Biochimica et Biophysica Acta 933 (1988) 12–21
Elsevier

BBA 42723

The binding and release of the inhibitor protein are governed independently by ATP and membrane potential in ox-heart submitochondrial vesicles

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(Received 8 December 1987)

Key words: Inhibitor protein; ATP synthase; ATP synthase inhibitor protein interaction; ATP; Membrane potential; Bovine heart submitochondrial vesicle

(1) The effects of membrane potential ($\Delta\psi$) and nucleotides on the interaction between the F_1-ATP synthase and its natural inhibitor protein (IF_1) are studied in ox-heart submitochondrial vesicles. (2) Membrane potential causes displacement of IF_1 from submitochondrial vesicles, as shown by measuring both $\Delta\psi$-dependent stimulation of ATPase capacity and release of ^{125}I-labelled IF_1 from the vesicles. These effects are abolished if ATP is included in the incubation. (3) There is a linear increase in the steady-state ATPase capacity of oxidising vesicles as $\Delta\psi$ is increased from 100 mV to 135 mV. Increasing $\Delta\psi$ above 140 mV leads to no further change. (4) At a constant membrane potential, ATP suppresses the increase in ATPase capacity, with a concentration for half maximal effect of 140 μM. This value is close to the K_m for ATP hydrolysis by membrane-bound F_1. This suppression is related to ATP concentration rather than to ΔG_P or ATP/ADP ratio. (5) The unidirectional on- and off-rates of IF_1 were measured separately. The off-rate of IF_1 is increased by membrane potential but unaffected by ATP. The on-rate, conversely, is increased by ATP. Thus, the suppression of the potential-dependent net release of IF_1 from submitochondrial vesicles by ATP results from an increase of the IF_1 on-rate above the off-rate.

Introduction

Under energised conditions, the mitochondrial ATPase inhibitor protein (IF_1) is released from its inhibitory site on F_1 into solution, allowing either ATP synthesis or ATP hydrolysis to proceed [1]. In ox-heart submitochondrial vesicles, we have shown that energy-dependent induction of both ATP synthetic and hydrolytic capacities follow the same time-course as the IF_1 release [2], suggesting a causal relationship between these processes. In other words, both ATP hydrolysis and synthesis are prevented by IF_1 binding to F_1 and either, depending on the conditions, is induced on IF_1

Abbreviations: IF_1, naturally occurring inhibitor protein of mitochondrial ATPase; [^{125}I]IF_1, radioiodinated inhibitor protein; MgATP SMP, submitochondrial vesicles prepared in the presence of ATP and magnesium; [^{125}I]IF_1 SMP, submitochondrial vesicles supplemented with [^{125}I]IF_1; Δp, protonmotive force = $\Delta\tilde{\mu}_H/F = \Delta\psi + (2.303\ RT/F)\ \Delta pH$, where $\Delta\psi$ is the electrical component and ΔpH the chemical component of the transmembrane difference of the electrochemical potential of protons ($\Delta\tilde{\mu}_H$); P_i, inorganic phosphate; $\Delta G_P = \Delta G^{O\prime} + RT$ ln ([ATP]/[ADP][P_i]); Ap_5A, P^1,P^5-di(adenosine-5′-)pentaphosphate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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release [1–3]. In the present work, we investigate quantitatively how, in submitochondrial vesicles, membrane potential, ADP and ATP affect IF_1–F_1 interaction. This information should allow us to construct a model for control of F_1 applicable to the mitochondrion itself.

IF_1 release from, or binding to, ox-heart mitochondrial inner membranes is followed, in these experiments, by measuring ATPase capacity of submitochondrial vesicles, a strict inverse correlation having been demonstrated previously between IF_1 content of these vesicles and their (uncoupled) ATPase activity [2,4]. Further, in some experiments, binding or release of IF_1 is measured directly using ^{125}I-labelled IF_1; this has the additional advantage of allowing unidirectional rates (IF_1 off- or IF_1 on-rates) to be followed under conditions where changes in net membrane content of IF_1 may be negligible.

We find that release of IF_1 from, and its rebinding to, F_1 are governed independently by different factors. In the absence of a Δp and added medium nucleotides, both release and rebinding of IF_1 have been found to be very slow processes [5]. In this paper we show that generation of a membrane potential stimulates IF_1 release from mitochondrial membranes, showing a very steep dependence over the range 100–135 mV. ATP, conversely, stimulates the binding of IF_1 with a concentration for half maximal effect at about 140 μM, which is roughly equal to the K_m for ATP hydrolysis by membrane-bound F_1 [6]. By varying ATP concentrations while keeping the phosphate potential ($\Delta G_P = \Delta G^{O'} + RT \ln([ATP]/[ADP][P_i])$) constant, we show that it is ATP concentration and not the value of ΔG_P which affects the IF_1 on-rate. The opposing effects of increasing membrane potential and increasing ATP concentrations thus lead to varying steady-state levels of F_1 activity in the mitochondrial inner membrane.

Materials and Methods

Ox-heart submitochondrial vesicles were prepared and incubated with malonate as described previously [2]. Protein was determined as described [2]. For measurements of ATPase activity, protonmotive force and phosphate potential, submitochondrial vesicles (2.0–3.5 mg/ml) were preincubated, under continuous stirring, at 25 °C for 5 min in a final volume of 3.3 ml, in the upper chamber of a flow dialysis cell, fitted with a Clark-type oxygen electrode and a thermostatically controlled water jacket. The medium (buffer A) contained 10 mM phosphoric acid, 21 mM Tris (pH 7.3), 5 mM magnesium acetate, 90 μM Ap_5A, 1 mg/ml bovine serum albumin and 20 mM glucose. Where indicated, FCCP was added at this stage. The final concentration of ethanol never exceeded 3% (v/v).

