INTERCONVERSION OF TWO KINETICALLY DISTINCT STATES OF THE MEMBRANE-BOUND AND SOLUBILISED H^+-TRANSLOCATING ATPase FROM *RHODOSPIRILLUM RUBRUM*

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1. Introduction

The ATP synthetase enzymes which catalyse the terminal reactions of oxidative and photophosphorylation may be dislodged from their membrane vesicles by a variety of techniques. Studies on ATPase activity of the solubilised enzymes will only provide information on the phosphorylation mechanism if a clear relationship with the membrane-bound enzymes can be established. In fact many differences in behaviour between membrane-bound and solubilised enzyme have been observed (see review by Pederson [1]).

The ATPase activity of *Rhs. rubrum* chromatophores is dependent on the presence of either Ca^{2+} or Mg^{2+} [2,3]. In contrast, the solubilised coupling factor (F_1-type) is strictly Ca^{2+}-dependent and is competitively inhibited by Mg^{2+} [4]. A similar change in divalent cation requirement takes place upon resolution of the chloroplast ATPase [5–8] and has been described as allotopic [7], implying that the substrate specificity of the enzyme is modified by membrane binding [9]. The experiments described in this paper show that the divalent cation requirement of the solubilised enzyme may be duplicated in the membrane-bound system provided that the chromatophores are treated with high concentrations of uncoupling agent. The conclusion is that the substrate specificity is determined, not by association with membrane components but by the level of the high-energy state across the enzyme. In the isolated, isotropic enzyme this state cannot exist and in the membranous particles it may be effectively collapsed with uncoupling agents.

Certain anions are known to increase the rate of ATP hydrolysis in sub-mitochondrial particles [10–12] and isolated F_1 [12,13]. Moyle and Mitchell [12] have argued that this is a consequence of the conversion of inactive to active catalytic centres in their ATPase preparation. Nelson et al. [8] showed that maleate and bicarbonate unmask latent Mg–ATPase in heat or trypsin activated chloroplast CF_1. We find that in the presence of anions such as sulphite, the divalent cation requirement of solubilised *Rhs. rubrum* ATPase or of highly uncoupled chromatophores reverts to that normally found in coupled chromatophores. Low concentrations of efrapeptin inhibit ATPase activity independently of the divalent cation, activating anion or uncoupling agent concentrations. It appears that the anions block the change in properties of the enzyme which normally accompanies severe uncoupling, or dislocation from the membrane.

2. Methods

The growth of the bacteria and preparation of chromatophores were carried out as previously described [3]. Bacteriochlorophyll was estimated from the chromatophore absorbance at 880 nm using the extinction coefficient given by Clayton [14] and protein was estimated by the tannin assay [15].

Abbreviation: FCCP Carbonyl, cyanide-*p*-trifluoromethoxy phenylhydrazone

The isolation of the chromatophore soluble ATPase was by a modification of the method described by Beechey et al. [16] for mitochondrial ATPase. The chromatophores were washed once in 10 mM Tris–sulphate, 1 mM EDTA, pH 7.6 and resuspended to give a bateriochlorophyll concentration of 0.25 mM. The suspension was shaken with a half-volume of chloroform and centrifuged to break the emulsion. The aqueous layer was centrifuged at room temperature for 70 000 × g, 150 min and the clear supernatant was supplemented with 10% glycerol after dialysis overnight, to remove residual chloroform. Judged by polyacrylamide gel electrophoresis and filtration through Sepharose 6 B the ATPase in this preparation was routinely 70–80% pure. The subunit structure and further purification by affinity chromatography will be described in a forthcoming report.

Specific activities were usually of the order of 1–3 μmol Ca–ATP/mg protein/min in 10 mM Tris–sulphate, pH 7.6 and the enzyme was stable at 4°C for over one week. The solution kinetics of the preparation were similar to those of the enzyme prepared by acetone powder extraction as described by Johansson et al. [4].

