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Preparation and characterisation of an $\alpha\beta$ heterodimer from the ATP synthase of *Rhodospirillum rubrum*

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1. An $\alpha\beta$ heterodimer of the F_1-ATPase of *Rhodospirillum rubrum* (RF_1) was isolated from extracts of chromatophores. This fragment of RF_1 is highly active in restoring ATP hydrolysis and ATP synthesis to LiCl-treated chromatophores, maximal activity being reached at approximately 2 mol $\alpha\beta$ per original RF_1 molecule. Both α and β subunits of the heterodimer bind to chromatophore membranes on reconstitution. It was concluded that the $\alpha\beta$ heterodimer was able to reconstitute RF_1 in these membranes. 2. Reconstitutive activity in LiCl extracts purifies with the $\alpha\beta$ heterodimer and away from the monomeric β subunit. Prolonged exposure to LiCl leads to irreversible dissociation of the heterodimer and loss of reconstitutive activity. It was concluded that the $\alpha\beta$ heterodimer was required for reconstitution in LiCl-treated membranes, and that the monomeric β subunit was inactive. 3. The $\alpha\beta$ heterodimer contains a non-catalytic nucleotide binding site. It can also catalyse CaATP and MgATP hydrolysis at similar rates, with K_m values similar to RF_1. However, hydrolysis is insensitive to activators/inhibitors of RF_1 such as sulphite, octyl glucoside and azide. It was concluded that the isolated heterodimer represents a functional unit of RF_1 but that, as it has a single catalytic site, catalytic cooperativity is absent.

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, with some 20 polypeptides in total. The complex can easily be 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 $\alpha_3\beta_3\gamma\delta\epsilon$ (for a review, see Refs. 1,2). It also shows functional complexity, containing three catalytic sites and three non-catalytic (high-affinity) nucleotide binding sites [3,4].

Attempts to simplify the system further have led to the demonstration of ATPase activity in $\alpha_3\beta_3\gamma$ [5], $\alpha_3\beta_3$ [6,7], $\alpha_1\beta_1$ [8] and isolated β [9,10] fragments of F_1. Furthermore, it has been shown that treatment of chromatophores from *R. rubrum* with LiCl yields a membrane fraction, incapable of ATPase or ATP synthetic activity, and a soluble fraction, containing the β subunits of RF_1. Mixing the soluble fraction (after removal of LiCl) with the membrane fraction was found to restore ATP hydrolysis and synthesis [11]. These observations all indicate a direct role for the β subunit in catalysis by F_1F_0 ATPases.

We have recently demonstrated that, in addition to β, an $\alpha\beta$ heterodimer can be identified in the soluble fraction resulting from LiCl treatment of *R. rubrum* chromatophores [12]. This $\alpha\beta$ heterodimer was shown to be highly active in restoring both ATP-hydrolytic and ATP-synthetic activities to the chromatophore membranes from which they were derived. In addition, the inherent Mg^{2+}-dependent ATPase activity of this $\alpha\beta$ preparation was as much as 2 orders of magnitude greater than that reported for the purified β subunit [12].

In the present paper, we investigate the relationship between binding of the $\alpha\beta$ complex to LiCl-treated chromatophores and reconstitution of ATPase and ATP synthetic activities. It is shown that both α and β subunits bind to the stripped chromatophores, and that maximal reconstitution requires stoichiometric amounts of both subunits. We also investigate the intrinsic ATPase activity of the $\alpha\beta$ heterodimer, showing that it is

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Abbreviations: F_1, catalytic portion of ATP synthase; RF_1, F_1 from *Rhodospirillum rubrum*; TF_1, F_1 from thermophilic bacterium PS3; HPLC, high performance liquid chromatography; seHPLC, size exclusion HPLC; DTT, DL-dithiothreitol; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; BChl, bacteriochlorophyll; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

distinguishable from that of RF_1 in metal ion dependence and inhibitor/activator sensitivity. It therefore represents a novel ATPase activity of this system.

We were able to isolate the $\alpha\beta$ heterodimer from chromatophores using a very similar procedure to that reported for the isolation of the β subunit [13]. It was, therefore, surprising that the heterodimer had not previously been identified. The present work also identifies factors which facilitate the isolation of the heterodimer, and presents possible reasons why it has escaped detection until now.

Materials and Methods

Protein / membrane preparations

Chromatophores were prepared by sonication of late log phase cultures of *R. rubrum* (Strain S1) as already described [12]. They were then extracted with LiCl, sedimented by centrifugation, and the soluble protein in the supernatant precipitated by addition of solid ammonium sulphate. The precipitate was dissolved in 100 mM Tricine, 4 mM ATP, 4 mM $MgCl_2$, 10% (v/v) glycerol (pH 8.0) (NaOH), reprecipitated and then redissolved in, and dialysed against, at least 100 vols. of the same buffer, as described by Gromet-Elhanan and Khananshvili [13]. Care was taken to minimise the time of exposure of proteins to LiCl, long exposures resulting in a lower yield of heterodimer (see below). Similar results were obtained when chromatophores were prepared using a French press, rather than by sonication.

