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Hydrolysis of Adenosine 5′-Triphosphate by the Isolated Catalytic Subunit of the Coupling ATPase from *Rhodospirillum rubrum*†

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ABSTRACT: The purified isolated β subunit of the coupling ATPase from *Rhodospirillum rubrum* (RF_1) has an intrinsic ATPase activity, albeit only about 0.1% of that of the intact enzyme. The activity can be separated from intact RF_1 by gel filtration and is not sensitive to bathophenanthroline–Fe, azide, or adenylyl imidodiphosphate, but it is as sensitive as the intact enzyme to aurovertin and efrapeptin. ATPase activity of the isolated subunit, but not of intact RF_1, is rapidly lost by standing at or above room temperature in the absence of either MgATP or dithiothreitol. Hydrolysis of ATP by the β subunit requires Mg^{2+} or Ca^{2+} and occurs with a K_m of around 10 μM, with V_m = 0.6 mol (mol of protein)$^{-1}$ min^{-1}. RF_1 shows a similar K_m and V_m for MgATP, but CaATPase activity occurs with a K_m some 100-fold greater and a V_m 5000-fold higher. The β subunit lacks the very tight (K_d = 10 nM) catalytic ATP binding site and also the cooperativity of catalysis, both of which characterize hydrolysis by intact RF_1. It is shown that product release does not limit ATP hydrolysis by the β subunit, as it does by intact RF_1, and it is suggested that the bond splitting step itself limits the rate of hydrolysis by this subunit.

The coupling ATPase (F_1-ATPase, ATP synthase) from a variety of sources has a common structure ($\alpha_3\beta_3\gamma\delta\epsilon$) and functions to couple ATP synthesis to a transmembrane proton flux and/or to couple ATP hydrolysis to a transmembrane proton pump. The catalytic sites of this enzyme appear to lie on the β subunits [for a review, see Amzel & Pedersen (1983)].

Rapid ATP hydrolysis by F_1 requires cooperation between the three β subunits. Binding of ATP to the three catalytic sites shows strong negative cooperativity, K_d for binding a first ATP molecule being several orders of magnitude less than for subsequent ATP molecules (Cross & Nalin, 1982). Conversely, hydrolytic activity at the three sites shows considerable positive cooperativity, hydrolysis of the first bound ATP being slow until the other active sites are filled, and product release also being stimulated by ATP binding to the second and third active sites (Cross et al., 1982; Gresser et al., 1982).

The remarkably tight binding of ATP to the first catalytic site of F_1 ($K_d \approx 10$ nM; Harris et al., 1981; Cross & Nalin, 1982) is of considerable theoretical importance, since the energy gained on binding ATP is an important factor in shifting the ADP, P_i/ATP equilibrium on the enzyme toward ATP. This is integral to a "conformational" (Boyer 1977) or "binding energy" (Harris, 1981) model for ATP synthesis on F_1. Since the α subunits of F_1 affect cooperativity between the β subunits (Wise et al., 1981, 1984) and also bear very tight, noncatalytic, nucleotide binding sites themselves (Dunn & Futai, 1980), it is advantageous to use an F_1 enzyme lacking the subunits to study the catalytic site.

One fruitful approach has been to study an F_1 molecule mutant in its α subunits, as has been done for the *Escherichia coli* enzyme (Wise et al., 1981, 1984). It is also possible to isolate functional β subunits from the rest of the F_1 molecule (Yoshida et al., 1977; Futai, 1977; Verschoor et al., 1977; Douglas et al., 1979). Here we use the β subunit isolated from the photosynthetic bacterium *Rhodospirillum rubrum*, which can be isolated directly from coupled membranes by treatment

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with LiCl (Khananshvili & Gromet-Elhanan, 1982). Conveniently, this subunit can be used to reconstitute phosphorylation in the stripped membranes (Philosoph et al., 1977), thus providing a simple check on its integrity.

We show that the isolated catalytic subunit of *R. rubrum* F_1 (RF_1)[1] has an intrinsic ATPase activity, although only about 0.1% that of the intact enzyme. Although the K_m^{ATP} for this hydrolysis is 2 orders of magnitude lower than that of the intact enzyme, the very tight binding characteristic of the "first" catalytic site of F_1 is absent from the β subunit. This appears to be due to the fast rate of ATP release from this subunit. Single-turnover experiments indicate that catalytic turnover by the β subunit is slow ($k = 0.01\ s^{-1}$) and limits hydrolysis by the isolated subunit. This can be contrasted with the intact enzyme, where turnover is fast and limited overall by product release (Harris et al., 1981; Cross et al., 1982).

Experimental Procedures

Chromatophores were prepared from late log phase *R. rubrum* cells, grown photosynthetically, as described by Baltscheffsky (1967). They were depleted of the β subunit of RF_1 as described by Philosoph et al. (1977), and the subunit was purified to homogeneity essentially as described by Khananshvili and Gromet-Elhanan (1982), except that DEAE-Sephacel was used for the first ion-exchange step and DEAE-Sephadex for the second. RF_1 was isolated from chromatophores by chloroform treatment and purified, as described by Khananshvili & Gromet-Elhanan (1983). For comparison, both preparations were stored at −20 °C in a buffer containing 100 mM Tricine, 4 mM $MgCl_2$, 4 mM ATP, and 10% glycerol, pH 8.0 (NaOH). The β subunit was stable to storage under these conditions, but they were not optimal for RF_1 storage, and the rather low value of specific activity of our RF_1 preparation (5 μmol min^{-1} mg^{-1}) reflects this.

Reconstitution of β subunit into membrane-bound F_1 was carried out as described by Philosoph et al. (1977). Photophosphorylation was measured by using luciferin/luciferase as described by Lundin et al. (1977). β-Depleted particles had phosphorylation rates of about 5% of that of the untreated chromatophores, and phosphorylation could be restored to 100–110% activity by addition of a crude β preparation (prior to the first ion-exchange column) but only to 60–70% with the purified β preparation. This may reflect some loss of the γ subunit in the stripped particles. Maximal phosphorylation rates (100% activity) were typically 4–6 mol of ATP (mol of bacteriochlorophyll)$^{-1}$ min^{-1}.

