitor protein on membrane-bound F_1. (7) The energisation of coupled submitochondrial particles by succinate oxidation or by ATP hydrolysis results in both the dissociation of inhibitor protein into solution, and the activation of ATP hydrolysis. At least 80% of the membrane-bound F_1-inhibitor complex responds to this energisation by participating in a new equilibrium between bound and free inhibitor protein. This finding suggests that a delocalised energy

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Abbreviations: Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; Nbf-Cl, 4-chloro 7-nitrobenzofurazan; Mops, 3-(*N*-morpholino)propanesulphonic acid; MgATP particles, phosphorylating particles prepared by sonication of ox heart mitochondria in the presence of 15 mM $MgCl_2$ and 1 mM ATP; state III particles, MgATP particles treated by sedimentation in the presence of 5 mM succinate, 10 mM potassium phosphate, 5 mM $MgCl_2$ and 1 mM ADP (pH 7.4 with KOH); AS particles, non-phosphorylating particles prepared by sonication of ox heart mitochondria in the presence of 2 mM EDTA (pH 9.2 with ammonia), followed by passage through a Sephadex G50 column.

pool is important in promoting inhibitor protein release from F_1. (8) Dissipation of the electrochemical gradient by uncouplers, or the binding of oligomycin or efrapeptin effectively blocks energised release of the inhibitor protein. Conversely, the addition of aurovertin or adenosine 5′ − [β,γ − imido]triphosphate enhances energy-driven release. The mode of action of various inhibitors on binding and energised release of the protein inhibitor is discussed.

Introduction

The ATPase inhibitor protein, first isolated from ox heart mitochondria by Pullman and Monroy [1], is a small basic protein which inhibits ATP hydrolysis by the mitochondrial F_1-ATPase. It has also been demonstrated to inhibit the onset of ATP synthesis in ox heart submitochondrial particles [2,3], and is thus implicated as a major control element involved in modulating the mitochondrial ATP synthase complex.

The amino acid sequences of inhibitor protein from ox heart [4] and yeast [5] have been published, and homologies exist between them [6]. The inhibitor protein seems to interact with the catalytic subunit (β) of the F_1-ATPase [7]. Despite there being three copies of the catalytic subunit per mole F_1 [21], a single mole of inhibitor protein is sufficient to inhibit completely ATP hydrolysis [8]. These features, and details of its protein chemistry have been recently reviewed [9,10].

Two basic features of the inhibitor protein's action remain undecided. First, although one mole of inhibitor protein per mole F_1 is needed for inhibition of ATPase activity, there may be other binding sites for this protein on F_1 or elsewhere on the mitochondrial membrane. The second feature concerns the response of the inhibitor protein to energisation of the membrane. In order to switch on phosphorylation, the inhibitor protein is presumably removed from its inhibitory site on F_1. This is seen as an increase in (subsequently measured) ATPase activity [11]. Since this increase is stable and reversed by addition of pure inhibitor protein, it has been proposed that it represents a dissociation of the inhibitor protein into solution [3,11]. However, it is also possible that the protein has simply moved to a non-inhibitory alternative binding site on the coupling membrane [12].

To investigate both these problems, an assay for inhibitor binding independent of its inhibitory activity was required. In this paper, we describe a fully active, highly radioactive inhibitor prepared by radio-iodination of its single tyrosine residue. This preparation was used to demonstrate both the existence of a single high-affinity binding site per ATPase complex in submitochondrial particles, and the energy-driven release of inhibitor protein from this site into solution.

These studies also allow the measurement of the intrinsic inhibitor protein pool of coupled submitochondrial particles, and the response of this endogenous inhibitor to energisation. Most (greater than 80%) of the intrinsic pool responds to energisation in the same way as inhibitor protein bound after isolation of the particles, suggesting that most ATP synthase complexes can be activated by membrane energisation in coupled particles.

The effects of 'artificial' inhibitors of F_1 on the inhibitor protein's binding to, and energised release from, submitochondrial particles were also studied. Inhibitors of ATPase turnover slow down combination of the protein with F_1 without affecting the ultimate level of binding. These results can be interpreted in terms of our previous model for ATPase-inhibitor combination [8].

Methods

Inhibitor protein was prepared by the method of Nelson et al. [13] as modified by Gomez-Fernandez and Harris [8], except that the heat treatment and precipitation with ammonium sulphate/ethanol in the procedure of Nelson et al. [13] were omitted. The preparation gave a single band on gel electrophoresis and thin-layer chromatography/electrophoresis (see below). F_1, inhibitor-depleted submitochondrial particles ('AS' particles), type II submitochondrial particles and state III submitochondrial particles were prepared, and ATPase activity measured, as described previously [8,14,15].

Inhibitor protein was iodinated as follows: 0.25 mg of protein were added to 50 μl Bio-Rad solid-phase iodination reagents (lactoperoxidase/glucose oxidase) in a final volume of 350 μl of 120

mM potassium phosphate, pH 7.2, containing 115 μM KI and 0.16 mCi ^{125}I (Amersham). The reaction was started by addition of glucose (final concentration 73 mM), and terminated after 90 min by loading onto two 1-ml columns of Sephadex G10 and centrifuging, as described by Penefsky [16]. The centrifugation was repeated once. Final recovery of protein was 30–50%, and greater than 95% of the iodine recovered was protein bound, as measured by trichloroacetic acid precipitation. The specific activity attained in such a preparation averaged $5 \cdot 10^5$ cpm/μg protein and could be varied by varying the amount of carrier iodide added.

Combination of F_1-ATPase and the inhibitor protein was performed at pH 6.5 as described previously [8] except that 50 mM KCl and 1 mg/ml bovine serum albumin or cytochrome *c* were included to decrease non-specific binding and 2 mM MgATP was present where indicated (see ref. 8). Energised release of inhibitor protein from particles was performed at pH 7.5 as described previously [15] except that 0.1 mg/ml cytochrome *c* was present in the buffer during energisation and centrifugation.

Inhibitory activity of the inhibitor protein was assayed by titration of AS particles at pH 6.5 as described by Horstman and Racker [14], except that, when very low concentrations were to be measured, AS particles (0.15 U ATPase) and inhibitor protein were incubated in a volume of 100 μl at 37°C rather than room temperature, since the dissociation constant is lower at higher temperatures [8].