Pure $\alpha\beta$ heterodimer was obtained from this crude LiCl extract by a combination of anion-exchange chromatography, using both DEAE-Sephacel and DEAE-Sephadex, and size-exclusion chromatography, using Sephacryl S-200, at 0–4°C, as outlined in [12]. The order in which these separation methods were applied was not critical, the final specific reconstitutive activity being in the range 30 to 40 μmol min^{-1} (mg protein)$^{-1}$ for all combinations tested. For the procedure described in Table I, 8 ml of an 18 mg ml^{-1} solution of the LiCl extract was applied to a column of DEAE-Sephacel (1.8 × 7 cm) equilibrated in buffer 1 (100 mM Tricine, 4 mM ATP, 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 100 mM NaCl (pH 8.0) (NaOH)) at a rate of 0.4 ml min^{-1}. The first protein peak was eluted using 80 ml of the same buffer and was without reconstitutive activity. Subsequent use of a linear 0.1–0.4 M NaCl gradient (in 160 ml of buffer 1) resulted in the elution of a broad protein peak containing a single (narrow) peak of reconstitutive activity. Fractions (4 ml) with a specific reconstitutive activity greater than 10 units (mg protein)$^{-1}$ were pooled, diluted 2.5-fold in buffer 2 (50 mM Tricine, 4 mM ATP, 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol (pH 8.0) (NaOH)) and applied to a column of DEAE-Sephadex A-50 (1.8 × 4 cm), equilibrated in the same buffer, at a flow rate of 0.5 ml min^{-1}. The column was first washed with 15 ml of 0.15 M NaCl in buffer 2, after which a single (symmetrical) protein peak with reconstitutive activity was eluted, using a linear 0.15–0.4 M NaCl gradient, in 80 ml of buffer 2. Fractions (1 ml) with a specific reconstitutive activity greater than 18 Units (mg protein)$^{-1}$ were pooled and then concentrated to 4 mg ml^{-1} by ultrafiltration (Amicon 8010 with YM5 membrane). Finally, the sample was applied to a column of Sephacryl S-200 (1.6 × 65 cm) equilibrated in buffer 2, supplemented with 1 mM dithiothreitol (DTT). The column was developed using the same buffer, at a flow rate of 0.125 ml min^{-1}. Fractions (1 ml) with a specific reconstitutive activity greater than 30 Units (mg protein)$^{-1}$ were pooled, concentrated by ultrafiltration (as before) to approx. 1 mg ml^{-1}, and stored at −70°C, after freezing in liquid nitrogen.

TABLE I

Purification of $\alpha\beta$ heterodimer from a (short exposure) LiCl extract of chromatophores

Purification of the heterodimer from *R. rubrum* chromatophores, and assay procedures were as described in Materials and Methods.

Purification stage	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Recovery	
				protein (%)	activity (%)
LiCl extract	616	140	4.4	100	100
DEAE-Sephacel (eluting at 0.16–0.20 M NaCl)	288	16	18	11	47
DEAE-Sephadex (eluting at 0.21–0.26 M NaCl)	93	3.7	25	2.6	15
Sephacryl S200 (eluting at $M_r \approx 100\,000$)	43	1.3	33	0.9	7

Extraction and storage of RF_1 was as described previously [12]. LiCl-treated chromatophores ('stripped chromatophores') were prepared according to Ref. 13.

Assays

Restoration of ATP hydrolysis to LiCl-treated chromatophores was essentially as described by Gromet-Elhanan and Khananshvili [13]. To 75 μl of reconstitution buffer {50 mM tricine, 50 mM $MgCl_2$, 8 mM ATP pH8.0 (NaOH)} was added 5 μl of LiCl treated chromatophores (containing 10–12 μg BChl), and ≤ 70 μl of LiCl extracted material, the total volume being made up to 150 μl by the addition of 50 mM tricine-NaOH, pH8.0. After 30 minutes at 35°C, the sample was assayed for recovered activity. This procedure was modified for reconstitution of photophosphorylation; chromatophores equivalent to 40 μg BChl were incu-

bated in a total volume of 200 μl, containing 100 μl of reconstitution buffer. We define 1 Unit (U) of reconstitutive activity to be equal to that amount of material that will restore 1 Unit (μmol min^{-1}) of ATPase activity, measured in the region of linear proportionality (generally $\leq 40\%$ of the maximum attainable rate).

The assays used in this work have been described in detail elsewhere [12]. In brief, photophosphorylation was determined by illuminating chromatophores in a phosphorylation medium, and continuously measuring medium pH. Chromatophore (Mg^{2+}-dependent) ATPase activity was determined by coupling ADP production to NADH oxidation using pyruvate kinase/lactate dehydrogenase [12]. Measurement of the Ca^{2+}-and Mg^{2+}-dependent ATPase activities of the soluble $\alpha\beta$ heterodimer was deduced from the rate of production of [^{32}P]P_i from [γ-^{32}P]ATP [14]. In studies on the soluble heterodimer, Me^{2+}-ATP was maintained at or above 100 μM, to minimise dissociation of the dimer in the assay buffer.

Tightly bound adenine nucleotides, associated with coupled and LiCl-treated chromatophores, were assayed using firefly luciferase. Removal of loosely-bound and medium nucleotides was achieved by 3 consecutive cycles of sedimentation (250000 $\times g$, 60 min)/resuspension in 10 ml aliquots of (0.2 M glycylglycine, 5 mM NaP_i, 2 mM $MgCl_2$ (pH 7.4) (NaOH)), at 0°C. Nucleotide release using 4% (w/v) $HClO_4$ and subsequent neutralisation was performed as described by Harris [15]. Bioluminescence was measured using an LKB luminometer (model 1251), and a proprietary ATP monitoring reagent (Labsystems Group (UK) Ltd.).

Size-exclusion HPLC utilised a Waters Protein Pak 300 SW (7.8 mm $\times$ 30 cm). The 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 continously at 220 nm.

SDS-PAGE was performed using the discontinuous buffer system of Laemmli [16], acrylamide/bisacrylamide concentrations of 10% and 0.5% respectively, and (unless otherwise indicated) was stained using Serva brilliant blue G. Protein concentrations were determined using the dye binding method of Bradford [17], using bovine serum albumin as standard. Bacteriochlorophyll (BChl) content of chromatophores was determined using an extinction coefficient at 880 nm of 140 mM^{-1} cm^{-1} [18]. A molecular mass of 106 kDa was assumed for $\alpha_1\beta_1$ [19].

Results

Isolation of reconstitutively active protein

A LiCl extract of chromatophores, partially purified on DEAE Sephadex, contains both α and β subunits of RF_1 with a preponderance of β subunit (Fig. 1, track 0). Further fractionation of this extract on DEAE-cellulose, as described by Gromet-Elhanan and Khananshvili [13], yields two major protein peaks (Fig. 1). The first peak eluted eluted at 0.16 M NaCl, and contained principally α and β subunits of RF_1. The second peak was eluted using a linear (0.16–0.40 M) NaCl gradient, and contained pure β subunit. Comparison of the protein and activity profiles in Fig. 1 shows that reconstitutive activity is found exclusively in the first peak, which contains both α and β subunits. Association of reconstitutive activity with fractions containing both α and β subunits, and its absence in fractions containing β alone, was also observed during separation on Sephacryl S-200 [12].