ATP hydrolysis was carried out at 30 °C in 250 μL volumes of 20 mM HEPES–1 mM $MgCl_2$, pH 8.0 (NaOH), for the β subunit or 20 mM HEPES–1 mM $CaCl_2$, pH 8.0 (NaOH), for the intact RF_1. The mixtures contained [γ^{32}P]ATP, added as its M^{2+} complex, at 100 μM (8000 dpm/nmol), or 1 mM (800 dpm/nmol), respectively. The reaction was started by addition of enzyme (5 μg of β subunit or 0.5 μg of RF_1) except where indicated and stopped after 5–60 min by addition of an equal volume of 10% trichloroacetic acid containing 1 mM potassium dihydrogen phosphate and 1 mM ATP. Extraction and counting of free phosphate was carried out according to Ernster et al. (1950). Immediately before use, the stored enzymes were freed from ATP by centrifugation through a Sephadex G-50 column, as described by Khananshvili & Gromet-Elhanan (1983), except that the centrifugation occurred at 22 °C and that the enzyme was subsequently stored in the presence of 1 mM dithiothreitol (see below).

Nitrophenyl phosphatase activity was measured by incubation of 25–50 μg of protein with 1 ml of 20 mM HEPES–1 mM $MgCl_2$, pH 8.0 (NaOH), containing 2 mM *p*-nitrophenol phosphate at 30 °C. Measurements of A^{400} were taken over a period of 24 h. In measurements with RF_1, $CaCl_2$ replaced $MgCl_2$.

Adenylate kinase activity was measured at 30 °C in the Mg-containing buffer above, except that *p*-nitrophenol phosphate was replaced by 10 units of hexokinase, 10 units of glucose-6-phosphate dehydrogenase, 10 mM glucose, and 0.1 mM $NADP^+$; 50 mM KCl was also present. The reaction was started by the addition of 1 mM ADP (ATP free) and NADPH formation followed fluorometrically on a Perkin-Elmer LS5 spectrofluorometer.

To measure nucleotide binding to the β subunit under conditions of hydrolysis, ATP-free β subunit [0.5 mg/mL in 20 mM HEPES–1 mM $MgCl_2$, pH 8.0 (NaOH)] was incubated at 23 °C with 5–200 μM MgATP labeled with ^{3}H (75×10^3 dpm/nmol) and ^{32}P (7.5×10^3 dpm/nmol). At intervals up to 5 min, 0.1-mL aliquots were removed and layered onto a Sephadex G-50 column (2-mL total volume) preequilibrated with the same buffer and then centrifuged immediately for 30 s. Protein and radioactivity were determined in the eluate. Recovery of protein was typically 60–70%.

Protein concentrations were determined with a proprietary reagent from Bio-Rad Laboratories, on the basis of the method of Bradford (1976). The molecular weight of the β subunit was taken as 50000 (Bengis-Garber & Gromet-Elhanan, 1979).

Radioactive nucleotides were obtained from Amersham, U.K., and [2,8-^{3}H]ATP was treated to remove traces of ADP as previously described (Rosing et al., 1975). Unlabeled nucleotides were obtained from Boehringer, and the luciferin/luciferase mixture was from LKB. Aurovertin and efrapeptin were kind gifts of Professor R.B. Beechey.

Results

Separation of RF_1 and β Subunit Activity. Isolation of the pure β subunit of *R. rubrum* F_1 followed essentially the method of Khananshvili & Gromet-Elhanan (1982). The subunit was released from washed chromatophores with 2 M LiCl and subjected to ion-exchange chromatography on DEAE-Sephacel (first ion-exchange step), eluting with a gradient of 100–400 mM NaCl. At this point, traces of membranes were removed from the preparation by centrifugation for 2 h at 150000*g*. The supernatant protein was concentrated by precipitation with 60% ammonium sulfate. At this stage (crude β preparation), the preparation restored phosphorylation to LiCl-washed chromatophores (see Experimental Procedures) and contained the β subunit as a major protein component (as shown by electrophoresis).

This crude β preparation (15–20 mg of protein) was applied to a Sephacryl S-200 gel filtration column. The pore size of Sephacryl S-200 is such that RF_1 (M_r 360000) will be excluded, while the β subunit (M_r 50000) should be included in the gel. This is confirmed by calibrating the column with a preparation of RF_1 mixed with bovine serum albumin (dotted lines), when RF_1 is seen to elute at the void volume.

The profile obtained from such a column is shown in Figure 1. A small protein peak (peak I), comprising about 1 mg of total protein, elutes at the void volume. This protein has ATPase activity (Table I) but is unable to restore phosphorylation (not shown) or ATPase activity (Table I) to LiCl-

[1] Abbreviations: RF_1, soluble fragment of the coupling ATP synthase from *R. rubrum*; AMP-PNP, 5′-adenylyl imidodiphosphate; ITP, inosine 5′-triphosphate; DEAE, diethylaminoethyl; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

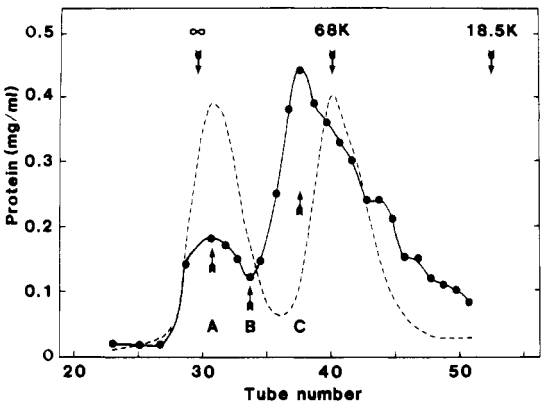

FIGURE 1: Gel filtration of partially purified β subunit of RF_1. Chromatophores (50 mg of bacteriochlorophyll) were depleted of RF_1 β subunit with 2 M LiCl, and the subunit was partially purified by ammonium sulfate fractionation, ultracentrifugation, and ion-exchange chromatography on DEAE-Sephacel (see Experimental Procedures). The preparation (2 mL containing 14 mg of protein) was applied to a Sephacryl S-200 column (1.4 × 100 cm) preequilibrated at 4 °C with 100 mM Tricine, 4 mM $MgCl_2$, 4 mM ATP, and 10% glycerol, pH 8.0 (NaOH). The column was run at 10 mL/h. Fractions of 2 mL were collected and tested for protein and ATPase activity as described under Experimental Procedures. Calibration with RF_1 (360 K) and bovine serum albumin (68K) was carried out under identical conditions (dotted lines). The positions of markers [and of blue dextran (∞)] are indicated by the downward arrows. Tubes A–C were further tested (see Table I).