To measure binding of ^{125}I-labelled inhibitor protein, particles were collected by centrifugation in a Heraeus hemofuge (20 min, $18000 \times g$) after addition of ammonium sulphate to 10% saturation. The pellets were washed once (without resuspension) with cold 250 mM sucrose, 50 mM KCl, 1 mg/ml albumin, 10 mM Mops, pH 6.5 (with NaOH). The supernatants contained less than 2% of the total ATPase activity added. Counts bound non-specifically to the tube and contents were less than 3% of counts added, and increased linearly with added radiolabelled inhibitor protein. Inhibitor protein bound to F_1 was calculated after correction for non-specific binding.

Protein content of particulate preparations was determined after haem extraction by the biuret procedure of Cleland and Slater [17]. Soluble protein was estimated by the procedure of Lowry et al. [46] as modified by Bensadoun and Weinstein [18], in which the protein, before addition of the colour reagents is precipitated by trichloroacetic acid in the presence of deoxycholate. The latter procedure gave good results for the inhibitor protein when calibrated using diethyl pyrocarbonate titration of histidine residues [19], assuming five histidine residues per molecule of inhibitor protein [4] and using spectrophotometric titration of tyrosine residues assuming one tyrosine residue per mol inhibitor protein [4]. Dye-binding methods e.g., the method of Bradford [20] gave values for inhibitor protein concentrations which were less than 50% of the true value (not shown).

Efrapeptin and aurovertin were kind gifts of Dr. B. Beechey, Shell Research, Sittingbourne, Kent, U.K. Cytochrome *c* (type III), hexokinase and albumin (from bovine serum, crystalline) were obtained from Sigma and used without further purification.

Results

Inhibitor protein content of membrane preparations

Inhibitor protein is released from membranes by heating to 100°C for 2 min. Most protein denatures and precipitates, while the inhibitor protein remains soluble. Titration of inhibitor-deficient submitochondrial particles (AS particles) with the supernatant enables us to detect as little as 0.1 μg inhibitor protein. Controls with added radiolabelled inhibitor indicate a recovery of greater than 90% by this procedure.

Table I shows the inhibitor protein content of mitochondria, and membrane fragments derived therefrom, as assayed by this procedure. Allowing for a 30% enrichment in F_1 content (mol/mg protein) in passing from mitochondria to submitochondrial particles [22], we find that fragments prepared by sonication of mitochondria in the presence of MgATP (MgATP particles) contain about 85% of the mitochondrial inhibitor protein, suggesting that this protein is not removed with soluble (e.g., matrix) mitochondrial proteins to any great extent. Similar observations have been made on rat liver mitochondria [23]. On the other

TABLE I

INHIBITOR CONTENT OF MITOCHONDRIAL PREPARATIONS

Mitochondria and submitochondrial particles were prepared as in Methods. MgATP particles (type II) were combined with a 5-fold molar excess of inhibitor protein as described, and then washed twice by centrifugation through 250 mM sucrose, 20 mM Hepes, 5 mM potassium phosphate, 2 mM $MgCl_2$ containing 0.1 mg/ml cytochrome *c* (pH 7.5 with NaOH). The final particles are referred to as 'MgATP particles reconstituted with inhibitor protein'. The inhibitor content of the preparations was assayed, after denaturation of other proteins at 100°C, as described in Methods. 1 U of inhibitory activity is defined as that amount of inhibitor which inhibits 0.2 U (μmol/min) ATPase by 50% [9].

	ATPase (μmol/min per mg)	Inhibitor content (U/mg protein)
Mitochondria	–	23.5
MgATP particles	4.2	26.7
AS particles	12.1	3.2
MgATP particles reconstituted with inhibitor	0.3	48

hand, particles prepared by sonication with EDTA at pH 9.2, followed by Sephadex filtration under high salt condition (AS particles), still retain most of their F_1-ATPase [24] but virtually none of their inhibitor (Table I) [8,14]. The latter fragments are those routinely used in inhibitor-binding assays. Pure F_1 also contains very little inhibitor protein (not shown).

MgATP particles derived from mitochondria prepared in the presence of succinate (type II particles [15]) had a significant ATPase activity (Table I), presumably the result of partial loss of inhibitor protein from these particles [15]. Addition of exogenous inhibitor decreased the ATPase activity and resulted in the binding of more inhibitor to the membranes (reconstituted particles, Table I).

These measurements allow us to compare the inhibitory activity per mg protein found associated with various membrane preparations. In order to quantitate the inhibitor on a molar basis, the radio-iodinated protein was used (see below).

Characterisation of iodinated protein

The inhibitor protein was iodinated up to levels above 1 mol iodine per mol inhibitor protein, using an immobilised glucose oxidase/lactoperoxidase system. Above about 0.8 mol iodine/mol protein, activity was lost, and so experiments were routinely performed on protein iodinated to a ratio of less than this.

After labelling, radioactive iodine is associated almost entirely with a single protein that comigrates with the inhibitor protein on both polyacrylamide gels and two dimensional thin-layer chromatography (not shown). This rules out the possibility of a highly labelled trace impurity in the preparation. Following tryptic digestion, the radioactivity is associated with one major peptide – presumably that containing tyrosine (Fig. 1). The two peptides containing histidine (localised by the Pauly diazo stain) are virtually unlabelled when the incorporation is less than 0.8 mol/mol. The total number of spots observed on the fingerprint (about 20) and the number of peptides containing histidine (two) and the presumed tyrosine (one) are consistent with the known protein sequence [4].

Fig. 2 compares the inhibitory activity (a) and binding affinity (b) of the protein labelled with 0.4 mol iodine/mol protein, to those of the same preparation diluted 1/50 with unlabelled inhibitor

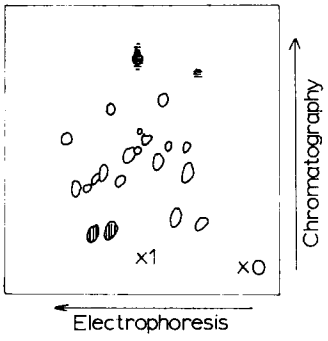

Fig. 1. Tryptic fingerprint of radiolabelled inhibitor protein. 100 μg inhibitor protein in water were incubated with 4 μg trypsin overnight, and the mixture heated to 100°C for 5 min, and then freeze-dried. Electrophoresis and chromatography were carried out as described in Ref 43. Peptides (open 'shapes') and histidine (vertical hatching) were located by staining [44,45] and iodine (horizontal hatching) by autoradiography. 0, origin; 1, position of undigested inhibitor.