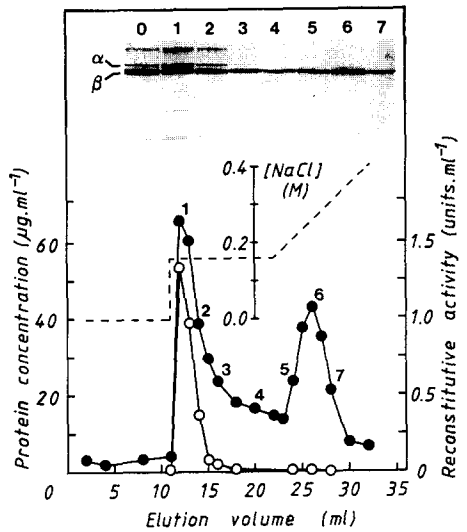

Fig. 1. Anion-exchange chromatography of a partially purified $\alpha\beta$ preparation. A partially purified LiCl-extract of chromatophores (following DEAE-Sephadex treatment, as described by Gromet-Elhanan and Khananshvili [13]) containing 3 U of reconstitutive activity, was applied to a 0.7$\times$ 4 cm column of DEAE-Sephacel, equilibrated in (50 mM Tricine-NaOH, 4 mM ATP, 4 mM $MgCl_2$, 10% (v/v) glycerol (pH 8.0)). Elution of reconstitutive activity (○) and protein (●) was effected by addition of NaCl to the buffer, as indicated (broken line). 10 μl samples from the numbered fractions were then subjected to SDS-PAGE, and the resolved proteins visualised by silver staining [44]. The initial composition of the sample is shown in track 0.

This finding is at variance with the conclusions of Gromet-Elhanan and coworkers, who have claimed that reconstitutive activity is associated with the β subunit of RF_1 [11,20]. In our hands, repeated attempts to purify β by their procedures [13] or modifications thereof [14] always yield pure β subunit which is inactive in reconstitution. This is not likely to be due to inactivation of β during our purification; in the experiment of Fig. 1, 2.7 U of the 3.0 U of reconstitutive activity applied to the column were recovered in the initial peak. Thus, very little loss of total activity occurred even though the resultant pure β subunit was inactive in reconstitution.

Table I summarises a protocol for purification of the reconstitutively active protein from an LiCl extract of chromatophores. The overall recovery of reconstitutive activity during such a large scale preparation was typically 6–9%. While this appears low compared to the recovery in the experiment of Fig. 1, it should be remembered that in Fig. 1, total recovered activity was pooled for measurement, while in Table I, only peak fractions were taken for further purification at each stage (see Methods).

The specific reconstitutive activity of our preparation rose about 8-fold during the purification, typically reaching 30–40 U (mg protein)$^{-1}$. Our final species is a pure $\alpha\beta$ heterodimer, as can be seen from Fig. 6. We have shown previously that this preparation contains less than 7% (i.e., 0.07 (mol(mol$\alpha\beta$)$^{-1}$) contamination with the smaller subunits of F_1 [12].

It is noteworthy that the *initial* specific activity in our LiCl extracts was greater that that recorded for a *purified* β preparation in Ref. 13, when calculated on a common scale. This seems to be due to our precautions to minimise $\alpha\beta$ dissociation in crude LiCl extracts of chromatophores (see later).

Effect of LiCl on the $\alpha\beta$ heterodimer

Prior to the recent investigations of our laboratory ([12] and present work), reconstitutively active $\alpha\beta$ heterodimer had not been identified in LiCl extracts from *R. rubrum* chromatophores. This seems to be due to a slight modification in our experimental protocol. In the procedure of Gromet-Elhanan and Khananshvili [13], incubation of chromatophores with LiCl for 30 min is followed by a prolonged (6 h) centrifugation, after which $(NH_4)_2SO_4$ is added to the supernatant (to 60% saturation) and precipitation allowed to proceed overnight. However, our LiCl treatment involves a much shorter centrifugation (1–2 h) and a shortened incubation with $(NH_4)_2SO_4$ (2–3 h). Products of these two procedures (referred to as 'long' and 'short' exposure extracts, respectively) were investigated.

The relative amounts of $\alpha\beta$ dimer and α,β monomers in both LiCl extracts was assessed by size-exclusion HPLC (Fig. 2). Long exposure (Fig. 2, solid line) yielded an extract composed predominantly of proteins which eluted from the column after 18.4 min (as does the purified β subunit). Short exposure extracts, in contrast, contained a peak at 17.1 min, corresponding to the $\alpha\beta$ heterodimer, as well as that coinciding with monomeric β (Fig. 2, broken line). Traces of membrane fragments eluting at the void volume (10.3 min) were also detected in the short exposure extracts, these being removed less efficiently using the relatively short centrifugation of this procedure.

SDS-PAGE revealed similar patterns of protein bands in both types of LiCl extract (Fig. 2, inset), including roughly equal proportions of RF_1 α and β

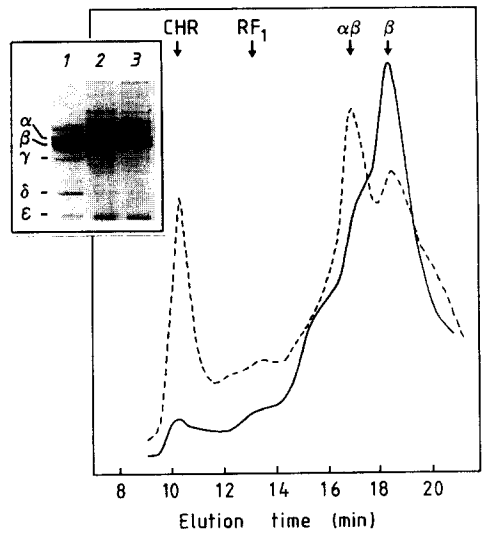

Fig. 2. Effects of LiCl exposure on the composition of chromatophore extracts. Chromatophores were incubated for 30 min at 4°C in a solution containing (2 M LiCl, 50 mM Tricine-NaOH, 0.25 M sucrose, 4 mM ATP, 4 mM $MgCl_2$ (pH 8.0)) after which the membranes were sedimented by centrifugation, either for 6 h at 170,000× g (solid line) or for 1 h at 260,000× g (broken line) prior to protein precipitation using $(NH_4)_2SO_4$, as described in the text. 50 μg of protein from each extraction was then applied to a Waters 300 SW seHPLC column, and subjected to gel permeation chromatography, as detailed under Methods. The positions of elution of β, $\alpha\beta$, RF_1 and chromatophores are as indicated. Inset: SDS-PAGE of chromatophore extracts resulting from such 'long' (track 2) and 'short' (track 3) periods of exposure to LiCl. Track 1: RF_1. 6 μg of protein was applied to each track.

subunits. We concluded that the protein composition of both extracts was similar, but that they differed in the state of association of their α and β subunits.

