Volume 310, number 2, 187–192 FEBS 11592 September 1992

Isolation and characterisation of a functional $\alpha\beta$ heterodimer from the ATP synthase of *Rhodospirillum rubrum*

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Received 23 June 1992; revised version received 14 August 1992

An $\alpha\beta$ heterodimer of the F_1-ATPase of *Rhodospirillum rubrum* was isolated by extraction of chromatophores with LiCl. Each $\alpha\beta$ heterodimer contains one tightly bound ADP, which is released upon removal of medium Mg^{2+}. The dimer can be reversibly dissociated by removal of Mg^{2+}-ions. The $\alpha\beta$ heterodimer restores both ATP-synthetic and -hydrolytic activities to LiCl-treated chromatophores, saturation being achieved at approximately 2 mmol $\alpha\beta$ · mol $BChl^{-1}$. The heterodimer itself hydrolyses Mg-ATP with an activity distinct from RF_1, being unaffected by azide or sulphite ions. The V_{max} and K_m (ATP) for this Mg^{2+}-dependent activity were 110 ± 10 nmol · min^{-1} · mg $protein^{-1}$ and 100 ± 30 μM, respectively. The K_m did not differ significantly from that of RF_1.

Rhodospirillum rubrum; Enzyme reconstitution; ATP synthase; F_1-ATPase; Subunit interactions; Heterodimer

1. INTRODUCTION

H^+-driven ATP synthesis, in coupling membranes, occurs on a transmembrane enzyme complex, the ATP synthase (F_1F_0-ATPase). This comprises at least 8 different polypeptides (dependent on species) with some 20 polypeptides in total (for a review see [1]). This complex can be readily dissociated into a soluble ATPase (F_1) and a transmembrane H^+-channel (F_0).

The resolved F_1 fragment of the ATP synthase still shows structural complexity, with a minimal structure of $\alpha_3\beta_3\gamma\delta\varepsilon$ [2,3]. It also shows functional complexity, containing 3 catalytic sites and 3 non-catalytic (high-affinity) nucleotide binding sites [4,5]. Furthermore, F_1 exhibits positive cooperativity in catalysis but negative cooperativity with respect to ATP binding, leading to an alternating site kinetic mechanism [6,7]. These properties are also exhibited by the membrane bound form of F_1 [8] and so are likely to be of physiological relevance.

Because of this complexity, few details of its catalytic mechanism have emerged from either structural or kinetic studies on F_1. Attempts to simplify the system further have led to the demonstration of ATPase activity in $\alpha_3\beta_3\gamma$ [9], $\alpha_3\beta_3$ [10,11], $\alpha_1\beta_1$ [12], an indeterminate $\alpha_n\beta_n$ species [13] and isolated β [14,15]. However, it is not clear how far the polymeric subfragments resemble the holoenzyme in terms of nucleotide binding and/or cooperativity, while the ATPase activity of the isolated β subunit, while clearly non-cooperative, is very low (<0.1% of the holoenzyme [14,15]) and difficult to compare to that of F_1. A report of a highly active β_3 complex from chloroplast F_1 [16] has recently been contested [17].

In the present work, we demonstrate the isolation of a stable $\alpha\beta$ heterodimer from chromatophores of the (mesophilic) photosynthetic bacterium, *Rhodospirillum rubrum*. This dimer has a native conformation, as judged by its ability to restore ATP-synthetic and -hydrolytic activities to LiCl-treated chromatophores. Furthermore, it is an active Mg^{2+}-ATPase in its own right, having a specific activity that is 8-times higher than holo-RF_1. This activity is azide-insensitive, indicating that it is non-cooperative in nature. The heterodimer also contains 1 slowly exchangeable ADP binding site. The relationship between the $\alpha\beta$ heterodimer and the organisation of subunits in RF_1 is discussed.

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Abbreviations: F_1, catalytic portion of ATP synthase; RF_1, F_1 from *Rhodospirillum rubrum*; EF_1, F_1 from *Escherichia coli*; CF_1, F_1 from chloroplast thylakoids; HPLC, high performance liquid chromatography; DTT, DL-dithiothreitol; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; BChl, bacteriochlorophyll; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone.