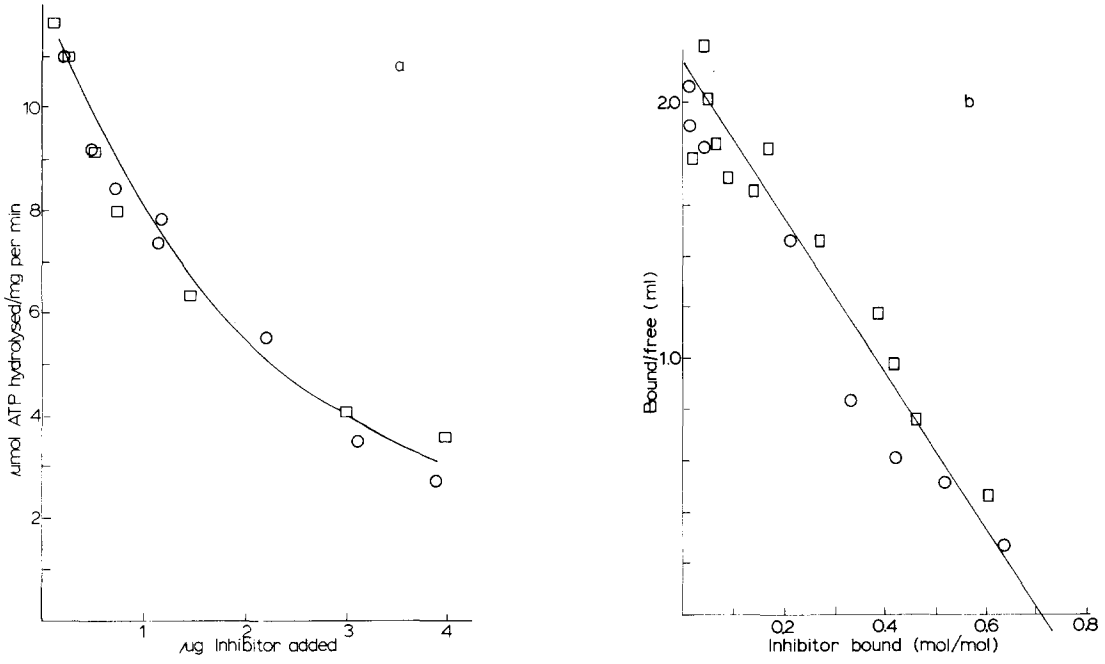

Fig. 2. Activity of the radiolabelled inhibitor protein. Varying amounts of inhibitor protein were combined with AS particles (0.4 mg protein) as described in Methods. After 5 min, a sample was taken for assay of ATPase activity (a) and the remaining solution adjusted to 10% saturation with ammonium sulphate. The solution was then centrifuged at top speed in a Hereus hemofuge for 15 min, and the supernatant removed by aspiration. (The ammonium sulphate aided the formation of a tight pellet, and slowed down further combination of F_1 with inhibitor.) The pellet was rinsed with 200 μl of the buffer used in the incubation, and then counted for ^{125}I with an Intertechnique gamma counter. The correction for non-specific binding of radio-iodinated inhibitor was routinely about 3% of free counts. AS particles were assumed to contain 0.36 nmol F_1/mg protein [24]. (□———□) Inhibitor at 0.4 mol iodine/mol inhibitor, (○———○) inhibitor diluted to 0.008 mol iodine/mol inhibitor.

protein. If the unlabelled inhibitor bound more strongly than the labelled, the amount of label bound would decrease more than expected on the basis of decreased specific radioactivity alone. Concurrently, the inhibition for a given amount of protein would increase. In fact the curves for highly labelled and diluted iodinated inhibitor solutions are indistinguishable within experimental error. Iodination thus does not appear to affect the functional activity of the inhibitor protein.

The iodinated inhibitor appeared to have a strong affinity for glass and plastic tubing and was thus routinely stored diluted with a carrier protein (albumin or unlabelled inhibitor protein) at concentrations of above 0.4 mg/ml.

Binding of inhibitor to submitochondrial particles

The binding of radioiodinated inhibitor to the virtually inhibitor-free AS particles in the presence of 50 mM KCl and 'carrier' protein (see Methods) indicates that only one high-affinity binding site for inhibitor protein is present on submitochondrial particles (Fig. 2b). The binding curves are plotted according to Scatchard with the ratio bound/free inhibitor on the ordinate and the amount of bound inhibitor on the abscissa. The K_d for binding is $96 \cdot 10^{-9}$ M, which is close to that obtained previously from inhibition studies under these conditions [8]. That this single high-affinity binding site is that responsible for inhibition of ATP hydrolysis by F_1 is confirmed in Fig. 3, which shows that the ATPase activity of AS particles is inversely related to the amount of inhibitor bound at this high-affinity site.

If the F_1-ATPase is removed from AS particles by urea treatment, the high-affinity binding site

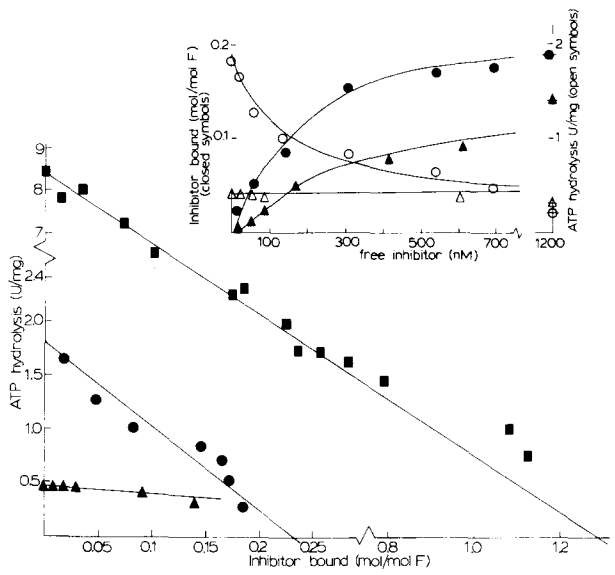

Fig. 3. Correlation of inhibitor protein binding with ATPase inhibition. ATPase activity and inhibitor combination with AS, MgATP and state III particles was measured as in Fig. 2. Typical titration curves showing increasing inhibitor binding and loss of ATPase activity are shown (for MgATP and state III particles) in the inset. MgATP particles and state III particles were assumed to contain 0.56 nmol F_1 per mg protein [24] (cf. AS particles, Fig. 2). (▲———▲) MgATP particles, (●———●) state III particles. (■———■) AS particles (note different scale).