The assays of chromatophore ATP hydrolysis were carried out as in ref. [3] using the glass-electrode technique or colorimetric determination of phosphate release as indicated in the figure legends. ATP hydrolysis by the solubilised enzyme was determined by colorimetric assay of phosphate release in a medium containing 10 mM Tris–sulphate, pH 7.6. Otherwise the experimental conditions were as described in the figure legends.

3. Results

3.1. *Equivalent divalent cation requirement for ATPase activity in uncoupled chromatophores and solubilised enzyme*

The Ca–ATPase activities of our solubilised enzyme preparation and of chromatophores treated with 16 μM FCCP are both competitively inhibited by free Mg^{2+} (fig.1). The K_m for Ca–ATP is 1.3 mM in each case and the K_i values are 100 μM and 250 μM Mg^{2+} respectively for the isolated and membrane bound enzyme. In contrast, chromatophores treated with less than 1.0 μM FCCP have a lower K_m for Ca–ATP (0.4 mM) and Ca–ATPase is activated by low

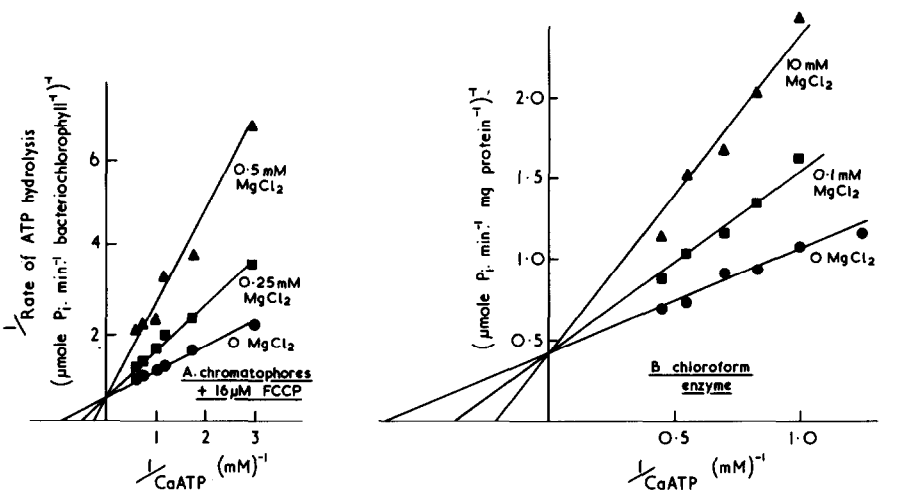

Fig.1. Competitive inhibition of Ca–ATPase by magnesium in solubilised and membrane-bound enzyme. A (left): Severely uncoupled chromatophores. The assay medium contained 50 mM KCl, 20 mM tricine, 0.83 mM succinate, 0.83 mM EDTA, 0.83 mM $CaCl_2$, 10% sucrose, 50 μM bacteriochlorophyll, 16 μM FCCP, final pH 7.6, in a total volume of 2.0 ml. The reaction was started adding Ca–ATP and $MgCl_2$ as indicated. The release of inorganic phosphate was measured colorimetrically after 3 min incubation at 30°C in complete darkness. B (right): Solubilised (chloroform) ATPase was assay at 30°C in 2.0 ml 10 mM Tris-SO_4 pH 7.6 from the colorimetric determination of phosphate release 3 minutes after the addition of Ca ATP. The total protein in each assay was 90 μg.

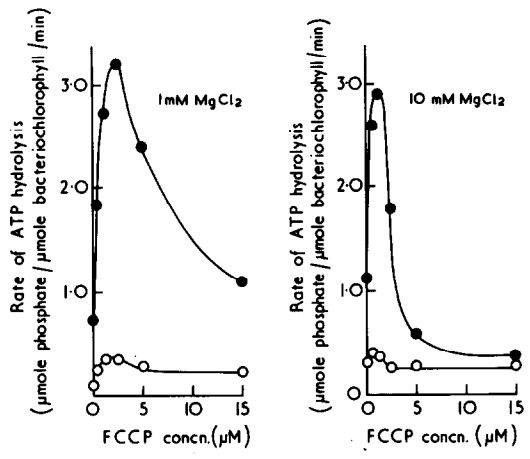

Fig.2. Oligomycin inhibition of chromatophore ATPase is independent of the concentration of uncoupler. ATPase activity was measured in the dark by the colorimetric assay of phosphate release as described in fig.1A, except that $CaCl_2$ was replaced by a final concentration of 1 mM $MgCl_2$ (left and 10 mM $MgCl_2$ (right). Reaction was started by the addition of 2.5 mM ATP and each sample contained 34 μM bacteriochlorophyll. (•) No further additions. (○) Oligomycin added to give a final concentration of 7.5 μg/ml.