2. MATERIALS AND METHODS

Chromatophores were prepared from late log phase cultures of *R. rubrum* (strain S1, grown anaerobically in the light) essentially as described by Gromet-Elhanan and Khananshvili [18], although cell rupture was effected by sonicating 50 ml batches of cells (containing 25–40 mg BChl) at 0–5°C at an amplitude of 20 μm for 4 periods of 30 s (MSE Soniprep 150). Chromatophores were treated with LiCl and protein precipitated from the supernatant, using ammonium sulphate, as described in [18], except that (i) the time between exposure of the chromatophores to LiCl and $(NH_4)_2SO_4$ precipitation was restricted

to less than 2.5 h, and (ii) the initial $(NH_4)_2SO_4$ precipitate was redissolved in buffer A (100 mM tricine-NaOH, 4 mM ATP, 4 mM $MgCl_2$, 10% (v/v) glycerol, pH 8.0) after standing for only 2–3 h at 0°C. Longer exposure to LiCl resulted in a lower yield of heterodimer.

Pure $\alpha\beta$ heterodimer was obtained from this extract by anion-exchange chromatography, using DEAE-Sephacel followed by DEAE-Sephadex [18]. The sample was concentrated by ultrafiltration (Amicon 8010 with YM5 membrane) and then subjected to chromatography on Sephacryl S-200 (Fig. 1) as described by Harris et al. [19]. Pure heterodimer was dissolved and stored in buffer B (50 mM tricine-NaOH, 4 mM ATP, 4 mM $MgCl_2$, 10% glycerol, 1 mM DTT, pH 8.0) at a concentration of 0.8–1.1 mg · ml^{-1}, and stored at −70°C, after freezing in liquid nitrogen.

RF_1 was extracted by chloroform treatment of chromatophores, as described by Norling et al. [20], precipitated by ammonium sulphate, and then subjected to gel-filtration chromatography, as described by Khananshvili and Gromet-Elhanan [21]. It was stored under identical conditions to the $\alpha\beta$ preparation.

Conditions for the restoration of ATP hydrolysis to LiCl-treated chromatophores were taken from [18]. We define 1 unit of reconstitutive activity to be equal to that amount of material that will restore 1 μmol · min^{-1} of ATPase activity, measured in the region of linear proportionality (generally ≤40% of the maximum attainable rate).

Photophosphorylation by reconstituted chromatophores was measured by the method of Nishimura et al. [22], continuously monitoring the medium pH. After addition of reconstituted chromatophores, the assay medium contained 10 μg BChl · ml^{-1}, 50 mM KCl, 5 mM $MgCl_2$, 4.5 mM tricine-NaOH, 4 mM NaP_i, 1 mM ADP, 50 μM *N*-methyl phenazonium methosulphate and 10 mM NaF (to prevent pyrophosphate synthesis [23]), pH 8.0 (NaOH).

Chromatophore ATPase activity was determined in the presence of 1 μM FCCP by a continuous spectrophotometric method [24] at 35°C. Thus, following the addition of reconstituted chromatophores, the assay medium contained 6.7 μg BChl · ml^{-1}, 4 mM ATP, 2.5 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 50 mM KCl, 50 mM tricine-NaOH, pH 8.0, 10 units · ml^{-1} of pyruvate kinase and 15 units · ml^{-1} of lactate dehydrogenase (Boehringer-Mannheim).

Mg^{2+}-dependent ATPase activity of the soluble $\alpha\beta$ heterodimer was deduced from the rate of production of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$. The assay was initiated by addition of heterodimer (50 μg · ml^{-1} final) to 200 μl of assay buffer, containing: 50 mM tricine-NaOH, pH 8.0, 10% (v/v) glycerol, 5 mM $MgCl_2$, at least 100 μM ATP, 0.4 μCi $[\gamma\text{-}^{32}P]ATP$ · ml^{-1} (Amersham), with other additions as indicated. The assay was conducted at 30°C for 6 min, before the addition of 200 μl of (10% (w/v) trichloroacetic acid, 1 mM NaP_i, 1 mM ATP). Extraction and counting of free phosphate was carried out as already described [19]. The stated concentrations of $\alpha\beta$, ATP and Mg^{2+} were required to limit dimer dissociation (see below).