Table I: ATP Hydrolysis by Eluate Fractions after Gel Filtration of Crude β Subunit[a]

	ATPase activities [nmol min^{-1} (mg of protein)$^{-1}$]		
fraction	MgATPase	CaATPase	MgATPase (after reconstitution)
A	37	165	42
B	9	33	ND[b]
C	24	23	442

[a] Aliquots of protein were taken from the tubes indicated A, B, and C on the elution profile in Figure 1 and ATPase activities assayed as described under Experimental Procedures. For reconstitution experiments (third column), 1 μg of protein was incubated with LiCl-washed chromatophores (2 μg of bacteriochlorophyll) in 50 μL of 30 mM Tris, 25 mM $MgCl_2$, and 3 mM ATP, pH 7.5 (HCl), according to Philosoph et al. (1977). After 30-min preincubation, the reaction was initiated with 0.9 mL of 20 mM HEPES, 1 mM $MgCl_2$, and 1 mM [γ-^{32}P]ATP (1000 dpm/nmol) and measured as described. [b] ND, not determined.

washed chromatophores. These findings, and the elution position of this protein, suggested that peak I contained RF_1 (free and/or membrane bound), and it was discarded. From Figure 1 and Table I, however, we can calculate that this peak contains only about 37 units of MgATPase activity.

The bulk of the protein (peak II) elutes from the gel behind the position of RF_1. This protein is capable of restoring phosphorylation (not shown) and ATPase activity (Table I) to LiCl-washed chromatophores, and thus contains the β subunit of RF_1.

Considerable (about 75 units) MgATPase activity is associated with the protein in peak II. ATPase activities measured at A (peak I), B (trough), and C (peak II) are given in Table I. Two peaks of MgATPase activity clearly occur, separated by a trough, suggesting that peak II contains a protein separate from peak I with its own intrinsic MgATPase activity. The low total MgATPase activity in peak I means that it is most unlikely that the activity in peak II derives from protein "trailing" from peak I, as does the difference in CaATPase/MgATPase ratio between the two peaks. Thus it appears that the β subunit of RF_1 purifies with a MgATPase activity

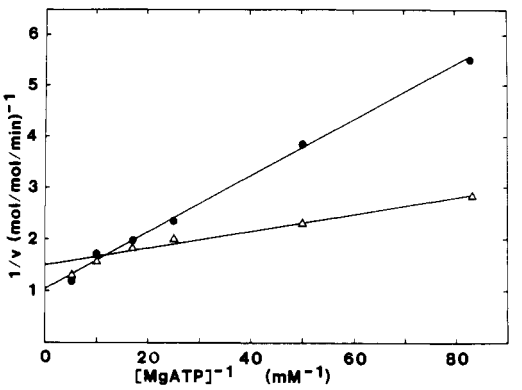

FIGURE 2: Kinetic parameters of the MgATPase activities of purified RF_1 and the isolated β subunit. ATP hydrolysis was measured at 30 °C as described under Experimental Procedures, except that 20 mM HEPES and 1 mM $MgCl_2$, pH 8.0 (NaOH), were used as the assay buffer throughout, 10-μg aliquots of both β subunit and RF_1 were used, and MgATP concentrations were varied as indicated. (●) RF_1; (△) β subunit.

associated with a protein smaller than RF_1.

Peak II was subjected to further ion-exchange chromatography (see Experimental Procedures), yielding a final (pure β) preparation that was homogeneous as judged by gel electrophoresis in dodecyl sulfate, as previously shown by Khananshvili & Gromet-Elhanan (1982). The ability of this protein to restore phosphorylation and ATPase activity to LiCl-washed chromatophores is retained through this purification step (see Experimental Procedures), as indeed is its own ATPase activity (see below). This purified preparation was used in all further experiments.

The β subunit runs behind RF_1 on a gel filtration column, as expected from its smaller molecular weight (Figure 1). Surprisingly, however, it runs ahead of albumin (M_r 68000). Since peak II contains almost pure β subunit, it appears that this polypeptide aggregates in solution to form dimers (M_r 100000) and/or trimers (M_r 150000). The asymmetric nature of this peak also suggests the presence of a mixture of aggregated forms. Aggregation of the isolated β subunit of *E. coli* has also been reported (Dunn & Futai, 1980).

Properties of the MgATPase Activity Associated with the β Subunit. These results strongly suggest that a reconstitutively active preparation of the pure β subunit of RF_1 contains a MgATPase activity. This activity is unlikely to be due to trace contamination with RF_1, first because the amount of RF_1 in the partially purified LiCl extract was very low and separable from the β subunit by gel filtration (Figure 1) and second because the CaATPase/MgATPase ratio of the pure β preparation was around 1 while the ratio for purified RF_1 under these assay conditions (very low inorganic anion concentration) is 1000 or greater (see Figure 2 and discussion thereof). [Note however that this ratio for RF_1 may decrease as Cl^- or SO_3^{2-} concentration in the assay buffer rises—see, e.g., Lucke & Klemme (1976).] Figure 2 shows that, to realize an MgATPase activity of 15 nmol min^{-1} mg^{-1}, 1 mg of β subunit preparation would need to be contaminated by over 1 mg of RF_1!