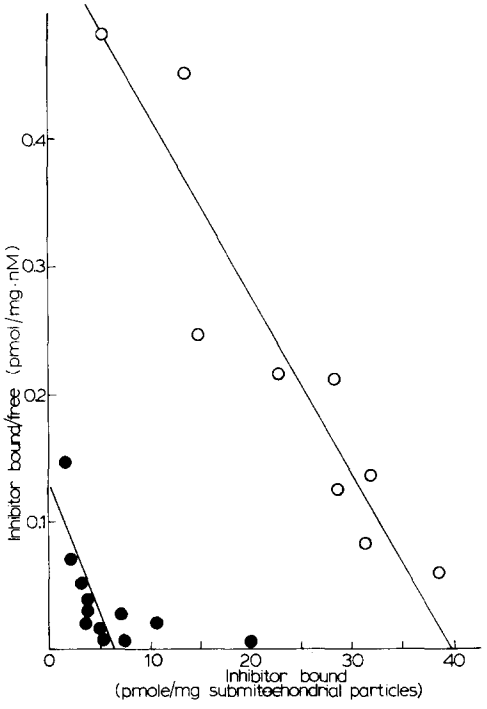

Fig. 4. Inhibitor protein binding to particles depleted of F_1. F_1 was removed from AS particles by treatment with 2 M urea [40] and reconstituted with pure F_1 as described therein. The ratio of F_1 to particle protein in the reconstitution was 1:10 (w/w). Free F_1 was removed by centrifugation. Inhibitor binding to stripped and reconstituted particles was measured as described in Fig. 2. The ATPase activity of the 'stripped' particles was 0.45 μmol/min per mg and of the reconstituted particles 2.8 μmol/min per mg. The oligomycin sensitivity in each case was greater than 75%. (●———●) Stripped particles, (○———○) reconstituted particles.

for inhibitor protein disappears. In Fig. 4, we find that when greater than 90% of F_1 is removed (as recorded by a drop in ATPase activity from 12 to 0.4 U/mg), the number of inhibitor-binding sites decreases from 280 pmol/mg particle protein (Fig. 2b) to 6 pmol/mg. Rebinding of F_1 to the membranes results in a partial recovery of the lost binding sites. In Fig. 4, the ATPase activity of particles on reconstitution rises from 0.4 to 2.8 U/mg, and the number of inhibitor-binding sites from 6 to 38 pmol/mg particle protein. The K_d for inhibitor protein binding to reconstituted particles is $60 \cdot 10^{-9}$ M – of the same order as that observed for binding to AS particles (above).

A single type of high-affinity binding site for the inhibitor protein is also observed with state III and MgATP submitochondrial particles (Fig. 5). Since these particles have considerable amounts of inhibitor protein already bound (Table I), it is anticipated that only a fraction of the membrane-bound F_1 molecules will bind added inhibitor protein. Indeed, only about 0.2 mol radiolabelled inhibitor/mol F_1 is bound. The dissociation constant observed with state III particles is comparable with that observed with AS particles – about $200 \cdot 10^{-9}$ M in this case.

It is interesting that MgATP particles, which have initially a much lower ATPase activity than state III particles, also bind inhibitor to a level of about 0.2 mol/mol F_1, but show a much larger apparent K_d. This is explained by the fact that radio-iodinated inhibitor binds to these particles without any significant drop in ATPase activity (Fig. 3, inset). Thus, it appears that iodinated inhibitor is simply exchanging with inhibitor al-

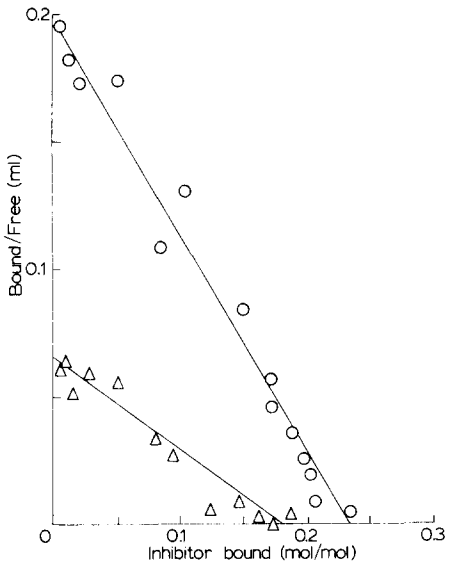

Fig. 5. Inhibitor protein binding to coupled submitochondrial particles. Inhibitor binding was measured as in Fig. 2. MgATP particles were prepared from type II mitochondria and state III particles by centrifugation of these particles under energised conditions as described previously [15]. (△——△) MgATP particles, (○——○) state III particles

ready present on the F_1 of the particles and the resulting decrease in specific activity of the inhibitor causes an apparent increase in K_d.

In these titrations, accurate values for free and membrane-bound inhibitor protein can be obtained up to levels as high as a 30-fold molar excess of inhibitor over F_1 (i.e., about 10% of total protein is added inhibitor protein). Under our conditions, this corresponds to an inhibitor concentration of about 15 μM. The linearity of the Scatchard plots (Figs. 2b, 4 and 5) up to these very high levels of inhibitor protein (even when the binding site on F_1 is largely occupied with endogenous inhibitor) allows us to rule out the possibility of other binding sites on F_1, or elsewhere on the mitochondrial membrane, with K_d values within about 2 orders of magnitude of the inhibitory binding site, i.e., any additional binding site must have $K_d > 10^{-5}$ M. The claim by Schwerzmann et al. [23] to find up to 3 mol inhibitor binding sites per mol F_1 in rat liver mitochondria is thus not confirmed in bovine heart mitochondria. This discrepancy may result from difficulties, in their case, in extrapolating the Scatchard plot to infinite inhibitor concentrations.

Effect of energisation of the F_1-inhibitor interaction

It has been suggested [11] that the inhibitor protein dissociates from submitochondrial particles on energisation. An experiment designed to test this point is shown in Table II. Submitochondrial particles, containing radiolabelled inhibitor protein as part of their total inhibitor pool, were energised and rapidly sedimented at 25°C by the method of Van de Stadt et al. [11]. The ATPase activity and radiolabelled inhibitor content of the sedimented particles, and the label released into the supernatant, were measured. (No ATPase activity was released from the particles under these conditions; not shown.)