Tightly bound adenine nucleotides were assayed using firefly luciferase [24]. Prior removal of loosely bound and medium nucleotides was achieved by passage of heterodimer (at a concentration of 0.8–1.1 mg · ml^{-1}) through a G-50 Sephadex centrifuge column [25] (equilibrated in 100 mM tricine-NaOH, pH 8.0, 10% glycerol and either 5 mM $MgCl_2$ or 1 mM EDTA). Nucleotide release using 4% (w/v) $HClO_4$ and subsequent neutralisation was performed as described by Harris [24]. Bioluminescence was measured using an LKB luminometer (model 1251), and a proprietary ATP monitoring reagent (Bio-Orbit).

Size-exclusion HPLC utilised a Waters Protein Pak 300SW (7.8 mm × 30 cm) column. This column was pre-equilibrated in, and the chromatogram developed using, 0.1 M NaP_i, pH 7.0, at a flow rate of 0.5 ml · min^{-1}, and the eluent monitored continuously at 220 nm.

N-terminal sequence analysis was carried out on an Applied Biosystems protein sequencer, by an automated Edman degradation procedure. SDS-PAGE was performed using the discontinuous buffer system of Laemmli [26], acrylamide/bis-acrylamide concentrations of 10% and 0.5%, respectively, and protein was stained using Coomassie brilliant blue (Serva). Protein concentrations were determined using the dye binding method of Bradford [27], using bovine serum albumin as standard. Bacteriochlorophyll (BChl) content of chromatophores was determined using an extinction at 880 nm of 140 $mM^{-1} \cdot cm^{-1}$ [28]. A molecular weight of 106 kDa was assumed for $\alpha_1\beta_1$ [29]. Our results were found to apply for two separate ($\alpha\beta$) protein preparations.

3. RESULTS AND DISCUSSION

3.1. *Characterisation of an αβ heterodimer*

Fig. 1 shows the elution profile of a partially purified, reconstitutively active, LiCl extract of *R. rubrum* chromatophores (following ammonium sulphate precipitation and ion-exchange chromatography) obtained by Sephacryl S-200 chromatography. The peak of reconstitutive activity (○) coincides with the major protein peak (●). Samples were taken at various points during protein elution (numbered 1–9 in Fig. 1) and their constituent proteins visualised by SDS-PAGE (Fig. 1, upper section). It can be seen that the samples containing almost pure β subunit (numbers 7, 8 and 9) had very little reconstitutive activity. However, the samples in the vicinity of the protein and activity peaks contained apparently equal amounts of 2 proteins, of approx. 50 kDa and 55 kDa. These bands were cut from an SDS-polyacrylamide gel, electroeluted, and subjected to N-terminal sequence analysis. With reference to the deduced amino acid sequence of the ATP operon of *R. rubrum* [29], and their apparent molecular weights, these two proteins were identified as the α and β subunits (Table I), although the N-terminal methionine of the β subunit was missing.

The position of elution of the active peak from Sephacryl S-200 (Fig. 1) suggested that the α and β subunits were associated, having an apparent molecular weight of 106 kDa, i.e. the predicted weight of an $\alpha\beta$ heterodimer. (Size-exclusion HPLC predicted the slightly lower value of 93 kDa for the $\alpha\beta$-containing species.) In addition, N-terminal analysis of this ($\alpha\beta$) protein indicated equal proportions of each subunit. We concluded that this protein was an $\alpha\beta$-heterodimer.

The possibility that the dimer peak consisted of a mixture of α_2 and β_2 dimers (rather than an $\alpha\beta$ heterodimer) was considered unlikely, since it proved impossible to separate the α and β subunits in our preparation using ion-exchange chromatography, affinity chromatography on ATP-Sepharose, or by chromatofocussing (data not shown). It was concluded, therefore, that the preparation was a true heterodimer of α and β subunits of RF_1.

None of the three smaller subunits of RF_1 (γ, δ or ε) could be detected in the purified $\alpha\beta$ preparations, even when the gel was overloaded for the α and β subunits (Fig. 2). By comparison with standard amounts of F_1 (Fig. 2), we estimate considerably less than 0.07 mol of any of the minor subunits per mol of heterodimer, in our preparation. It was concluded that the purified $\alpha\beta$ heterodimer was not associated with the smaller subunits of RF_1.