At $t = 0$, water-saturated oxygen was blown over the suspension and 50 mM sodium succinate (pH 7.3) was added. 5 μM potassium S[^{14}C]N was also added at this point, as the magnitude of $\Delta\psi$ was determined from the extent of uptake into the particles of S[^{14}C]N$^-$ [7]. The composition of buffer A was such that the ΔpH contribution to the protonmotive force (Δp) was vanishingly small so that $\Delta p = \Delta\psi$ [8]. Where indicated, 25 U/ml of yeast hexokinase, 180 μM sodium ADP or different concentrations of sodium ATP were added just prior to succinate addition. For $\Delta\psi$ determinations, the dialysate was collected at fixed intervals and counted for S[^{14}C]N$^-$ [7]. 20 μM FCCP or 28 μM CCCP was added 6–12 min after succinate addition in order to cause the efflux of S[^{14}C]N$^-$ from the particles and thus to allow $\Delta\psi$ determination [7]. The internal volume was taken as 1.3 μl/mg protein of submitochondrial vesicles [8]. For estimation of ATPase activity, 30 μl samples were withdrawn from the flow-dialysis suspension and transferred to test tubes containing 60 μl of 10 mM phosphoric acid, 21 mM Tris (pH 7.3), 5 mM magnesium acetate, 10 μM FCCP and 7.5 mM EDTA (disodium) and incubated with glucose oxidase as described in Ref. 2. ATPase activity was measured at 30 °C as in Ref. 2. For ΔG_P determination, 1 ml samples were taken from the suspension 5.5 min after succinate addition, quenched with perchloric acid and neutralised as in Ref. 9. ATP, ADP and P_i were determined spectrophotometrically according to Refs. 10 and 11, respectively. $\Delta G^{O'}$ was taken as 30.6 kJ/mol [12].

IF_1 release from membrane bound F_1 was measured using [^{125}I]IF_1 supplemented submitochondrial vesicles as in Ref. 2, except that,

where indicated, unidirectional rates were measured by including unlabelled IF_1 (18 μg/ml) in the incubation medium to prevent rebinding of released [^{125}I]IF_1. IF_1 binding was measured essentially similarly, except that untreated submitochondrial vesicles (1.4 mg protein/ml) replaced [^{125}I]IF_1 SMP in the incubation. At $t = 0$, with 50 mM sodium succinate, [^{125}I]IF_1 was added to a final concentration of 3.7 μg/ml (7800 cpm/μg). Then, 150 μl samples were taken at intervals and the binding reaction quenched as previously [2]. 140 μl of the quenched suspension were centrifuged in a Beckman airfuge for 5 min at 148000 × *g*. The resulting pellet was washed once with 140 μl 21 mM Tris (pH 7.3), 10 mM phosphoric acid, 7.5 mM EDTA (disodium), 0.2 mg/ml horse heart cytochrome *c* and counted for ^{125}I. In the absence of mitochondrial membranes, less than 1% of added [^{125}I]IF_1 adhered to the sides of the tubes when subjected to the above procedures.

FCCP was from Fluka AG (Switzerland). Ap_5A (as lithium salt) was purchased from Sigma. Glucose oxidase (from *Aspergillus niger*, grade II, lyophilised) and CCCP were from Boehringer (Mannheim, F.R.G.). Potassium S[^{14}C]N and ^{125}I were purchased from the Radiochemical Centre (Amersham, Bucks., U.K.).

Results

Effect of membrane potential and of nucleotides on IF_1–F_1 interaction

In submitochondrial vesicles generating a protonmotive force, IF_1 is released into solution from its binding site on F_1 in an exponential process [2]. Under our conditions, only about 30% of bound IF_1 is released maximally from the vesicles [2]. Since we have previously shown that bound IF_1 behaves as a uniform pool on energisation (i.e., there is no fraction of IF_1 especially sensitive to energisation [4]), it appears that the maximal release represents a steady state (see Fig. 2) where IF_1 molecules are released from F_1 and rebind to it at equal rates.

Fig. 1 shows the dependence of the steady-state level of F_1 activation in submitochondrial vesicles as a function of steady-state membrane potential. $\Delta\psi$ was varied by addition of small amounts of uncoupler (0.1–0.5 μM FCCP) 5 min prior to activation by succinate. In these vesicles (initial IF_1 content 0.75–0.8 mol per mol F_1 [4]) there is a linear increase in the level of ATPase activation around 100–135 mV. Increasing $\Delta\psi$ above 140 mV leads to no further change of the hydrolytic capacity of the vesicles. With the concentration of vesicles used in this type of experiment, the flow dialysis technique does not allow an accurate estimation of the magnitude of $\Delta\psi$ below approx. 100 mV. However, if a linear extrapolation of the plot of Fig. 1 is justified (dashed trace of Fig. 1), it