Fig. 3 shows further studies on the effects of LiCl exposure on the interaction between the subunits of the heterodimer. These experiments utilised pure $\alpha\beta$ heterodimer and were conducted at 25°C, rather than at 4°C (used during the extraction of chromatophores). $\alpha\beta$ Heterodimer was exposed to 2 M LiCl for varying lengths of time, after which the solution was diluted to 0.25 M LiCl (by adding water) and the proportions of heterodimer (D) and monomer (M) assessed by size exclusion HPLC. Fig. 3(I) shows the proportions of dimer and monomer in a sample of heterodimer simply diluted into water (control); the dimer clearly predominates. After only 10 min in 2 M LiCl (without subsequent dilution of LiCl) the dimer had totally dissociated (Fig. 3(II)). However, after dilution of LiCl, reassociation to form heterodimer can occur (Fig. 3,III). Fig. 3 (III-VI) shows that, as the period of exposure to 2 M LiCl increases, the subunits lose their ability to reassociate after dilution. After 2 h, for example, more than 90% of the dissociated subunits are unable to reassociate. Thus it appears that reversible dissociation of the $\alpha\beta$ complex in LiCl is rapid, but that subsequently a slow inactivation occurs in one (or both

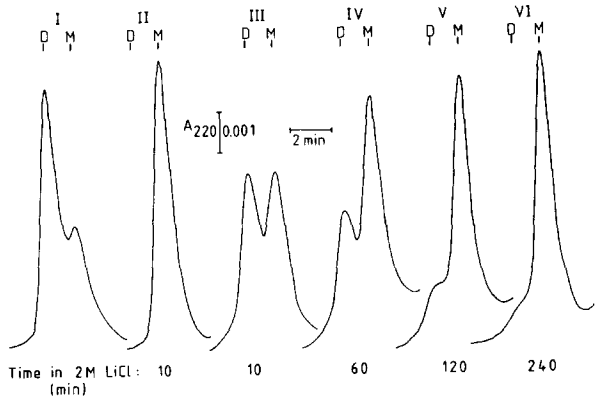

Fig. 3. Effect of LiCl on the association of α and β subunits. 5 μl aliquots of a 0.80 mg ml^{-1} solution of $\alpha\beta$ heterodimer in (50 mM Tricine-NaOH, 4 mM ATP, 4 mM $MgCl_2$ and 10% (v/v) glycerol, (pH 8.0)) were mixed with an equal volume of 4 M LiCl (dissolved in the same buffer). After the indicated times (samples III, IV, V and VI), 70 μl of pure water was added, and a 20 μl sample was immediately analysed by seHPLC, to find the relative proportions of $\alpha\beta$ heterodimer (D) and dissociated α and β monomers (M). In trace I, water was added instead of LiCl, whereas in trace II, a solution of 2 M LiCl was added instead of water. Protein was eluted from the column 16 to 20 min after sample application.

subunits) which prevents reassociation. This change is not prevented by Mg^{2+} or ATP (which were present throughout) or DTT (data not shown).

Fig. 4 shows that the reconstitutive activity of the $\alpha\beta$ preparation falls during exposure to LiCl. The time course shows that loss of activity was not due to re-

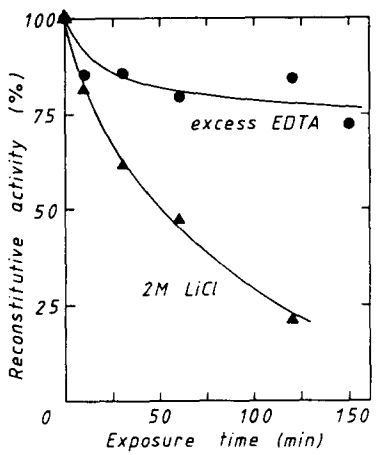

Fig. 4. Effect of LiCl- and EDTA-promoted dissociation of the $\alpha\beta$ heterodimer on its reconstitutive activity. Purified $\alpha\beta$ heterodimer was-treated with LiCl (▲) as described in Fig. 2, except that dilution (at the indicated times) was effected using 70 μl of {50 mM tricine-NaOH, pH 8.0, 10% (v/v) glycerol, 1 mM DTT, 1 mM ATP} instead of water. EDTA treatment (●) involved incubating 5 μl of heterodimer (0.8 mg ml^{-1}) with 70 μl of the same buffer, to which 2 μl of 0.1 M EDTA (pH 8.0), had also been added. This causes dissociation of the heterodimer [12]. At the indicated times, reassociation was then promoted by the further addition of 2 μl of 0.2 M $MgCl_2$. All incubations were conducted at room temperature. 40 μl of each sample was then assayed for (ATPase) reconstitutive activity, as described in Methods.