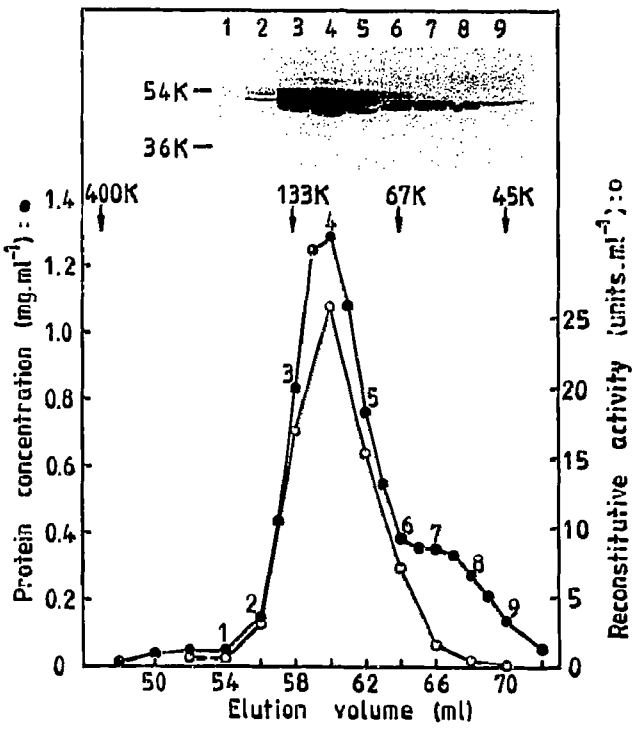

Fig. 1. Gel filtration of partially purified $\alpha\beta$ from RF_1. 1 ml of a 10 mg · ml^{-1} solution of the partially purified LiCl extract was applied to a Sephacryl S-200 column (1.6 × 65 cm), as described in section 2. Fractions of 1 ml were collected and assayed for protein (•) and reconstitutive (ATPase) activity (o). Column calibration was with CF_1, BSA and ovalbumin. The subunit composition of the numbered fractions (1–9) was determined by SDS-PAGE (upper section). 5 μl aliquots of these fractions were combined with an equal volume of tracker dye and loaded to consecutive wells, as indicated.

The $\alpha\beta$ heterodimer, isolated here, was not previously detected in LiCl extracts from *R. rubrum* chromatophores. This appears to be due to the prolonged exposure to LiCl used in previous extraction procedures [18,19] compared to the present procedure. We find that exposure to LiCl for periods longer than 2–3 h considerably decreases the yield of the $\alpha\beta$ heterodimer (data not shown).

3.2. *Nature of the α–β association*

The $\alpha\beta$ heterodimer, as prepared, was stable for more than 24 h at room temperature, provided that 1 mM MgATP was present, showing only traces of α and β monomers (Fig. 3A). However, some dissociation ($\leq$25%) into monomers accompanied heterodimer dilution (Fig. 3B). Moreover, if the ATP was removed by centrifugation through Sephadex G-50, or the Mg^{2+}-ions removed by addition of EDTA, and the protein concentration (in either case) diluted to $\leq 50\ \mu g \cdot ml^{-1}$, dissociation into the component monomers occurred within 45 min (Fig. 3C). Dissociation accompanying Mg^{2+} sequestration was reversed on addition of excess Mg^{2+}-ions (a 22.5 mM excess of Mg^{2+} over EDTA was used in Fig. 3D). The superimposed SDS-PAGE results (Fig. 3B–D) show the subunit composition of the dimer peak and, in particular, highlight the reappearance of both the α and β subunits, following heterodimer reassembly. By comparison, heterodimer from which both ATP and free Mg^{2+} had been removed, showed complete dissociation which was not reversed by readdition of either ATP and/or Mg^{2+} (not shown). It was concluded that divalent metal cations and nucleotide promote association between the α and β subunits of RF_1. We found that Mn^{2+}-ions were as effective at promoting reassociation as Mg^{2+}-ions, while Ca^{2+}-ions were far less effective, causing no more than 50% of the monomers to reassociate.

Mg^{2+}-ions and non-exchangeable adenine nucleotides are believed to play a structural role in the F_1 moiety [30]. Analysis of the $\alpha\beta$ heterodimer, after separating it from medium nucleotides (see section 2) showed it to contain tightly bound ADP (approx. 1 mol/mol dimer; Table II). However, addition of EDTA prior to removal of medium nucleotides led to the loss of this tightly bound nucleotide (Table II). The requirement for both Mg^{2+} and ATP for dimer integrity, together with the identification of a (Mg^{2+}-dependent) tight nucleotide binding site, is consistent with the idea that a non-exchangeable nucleotide binding site occurs at the $\alpha\beta$ interfaces in holo F_1 [3] and that this site is present in the $\alpha\beta$ heterodimer. Besides this 'non-exchangeable' nucleotide binding site, the $\alpha\beta$ heterodimer possesses a rapidly exchangeable (catalytic) ATP binding site, as evidenced by its ATP hydrolytic activity (below).