The MgATPase activity of the pure β subunit is low (14–24 nmol min^{-1} mg^{-1}) when compared to the CaATPase activity of RF_1 (1500–5000 nmol min^{-1} mg^{-1} in our preparation), but it is retained through several purification steps. The activity is associated with a protein, since it is destroyed by incubation at 100 °C (Table II), and requires a divalent cation, since it is abolished by EDTA (Table III).

To confirm that the measured ATPase activity was indeed associated with the isolated β subunit, its stability was com-

Table II: Effects of Preincubation on RF_1 and β Subunit ATPase Activities[a]

pretreatment	ATPase activity [nmol min^{-1} (mg of protein)$^{-1}$]	
	RF_1	β subunit
none	1535	14
(i) 60 min at 37 °C (0.16 mg of protein/mL)	ND	6.8
as (i), except 2 mM MgATP present throughout	ND	12.9
(ii) 60 min at 22 °C (0.05 mg of protein/mL)	1800	0
as (ii) except incubation followed by 10 min with 1 mM dithiothreitol	1550	21
5 min at 100 °C	0	0

[a] ATPase activity was measured as described, except that prior to initiating the reaction the proteins were incubated under the conditions shown in 50 μL of 50 mM Tricine, pH 8.0 (NaOH). The values given are for CaATPase (RF_1) and MgATPase (β subunit).

Table III: Effectors on ATP Hydrolysis by RF_1 and Its β Subunit[a]

addition	activity remaining (% control)	
	RF_1	β subunit
none	100	100
aurovertin (2 μM)	28	21
efrapeptin (10 μg/mL)[b]	49	32
EDTA (5 mM)	0	0
bathophenanthroline–Fe (2 μM)[b]	16	75
azide (1 mM)	12	82
sulfite (4 mM)	188	86
diadenosine pentaphosphate (40 μM)	ND	85

[a] ATPase activity was measured as described under Experimental Procedures, except that the indicated inhibitors were also included in the assay medium. 100% activities are given in Table II. [b] A 5-min incubation with the inhibitor preceded initiation of the reaction (see Figure 3).

pared with the stability of the reconstitutive activity of the subunit. Khananshvili & Gromet-Elhanan (1983) have shown that the latter activity is lost during incubation at or above room temperature in the absence of MgATP. Table II shows that the ATPase activity of the β subunit is also lost during incubation in the absence of ATP. Loss is prevented by the presence of 2 mM MgATP in the incubation medium, just as this nucleotide stabilizes the reconstitutive activity. The rate of loss of activity is strongly dependent on protein concentration and temperature (Table II).

In contrast, in agreement with other workers (Johansson et al., 1973), we find that incubation with or without MgATP has little effect on the ATPase activity of intact RF_1 (Table II). The sensitivity of the ATPase of the β preparations to preincubation thus confirms that this activity cannot be due to RF_1 contamination. A similar consideration rules out contamination by membrane fragments, whose ATPase is also very stable to preincubation (J. Boork and M. Baltscheffsky, unpublished results).

Table II also shows that the MgATPase activity of the β subunit is regained after a short incubation with 1 mM dithiothreitol, even if MgATP is absent. Thus MgATP binding appears to exert its effect on the β subunit by protecting –SH groups from exposure (and possible oxidation). In the light of this finding, further experiments involving incubation of the subunit without ATP included 1 mM dithiothreitol in the incubation medium. Dithiothreitol or mercaptoethanol is also included in the buffers for isolation and stabilization of the *E. coli* F_1 subunits [see, for example, Dunn & Futai (1980)]. Dithiothreitol does not appear to affect RF_1 activity (Table II).

Effects of Inhibitors on the ATPase Activity of the β Subunit. The above experiments show that the MgATPase activity of the subunit preparation is unlikely to be due to contaminating RF_1, either free or membrane bound. In summary, the experiments of Figure 1 and Table I show that even impure LiCl-extracted β subunit contains very little RF_1 activity (measured as CaATPase) and that RF_1 runs ahead of β on the gel filtration column used in the purification of the β subunit. Table II shows that the stability of the β subunit MgATPase activity is similar to that reported for the reconstitutive activity of β (Khananshvili & Gromet-Elhanan, 1983), while RF_1 itself is much more stable. Finally, as shown below (Figure 2), the kinetic parameters of the two activities differ—the CaATPase/MgATPase activity ratio is far higher for RF_1 than for the ATPase, and the K_m values for both substrates are also far higher on RF_1.

However, one may question whether the ATPase activity in the β preparation is in fact due to a derivative of RF_1: is it perhaps due to slight contamination with a totally different enzyme? Assays for nitrophenyl phosphatase activity in β and F_1 preparations, under the conditions used for the ATPase assays, indicate very low activities [$\leq$0.1 nmol min^{-1} (mg of protein)$^{-1}$], suggesting that the ATPase activity is not due to a nonspecific phosphatase. This is supported by the observation that ATP hydrolysis is abolished by EDTA (Table III).

Table III also shows that both aurovertin and efrapeptin inhibit ATP hydrolysis by the isolated β subunit and by RF_1 at comparable concentrations. Since these are both very specific inhibitors of F_1 species, we conclude that the MgATPase activity observed in the preparation does indeed arise from an RF_1-derived protein. Interestingly, these are the inhibitors that are known to bind to the β subunit in intact F_1 (Douglas et al., 1979; Harris & Baltscheffsky, 1981), and thus the interaction between the inhibitor binding site and the catalytic site seems to be maintained in these cases when the β subunit is isolated.

Table III also shows that RF_1 ATPase activity responds to the effectors bathophenanthroline–Fe and azide (inhibitory) and sulfite (stimulatory) as do intact F_1 enzymes from other species. However, these compounds seem to have little or no effect on the ATPase activity of the β subunit. The finding that the MgATPase activity of the isolated β preparation is unaffected by sulfite provides further evidence that this activity is not due to trace contamination by RF_1—whose MgATPase activity is massively stimulated (10–20-fold) by sulfite (Webster et al., 1977). It is possible either that these effectors do not interact with the isolated β subunit at all or that they affect steps in the reaction mechanism that are limiting to hydrolysis by F_1 but not in the (much slower) hydrolysis by β (see below). It is interesting that azide does not affect hydrolysis by the α-modified mutants of *E. coli*, F_1, while it is a powerful inhibitor of intact EF_1 (Wise et al., 1984).