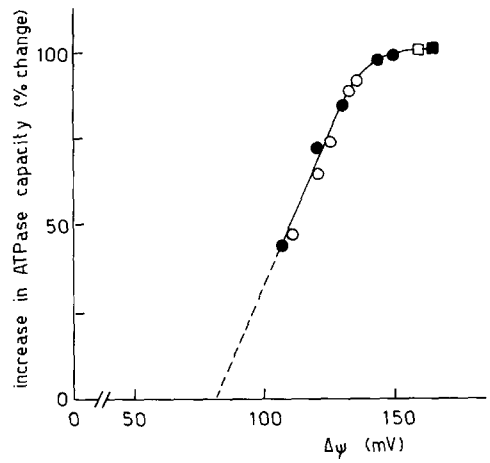

Fig. 1. Dependence of the increase in ATPase capacity on $\Delta\psi$ in submitochondrial vesicles. 3.5 mg/ml MgATP-SMP were suspended in 3.3 ml of buffer A (without bovine serum albumin) in the upper chamber of a flow-dialysis cell (see Materials and Methods) for 5 min in the absence (□, ■) or presence (○, ●) of concentrations of FCCP between 0.1 and 0.5 μM. 25 U/ml hexokinase, 5 μM potassium S[^{14}C]N and 50 mM sodium succinate were then added. Just before and within 7 min after succinate addition, 30 μl samples were taken for the estimation of the ATPase activity (see Materials and Methods), which was found to be maximal after approx. 5 min (see Fig. 2). The difference between the ATPase activity found after more than 5 min of energisation with FCCP present and that at $t = 0$ (no energisation) was calculated and expressed relative to the difference in ATPase activity found at the same times but in the absence of FCCP (100%) (□, ■). 7.5 min after succinate addition, CCCP was added to allow estimation of $\Delta\psi$ (see Materials and Methods). The ATPase activity of MgATP SMP at time zero was about 2 μmol ATP hydrolysed/min per mg total protein, while the maximal activity after 5 min of incubation with succinate, in absence of FCCP, was 6 μmol ATP hydrolysed/min per mg total protein. Temperature: 25° C. Open and closed symbols relate to data obtained with different vesicle preparations.

can be seen that a threshold membrane potential of about 85 mV is required to activate any F_1 molecule.

The experiments of Fig. 1 were carried out in the absence of added nucleotides and in the presence of a glucose-hexokinase trap, to eliminate any ATP, possibly originating from nucleotide carried over from the preparation of the vesicles. Fig. 2 shows that nucleotides, in addition to membrane potential, can influence net IF_1 release. The The curves show the increase in ATPase capacity under conditions where $\Delta\psi$ is varied between 165–135 mV with FCCP, and ADP and ATP concentrations are also varied. In the upper curves, ADP and the glucose-hexokinase trap were present, so as to allow the maximal net rates of phosphorylation (compatible with the imposed $\Delta\psi$) by the vesicles and to maintain virtually no ATP free in solution. In the lower set of curves, no

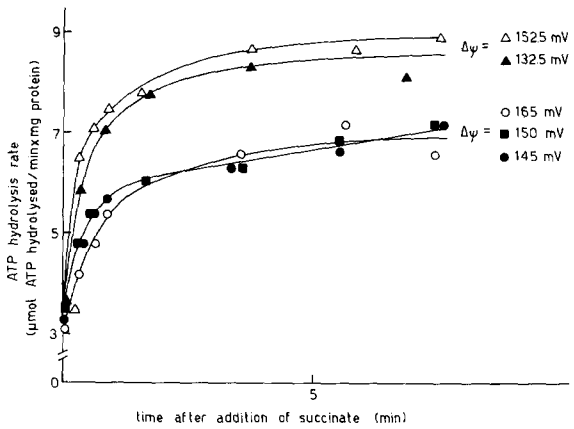

Fig. 2. Time-course of increase in ATPase capacity after generation of $\Delta\psi$ in submitochondrial vesicles during, and in the absence of, net phosphorylation. 2.0 mg/ml MgATP SMP were incubated at 25 °C in 3.3 ml of buffer A (without bovine serum albumin) in the upper chamber of a flow dialysis cell (see Materials and Methods). FCCP (0–0.16 μM) was added as indicated (see Materials and Methods). After 5 min, 5 μM potassium S[^{14}C]N was added and oxidation initiated by addition of 50 mM sodium succinate. To induce phosphorylation, 180 μM sodium ADP and 25 U/ml hexokinase were added just prior to succinate (△, ▲). Aliquots were withdrawn at intervals for measurement of ATPase activity (see Materials and Methods). 8.5 min after succinate addition 20 μM FCCP was added to allow determination of the magnitude of $\Delta\psi$ (see Materials and Methods). The values obtained are given on the diagram. △, hexokinase and ADP, no FCCP; ▲, hexokinase, ADP and 0.16 μM FCCP; ○, no addition; ■, 0.06 μM FCCP; ●, 0.1 μM FCCP.

nucleotide was added and hexokinase omitted, thus allowing small amounts of ATP to accumulate (see above). In agreement with Fig. 1, Fig. 2 shows that, at high values of $\Delta\psi$, variation of $\Delta\psi$ over a range of 20 mV does not change the level of ATPase activation at one nucleotide concentration. However, changing from one nucleotide composition to the other does greatly affect the increase in ATPase capacity, the increase in steady-state level with ADP and hexokinase being about two-fold higher than the value if both were omitted.