It seems likely that the association between α and β represents a functional interaction within the F_1 molecule. One tight nucleotide per $\alpha\beta$ dimer corresponds

Table I

N-terminal amino acid sequences of proteins of apparent molecular weights 50 and 55 kDa (as in Fig. 1). For comparison, the corresponding deduced amino acid sequences for the α and β subunits of RF_1 are shown (from Falk et al. [29]). For further details, see text

Residue number:	1	6	11	16
α-Subunit:	M E I R A	A E I S A	I L K E Q	I A N F G
55 kDa band:	M E I R A	A E I S A	I L K E Q	
β-Subunit:	M A K N N	L G T I T	Q V T G A	V V D V K
50 kDa band:	A K N N	L G T I T	Q V T G A	V V D V K

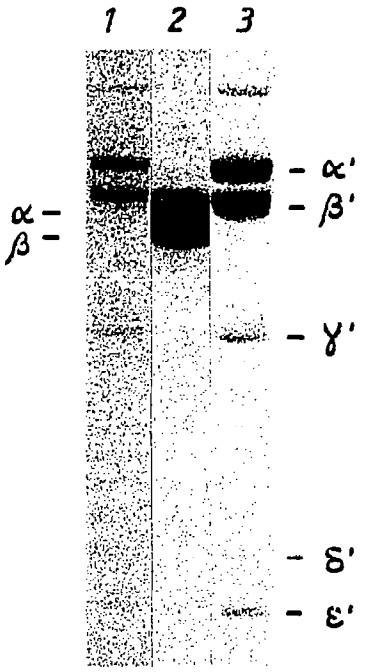

Fig. 2. Subunit composition of purified heterodimer. 14 μg of heterodimer, purified from LiCl-treated chromatophores (track 2), was resolved by SDS-PAGE, together with 1.8 μg (track 1) and 7.0 μg (track 3) of CF_1 (whose subunits, α'–ε', have similar molecular weights and staining characteristics to those of RF_1).

with the 3 non-exchangeable sites observed in the $\alpha_3\beta_3$ assembly in F_1. Furthermore, cross-linking, neutron scattering and electron microscopic investigations all suggest a structural αβ interaction [31–33]. In effect, therefore, we can look at the arrangement of the large subunits in F_1 as a trimer of 3 αβ pairs.

3.3. *Activity of the αβ heterodimer*

LiCl washed chromatophores from *R. rubrum* have lost the capacity for photophosphorylation and ATP hydrolysis that is exhibited by untreated chromatophores. Both activities have been shown to be restored to the treated chromatophores by adding back soluble components isolated from the LiCl wash [34].

The αβ heterodimer, as isolated here, was effective in restoring both ATP synthesis and hydrolysis to LiCl-treated chromatophores (Fig. 4). Indeed, on a molar basis it was at least 50 times more effective in reconstituting either activity than previously reported preparations. As can be seen from Fig. 4, 0.20 μg αβ · μg $BChl^{-1}$ (equivalent to 1.9 mmol αβ · mol $BChl^{-1}$) is required for saturation, as compared to between 5 and 20 μg β · μg $BChl^{-1}$ (equivalent to 0.1–0.4 mol β · mol $BChl^{-1}$) reported elsewhere, using preparations of purified β subunit [35–37]. Thus, the αβ heterodimer is a highly active species and, presumably, represents a subfragment of F_1 in its native conformation. Both reconstituted activities have a similar (αβ) concentration dependence, and are therefore likely to be catalysed by the same reconstituted entity. The reconstituted activity was associated with the chromatophore vesicles, being removed from solution by centrifugation. Moreover, RF_1 itself (1 μg RF_1 · μg $BChl^{-1}$) did not restore any capacity to syn-