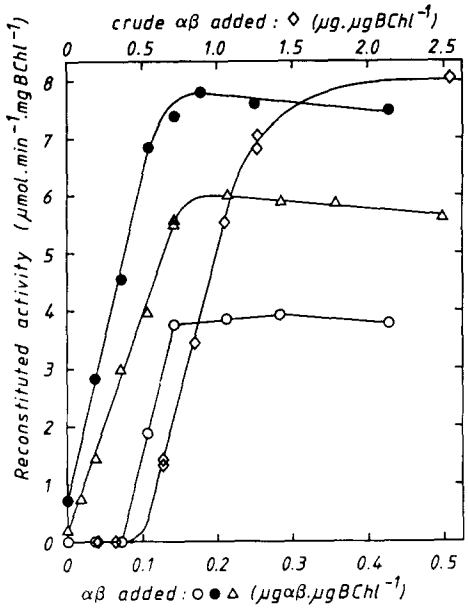

Fig. 5. Reconstitution of ATP-hydrolytic and -synthetic activity in LiCl-treated chromatophores. The indicated amounts of purified heterodimer (○, △) or a short exposure LiCl extract (◇) were used to restore the capacity of LiCl-treated chromatophores to hydrolyse (△) or to synthesise (○, ◇) ATP (see Materials and Methods). The combined effect of a long exposure LiCl extract (1.2 μg protein.μg BChl^{-1}) and increasing amounts of $\alpha\beta$ heterodimer, is also illustrated (●).

versible subunit dissociation (complete within 10 min in LiCl), but followed the loss of ability of the subunits to reassociate. This would explain the low reconstitutive activity observed in long exposure extracts; in the experiment of Fig. 2, for example, the reconstitutive activities of the extracts given short and long exposures were 6.4 and 1.7 U^{-1} (mg protein)$^{-1}$, respectively.

The $\alpha\beta$ heterodimer also dissociates in the presence of EDTA, in excess over Mg^{2+} (more than 90% having dissociated after 30 min). Reassociation occurred on addition of excess Mg^{2+} [12], but in this case reversibility was maintained for several hours. Fig. 4 shows that the reconstitutive activity of the $\alpha\beta$ heterodimer was little affected by prolonged periods of separation in EDTA. Thus, irreversible inactivation is not due to instability of the separated subunits *per se*, but requires the presence of 2 M LiCl as an inducing (possibly denaturing) agent.

Reconstitution of RF_1 activities

The reconstitution of ATP synthesis and hydrolysis in LiCl-treated chromatophores by the $\alpha\beta$ heterodimer was investigated. Reconstitution of ATPase activity increased linearly with addition of $\alpha\beta$ heterodimer (Fig. 5 (△)), with saturation at 0.15 μg $\alpha\beta$ (μg BChl)$^{-1}$. In contrast, reconstitution of photophosphorylation required a threshold concentration of $\alpha\beta$ heterodimer (approx. 0.07 μg $\alpha\beta$ (μg BChl)$^{-1}$), below

which no reconstitution (net ATP synthesis) was apparent (Fig. 5 (○)). Saturation of ATP synthesis was then attained following the addition of a further 0.07 μg $\alpha\beta$ (μg BChl)$^{-1}$. In spite of this threshold, maximal ATP synthesis was restored by the same amount of heterodimer as was maximal ATP hydrolysis viz. 0.15 μg $\alpha\beta$ (μg BChl)$^{-1}$. This threshold value was not detected over the concentration range of $\alpha\beta$ tested previously [12].

We know, from the reconstitution of ATPase activity (Fig. 5 (△)), that even below the threshold value for photophosphorylation, protein is binding to the stripped chromatophores. The difference in sensitivity of the two processes, thus, does not reflect differences in total numbers of RF_1-F_0 complexes reassembled. More likely is the explanation that ATP hydrolysis can take place on any intact RF_1 (and thus parallels protein binding) while synthesis requires, in addition, a reasonably intact chromatophore to avoid proton leakage (uncoupling). Thus a large number of RF_1 molecules on any single chromatophore need to be assembled before any can synthesise ATP, and a threshold value is observed. Other explanations, however, cannot be ruled out.

Another feature observed during the reconstitution of ATP synthesis with purified $\alpha\beta$, was its inability to restore synthetic capacity to the level observed in untreated chromatophores. In the experiment of Fig. 5 (○), chromatophores reconstituted with pure $\alpha\beta$ reached activities of almost 4.0 μmol min^{-1} (mg BChl)$^{-1}$ as compared to a value of 10.5 μmol min^{-1} (mg BChl)$^{-1}$ obtained with control, untreated membranes. Higher levels of reconstitution – up to 75% of control values – were observed when a crude LiCl extract of chromatophores was used (Fig. 5 (◇))

It appeared, therefore, that some factor which promotes phosphorylation, in addition to $\alpha\beta$, was present in the LiCl extract. That this was indeed the case is shown in Fig. 5 (●) where a constant, threshold amount of crude extract (in which $\alpha\beta$ had been largely inactivated by prolonged exposure to LiCl) was added to stripped chromatophores, together with increasing amounts of pure $\alpha\beta$. The 'factor' in the LiCl extract complemented the purified heterodimer and allowed restoration of photophosphorylation up to 75% of control values. This behaviour is reminiscent of that observed previously by Husain et al. [22], who attributed such enhanced reconstitution to the presence of the γ subunit of RF_1 in the extract.

Both α and β subunits bind to LiCl-treated chromatophores

Addition of $\alpha\beta$ heterodimer to LiCl-treated chromatophores restored their ATPase activity (see above). Affinity of the chromatophores is high; titration was virtually linear until saturation at 0.15 μg $\alpha\beta$ (μg BChl)$^{-1}$. However, a requirement for $(\alpha+\beta)$ – as demonstrated above – does not necessarily imply that both subunits occupy a membrane binding site after reconstitution. It has been suggested, for example, that the α subunit might stabilise and/or guide the insertion of the β subunit into its binding site, without itself binding, in a 'chaperonin-like' mode of action [23].