Table II shows that on energisation by either succinate oxidation or MgATP hydrolysis, the ATPase activity of these particles rises and inhibitor is lost from the membranes into the solution. A rough correlation between the amount of radiolabel lost and ATPase activity induced (using the ATPase activity of AS particles as the maximal attainable) suggests that virtually the entire pool of F_1-bound inhibitor protein is involved in this energised release, i.e., it is not simply the pre-bound radiolabelled inhibitor (about 0.15 mol/mol F_1) which is relased on energisation but the also the native, endogenous inhibitor. If the radiolabelled inhibitor only were released, a loss of 30% of label (i.e., 0.05 mol radiolabelled inhibitor per mol F_1) would induce a rise of only 0.6 μmol/min per mg (5% maximal rate) in ATPase activity.

This is further investigated by the experiment of Table III. The first column shows that, when MgATP particles, preincubated with iodinated inhibitor, are energised, the total units of inhibitory activity associated with the membrane decrease by an amount equal (within experimental error) to the amount of activity appearing in the supernatant. FCCP-treated submitochondrial particles, used as controls, released very little inhibitory activity when sedimented with succinate present. The smaller energisation-dependent stimulation of ATPase observed in this experiment (Table III, last column) compared to that reported in Table II is probably due to the use of higher particle concentrations here, as required for direct assays of

TABLE II

EFFECT OF ENERGISATION ON RELEASE OF PREBOUND IODINATED INHIBITOR PROTEIN FROM SUB-MITOCHONDRIAL PARTICLES

MgATP particles were prepared, combined with a 2-fold molar excess of radiolabelled inhibitor (22 400 cpm/μg), and washed to remove excess inhibitor as described in Table I. Aliquots of about 4 mg protein were diluted to 8 ml in a buffer at 30°C, containing 250 mM sucrose, 20 mM Hepes, 5 mM potassium phosphate, 2 mM $MgCl_2$, 0.1 mg/ml cytochrome *c* brought to pH 7.5 with NaOH. To one tube was added FCCP to a final concentration of 2 μM, and then to this and a second tube 50 μmol sodium succinate, 10 μmol ADP, 2 U catalase and 1 μl H_2O_2 (100 vol). To the third tube 40 μmol MgATP alone were added. Immediately after mixing, the tubes were spun for 10 min at 150000 × *g* at room temperature to sediment the particles. Aliquots were taken from the resuspended pellets and supernatants for determination of radioactivity, protein and ATPase activity. 100% ATPase activity is taken as 12.1 μmol/min per mg – see Table I.

Treatment	ATPase (μmol/min per mg)	Bound ^{125}I inhibitor (cpm in pellet) ($\times 10^{-3}$)	Released ^{125}I inhibitor (cpm in supernatant) ($\times 10^{-3}$)	ATPase (% max)	^{125}I released (% total)
Succinate + FCCP	0.41	78.5	8.2	3.4	9
Succinate	4.23	53.1	33.4	35	39
MgATP	3.74	58.7	24.0	31	29

TABLE III

SIZE OF INHIBITOR PROTEIN POOL RESPONSIVE TO ENERGISATION

MgATP particles were combined with radiolabelled inhibitor, and allowed to oxidise succinate ± FCCP as described in Table II, except that 11 mg protein were present in each 8 ml sample and the specific radioactivity of the inhibitor protein was $7.6 \cdot 10^3$ cpm/μg. The supernatant obtained from the energised particles was dialysed against two 100-vol. portions of 1 mM Tris, 0.1 mM EDTA, pH 7.5, freeze dried and redissolved in 0.5 ml of 250 mM sucrose, 10 mM Mops-Na, pH 6.5. The inhibitory activity of this extract was found as described in Methods. Control samples of radiolabelled inhibitor (1 μg/ml in 0.1 mg/ml cytochrome *c* solution) and cytochrome *c* alone were run in parallel to the supernatants through the dialyses and drying steps.

Sample	Inhibitor (U)	Specific activity (cpm/U inhibitor)	ATPase (μmol/min per mg)
^{125}I-labelled inhibitor	–	648	–
Particles before energisation	700	71	0.21
Particles after energisation	666	43	1.71
Supernatant after energisation	51	95	–
FCCP supernatant	< 15	> 430	–

inhibitory activity in the supernatant.

The specific radioactivity (cpm/U inhibitory activity) of the released and bound inhibitor was also measured. We see that the specific radioactivity of the bound inhibitor is about 10-times less than that of the initial radiolabelled inhibitor. This implies that the amount of radiolabelled inhibitor that binds is equal to about one-tenth of the amount of endogenous inhibitor. Since the amount of radiolabelled inhibitor bound was 0.075 mol/mol F_1 (calculated as in Table II), the endogenous inhibitor pool in these particles appears to be about 0.75 mol/mol F_1.

On energisation, the specific radioactivity of the released inhibitor is slightly higher than, but quite similar to, that of the inhibitor retained by the particles. This implies that most (greater than 80%) of the endogenous pool of inhibitor participates in the energy-dependent displacement equilibrium – not just the newly bound inhibitor.

Although a significant amount of radiolabel is released from the particles when they are washed in the presence of FCCP, the amount of inhibitory activity released is at the lower limit of detection, and the specific radioactivity thus high (Table III) – close, in fact, to that of the added radiolabelled inhibitor. It is concluded, therefore, that a little radiolabelled inhibitor (less than 0.05 mol/mol F_1) is bound to the particles non-specifically, and can

be washed off under uncoupled conditions. This label does not equilibrate with the endogenous pool of inhibitor as does the labelled inhibitor bound at its specific, inhibitory, energy-dependent binding site. In calculating the specific activity of the inhibitor released on energisation (Table III), the total counts released on energisation were corrected for counts thus (non-specifically) washed off the particles.

Effect of ATPase inhibitors on F_1-inhibitor protein interaction

Hydrolytic turnover of free or membrane-bound F_1 under uncoupled conditions (cf. Table II) promotes inhibitor protein binding [8,14,28]. We have postulated [8] that during turnover, the ATPase exists briefly in a state capable of binding the inhibitor protein. The ability to measure inhibitor protein binding independently of ATPase inhibition using the radiolabelled protein allows the study of inhibitor protein binding in the absence of ATPase turnover.

Fig. 6 shows that oligomycin, which prevents ATPase turnover without binding to F_1 itself, allows F_1 to bind to inhibitor protein to about the same level as attained during ATPase turnover. However, the rate of binding is much slower ($t_{1/2}$ increases from 30 s to 90 min). Inhibitor protein binding to oligomycin-inhibited particles shows an exponential time course if the protein is in excess (Fig. 7, solid squares), consistent with there being just one type of inhibitor protein-binding site on the particles.