concentrations of magnesium [3]. Mg–ATP may of course also act as a substrate (K_m = 0.2 mM) at these suboptimal concentrations of uncoupler [3,4]. Two explanations may be advanced to account for these results. Either (a) uncoupling agents induce the dislocation of the enzyme from the membrane and the divalent cation-dependence changes as a consequence (i.e., allotopically) or (b) the divalent cation requirement is controlled by the membrane high-energy state; this state is dissipated by proton-conducting uncoupling agents and is not developed by the solubilised enzyme.

Following the work of Melandri [17] et al. on the light-stimulated ATPase of *Rps. capsulata* we proposed [3] that magnesium ion inhibition of chromatophore ATPase activity at high concentrations of uncoupling agent is mediated via a regulatory, divalent cation binding site. When present in slight excess and when the H^+-permeability of the membranes is increased with uncoupler, Mg^{2+} inhibits ATP hydrolysis non competitively. Mg^{2+} is not inhibitory at low concentrations of uncoupler. Figure 2 shows that oligomycin sensitivity is retained at high concentrations of FCCP. Since oligomycin inhibits a membrane-bound component of the chromatophore ATPase [18] this suggests that treatment with uncoupling agent does not induce dislocation of the ATPase from the membrane. This is supported by experiments in which chromatophores in 15 μM FCCP were centrifuged for 100 000 × *g*, 90 min. Over 90% of the ATPase activity was recovered in the resuspended chromatophore fraction.

The existence of Ca–ATPase in the solubilised enzyme may be related to the fact that free-Ca^{2+}, unlike free-Mg^{2+}, is not inhibitory in severely uncoupled chromatophores. If in fact the enzyme conformation in these two situations is similar then it follows that the total activity of a sample of chromatophores assayed with Ca–ATP as a substrate and at a high FCCP concentration should be recoverable after separation of the enzyme from the membranes. This is found to be the case for two methods of separation (table 1).

Table 1
Recovery of Ca–ATPase from severely uncoupled chromatophores[a] after dislocation from the membranes

Dislocation technique	Ca–ATPase in native chromatophores[b]	Ca–ATPase in resolved chromatophores[b]	Ca–ATPase in solubilised enzyme[c]	Recovery (%)
Chloroform	1.8	–	1.6	89
Sonication[d]				
preparation 1	1.35	0.49	0.70	88
preparation 2	1.18	0.65	0.69	114

[a] Chromatophore ATPase was assayed with 2.5 mM Ca–ATP in the presence of 50 μM FCCP
[b] Rates are expressed as μmol phosphate min^{-1} in a sample containing 1 μmol bacteriochlorophyll
[c] Rates are calculated for the amount of solubilised enzyme (by vol.) that was derived from 1 μmol bacteriochlorophyll
[d] A sample of chromatophores was sonicated at 0°C for 3 × 30 sec at full power on a Rapidis 180, Ultrasonics Ltd. England

3.2. *The antagonism of 'activating anions' and uncoupling agents*

The addition of mM concentrations of sodium sulphite to the assay medium unmasks a 'latent' Mg–ATPase activity and stimulates Ca–ATPase activity in the chloroform enzyme preparation from *Rhs. rubrum* chromatophores. Sulphite, chromate and selenite are about equally effective but maleate and bicarbonate are less effective (data not shown). These potencies concur with the activation of mitochondrial ATPase by anions [11,12]. The acetone-powder enzyme prepared according to Johansson et al. [4] behaves similarly with activating anions (not shown). In direct agreement with our thesis that the solubilised enzyme has similar properties to highly-uncoupled chromatophore ATPase, fig.3A shows that membrane-bound Mg–ATPase activity which had been abolished by the addition of FCCP and excess Mg^{2+} may be restored by sulphite. Ca–ATPase activity in chromatophore preparations is also stimulated by sulphite in parallel with the solubilised ATPase.