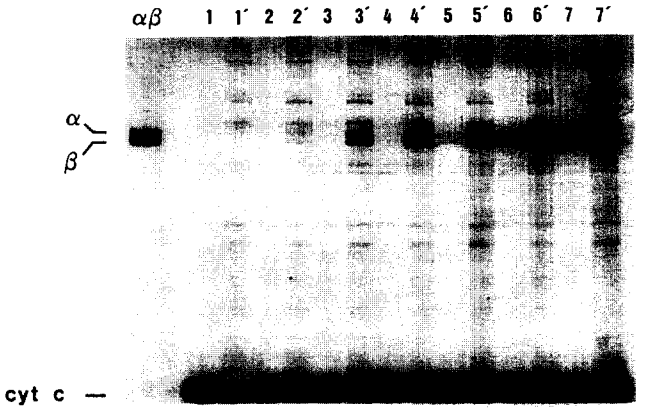

Fig. 6. Both the α and β subunits of the $\alpha\beta$ heterodimer bind to LiCl-treated chromatophores. Untreated (tracks 1′ to 7′) and LiCl-treated chromatophores (tracks 1 to 7) – equivalent to 60 μg BChl – were incubated with increasing amounts of the purified $\alpha\beta$ heterodimer, as in a reconstitution assay (see Methods). 0.5 mg ml^{-1} cytochrome c – which was found not to interfere with reconstitution – was also present, to prevent non-specific binding. Heterodimer equivalent to 0% (1,1′); 33% (2,2′); 67% (3,3′); 100% (4,4′); 133% (5,5′); 200% (6,6′) and 267% (7,7′) of the quantity required for saturation was used. After incubation, soluble and membrane bound components were separated by centrifugation (for 10 min at 80,000 rpm using a Beckman Airfuge) and 10 μl aliquots of the supernatant were analysed by SDS-PAGE, followed by silver staining [44].

Direct binding studies show this not to be the case. In the experiment of Fig. 6, LiCl-treated chromatophores were titrated with $\alpha\beta$ heterodimer at levels between 33% and 270% of saturation. After sedimentation of the membranes, unbound (soluble) protein was assessed by SDS-PAGE.

Lanes 1 and 1′ (Fig. 6) represent the supernatant after sedimentation of LiCl-treated and untreated chromatophores, respectively. Small amounts of some peptides were observed (as well as cytochrome c, added to minimise non-specific protein binding) but nothing was observed at the position of the α and β subunits of RF_1. With untreated chromatophores (lanes 2′,3′,....7′), even low levels of added $\alpha\beta$ heterodimer were detected in this supernatant, indicating that non-specific binding of $\alpha\beta$ by chromatophore membranes was negligible under the conditions used. Titration of LiCl-treated chromatophores, in contrast, showed binding of both α and β subunits until saturation was reached (lane 4), after which both subunits were detected in the supernatant. It is clearly not only the β subunit that becomes membrane bound, as would be predicted from the 'chaperonin' model. It was con-

cluded that both subunits of the $\alpha\beta$ heterodimer are bound to the membrane during reconstitution.

To investigate further the possibility that β subunit binds to LiCl-treated membranes, such membranes were incubated with sufficient $\alpha\beta$ to restore 30% maximal levels of ATPase activity. The inclusion of up to a 60-fold molar excess of β subunit with this amount of $\alpha\beta$, resulted only in a small decline in the resulting rate of reconstituted activity (Table II). In other words (a) the presence of α did not stimulate reconstitutive activity of isolated β subunit, as suggested in [23], and (b) the β subunit did not compete (with significant affinity) for the $\alpha\beta$ binding site on the stripped membranes. Thus, there is no indication that purified β subunit alone can rebind to LiCl treated chromatophores in our experiments.

TABLE II

Effects of $\alpha\beta$ heterodimer and β subunit on the reconstituted ATPase activity of LiCl-treated chromatophores

Reconstitution of LiCl-treated chromatophores with the indicated amounts of $\alpha\beta$ heterodimer was carried out as in Methods, except that, where indicated, the purified β subunit was also included in the reconstitution medium in the amounts shown. The resultant ATPase activity was determined as in Materials and Methods. % reconstitution is expressed relative to the value observed using 10 μg BChl and 1 μg (non saturating levels of) $\alpha\beta$ alone in the reconstitution.

LiCl-treated chromatophores plus		ATPase activity (μmol min^{-1} (mg BChl)$^{-1}$)	% Reconstitution
μg $\alpha\beta$	μg β		
0	0	0.19	0
0	15	0.19	0
1	0	2.07	100
1	10	1.49	69
1	20	1.57	73
1	30	1.63	77

TABLE III

Tightly bound ATP and ADP associated with coupled and LiCl-treated chromatophores

Chromatophores, and the $\alpha\beta$ heterodimer were prepared and freed from ambient nucleotides as described in Materials and Methods. Nucleotides were released using perchloric acid and assayed using luciferase as previously [12]. Values are given as mean ± S.D. ($n = 6$).

Preparation	Nucleotide tightly bound (mmol (mol BChl)$^{-1}$)	
	ATP	ADP
Untreated chromatophores	1.76 ± 0.03	1.38 ± 0.09
LiCl-washed chromatophores	0.91 ± 0.03	1.44 ± 0.06
$\alpha\beta$ heterodimer	< 0.01	1.12 ± 0.11 [a]

[a] mol nucleotide per mol heterodimer

Tightly bound nucleotide associated with chromatophores

Native F_1 from all species contains 2 mol ATP 'tightly bound' per mol F_1 at so called 'non-catalytic' sites [24]. This ATP content is very stable in membrane preparations (although varying amounts may be lost from isolated soluble F_1), and represents the only such ATP bound in coupled membranes; it can thus be used to quantitate the F_1 content of coupled membranes [25]. Many F_1 preparations also contain ADP trapped at a catalytic site [24].

Table III shows the levels of tightly bound ATP and ADP associated with washed, coupled chromatophores. Assuming an ATP/RF_1 ratio of 2, we can estimate that the F_1 content of these membranes is approx. 0.9 mmol RF_1 (mol BChl)$^{-1}$. This value is rather lower than that reported by Harris and Baltscheffsky [26]. This is probably due to the lower light intensities at which our *R. rubrum* cultures were grown, leading to an increase in BChl relative to other membrane components. Table III also shows that LiCl-treated chromatophores, depleted of $\alpha\beta$ subunits, still contain about half the initial levels of ATP i.e. about 1 mol ATP per mol of original RF_1. This cannot reflect a partial (e.g. 50%) removal of entire RF_1 molecules, since the resulting membranes have < 5% of the ATPase activity of the original membranes. Rather, this indicates that each RF_1 molecule partially dissociates on LiCl treatment, leaving some α and β subunit (on which the nucleotide binding sites reside) attached to the membrane.

Finally, Table III shows that, as determined previously [12], the $\alpha\beta$ heterodimer contains one tight (non-exchangeable) nucleotide binding site which is stable to size-exclusion chromatography. This site is occupied by ADP in our preparations. It is shown below that a catalytic nucleotide binding site is also present on the heterodimer.