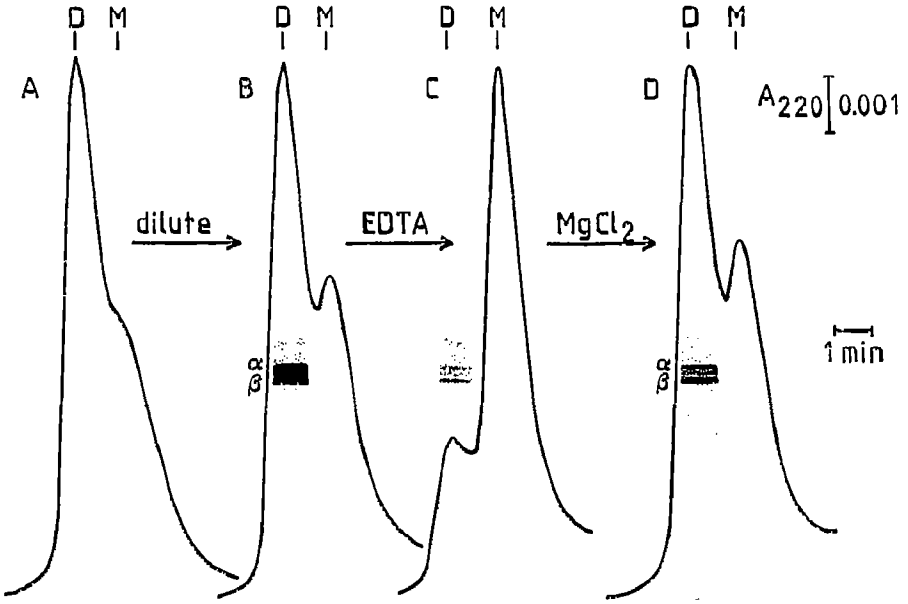

Fig. 3. Dissociation and reassociation of the αβ heterodimer. Size-exclusion HPLC was used to separate αβ heterodimer (D) from dissociated α and β monomers (M). Profiles shown were eluted from the column 14–17 min after sample application. 20 μl samples of αβ dimer were used throughout. (A) 0.82 mg protein · ml^{-1}; (B) protein diluted to 50 μg · ml^{-1} by addition of (0.1 M tricine-NaOH, pH 8.0, 10% glycerol, 1 mM ATP, 1 mM DTT); (C) sample diluted as in (B), followed by a 45 min incubation with 2.5 mM EDTA; (D) sample treated as in (C), followed by a 40 min incubation with 25 mM $MgCl_2$. Fractions were collected at 30 s intervals, freeze-dried, subjected to SDS-PAGE and silver stained [43]. Thus, in traces B, C and D, the subunit composition of the dimer peak is shown.

Table II

ATP and ADP tightly bound to $\alpha\beta$ dimer. As described in section 2, loosely associated and medium nucleotides were removed using a G-50 Sephadex centrifuge column, pre-equilibrated with buffer containing tricine, glycerol and either 5 mM $MgCl_2$ or 1 mM EDTA, as indicated

Column buffer containing	Nucleotide content (mol nucleotide · mol $\alpha\beta^{-1}$)	
	ATP	ADP
5 mM $MgCl_2$	<0.01	1.12 ± 0.11 (6)
1 mM EDTA	<0.01	0.04 ± 0.02 (6)

Table III

Comparison of Mg^{2+}-dependent ATPase activities of soluble $\alpha\beta$ and RF_1. The effects of NaN_3 and Na_2SO_3 were assessed (in duplicate) using 200 μM ATP, for which the corresponding control activities (1.00) were: 10 nmol · min^{-1} · mg^{-1} (RF_1) and 92 nmol · min^{-1} · mg^{-1} ($\alpha\beta$). For experimental details, see section 2

Sample	K_m (μM)	V_{max} (nmol · min^{-1} · mg^{-1})	Relative rate	
			+2 mM NaN_3	+10 mM Na_2SO_3
RF_1:	131 ± 19 (5)	14 ± 0.5 (5)	0.07	48.5
$\alpha\beta$:	98 ± 32 (5)	109 ± 8 (5)	0.90	1.35

thesise ATP in LiCl-treated membranes (results not shown) and so this property of the heterodimer is not due to contamination with RF_1 or therefore, with the minor subunits (γ, δ, ε) or RF_1.

The isolated $\alpha\beta$ heterodimer is itself capable of ATP hydrolysis. When its Mg-ATPase activity was measured by release of P_i from ATP (see section 2), a V_{max} of 109 nmol · min^{-1} · mg^{-1} was determined (Table III). This is considerably higher than the Mg-ATPase activity of its parent RF_1 (14 nmol · min^{-1} · mg^{-1}; Table III). However, in agreement with others [38], our RF_1 had a high Ca^{2+}-dependent ATPase activity (some 200-fold greater than the Mg-ATPase activity).