Fig. 7 also shows the effects of Ado*PP*[NH]*P*, ADP ($+P_i$), and Nbf-Cl on inhibitor protein binding to F_1. In all cases, reagents were added in amounts sufficient to inhibit the ATPase by more than 90%. Both Ado*PP*[NH]*P* binding and Nbf-Cl modification slow down the combination of F_1 with inhibitor protein even more – $t_{1/2}$ increasing to above 200 min with Ado*PP*[NH]*P*, for example. The effect of Ado*PP*[NH]*P* is reversed by the addition of ATP, with which it competes for the active site of F_1 [32], and the effect of Nbf-Cl is reversed by dithiothreitol, which displaces it from the enzyme [25] (results not shown). ADP ($+P_i$) on the other hand, cause the inhibitor protein to bind about 4-times faster than in the presence of oligomycin (Fig. 7).

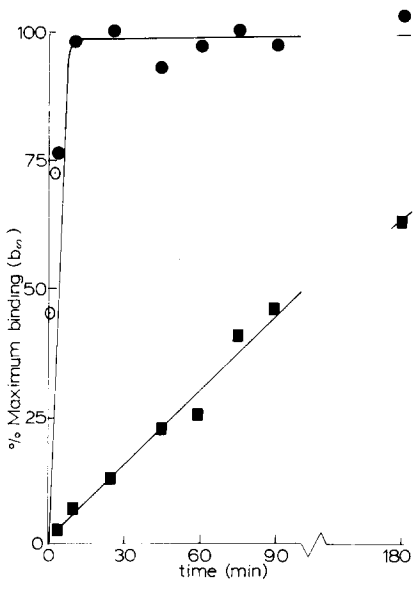

Fig. 6. Time course of inhibitor protein binding to AS particles. Inhibitor binding to AS particles, in buffer containing 2 mM MgATP, was measured as in Fig. 2, except that incubations were carried out in a volume of 1 ml containing 1.56 mg AS particles and 37 μg (excess) radiolabelled inhibitor. Where indicated, the AS particles were preincubated with oligomycin (25 μg) in the absence of MgATP. 0.1-ml aliquots were removed at times varying between 5 and 180 min. (The shorter time points were obtained by activity measurements rather than binding measurements [8] – open circles.) (●———●) Control, (■———■) preincubated with oligomycin.

If we extrapolate the time course of binding to infinite time using an inverse plot (Fig. 8), we detect only one binding site for inhibitor protein per F_1 irrespective of whether binding occurs in the presence or absence of ATP hydrolysis. We have demonstrated above that, in the presence of MgATP, submitochondrial particles bind inhibitor protein at only one high-affinity binding site, its inhibitory site on F_1. It thus seems likely that in the absence of ATP hydrolysis, the same site is occupied by bound inhibitor protein. To test this hypothesis, we measured inhibition of ATP hydrolysis as a function of inhibitor protein binding to AS particles as in Fig. 3, except that ADP, hexokinase and glucose ($\pm$15 mM AMP) replaced ATP in the incubation. The plot of inhibition versus binding obtained when ADP is present was indistinguishable from that obtained in the presence of ATP (not shown) i.e., the binding observed

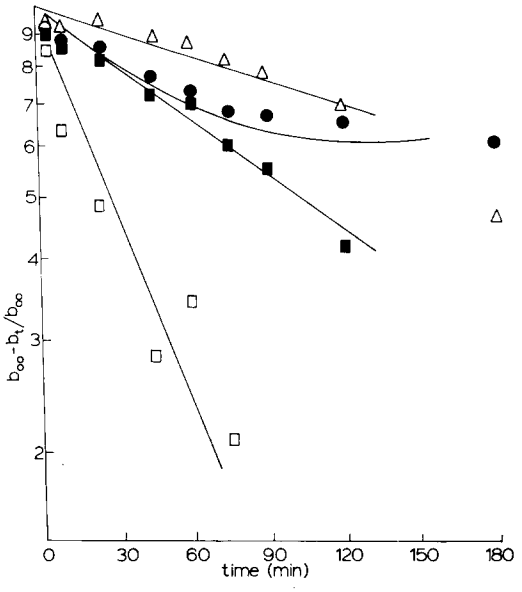

Fig. 7. Effect of ATPase inhibitors on inhibitor protein binding. Nbf-Cl treated AS particles were prepared as follows. 400 μg AS particles were suspended in 200 mM sucrose, 50 mM triethanolamine hydrochloride (brought to pH 8.0 with NaOH) and 20 nmol Nbf-Cl added from a concentrated solution in ethanol. The mixture was incubated for 60 min in the dark at room temperature, and 6 μmol Tris-HCl, pH 8.3, added to stop the reaction. The particles were spun down and resuspended in sucrose-Tris at pH 7.5. Their ATPase was less than 5% of the original value. Binding was measured as in Fig. 6, except that AS particles (0.92 mg) were pretreated with Nbf-Cl as above (●———●) or 80 μg oligomycin (■———■) or 1 mM Ado*PP*[NH]*P* (△———△). In the last two cases, pretreatment was for 5 min prior to the addition of inhibitor protein. Either 2 mM MgATP (closed symbols) or 10 mM glucose/50 U hexokinase/2 mM $MgCl_2$ (open symbols) was added with the inhibitor protein, and in one case (□———□) 2 mM ADP/10 mM potassium phosphate was added in addition to the glucose/ hexokinase mixture. The ordinate is plotted as $b_\infty - b_t/b_\infty$, where b_∞ represents counts bound after 10 min in the presence of MgATP alone, and b_t, counts bound at time t in the presence of the compounds indicated. A straight line is characteristic of a pseudo-first-order reaction.

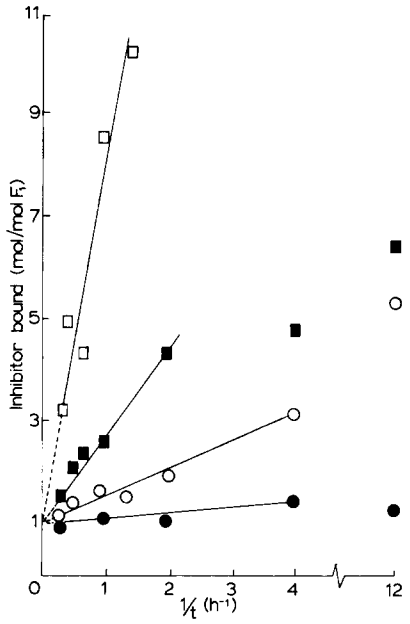

Fig. 8. Stoicheiometry of inhibitor protein binding in the absence of ATP hydrolysis. The time course of inhibitor protein binding was measured, at an AS particle concentration of 0.79 mg/ml, as described in Fig. 7. 2 mM MgATP (closed symbols) or 2 mM MgADP/10 mM potassium phosphate/ 10 mM glucose/50 U hexokinase (open symbols) were added with inhibitor protein (25 μg/ml). (circles) No further addition: (squares) preincubated with 50 μg oligomycin.

in the presence of ADP, at least, although slower than in the presence of MgATP, was to the same inhibitory site on F_1.