ATPase activity of the purified $\alpha\beta$ heterodimer

We have shown previously that the purified $\alpha\beta$ heterodimer has a significant Mg^{2+}-dependent ATPase activity, of 0.11 μmol min^{-1} (mg $\alpha\beta$)$^{-1}$ [12]. This is 8-fold higher than the MgATPase activity of purified RF_1 (which shows a strong preference for the substrate CaATP) or of the β subunit of RF_1 [14]. Contamination by these species can thus be ruled out as a source of activity.

Further properties of the ATPase activity of $\alpha\beta$ are shown in Fig. 7 and Table IV, in comparison to those of the holoenzyme, RF_1. Several points emerge. (1) The $\alpha\beta$ heterodimer showed little discrimination between CaATP and MgATP (CaATPase/ MgATPase $\approx$ 2), whereas RF_1 shows strong discrimination (CaATPase/MgATPase $\approx$ 160). (2) The K_m values for MgATP for both the heterodimer and RF_1 – at 130 μM and 100 μM respectively – were identical within

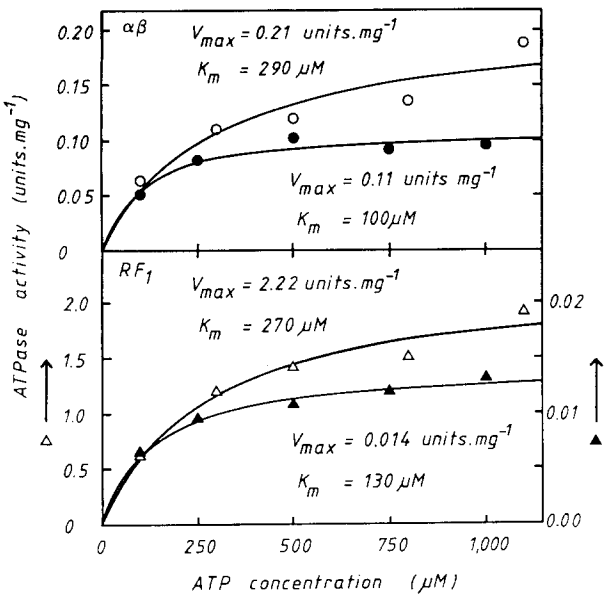

Fig. 7. Substrate [ATP] dependence of ATPase activity of $\alpha\beta$ heterodimer and RF_1. Both Ca^{2+}-dependent (open symbols) and Mg^{2+}-dependent (closed symbols) activities were measured, using [γ-^{32}P]ATP (varied as indicated) and excess (5 mM) Me^{2+}. Upper panel, ATPase activity of heterodimer. Lower panel, ATPase activity of RF_1. Estimates of V_{max} and K_m were obtained by non-linear regression analysis, and are shown by the corresponding curves (e.g. upper values refer to upper curves). For experimental details, see Methods.

experimental error. The K_m values for CaATP for both species, while some 3 times greater, show a similar identity. (Note that experimental error on the measured K_m(MgATP) values is relatively large, as substrate concentrations could be varied only above 100 μM. The heterodimer showed significant dissociation at lower Me^{2+}-ATP concentrations). (3) Agents that selectively stimulate MgATPase activity of RF_1 (sulphite, octyl glucoside, ethanol) hardly (if at all) stimulated the MgATPase activity of $\alpha\beta$. (4) Azide, which inhibits multisite catalysis by F_1 from various species [27–29] strongly inhibited both the MgATPase and CaATPase activity of RF_1. It had little effect on $\alpha\beta$ activity, as would be expected if only one catalytic site was present per dimer. (5) Overall, the pattern of sensitivity of the $\alpha\beta$ complex to inhibitors/activators was very different to that of holo-F_1, confirming that contamination with RF_1 cannot explain the observed activities.

The inhibition pattern observed for $\alpha\beta$ activity was similar to that reported for isolated β [14], whose activity is an order of magnitude lower. Indeed, we consider it likely that the bulk of ATPase activity observed in previous 'β' preparations was due to contaminating $\alpha\beta$. Recent preparations of β, from which all traces of $\alpha\beta$ had been removed (having no detectable reconstitutive activity), show that it may, nonetheless, have a low intrinsic (Mg^{2+})ATPase activity. For example, the purified β subunit from Fig. 1 (track 6 of SDS-polyacrylamide gel) had an activity of 0.6 ± 0.2 nmol min^{-1} (mg β)$^{-1}$. This level of activity is similar to that recorded for the β subunit of *E.coli* [9] and CF_1 [10].

TABLE IV

Effect of activators and inhibitors on the ATPase activity of RF_1 and $\alpha\beta$ heterodimer

ATPase assays were performed as in Fig. 7, at concentrations of ATP and divalent metal cation of 200 μM and 5 mM, respectively, with additions as indicated. Values (%) are expressed relative to control values (no additions). These were, for the Ca^{2+}- and Mg^{2+}-dependent activities respectively, 85 and 90 nmol min^{-1} (mg protein)$^{-1}$ for $\alpha\beta$, and 1420 and 10 nmol min^{-1} (mg protein)$^{-1}$ for RF_1. Each value is the mean of two determinations.

Additions	Ca^{2+}-ATPase		Mg^{2+}-ATPase	
	$\alpha\beta$	RF_1	$\alpha\beta$	RF_1
None	100	100	100	100
Na_2SO_3 (10 mM)	116	289	135	4850
Octyl glucoside (17.5 mM)	9	29	49	3110
Ethanol (10% v/v)	25	13	81	176
NaN_3 (2 mM)	103	16	90	7
EDTA (10 mM)	5	1	–	–

Discussion

An $\alpha\beta$ heterodimer from the R. rubrum coupling ATPase

An $\alpha\beta$ heterodimer (free of other RF_1 subunits) can be isolated from LiCl extracts of *R. rubrum* chromatophores. This complex is stable to a variety of chromatographic procedures, and to precipitation with ammonium sulphate. It contains a single non-exchangeable nucleotide (as ADP) per dimer (Table III) and a catalytic ATP binding site (Fig. 7). The dimer dissociates in 2 M LiCl (above) or excess EDTA [12], but will reassociate on removal of LiCl (Fig. 3) or on addition of excess Mg^{2+}, respectively. However, prolonged exposure to LiCl leads to irreversible changes in structure of either the α or β subunit, which prevents reassociation (Fig. 3) and is accompanied by loss of activity (Fig. 4).