An increased IF_1 release during net phosphorylation (in the presence of hexokinase) was demonstrated in ox-heart submitochondrial vesicles by Van de Stadt et al. [13] and the regulation of IF_1 release was attributed primarily to the ATP/ADP ratio (or ΔG_P) [13]. However, the data of Fig. 3 show that such explanation cannot account for our results. The experiments of Fig. 3 were designed to ascertain how different concentrations of ATP affect the steady-state level of F_1 activation at similar equilibrium values of ΔG_P. Different amounts of ATP (approx. 0.1–0.8 mM) were added to the vesicles along with succinate (in the absence of hexokinase). After thermodynamic equilibrium between Δp and ΔG_P had been reached (5.5 min incubation), the actual concentrations of ATP, ADP and phosphate were measured directly by analysis of the reaction mixtures. As expected for the similar value of $\Delta\psi$ found in all cases (170 mV), a similar value of ΔG_P was also determined (43.9 kJ/mol), despite the different steady-state concentrations of ATP (which are shown in the figure).

As in Fig. 2, in all cases of Fig. 3, oxidation leads to an increase in ATPase capacity to a steady-state level, which is reached within about 5 min of succinate oxidation. Moreover, Fig. 3 shows that increasing amounts of ATP suppress the succinate-induced increase in ATPase capacity and that this effect is virtually complete above approx. 500 μM steady-state concentration of ATP. Fig. 3 also shows that if ATP is removed by hexokinase after the steady state is reached, the ATPase capacity of the vesicles is immediately stimulated to approximately the value measured when no ATP was initially added (upper curve).

It is important to note that ADP has no major

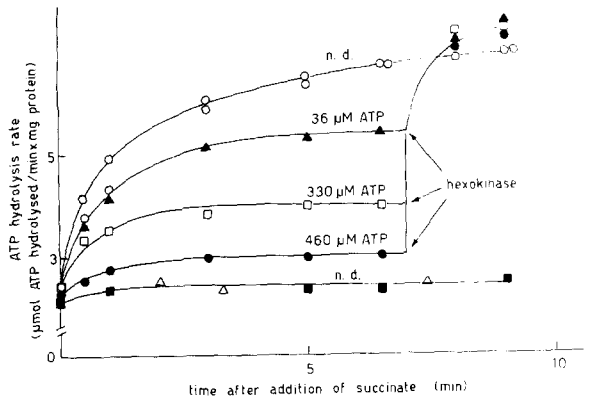

Fig. 3. Time-course of increase in ATPase capacity in submitochondrial vesicles generating a $\Delta\psi$, as a function of ATP concentration. MgATP SMP (2.8 mg/ml) were incubated in buffer A (without bovine serum albumin) (see Materials and Methods). At $t = 0$, 50 mM sodium succinate and 5 μM potassium S[^{14}C]N were added. Other additions, just prior to succinate, were as follows: ○, 25 U hexokinase per ml; ▲, 120 μM sodium ATP; □, 580 μM sodium ATP; ●, 780 μM sodium ATP; ■, 780 μM sodium ATP and 28 μM CCCP (CCCP being added in the preincubation stage); △, 780 μM sodium ATP (with no succinate and CCCP). Where indicated, hexokinase (25 U/ml) was added at $t = 7$ min to remove ATP. Samples were taken at intervals for the determination of ATP hydrolysis rate and, at 5.5 min from succinate addition, for the determination of the concentrations of ATP, ADP, and P_i (ΔG_P) (see Materials and Methods). ATP concentrations, after 5.5 min incubation, are given on the figure. The steady-state membrane potential was measured as described in Materials and Methods and found to be 170 mV in all cases (within 7 min from succinate addition). When hexokinase was added ($t = 7$ min), $\Delta\psi$ fell to 150 mV. $\Delta\psi$ was not measured in the experiments shown in the lower trace. n.d., ATP concentration was not determined.

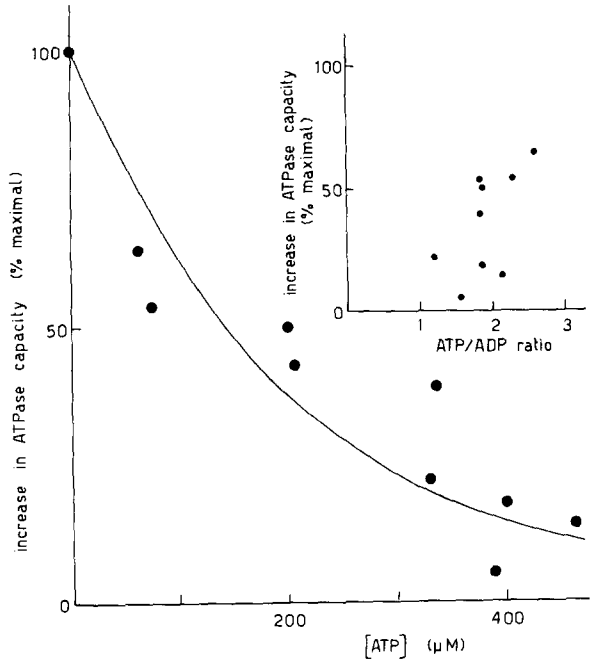

Fig. 4. Suppression of $\Delta\psi$-dependent increase in ATPase capacity by ATP. Data points are derived from experiments run as in Fig. 3, and have been fitted by a regression analysis to an exponential function ($y = e^{-0.0048x}$). The concentration range of sodium ATP (added just before succinate) varied from 0 to 780 μM. The x axis shows the steady-state concentration of ATP, found after 5.5 min from succinate addition. The y-axis shows the steady-state stimulation of ATPase capacity relative to the maximal stimulation obtained after 5.5 min energisation by succinate (in the presence of hexokinase (25 U/ml) and with no added ATP) minus the control value (ATPase activity at 5.5 min after incubation with succinate, in the presence of CCCP (28 μM) and ATP (780 μM)). The maximal stimulation (about 4 μmol ATP hydrolysed/min per mg total protein, depending on preparation) was taken as the 100% value. Inset: values in y-axis are those of y-axis of Fig. 4, and values on x-axis are the ATP/ADP ratios found after 5.5 min from succinate addition in the same experiments.