The effects of inhibitors on energy-dependent release of inhibitor protein from submitochondrial particles were studied in the experiments of Table IV. Both oligomycin and efrapeptin, while allowing (and even enhancing) energisation of the membrane as a whole, prevent inhibitor protein release.

TABLE IV

EFFECT OF ATPase INHIBITORS ON THE ENERGY-DEPENDENT RELEASE OF THE NATURALLY OCCURRING INHIBITOR PROTEIN

MgATP particles were combined with radiolabelled inhibitor protein and allowed to oxidise succinate as described in Table II, except that the indicated amounts of inhibitors were added 5 min prior to energisation of the particles. Counts released from the particles were quantitatively recovered in the supernatant (not shown), as in Table II. MgATP particles reconstituted with inhibitor protein had an ATPase activity of 0.4 μmol/min per mg prior to incubation.

Addition	Inhibitor released (% counts bound)	ATPase (μmol/min per mg)
Succinate alone	30	4.23
+ FCCP (2 μM)	0	0.41
+ oligomycin (1 μg/mg)	0	< 0.1
+ efrapeptin (0.5 μg/mg)	5	< 0.1
+ aurovertin (2 μM)	47	4.16
+ Ado*PP*[NH] *P* (40 μM)	39	1.79

Presumably, therefore, they prevent some conformational change in F_1, normally the result of energisation, necessary for inhibitor protein to be released.

On the other hand, Ado*PP*[NH]*P* and aurovertin not only allow but also promote loss of inhibitor protein. The inability of Ado*PP*[NH]*P* to block inhibitor protein release from energised F_1 may be due to its inability to bind to either the energised F_1 (see Refs. 27 and 42) or to the F_1-inhibitor protein complex [28], while in the case of aurovertin, which does bind to the energised F_1 [29], the latter possibility is most likely. These direct binding studies confirm previous indications that aurovertin enhances energy-dependent release of the inhibitor protein from submitochondrial particles [29]. The relatively low ATPase rate observed after energisation in some cases is presumably due to the inhibitory ligand being bound either in addition to (oligomycin, efrapeptin) or in partial replacement of (Ado*PP*[NH]*P*) the inhibitor protein.

Discussion

Inhibitor-binding sites on submitochondrial particles

The binding experiments described above suggest that submitochondrial particles contain a single, high-affinity, binding site for the ATPase inhibitor protein per F_1 molecule (Fig. 2), that this site is on the F_1 molecule (Fig. 4), and that occupancy of this site inhibits enzyme activity (Fig. 3 and Table III). Inhibitor protein binding is directly correlated with inhibition of ATPase activity, whether or not binding is induced by MgATP (Fig. 3 and last section of Results). Combined with previous demonstrations that 1 mol of inhibitor protein is needed to inhibit 1 mol of F_1 [7,8], this leads us to conclude that no significant number (less than 0.2 mol/mol F_1) of sites capable of binding inhibitor with an affinity of within 2 orders of magnitude of the high-affinity site on F_1 exists on the membrane. Furthermore, the finding (Table I) that most of the mitochondrial inhibitor protein is associated with the membrane suggests that there is no significant pool of inhibitor in mitochondria associated with proteins other than F_1. In particular, our results do not support previous suggestions of a non-inhibitory inhibitor protein-binding site either on the membrane or on soluble matrix proteins of the mitochondrion (see, e.g. ref. 30).

MgATP particles, even those derived from mitochondria prepared in the presence of succinate [15], retain significant amounts of bound inhibitor protein (the amount decreasing on storage of the mitochondria at $-70°C$, data not shown). By measuring the dilution in specific activity on binding radio-iodinated inhibitor to particles, we are able to estimate that the MgATP particles used in the experiments of Table III retained about 0.75 mol inhibitor per mol F_1. Alternatively, knowing the specific radioactivity of our stock iodinated inhibitor in terms of protein (7600 cpm/μg) and inhibitory activity (648 cpm/U inhibitory activity), we calculate the specific activity of the inhibitor to be $11.6 \cdot 10^3$ U/mg protein. (This is close to the values obtained with purified samples of non-iodinated inhibitor – see Methods). Using this as a calibration factor, we calculate that the inhibitor-supplemented particles of Tables III and I contain about 5 μg inhibitor/mg protein (0.9 mol/mol F_1), and the MgATP particles of Table I slightly (less about 0.6 mol/mol). The similarity of these values to those obtained for the percentage of inactive F_1 (see Table II) – between 65 and 85% in different preparations – again confirms that inhibitor protein is bound at a single high-affinity, inhibitory site on F_1. Such calculations, using the data of Table I, also indicate that in bovine heart mitochondria, unlike rat liver mitochondria [23], F_1 and inhibitor protein are normally present in an approx. 1:1 molar ratio.

Mechanism of inhibitor binding to F_1

It has been proposed previously [8] that combination of F_1 with its inhibitor involves the participation of a transient intermediate during ATPase turnover. The results of Fig. 6 indicate that, in the absence of turnover, inhibitor protein still binds to F_1, but very much more slowly. This is consistent with our previous model, since any conformation of F_1 occurring during turnover must occur, albeit with much lower probability, in the absence of turnover. Since the rate of combination drops by 2 orders of magnitude in the presence of oligomycin ($t_{1/2}$ falls from 30 s to 90 min), existence of the 'productive' conformation appears

100-times less likely in the absence of turnover.