Chromatophores of *R. rubrum* contain an active adenylate kinase (Lundin et al., 1979), and some adenylate kinase activity is associated with our preparations [0.5–4 nmol min^{-1} (mg of protein)$^{-1}$ according to preparation]. We have not yet firmly established whether this associated activity is an intrinsic property of the β active site (Moudrianakis & Tiefert, 1976) or a contaminating activity, although we favor the latter explanation. However, Table III shows that a specific adenylate kinase inhibitor, diadenosine pentaphosphate, has little effect on ATP hydrolysis by the β subunit, and so adenylate kinase

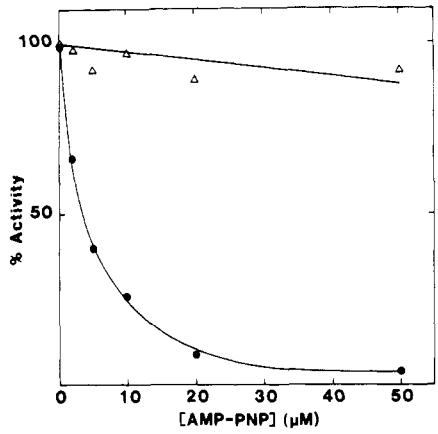

FIGURE 3: Effect of AMP-PNP on ATPase activities of RF_1 and its β subunit. Preincubation of protein with AMP-PNP was carried out at 30 °C in 50 μL of the appropriate (Ca^{2+}- or Mg^{2+}-containing) assay buffer for 5 min before the reaction was initiated by addition of 200 μL of the same buffer containing M^{2+}ATP (see Experimental Procedures). The final concentration of AMP-PNP (i.e., the concentration in the assay medium) is shown on the abscissa. (●) RF_1; (△) β subunit.

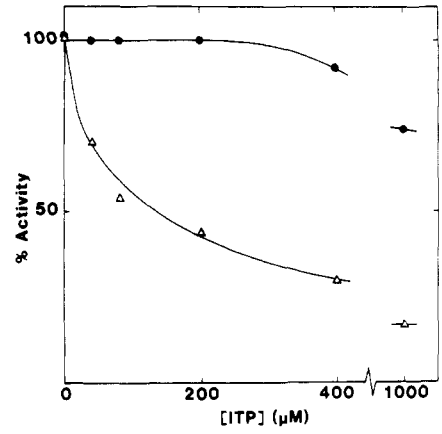

FIGURE 4: Effect of ITP on ATPase activities of RF_1 and its β subunit. ATP hydrolysis was measured as described except that 10 μM M^{2+}ATP (120 000 dpm/nmol) and the indicated concentrations of M^{2+}ITP replaced 1 mM CaATP or 100 μM MgATP in the reaction mixture. (●) RF_1; (△) β subunit.

activity can at any rate be ignored in the analysis of ATP hydrolysis given below.

Kinetic Parameters of Hydrolysis by the β Subunit. It is evident from above that the hydrolysis of MgATP by the purified subunit preparation cannot be due to contaminating enzymes. The same is probably true for its CaATPase activity, although this is rather more difficult to rule out due to the much higher CaATPase activity of RF_1.

K_m values for MgATP for RF_1 and its β subunit are measured in Figure 2. Both RF_1 and β give apparently linear $1/v$ vs. $1/s$ plots, although some curvature may be present at high ATP levels in the β preparation. The curvature may reflect binding to a second, weaker site on each β subunit, as reported by Gromet-Elhanan & Khananshvili (1984). In this case, the weaker site observed by these workers would be a positive "regulator" of the stronger catalytic site rather than, as they propose, the catalytic site being the weaker binding site. However, in view of the difference between their and our binding studies (see below), this assignment cannot be made with certainty. It is also possible that the curvature may reflect effects of different aggregation states (see Figure 1) on β subunit activity.

The K_m values measured are 9.6 μM for the β subunit and 56 μM for RF_1. These values can be compared with respective values of 20 μM (data not shown) and 1.2 mM (Johansson et al., 1973) for CaATP. K_m values for hydrolysis on the β subunit thus appear far lower than those on the intact RF_1.

V_m^{MgATP} values for the two preparations are, surprisingly, comparable, at 0.63 mol mol^{-1} min^{-1} (13 nmol min^{-1} mg^{-1}) for the β subunit and 0.95 mol mol^{-1} min^{-1} (2.7 nmol min^{-1} mg^{-1}) for RF_1. This is remarkable when one considers that V_m^{CaATP} varies by several orders of magnitude (13 nmol min^{-1} mg^{-1} for the β subunit and 5000 nmol min^{-1} mg^{-1} for RF_1). It may be that MgATP hydrolysis occurs at a single β subunit in intact RF_1, although other explanations are of course possible at our present state of knowledge. In this regard, it is interesting that the CaATPase activity of isolated chloroplast F_1, in contrast to its MgATPase activity, does not show P_i–$H_2{}^{18}O$ exchange (Kohlbrenner & Boyer, 1983), suggesting some change in intersubunit cooperativity between the two activities.

Nucleotide Analogues on ATP Hydrolysis by the β Subunit. The nonhydrolyzable ATP analogue AMP-PNP is a powerful inhibitor of the F_1-ATPase from a variety of sources. This is because it binds to the active site of F_1 (Harris et al., 1981), and binding of a single AMP-PNP molecule to just the first, very tight catalytic site of F_1 is sufficient to inhibit the whole enzyme molecule (Cross & Nalin, 1982). In a sense, an AMP-PNP molecule can be said to "trap" the F_1 molecule in an inactive state (Harris, 1982).