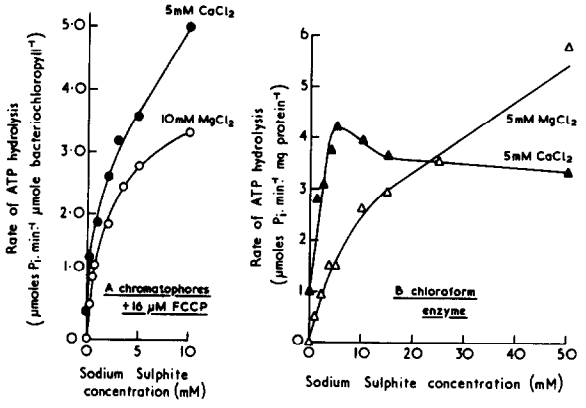

Fig.3. The effect of sulphite on ATP hydrolysis in chromatophores and solubilised enzyme. A (left): Severely uncoupled chromatophores. The rate of ATP hydrolysis was measured by the glass electrode technique in a medium containing 50 mM KCl, 1.0 mM tricine, 0.83 mM succinate, 0.83 mM EDTA, 10 μM FCCP, 10% sucrose, 40 μM bacteriochlorophyll and divalent cations as shown, at final pH 7.4, in a total volume of 3.0 ml, at 30°C, in complete darkness. Sodium sulphite was present at the concentrations indicated. The rates were estimated during the second minute after addition of 0.83 mM ATP. B (right): Chloroform ATPase. Rates were measured by the colorimetric determination of phosphate release in a medium containing 10 mM Tris–SO_4, 1 mM EDTA and $CaCl_2$ or $MgCl_2$ as indicated, at a final pH of 7.6, during a 3 min period after the addition of 2.5 mM ATP. The total protein in each assay was 47 μg.

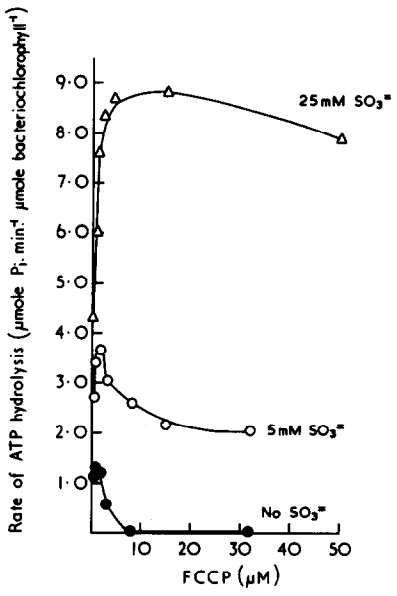

Fig.4. Reversal of Mg^{2+}/uncoupler-induced inhibition of chromatophore ATPase by sulphite. The rate of ATP hydrolysis in the absence of sulphite and at 5 mM sulphite were measured by the glass-electrode method (see fig.3A). The bacteriochlorophyll concentration was 43 μM. ATPase was determined colorimetrically (see fig.1A) in the presence of 25 mM sulphite owing to the high buffering capacity of this anion. In this case 55 μM bacteriochlorophyll was present. In all experiments the concentration of $MgCl_2$ was 10 mM.

Figure 4 shows that inhibition of Mg–ATPase by excess Mg^{2+} at high concentrations of uncoupler is prevented in the presence of 1 mM Na_2SO_3. Consequently, sulphite has a much greater stimulatory effect on ATPase activity when assayed in severely uncoupled chromatophores in the presence of an excess of free-Mg^{2+}. Nevertheless sulphite increases V for Mg–ATP hydrolysis under all the experimental conditions that we have tested.