Although the form of F_1 responsible for its combination with the inhibitor protein exists only transiently during turnover, its lifetime must be long enough for collision between the two species to occur. This implies that the combining state of F_1 is very probably a kinetically significant intermediate along the reaction pathway. Several intermediate forms of F_1 have been identified kinetically as participating in hydrolysis [32–34]. These are labelled a–f in the scheme below.

$$\underset{a}{E+ATP} \rightleftharpoons \underset{b}{E\cdot ATP} \rightarrow \underset{c}{E^*\cdot ATP} \rightleftharpoons \underset{d}{E^*\cdot ADP\cdot P_i} \rightarrow$$

$$\underset{e}{E^*\cdot ADP} \rightarrow \underset{f}{E\cdot ADP} \rightleftharpoons E+ADP$$

Ado*PP*[NH]*P* binds very tightly to the active site of F_1, apparently trapping the enzyme in form c (since hydrolysis is impossible and dissociation thermodynamically unfavourable) [41]. The Nbf enzyme complex , on the other hand, can hydrolyse ATP but the products are not released [25] so the enzyme form d is apparently attained in the presence of MgATP. Since the inhibitor protein binds very slowly to F_1 alone [8], F_1 inhibited with Ado*PP*[NH]*P* or, in the presence of MgATP, to F_1 inhibited with Nbf-Cl (Fig. 7), we conclude that its combination with inhibitor protein is not favoured by enzyme conformations a–d.

Since saturating levels of ADP and P_i also promote only a slow binding of inhibitor protein, it appears that conformations available by simple reversal of ADP binding to F_1 are also not those to which the inhibitor protein binds rapidly and strongly. Kinetic studies on F_1 [32–34] suggest that ADP release is slow relative to the catalytic step, effectively irreversible and thus may be limited by an enzyme conformational change (e to f in our model). One possibility, therefore, consistent with our data, is that the inhibitor protein combines with form e in the kinetic scheme above.

Since iodination of the inhibitor protein does not decrease its potency (Fig. 2), nor affect the response of bound inhibitor to energisation (Tables II and III), its single tyrosine residue is presumably not essential for activity. This is consistent with the absence of an analogous tyrosine in the sequence of yeast inhibitor protein [5].

Effects of energisation on F_1-inhibitor interaction

In the experiments of Tables II and III, we demonstrate directly that when submitochondrial particles are energised, inhibitor protein is displaced from its binding site on F_1 into the surrounding solution. This is accompanied by (a) an increase in ATPase activity of the particles [8,11], (b) a loss of radiolabelled inhibitor protein to the solution (Table II), and (c) a recovery of radiolabelled inhibitor and inhibitory activity from the supernatant (Table III). All these changes are prevented by uncoupler. These findings, although following directly from our inability to demonstrate non-inhibitory binding sites for the inhibitor protein, are the first direct demonstration of displacement of inhibitor from F_1 into solution on energisation.

From our results of Tables II and III, we conclude that both endogenous inhibitor protein, retained by submitochondrial particles during isolation, and iodinated inhibitor, bound to the particles subsequently, respond in a similar way to energisation of the membrane. Indeed, from the dilution of the specific radioactivity of the inhibitor protein released from energised membranes, we estimate than more than 80% of the total inhibitor pool takes part in the energy-dependent equilibrium. The actual amount displaced, of course, depends on the equilibrium constant – at low protein concentrations (Table II), about 30% of the ATPase molecules are activated, while at higher concentrations (Table III), only about 10%. Since in intact mitochondria, the F_1 and inhibitor protein concentrations in the matrix will be fairly high, it is possible that not all ATPase molecules become activated during steady-state phosphorylation. However, it is possible that a higher membrane potential, or the involvement of other effectors such as Ca^{2+} [2], in intact mitochondria might lead to a higher percentage displacement in vivo than that predicted from studies on submitochondrial particles.

Conflicting conclusions as to whether the energised release of inhibitor protein precedes phosphorylation are found in the literature. Harris and Crofts [35], studying chloroplasts, show that induction of ATPase and initiation of phosphorylation both occur within four flashes (0.04 s), and they conclude that release of an inhibitor protein

precedes phosphorylation. In contrast, Schwerzmann and Pedersen [12], report that the onset of phosphorylation can precede ATPase induction in rat liver submitochondrial particles.

One explanation for the slow release observed by the latter workers may be that their attempts to measure release of inhibitor protein were performed under low salt conditions, when the (positively charged) inhibitor will tend to associate with negatively charged membrane phospholipid. This would explain their apparent non-inhibitory 'binding site' for the inhibitor protein from which the inhibitor can readily migrate to its inhibitory site on F_1. In Ref. 12, no precautions were taken to prevent this migration prior to measuring ATPase activity, while in ref. 35. dithiols were added to prevent decay of the active state. In the present studies, when the ionic strength is sufficiently high to prevent non-physiological charge interactions, no evidence for a non-inhibitory binding site is obtained.

Since nearly all bound inhibitor protein responds to energisation by succinate oxidation, we conclude that nearly all F_1 molecules experience the effects of membrane energisation, even though there is only about 0.1 mol succinate dehydrogenase per mol ATPase on average [36]. Table II shows that energisation by ATP hydrolysis is also felt by nearly all F_1 molecules of coupled submitochondrial particles, even though initially only about 2–5% of the total ATPase molecules may be active. (At this temperature, in the absence of energisation, inhibitor release from F_1 would be about 1% per h [15].) These results are thus best explained on the basis of a response of membrane-bound inhibitor protein to a delocalised energy pool (e.g., a bulk membrane potential [35]) communicating between different ATPase molecules (and the electron-transfer chain) (cf. Ref. 37).

The effects of inhibitors of oxidative phosphorylation on inhibitor protein release are shown in Table IV. Both oligomycin and efrapeptin, at various concentrations (titration not shown here), allow development of the electrochemical gradient during substrate oxidation, yet they prevent energised release of the inhibitor protein. Similar results, using Nbf-Cl and bathophenanthroline-Fe have been reported by Gomez-Puyou et al. [38]. In the case of efrapeptin, Nbf-Cl and bathophenanthroline, which bind directly to F_1, prevention of inhibitor protein release may be accomplished by 'locking' the ATPase into a refractory conformation. This is less likely to be the case with oligomycin, since it binds to the F_0 portion of the ATP synthase complex. This result might indicate that it is necessary for protons to enter the proton channel of F_1F_0 in order for F_1 to release the inhibitor protein, rather than F_1 directly sensing a bulk change in the gradient. Some conformational effect of oligomycin on F_1 cannot, however, be ruled out. We can also confirm the finding [39] that efrapeptin is not displaced from F_1 on energisation (Table IV).

Ado*PP*[NH]*P* and aurovertin, in contrast, appear to stimulate inhibitor protein release (Table IV). In both cases, the ligand is unlikely to bind to F_1 simultaneously with the inhibitor protein (see above). Thus, once inhibitor protein is released on energisation, the ligand can bind and prevent the inhibitor protein rebinding.

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