A number of other differences between the $\alpha\beta$ and RF_1 Mg-ATPase activities are shown in Table III. RF_1-ATPase is strongly stimulated by anions such as sulphite [19,39] while the heterodimer ATPase is not. Conversely, RF_1 (like all F-type ATPases) is strongly inhibited by azide [19] while the heterodimer is again unaffected. An absence of azide inhibition is characteristic of non-cooperative ('unisite') catalysis by F_1 [40,41] and this is consistent with the view that the $\alpha\beta$ dimer contains a single catalytic site. The relatively high ATPase activity of this $\alpha\beta$ preparation, together with the negligible effect of either azide or sulphite, mean that the heterodimer cannot be significantly contaminated with RF_1.

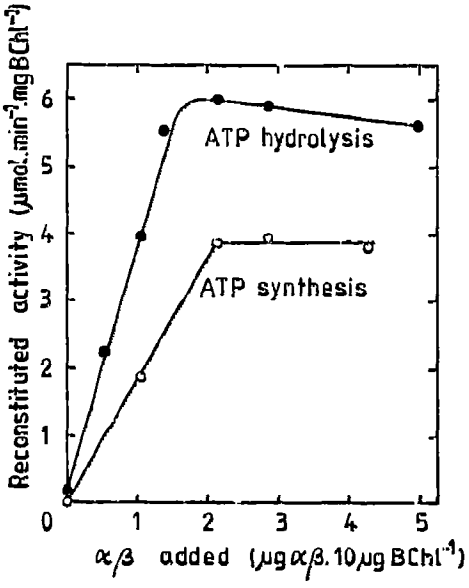

Fig. 4. Reconstitution of ATP-hydrolytic (●) and -synthetic (○) activity in LiCl-treated chromatophores, using $\alpha\beta$ heterodimer. For comparison, coupled chromatophores displayed rates of approx. 10.5 μmol · min^{-1} · mg $BChl^{-1}$ for both ATP synthesis and hydrolysis. For experimental details, see section 2.

Another feature of the $\alpha\beta$ ATPase is that its K_m(ATP) is, within experimental error, identical to that of holo-RF_1 (Table III). Since the K_m measured for the holoenzyme corresponds to the lowest affinity of the negatively cooperative enzyme, it appears that – when removed from the constraints of the other subunits – the 'natural' (or 'relaxed') conformation of the catalytic site is the low affinity form.

The specific reconstitutive and ATPase activities of the present preparation are both very much greater than the activities of the β monomer, purified as previously described [19,34–36]. The cation dependence and inhibitor/activator sensitivities of the ATPase activity of both preparations (Table III and [19]) are, however, similar to each other, albeit different to those of RF_1. Thus, it might be possible to explain the ATPase activity of a 'purified β' preparation [19] by the presence of some contaminating $\alpha\beta$ heterodimer (2–4% (w/w)). Preliminary experiments suggest that this could account for most of the ATPase activity of such preparations, but that pure β subunit from *R. rubrum* does have a low intrinsic ATPase activity, of the order of 0.6 nmol · min^{-1} · mg^{-1}, comparable to that of β from EF_1 and CF_1 [14,15]. In contrast, all $(\alpha\beta)_n$ preparations so far reported ([12,13] and the present work) show an ATPase activity of 100–200 nmol · min^{-1} · mg $protein^{-1}$.

It is not clear whether previous reconstitution experiments using *R. rubrum* β can also be explained on the basis of contamination with $\alpha\beta$ dimer, not least because it is not known whether both α and β subunits of the heterodimer, or just the β subunit, become attached to

the chromatophore membrane. In the latter case, the high reconstitutive activity of our $\alpha\beta$ heterodimer might be explained by a stabilising effect of α on the β component of the dimer (cf. [42]). Further work is necessary to resolve these possibilities.

Acknowledgements: We would like to thank Professors M. and H. Baltscheffsky for fruitful discussions; Tony Willis of the MRC Immunochemistry Group, for the N-terminal sequence analyses (Table I); Ken Johnson for his photographic expertise and Valerie Laux, for her excellent technical assistance. Financial support from the SERC (Grant GR/E94036) is gratefully acknowledged.

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