Sulphite not only increases the V for Ca–ATPase but also, in the isolated enzyme or in highly uncoupled chromatophore it produces a significant decrease in the K_m for Ca–ATP. In chromatophores treated with sub-optimal concentrations of FCCP, sulphite increases V but has little effect on K_m (Ca–ATP).

These experiments suggest that 'activating anions' interfere with the control mechanism operated by the

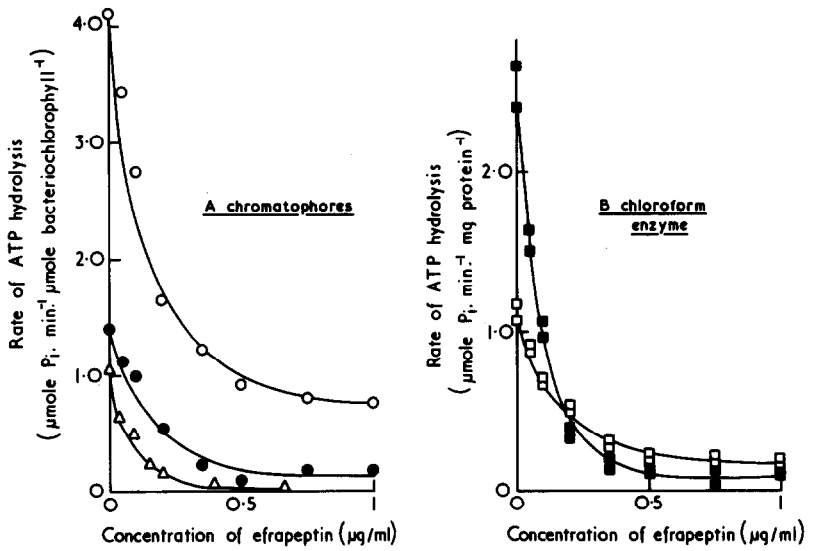

Fig.5. Inhibition of chromatophore ATPase and solubilised ATPase by efrapeptin. A (left): Chromatophore ATPase. Glass-electrode assay in standard medium (fig.3A) containing additionally 10 mM $MgCl_2$, no FCCP and no sulphite, 25 μM bacteriochlorophyll. Colorimetric assay in standard medium (fig.1A) containing additionally 10 mM $MgCl_2$. 32.5 μM FCCP, 10 mM sodium sulphite. 36 μM bacteriochlorophyll. Colorimetric assay in standard medium containing additionally 2.5 mM $CaCl_2$, 32.5 μM FCCP, no sulphite, 36 μM bacteriochlorophyll. (B (right): Chloroform ATPase. Colorimetric assay in standard medium (fig.1B) containing additionally 5 mM $CaCl_2$, no sulphite, 50 μg protein. Colorimetric assay in standard medium (fig.1B) containing additionally 5 mM $MgCl_3$, 5 mM sodium sulphite, 50 μg protein. Efrapeptin was added as a solution in methanol. In each type of experiment reaction was started by the addition of 2.5 mM ATP.

membrane high-energy state and the level of the divalent cations. Less likely, in view of the present results is one of the possibilities suggested by Ebel and Lardy [11] and by Pederson [19] working with rat-liver mitochondria, that the anions 'open' an alternative site for ATP hydrolysis. Supporting the contention that only one catalytic site is involved we find that the F_1-inhibitor efrapeptin inhibits ATP-ase activity in the presence and absence of sulphite and independently of the prevalent divalent cation in coupled and uncoupled chromatophores and in solubilised enzyme (fig.5). In each case a similar low concentration of efrapeptin is effective approximately one molecule of inhibitor (assumed mol.wt 1500) per 150 bacteriochlorophyll for 90% inhibition in the case of chromatophores.