role in stimulating ATPase capacity. This is clear from Fig. 3 because, upon addition of hexokinase to the incubation mixtures with different steady-state concentrations of ATP and ADP, both nucleotides are transformed in recycling ADP. Hence, equal ATPase activities are induced whatever the concentration of ADP, as long as ATP is absent from the incubation.

Fig. 4 shows the relationship between the steady-state values of medium ATP concentration and the induced ATPase capacity of the vesicles. ATP suppresses the induction of ATPase capacity with a concentration for half maximal effect of about 140 μM, which is close to the K_m for ATP hydrolysis by membrane-bound F_1 [6]. It seems likely, therefore, that this suppression involves interaction of ATP with the catalytic site of F_1.

As the nucleotide levels were varied, in the experiments of Fig. 4, nonetheless $\Delta\psi$ developed by the vesicles was almost unaltered (160–170 mV) and correspondingly, the ΔG_P was found also to be approximately constant (42.7–44.4 kJ/mol). Although ΔG_P was nearly constant in these incubations, it might be argued that this parameter is not very sensitive to significant changes in ATP/ADP ratio, which indeed did vary about

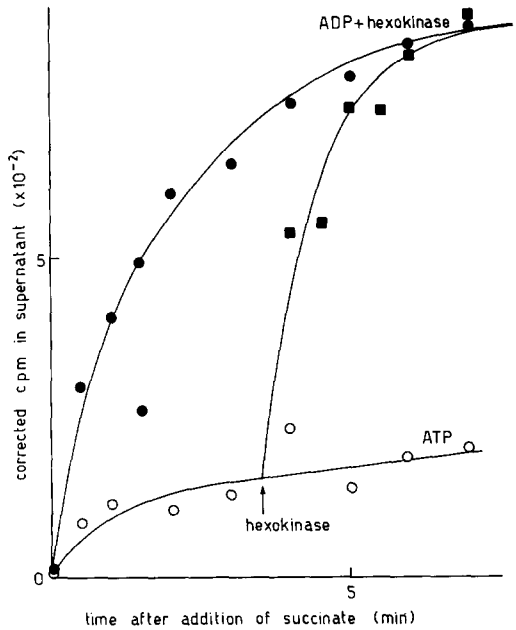

Fig. 5. Effect of nucleotides on net IF_1 release from submitochondrial vesicles oxidising succinate. [^{125}I]IF_1 SMP (1 mg/ml, 39000 cpm/mg total protein) were incubated in buffer A (see Materials and Methods) at 37 °C for 5 min, in a final volume of 2 ml. Oxidation was initiated by addition of sodium succinate (50 mM). Just prior to succinate addition, sodium ADP (180 μM) and hexokinase (25 U/ml) (●) or sodium ATP (2 mM) (○) were added. 150 μl aliquots were withdrawn at intervals and added to 30 μl quenching medium [2]. After centrifugation, 100 μl of the supernatant were counted for ^{125}I, to determine the amount of IF_1 released from the membranes [2]. In one sample, hexokinase (25 U/ml) was added to the incubation with sodium ATP at $t = 3.5$ min, to convert ATP to ADP (■). The corrected cpm were calculated by subtracting, from the measured values, the cpm detected in the supernatants before the addition of succinate.

two-fold (from 1.2 to 2.6), as ATP was varied. However, this variation in ATP/ADP ratio did not correlate with steady-state ATPase capacity, as shown in Fig. 4 (inset). It is evident that suppression of ATPase activation is not a function of increasing ATP/ADP ratio; in fact, within experimental error, the two parameters do not appear to be related. It seems thus that in submitochondrial vesicles, at a constant $\Delta\psi$ value, the extent of $\Delta\psi$-dependent ATPase activation is inversely related to ATP concentration and not to either ΔG_P or ATP/ADP ratio.

It is shown above that ATP suppresses the $\Delta\psi$-dependent activation of F_1. Fig. 5 demonstrates that, as expected, this is achieved by suppression of net IF_1 release from the vesicles. IF_1 release from [^{125}I]IF_1 SMP was measured during succinate oxidation, with ADP and hexokinase (upper curve) or ATP (lower curve) present in the reaction medium. During continuous phosphorylation (upper curve) (no free ATP), about 30% of bound IF_1 is released from oxidizing vesicles into the supernatant; in the presence of 2 mM added ATP (lower curve), less than 5% is released. Further, addition of hexokinase to remove ATP in the latter experiment leads to a stimulation of IF_1 release from the lower to the higher value. This confirms that suppression of net IF_1 release is simply a consequence of the presence of ATP in the reaction medium. All of these effects are paralleled by changes in ATP hydrolytic capacity (see Fig. 3).