Figure 3 shows that RF_1 is very strongly inhibited by AMP-PNP; the concentration required for half-maximal inhibition is around 3.5 μM (in the presence of 1 mM competing ATP as substrate). This is similar to other F_1-ATPases. However, the β subunit seems to have a far lower affinity for AMP-PNP, very little inhibition (<15%) being observed even as AMP-PNP concentrations approached the substrate concentration. It was concluded that the β subunit, despite its low K_m^{MgATP}, lacked the very tight catalytic binding characteristic of the intact F_1 molecule.

Because of the large difference in K_m values, at very low ATP concentrations the activities of RF_1 and its β subunit are comparable in magnitude. At 10 μM M^{2+}-ATP, for example, the β subunit cleaves ATP at 5.6 nmol min^{-1} mg^{-1}, and RF_1 cleaves it at 16 nmol min^{-1} mg^{-1}. At such low ATP concentrations, beef heart F_1 exhibits an unusual phenomenon—the stimulation of ATP hydrolysis by an alternative substrate (Schuster et al., 1976). This can be explained by the positive cooperativity of the enzyme with regard to catalysis, ITP (the alternative substrate) binding to the second and third catalytic sites stimulating ATP hydrolysis at the first (Cross et al., 1982; Gresser et al., 1982).

Figure 4 shows the effect of ITP on ATP hydrolysis by intact RF_1 and its isolated β subunit. In contrast to the effects seen in Figure 3, where a nonhydrolyzable analogue was employed, ITP appears to be a good inhibitor of ATP hydrolysis by the β subunit. Inhibition shows a simple hyperbolic dependence on ITP and is half-maximal when the ITP/ATP ratio is 10.

ITP, however, seems to inhibit ATP hydrolysis by RF_1 rather poorly at low ATP concentrations. This is despite the facts that (a) ITP is a good substrate for RF_1 (Harris & Baltscheffsky, 1979) and (b) K_m^{ATP} is much higher for RF_1 than for the β subunit (see above). In addition, inhibition of ATP hydrolysis does not show a simple hyperbolic pattern but a convex plot, little or no inhibition being observed below about 200 μM (Figure 4). This is consistent with a complex pattern of interaction of ITP with F_1, in which low concentrations of ITP stimulate ATPase activity in addition to competing with ATP for the catalytic sites of the enzyme. If ATP concen-

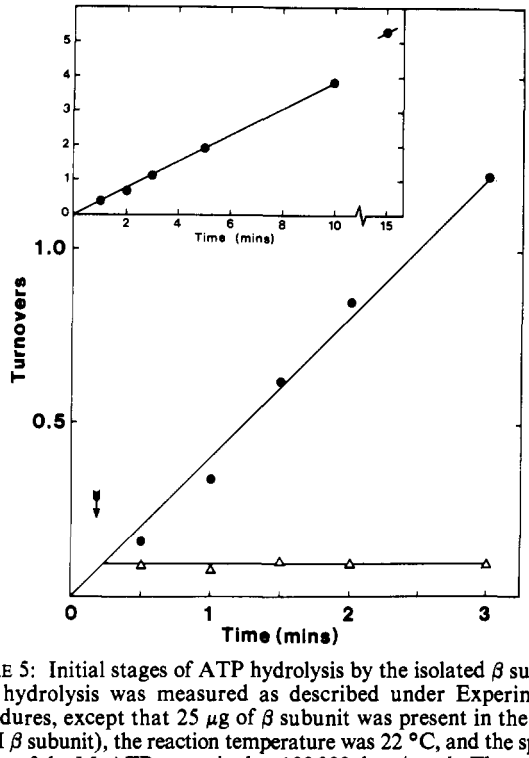

FIGURE 5: Initial stages of ATP hydrolysis by the isolated β subunit. ATP hydrolysis was measured as described under Experimental Procedures, except that 25 μg of β subunit was present in the assay (2 μM β subunit), the reaction temperature was 22 °C, and the specific activity of the MgATP was raised to 120000 dpm/nmol. The reaction was quenched at 30-s intervals over the first 2 min (main figure) and then less frequently over a 15-min time span (insert). The arrow indicates the addition of unlabeled MgATP (2 mM). Subsequent production of labeled P_i is represented by the open triangles. Solid circles represent production in the absence of added, unlabeled ATP.

trations are reduced below 10 μM, some stimulation of ATP hydrolysis by ITP with F_1 is indeed observed at ITP levels less than 200 μM (not shown).

Hydrolysis of ATP at Stoichiometric β and ATP Concentrations. In an attempt to elucidate the mechanism of ATP hydrolysis, the time course of ATP hydrolysis by the isolated β subunit was measured over a period corresponding to 0.2–5 turnovers of the enzyme. Figure 5 shows that, at a 50-fold excess of substrate, ATP hydrolysis is linear over the first five turnovers, at a rate of 0.36 turnover/min. This is equivalent to a rate of 7.2 nmol min^{-1} (mg of protein)$^{-1}$, very close to the steady-state rate observed for this enzyme. (This value is rather lower than the rate measured above because measurement here was at 22 °C rather than 30 °C above). In keeping with the low K_m, the initial rate is scarcely altered as the ATP concentration is lowered to 5 μM, although after the first turnover, hydrolysis rates rapidly fall off at very low ATP concentrations (not shown).

If the release of product limits ATP hydrolysis for the isolated subunit, as it probably does for F_1 (Harris et al., 1981; Cross et al., 1982), we would expect a rapid burst of appearance of [^{32}P]P_i, up to 1 mol/mol of enzyme, followed by a much slower phase of turnover [see, e.g., Lymn & Taylor (1970)]. The linearity observed here, up to more than five turnovers and the comparability of the first turnover and steady-state hydrolysis rates suggest that hydrolysis by the β subunit is limited either by the binding of ATP or by the catalytic step on the enzyme.

Studies of the binding step were attempted in two ways. Information on the ATP off-rate from the catalytic site can be investigated by incubating the enzyme with [γ-^{32}P]ATP and then, before the first turnover is complete, adding a large excess of unlabeled ATP. If k_{off} for ATP is slow relative to turnover, as has been observed for intact beef heart F_1 (Harris et al., 1981; Grubmeyer et al., 1982), [γ-^{32}P]ATP already bound will be "committed" to hydrolysis, and the first turnover will be completed with radioactive ATP.