4. Discussion

The change in the divalent cation requirement of ATPase upon dislocation from the chromatophore membrane is not allotopic as defined by Racker [9] but appears to result from a failure in the feed-back mechanism of the high-energy state. In coupled chromatophores the free-energy released during ATP hydrolysis is conserved as a high-energy state, most probably on electrochemical activity gradient of H^+ across the membrane, which prevents inhibition of the ATPase by Mg^{2+} and lowers the K_m for Ca–ATP. Intermediate sensitivity to Mg^{2+} is observed as the H^+ conductivity of the chromatophores is progressively increased with uncoupling agent. In the solubilised enzyme or in highly uncoupled chromatophores the free-energy of ATP hydrolysis is rapidly dissipated so that magnesium-inhibition is complete and the K_m for Ca–ATP is high. The changing divalent cation sensitivity and K_m-values must be consequent on a shift in ATPase conformation. Melandri et al. [20] have shown that the conformation of *Rps. capsulata* ATPase, as judged by *N*-ethyl maleimide sensitivity does change upon energisation of the chromatophore membranes.

Mg–ATPase activity and the low K_m for Ca–ATP are restored in the solubilised enzyme or in highly uncoupled chromatophores by the inclusion of 'activating anions' in the assay medium. These anions may give rise to a similar enzyme configuration to that which normally exists in coupled chromatophores. In this respect uncoupling agents and activating anions behave antagonistically. The effects of these anions have been studied in detail on the mitochondrial ATPase. The few anions that we have investigated show very similar potencies to those described for the mitochondrial system [11,12]. Of particular significance to our results is the finding of Moyle and Mitchell [12] that anions such as sulphite increase the proportion of active catalytic centres in their ATPase preparations particularly in Mg^{2+}-inhibited samples. These experiments were performed with either uncoupled sonic particles (plus 1 μM FCCP) or with isolated F_1 and so the significance of the energised state of the enzyme/membrane may have been overlooked. We note however that Ebel and Lardy [11] observed an eight-fold stimulation of F_1-ATPase by sulphite but only a two-fold increase in the case of coupled sonic particles with the same concentration of anion. In the light of our findings with chromatophore ATPase, these experiments may indicate an energised state-dependence of Mg^{2+}-sensitivity in mitochondrial ATPase.

Nelson et al. [8] have shown that activating anions unmask a latent Mg–ATPase in isolated chloroplast CF_1. Although their experiments were carried out on the trypsin or heat-activated enzyme, the results are similar to those reported above. Although the chloroplast ATPase is more complex than that of chromatophores viz. the requirement in the former for heat, trypsin or dithiothreitol and the stability of the activated enzyme after illumination [21], there are some indications that this explanation of the changing divalent cation-dependence with the energised state may also apply to the green-plant system. In particular it has been found that light-activated chloroplast Ca–ATPase is much less sensitive to inhibition by NH_4Cl than Mg-dependent ATPase [7], that chloroplast Ca–ATPase is competitively inhibited by Mg^{2+} [22] and that magnesium accelerates the rate of deactivation of the chloroplast ATPase following a period of illumination [21].

Consideration of the anion structures which promote ATPase activity has not led to any clear understanding of the mechanism of activation. Selwyn [23] found that the degree of activation by the anion dinitrophenate is related to the water exchange-rate of the supporting divalent cation. Moyle and Mitchell [12] doubted the relevence of this finding since their data indicated that the anions increase the proportion of enzyme in the active ATPase configuration but do not modify the catalytic mechanism of $[metal]^{2+}$ ATP hydrolysis. If, however, our assertion that divalent cations are also effective through a regulatory site on the enzyme is correct, then Selwyn's relationship should be re-considered.

Nelson et al. for the chloroplast ATPase [8] and Ebel and Lardy [11] for the mitochondrial system have speculated that the anions increase the rate of product-release from the enzyme complex. Moyle and Mitchell [12] have also drawn special attention to the influence of Mg^{2+} anions and ADP on the K_i (ADP) for mitochondrial ATPase. It is inherent in our explanation that the enzyme must turn over before control can be exerted. We propose that when the high-energy state is rapidly dissipated and when Mg^{2+} is bound to its regulatory site, product-ADP remains tightly bound to the catalytic site and inhibits further reaction. Sulphite and similar anions, or membrane energisation via ATP hydrolysis or cyclic electron-flow, give rise to an enzyme conformation in which the release of bound ADP is facilitated and net ATP hydrolysis proceeds unimpeded.

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