Mechanism by which the membrane potential and ATP regulate IF_1–F_1 interaction

Suppression of net IF_1 release could occur by two mechanisms. ATP might decrease the rate of (unidirectional) IF_1 release from the membrane bound F_1-IF_1 complexes ('off-rate'). Conversely, ATP might stimulate the rate of recombination of free IF_1 with F_1 ('on-rate'). Fig. 6 shows the effect of ATP on the IF_1 off-rate. In the presence of ATP and succinate, as above, net IF_1 release is small (lowest curve). When unlabelled IF_1 is included in the medium, to dilute the specific radioactivity of released [^{125}I]IF_1 and prevent its rebinding, much more label appears in the supernatant (upper curve). It appears, therefore, that although the net release of IF_1 is suppressed in the presence of ATP, its off-rate is still high. The uppermost curve in Fig. 6 shows the time course of unidirectional IF_1 release when ATP is absent. Comparing the two upper curves in this figure we conclude that ATP has little effect on the off-rate of IF_1.

The small difference between the two upper curves can be explained, since, due to the dilution factor used (50 μg unlabelled IF_1 added to vesicles with a total intrinsic IF_1 content of about 14 μg), rebinding of IF_1 is reduced rather than completely abolished. These curves thus slightly underestimate the off-rate of IF_1 from the membranes and this underestimation is greater if ATP is present

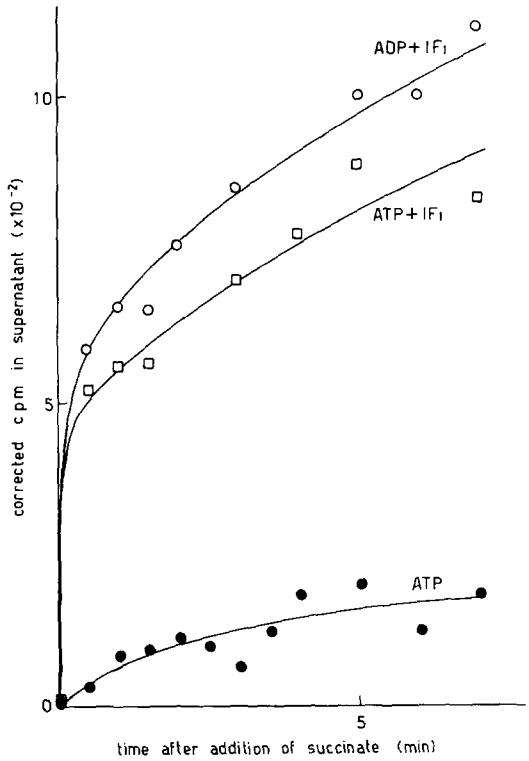

Fig. 6. Effect of nucleotides on IF_1 off-rate in submitochondrial vesicles oxidising succinate. [^{125}I]IF_1 SMP (0.93 mg/ml) were incubated in 3 ml buffer A, and release of [^{125}I]IF_1 measured as in Fig. 5, except that additions (made just prior to succinate addition) were as follows: ●, 1 mM sodium ATP; □, 18 μg/ml IF_1 and 1 mM sodium ATP; ○, 18 μg/ml IF_1, 1 mM sodium ADP and 25 U/ml hexokinase. The corrected cpm were calculated as in the legend of Fig. 5.

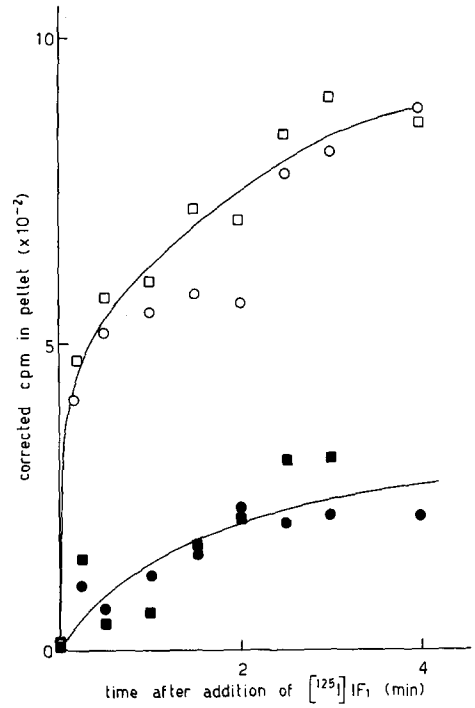

Fig. 7. Effect of nucleotides on the rate of IF_1 binding to submitochondrial vesicles. Submitochondrial vesicles (1.4 mg/ml) were incubated in 3 ml of buffer A (see Materials and Methods) at 37 °C for 5 min. At $t = 0$, [^{125}I]IF_1 (11 μg, 7800 cpm/μg) was added to the solution, simultaneously with the addition of: sodium ATP (1 mM), sodium ADP (1 mM) and sodium succinate (50 mM) (□); sodium ATP (1 mM) and sodium succinate (50 mM) (○); sodium ADP (1 mM), hexokinase (25 U/ml) and sodium succinate (50 mM) (■); no addition (●). Aliquots were taken at intervals for determination of membrane-bound IF_1 and ATPase activity as described in Materials and Methods and in Ref. 2. The initial ATPase activity of the vesicles was 3.8 μmol ATP hydrolysed/min per mg total protein, whereas, after 4 min from [^{125}I]IF_1 addition ,the values were as follows: ○, 1.2; ■, 5.8; ●, 3.3 μmol/min per mg total protein.

(since ATP stimulates rebinding of IF_1 – see below). The effects of both ADP and ATP on the IF_1 off-rate are thus concluded to be identical and probably small.