This is clearly not the case with the isolated β subunit. Figure 5 shows that addition of unlabeled ATP after only 0.1 turnover quenches hydrolysis immediately on the time scale of the experiment (within 5 s). This indicates that any bound ATP can rapidly dissociate from the active site of the β subunit before the pyrophosphate bond is hydrolyzed.

Rapid dissociation of ATP from the β subunit was confirmed when ATP binding was measured by the "centrifuge column" technique (Penefsky, 1977). β subunit (1 μM) was incubated with up to 200 μM [2,8-^{3}H, γ-^{32}P]ATP for up to 5 min, and aliquots (0.1 mL) were subjected to centrifugation through Sephadex G-50 as described under Experimental Procedures. Hydrolysis of [^{3}H,^{32}P]ATP was measured in a parallel experiment. Even after 5-min incubation (two enzyme turnovers), we found only 0.1 mol of ATP associated with 1 mol of β subunit after centrifugation. Since this procedure detects only nucleotides that do not dissociate from the enzyme within the 10–30-s passage through the column, this result also suggests that the release of ATP from the active site of the β subunit is rapid.

Discussion

Nature of the Preparation. It is shown here that the isolated, purified β subunit of RF_1 bears an ATPase activity that is about 0.1% of the maximal ATPase activity of the intact RF_1. This is comparable with ATPase activities recorded for the F_1 isolated from *E. coli* mutant strain uncA 401 (Wise et al., 1984), which has a defect in the α subunit of F_1, and also for the *E. coli* β subunit as isolated from the intact EF_1 (Futai, 1977). The advantage of using the *R. rubrum* β subunit for studies on activity is 2-fold. First, the subunit is isolated directly from the coupled membrane fragments of *R. rubrum*, without the intermediate stage of isolating RF_1. Second, isolated RF_1 shows a very low MgATPase activity, its predominant activity being as a CaATPase. Both these factors help to eliminate the possibility that ATPase activity in the preparation is due to contaminating RF_1. In addition, the ability to reconstitute photophosphorylation with an isolated subunit in *R. rubrum* gives us an independent means of characterizing the integrity of our preparation.

The hydrolytic activity of the β subunit was inhibited by both aurovertin (which also inhibits uncA 401 F_1) and efrapeptin (which does not) at concentrations comparable to those required to inhibit intact F_1 (Table III). Both these specific inhibitors of F_1 are known to bind to its β subunit, and this inhibition thus confirms that the conformation of the isolated β subunit and its ATPase activity are characteristic of the native form of this subunit.

An interesting contrast is provided by the effects of azide, a well-established inhibitor of F_1. This has little or no effect on hydrolysis by the isolated β subunit. Similarly, azide inhibits wild-type *E. coli* F_1, but not F_1 from a mutant (uncA 401) with a defective α subunit (Wise et al., 1984). This could be explained if azide exerts its effects by blocking catalytic cooperativity in intact F_1. Thus, catalytic cooperativity ("β–β interaction") is apparently lost in the absence of a functional subunit, both in mutants with a defective α subunit (Wise et al., 1984) and when the subunit is removed (this paper). In both these cases, loss of cooperativity yields preparations with (a) a low ATPase activity and (b) no sensitivity to azide.

Binding of Nucleotides to the β Subunit. The experiments described above show that the isolated β subunit of RF_1

contains at least one nucleotide binding site. This is a catalytic site for ATP hydrolysis, and probably, by virtue of its inhibitor sensitivity, it is also the active site of the intact RF_1-ATPase. Since the hydrolysis rate by the β subunit is so low, the E·ATP complex is in equilibrium with free ATP and $K_m \approx K_d \approx 10$ μM. This estimated value for K_d suggests that this site is equivalent to the ATP/ADP binding site with $K_d^{ATP} = 4.4$ μM demonstrated on β by Gromet-Elhanan & Khananshvili (1984). Whether this is also the ATP binding site responsible for protection of the subunit against denaturation (Table II) is at present unknown.

This value of K_d is considerably higher than that recorded for ATP binding to the first catalytic site in intact F_1, which is in the nanomolar range (Harris et al., 1981; Cross & Nalin, 1982). This view is confirmed by the experiment of Figure 3, where AMP-PNP is shown to be a powerful inhibitor of intact RF_1, but not of hydrolysis by the β subunit. Since AMP-PNP inhibits F_1 by binding to the first catalytic site, we conclude that a site with comparable high affinity is absent from the isolated β subunit.

Thus it seems that, to attain the very tight ATP binding necessary for ATP synthesis on F_1, cooperation between more than one subunit is required. In this case, it seems that in an $\alpha_3\beta_3$ trimer the first catalytic site occupied on a β subunit is modified to give tighter binding, and the third modified to give weaker binding, than the isolated subunit. Interestingly, mutants of *E. coli* with decreased β–β cooperativity (due to a modified α subunit) also show a decreased affinity for AMP-PNP at the first catalytic binding site (Wise et al., 1984).

A major factor contributing to the tightness of nucleotide binding to the intact F_1 is the very slow rate of ATP release from the catalytic site (Harris et al., 1981; Grubmeyer et al., 1982). This feature is absent from the β subunit, where the release of ATP seems rapid as judged by the pulse/quench experiment of Figure 5 and by the lack of ATP bound to the subunit after centrifugation through a centrifuge column (above). Thus, one aspect of the negative cooperation in binding seen in the intact F_1 might be the restriction of ATP release from its binding site on the β subunit due to conformational hindrance by the other (α?) subunits.

Our finding of rapid ATP release from the β subunit of RF_1 is in accord with the findings reported by Hirano et al. (1984), which showed that little bound nucleotide survived centrifugation of the β subunit of EF_1 through a Sephadex column. However, Gromet-Elhanan & Khananshvili (1984) report that nucleotide binding to RF_1 β subunit is indeed stable to column centrifugation in the absence of added nucleotide. The reason for this discrepancy is unknown, but the slow off-rate observed in their experiments may result from their chilling the centrifuge columns and incubation mixture to 4 °C prior to centrifugation and/or the absence of Mg^{2+} ions in their column buffer. In our experiments, binding measurements were carried out under the same conditions as ATP hydrolysis (Figure 5)—at 23 °C, in 20 mM HEPES–1 mM $MgCl_2$, pH 8.0—so that results are directly comparable.