The (unidirectional) binding of IF_1 to submitochondrial vesicles can be measured by incubating the untreated vesicles with [^{125}I]IF_1 and following the association of ^{125}I with the particulate fraction. Fig. 7 shows experiments where IF_1 binding is measured while the membranes are oxidising succinate. The initial slopes of the curves in Fig. 7 are indicative of the rates of IF_1 binding under the different conditions used. In the absence of ATP (lower curve), under continuous phosphorylating conditions, association of [^{125}I]IF_1 with the membrane is very slow. A similar low on-rate for IF_1 is observed in the absence of ADP and succinate (Fig. 7, lower curve).

In the presence of ATP and succinate, however, IF_1 binds rapidly to the membranes. After 4 min, in fact, there has been complete equilibration of bound and free IF_1. It appears, therefore, that ATP suppresses net IF_1 release by stimulating the IF_1 on-rate, the off-rate being left unchanged. As postulated above (Fig. 4), this effect appears to be due to ATP and not to depend on the ATP/ADP ratio; addition of ADP, in presence of ATP and succinate, does not apparently affect the IF_1 on-rate (Fig. 7, upper curve).

The legend of Fig. 7 also gives the final ATPase capacities in the above experiments. The initial value in each case was 3.8 μmol ATP hydrolysed/min per mg total protein. Addition of ATP and [^{125}I]IF_1 (plus succinate) leads to a very low final ATPase capacity (1.2 μmol ATP hydrolysed/min per mg), the fast IF_1 on-rate having outstripped the succinate-stimulated off-rate. The opposite is true if ATP is omitted: the succinate-stimulated IF_1 release outstrips the slow IF_1 binding and a high ATPase capacity results (5.8 μmol ATP hydrolysed/min per mg). If both ATP and succinate are omitted, both IF_1 on- and off-rate are very slow, and under these conditions (three-fold excess of added IF_1) a slow decline in ATPase capacity is the result (3.3 μmol ATP hydrolysed/min per mg).

Discussion

Membrane potential and ATP synthase activity

Generation of a Δp in submitochondrial vesicles, during substrate oxidation, leads to an increase in their ATP hydrolytic capacity, concomitant with a release of IF_1 from F_1 [2,4,14,15]. On the basis of studies with ionophores, it has been suggested that the membrane potential, rather than the pH gradient component of the protonmotive force, is responsible for inducing IF_1 release [16,17]. This work confirms that the membrane potential alone is sufficient to induce IF_1 release since, in the Tris-phosphate buffer used, a transmembrane pH gradient is undetectably small (less than 0.3 pH units) [8].

The dependence of net IF_1 release on membrane potential is shown in Fig. 1. Dependence is steep over the range 100–135 mV, but above about 140 mV net release is independent of membrane potential (see also Fig. 2). This dependence is different from that reported for phosphorylation: negligible phosphorylation rates have been measured in ox-heart submitochondrial vesicles at $\Delta j = 100$ mV [18], while the ATP synthase is already at 40% maximal activation (Fig. 1).

The observed increase in net IF_1 release is brought about by an increase in the off-rate of IF_1 from the membranes (Fig. 6). Control experiments (without succinate or with uncoupler) show that the IF_1 off-rate is negligible over the period of these experiments (7 min) in the absence of a membrane potential (Fig. 3) (cf. also Refs. 4 and 5). $\Delta\psi$ does not appear to affect the rate of IF_1 binding to the membranes (Fig. 7), nor is its effect on the off-rate affected by nucleotides (within experimental error) (Fig. 6).

We may only speculate as to how a transmembrane potential can affect the association of two proteins on one side of the mitochondrial membrane. Any model must take into account the observations that (a) the rate of IF_1 release is unaffected by altering the membrane surface charge with alkyl guanidines [16], suggesting no direct effect of surface potential; (b) IF_1 release is slowed only 2–3-fold by binding to it an anti-IF_1 antibody some 20-times its size [1], thus ruling out simple electrophoretic movement; and (c) IF_1 release, but not the development of $\Delta\psi$, is inhibited by oligomycin [4]. The last observation suggests that the effect of membrane potential is in fact on F_1 (possibly via the transmembrane F_0 channel), with F_1 changing in conformation under the influence of $\Delta\psi$ so as to abolish the IF_1 binding site. An alternative explanation, where IF_1 itself changes from a binding to a non-binding conformation, is suggested by the demonstration of two conformational states of IF_1 in both the yeast and ox-heart systems [19,20], but how the necessary conformational change could be triggered by a transmembrane potential is unclear.

Nucleotide levels and ATP synthase activity

It has long been known that, in uncoupled submitochondrial vesicles and in isolated F_1, ATP stimulated IF_1 binding to F_1 [5,21]. The situation in coupled vesicles, however, is less clear: ATP has been reported both to stimulate or to reduce IF_1 binding [4,22–24]. In agreement with Gómez-Puyou et al. [3] we show here that the succinate-induced increase in ATPase capacity observed in coupled submitochondrial vesicles is suppressed by ATP (Figs. 3 and 4). Furthermore we demonstrate that this suppression is due to ATP decreasing the net loss of IF_1 from the vesicles (Fig. 5) by increasing the IF_1 on-rate (Fig. 7), while having no effect on the ($\Delta\psi$-dependent) off-rate (Fig. 6). Thus ATP acts similarly on the IF_1 equilibrium in coupled and uncoupled vesicles. Varying resultant effects observed when coupled

vesicles are treated with ATP alone [4,24,25] are due, presumably, to a balance between ATP not only promoting IF_1 binding but itself inducing a transmembrane potential (which increases IF_1 release).

A possible mechanism by which ATP stimulates the rates of IF_1 binding to F_1 has been given elsewhere [4]: in principle, IF_1 is thought to bind to an enzyme species which occurs as a transient intermediate in ATPase turnover. This model is supported by the data of Fig. 4, where the concentration of half-maximal suppressive effect of ATP is shown to be about equal to the K_m for ATP hydrolysis by (membrane-bound) F_1, if ΔG_P is kept approximately constant.

These data also rule out a model in which the level of ATP synthase capacity is controlled by ΔG_P (cf. Ref. 13). Indeed, it seems that IF_1 release from the ATP synthase is controlled by the parameters $\Delta\psi$ and [ATP]. This dependence can be explained as in Scheme I:

$$IF_1 - F_1 \overset{\Delta\psi}{\rightleftharpoons} IF_1 + F_1^* \underset{ATP}{\rightleftharpoons} IF_1 + F_1$$

IF_1 binding (reverse direction) is limited by the amount of the unstable form F_1^*; however, this occurs transiently during hydrolysis and thus ATP stimulates IF_1 binding. The relaxation of $IF_1 + F_1^*$ to IF_1-F_1 is not ATP dependent [5] and is presumably a downhill process. IF_1 release (forward direction) also requires generation of F_1^*, and this (since it is a thermodynamically uphill process) is postulated to require $\Delta\psi$. The final step in IF_1 release is postulated not to require $\Delta\psi$; it will certainly be a spontaneous process but an indirect role of $\Delta\psi$ cannot, at this stage, be ruled out. Elements of this scheme have been proposed previously [4,5,16], but this is the first demonstration that they hold simultaneously in coupled phosphorylating vesicles.

The dependence of IF_1 release on the simple parameters $\Delta\psi$ and ATP contrasts with the proposed dependence of phosphorylation rates on the thermodynamic functions $\Delta\tilde{\mu}_H$ and ΔG_P [26]. To construct a complete model for the control of phosphorylation rates, therefore, both effects must be taken into account. First, $\Delta\psi$ and ATP, via IF_1, will control the number of active F_1 molecules available for turnover. Then, the net available energy ($n\Delta\tilde{\mu}_H - \Delta G_P$) would presumably control the rate of turnover of each individual active F_1 molecule. Analysis of this type of system has been attempted in detail for the chloroplast system by Junesch and Gräber [27].

Regulation of ATP synthase in vivo

Recent work provides an increasing body of evidence that ATP synthase capacity in vivo, as well as turnover rate, may alter with physiological changes. In mitochondria, transient changes in internal pH, observed by ^{31}P NMR [28], have been interpreted [29] as due to relatively slow changes in ATP synthase capacity limiting the utilisation of Δp. More directly, changes in ATP synthase capacity in heart mitochondria, linked to changes in oxidation rates, have been demonstrated in perfused heart muscle, either by subsequent isolation of mitochondrial membranes with different ATPase capacities [30] or by measurement in situ of (non-energy requiring) exchange reactions [31].

The energy-dependent responses of IF_1 demonstrated above and the time-course of such responses [2], make IF_1 an attractive candidate for such regulatory mechanism. At first site, however, the quantitative characteristics of the responses seem unsuitable for physiological regulation. Fig. 1, for example, shows that the net ATP synthase capacity varies with $\Delta\psi$ only up to 135 mV, while isolated mitochondria are believed to achieve values of 160–200 mV [32]. However, recent measurements of $\Delta\psi$ in mitochondria within cells suggest that the maximal attainable value is around 130 mV [33] (but see Ref. 34).

Perhaps a more serious objection to the incorporation of IF_1 into a model for ATP synthase regulation in vivo is evident from Figs. 3 and 4. Here 0.5 mM ATP is sufficient to suppress almost completely IF_1 release from F_1. Since intramitochondrial ATP levels presumably are above 1 mM, one might expect that ATP synthase in vivo to be permanently switched off!

This paradox may, in fact, be the result of our experimental conditions. Under low salt conditions, as used here to facilitate $\Delta\psi$ measurements, and using inverted inner mitochondrial membranes (leading to a low free IF_1 concentration),

IF_1 on-rates may be artificially high. High salt conditions are known to slow IF_1 binding to F_1 [5,25] and high concentrations of IF_1 to lead to self-aggregation [35,36], which may reverse only slowly. Both of these factors, therefore, may lead to a slower IF_1 on-rate in vivo (where intramitochondrial IF_1 will be around 0.2–0.5 mM) than the rate we measure here. Thus the IF_1-F_1 equilibrium in vivo will be displaced further towards free IF_1 than may be evident from these investigations, allowing modulation by ATP in the physiological range. Other factors, not considered above (e.g., Ca ions [37], the redox state of the mitochondrial membrane [38] or accessory protein factors [39,40]), may also be involved in affecting IF_1 binding or release rates. A definitive answer as to how (and if) IF_1 regulates the mitochondrial ATP synthase in vivo thus awaits further studies.

Acknowledgements

D.A.H. is supported by the Medical Research Council (U.K.) and by the British Heart Foundation. G.L. thanks the European Molecular Biology Organisation for the award of a short-term fellowship. The authors thank Ms. Monica Vettore for excellent secretariat help.

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