Kinetics of ATP Hydrolysis by the β Subunit. The isolated β subunit hydrolyzes ATP at a very slow rate—at 30 °C V_m = 0.6 mol of ATP (mol of subunit)$^{-1}$ min^{-1} (Figure 2). When reconstituted into the membrane (hydrolysis restrained by H^+ leakage?), turnover rates are some 20-fold higher (Table I), and in the isolated, intact RF_1, turnover may be 3000 times faster during CaATP hydrolysis.

The β subunit can therefore be considered to have an intrinsic, low, ATPase activity, which is enhanced when this subunit is assembled into an F_1 molecule. Enhancement appears to be partly due to assembly itself—catalytic rates of 0.5–5 s^{-1} have been reported for hydrolysis of ATP at a single catalytic site on an assembled F_1 molecule (Grubmeyer et al., 1982; Gresser et al., 1982; Wise et al., 1984), which is some 100 times faster than we observed for the isolated β subunit. However, this rate is further increased (50–100 times) by positive cooperative interactions when ATP fills the second and third binding sites on the intact F_1 molecule (Cross et al., 1982; Gresser et al., 1982), interactions that are presumably absent in the isolated β subunit in solution. The positive cooperative effects on catalysis are accompanied by even larger effects on product release, which is stimulated some 10^4 times when passing from single-site to multiple-site catalysis on intact F_1 (Cross et al., 1982; Gresser et al., 1982).

Positive cooperativity in hydrolysis can be demonstrated, in intact F_1, by measuring the effects of a second substrate (here ITP) on ATP hydrolysis at very low ATP concentrations. As observed for beef heart F_1 (Schuster et al., 1976), ITP effects on ATP hydrolysis by RF_1 are mixed (Figure 4). ITP will, of course, compete for catalytic sites with ATP, leading to inhibition of [γ-^{32}P]ATP hydrolysis. However, in addition, ITP will bind to unoccupied second and third catalytic sites, stimulating ATP hydrolysis by positive cooperative interactions, and thus a convex inhibition curve is observed (Figure 4). Since multisite cooperativity is impossible in the isolated β preparation, only the inhibitory effect of ITP is observed (Figure 4). From this curve, it seems that the affinity of the active site for ATP is about 10-fold higher than that for ITP.

It is of interest to investigate the factor limiting hydrolysis by the β subunit—is it the ATP binding step, the catalysis itself, or product release? The lack of stimulation of hydrolysis by sulfite, which like other oxyanions stimulates product release by F_1 (Harris et al., 1981), might indicate that product release does not limit ATP hydrolysis by the β subunit, in contrast to hydrolysis by RF_1 (Table III).

This is confirmed by measurement of ATP hydrolysis rates over the first few turnovers of the enzyme (Figure 5). The turnover of the enzyme is shown to be linear in time, from 0.24 to 5 turnovers, and to be equal in rate to the steady-state rate of hydrolysis. A product release step slow relative to turnover, which would lead to a single turnover "burst" of [^{32}P]P_i, is thus ruled out.

It is more difficult to determine which of the other two steps in the reaction is limiting to hydrolysis—a slow ATP on-rate or a slow bond fission. In the first case, a lag would be expected to precede the linear steady-state rate, and such a lag is not observed here (Figure 5). Its absence, the rapid rate of ATP release from the catalytic site, and the affinity of this site for ATP ($K_d \approx 10$ μM) all lead us to suggest that the bond fission is the slow step limiting ATP hydrolysis by the β subunit. However, Gromet-Elhanan & Khananshvili (1984) indicate that ATP binding to the β subunit may take several minutes under their conditions, and so this matter must be left unresolved at the present time.

In summary, therefore, we conclude that the β subunit of the RF_1-ATPase bears a catalytic nucleotide binding site with $K_d \approx 10$ μM and capable of a low rate of hydrolysis (0.6 turnover/min). When assembled in the F_1 molecule, the affinity of this subunit for ATP increases considerably (K_d falls to $\leq$10 nM, largely by virtue of a decreased off-rate for ATP) and the catalytic turnover rate increases several fold. However, hydrolysis rates are still very low, as product (ADP, P_i) release is very slow. Finally, on filling the remaining catalytic ATP binding sites on F_1, the turnover rate is further increased, and

Table IV: Orders of Magnitude of Binding and Kinetic Parameters for F_1 Assemblies

subunit assembly	source	K_d	catalytic rate (s^{-1})	product release (s^{-1})
isolated β	this paper	10 μM	10^{-2}	1
$(\alpha\beta)(\alpha_2\beta_2)$ (1 site active)	Grubmeyer et al. (1982)	10 nM	10	10^{-4}
$\alpha_3'\beta_3$ (α mutant)[a]	Wise et al. (1984)	1 μM	1	10^{-2}
$\alpha_3\beta_3$ (all sites active)	Harris et al. (1981)	1 mM[b]	10^4	10^3

[a] Differs between mutants. Values for uncA 401 taken here. [b] K_m value for the third binding site.

product release is also greatly enhanced, so that the hydrolysis rate of the free enzyme is attained. These effects are summarized in Table IV.

It is unusual for an allosteric enzyme to be modified toward higher "activity" (tighter binding, faster catalysis) in the assembled state. The affinity of dissociated hemoglobin subunits for oxygen, for example, is much higher than that of the affinity of the first binding site in the tetramer (Antonini & Brunori, 1977), as if assembly held the molecule in an unfavorable conformation that subsequently collapsed to the native one. Why F_1 should be unusual in this respect remains uncertain, but may be related to the importance in energy coupling of the large energy changes involved in binding and the need to transmit these changes to a proton-pumping mechanism.

Registry No. ATPase, 9000-83-3; MgATP, 1476-84-2.

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