Since $\alpha\beta$ association occurs specifically and spontaneously in the presence of MgATP [12], yielding a dimer with a non-exchangeable nucleotide binding site, it seems likely that the association represents the functional association of these subunits in holo F_1. This is borne out by the requirement for stoichiometric amounts of the $\alpha\beta$ dimer in reconstituting RF_1 (see below) and by the significant ATPase activity of the preparation (Fig. 7) with K_m values similar to RF_1.

In fact, the K_m values for both Mg^{2+}- and Ca^{2+}-dependent ATP hydrolysis by the heterodimer are comparable to those of RF_1 over the region 100–1,000 μM ATP (in the presence of excess Me^{2+}) i.e. to the

highest of the 3 K_m values of this cooperative enzyme [30]. Similarly, turnover rates are as much as 3 orders of magnitude faster than those of unisite catalysis by F_1 [2]. Nonetheless, the $\alpha\beta$ heterodimer turns over non-cooperatively (which is consistent with the presence of a single active site) as evidenced by the lack of azide inhibition (Table IV). Thus, to create the higher affinity nucleotide binding site ($K_d < 10^{-10}$M) for unisite catalysis ($k_{cat} \approx 10^{-3}$ s^{-1}) on holo-F_1, constraints must be placed on the $\alpha\beta$ heterodimer during its assembly into F_1. It is likely that interaction with the smaller subunits induces such changes.

Sulphite ions have been found to promote the MgATPase activity of CF_1, by reducing the inhibitory effect of free Mg^{2+} [31]. This effect of sulphite has also been demonstrated on RF_1 ([32] and Table IV). Thus, the relatively high, sulphite-insensitive MgATPase activity of the heterodimer (Table IV) might indicate that inhibition by Mg^{2+} is, like inhibition by azide, a property only of the cooperative mode of turnover. Azide and sulphite ions have also been shown to have little effect on the ATPase activity of an $\alpha_3\beta_3$ complex isolated from the thermophilic baterium PS3 [6]. However, recent observations by Ohta et al. [33] indicate that both the activity and azide-insensitivity of this complex may be due to its dissociation into an $\alpha\beta$ heterodimer.

Isolated β subunits from F_1 species bear the catalytic site for ATP synthesis, but can hydrolyse ATP only slowly (< 1 nmol min^{-1} (mg protein)$^{-1}$) ([9,10] and above). The $\alpha\beta$ dimer from RF_1 reported here has a k_{cat} 2–3 orders of magnitude higher than this (Fig. 7), being 110 nmol min^{-1} (mg protein)$^{-1}$ for MgATP. Other preparations containing $\alpha\beta$ subunits only, from TF_1 [8] or CF_1 [34] have similar activities, although in only the first case has the active species been shown to be a heterodimer. It seems, therefore, that creation of an $\alpha\beta$ interface (containing non-exchangeable nucleotide) promotes catalytic turnover by the β subunit. This is consistent with recent indications that occupation of non-exchangeable binding sites is required for catalysis by holo-CF_1 [35].

Quantitative aspects of RF_1 dissociation

Untreated chromatophores from *R. rubrum* catalyse ATP hydrolysis and ATP synthesis (at saturating light intensities) at approx. 10.5 μmol min^{-1}.mg $BChl^{-1}$. On the basis of their tightly bound ATP (Table III), we estimate an RF_1 content in these membranes of 0.9 mmol (mol BChl)$^{-1}$. LiCl treatment of chromatophores removes only half of this bound ATP (Table III) and we conclude, therefore, that some of the nucleotide binding sites on RF_1 remain on the membrane. Since all ATP hydrolysis is abolished, however, we conclude that no complete RF_1 ($\alpha_3\beta_3\gamma\delta\epsilon$) is present on the-treated membranes. Since tight nucleotide binding requires an $\alpha\beta$ interface (above), it appears that at least one $\alpha\beta$ complex remains bound at each RF_1 site on the stripped membranes. This may represent that single $\alpha\beta$ complex associated (asymmetrically) with the smaller F_1 subunits, as seen in electron micrographs [36]. The small subunits are believed to mediate F_1 interaction with the membrane sector, F_0 [37,38].

From the data of Table III, it is not possible to decide whether the residual complex, derived by LiCl treatment of membrane bound RF_1, contains one or two $\alpha\beta$ pairs. Addition of $\alpha\beta$ to stripped membranes, in reconstitution experiments, shows that 0.15 μg $\alpha\beta$ (μg BChl)$^{-1}$ (corresponding to 1.4 mmol $\alpha\beta$ (mol BChl)$^{-1}$) can bind functionally to stripped membranes (Fig. 5). This is equivalent to 1.6 mol $\alpha\beta$ per mol original RF_1 at saturation. Since reconstitution typically restores only 75% of the activity of untreated membranes – presumably because some RF_1 sites are irreversibly damaged – this value is likely to represent an underestimate of potential $\alpha\beta$ binding sites. Hence we deduce that 2 mol $\alpha\beta$ heterodimer are required to reassemble each RF_1 molecule. In other words, LiCl treatment probably removes 2α and 2β subunits from each RF_1 molecule, leaving a single $\alpha\beta$ pair attached to the smaller subunits/F_0 complex. This $\alpha\beta$ pair contains a single, non-exchangeable ATP binding site. Table III indicates that it also contains an ADP molecule, presumably trapped at a catalytic site, as has been reported for CF_1 [39,40]. The release of RF_1 subunits from chromatophores by LiCl treatment, the formation of $\alpha\beta$ heterodimers and the reconstitution of stripped chromatophores, are illustrated schematically in the model of Fig. 8. An additional feature, highlighted by this model, is the non-equivalence of the $\alpha\beta$ and $\alpha\beta'$ interfaces in holo-RF_1.

Relationship with previous preparations

A series of papers have chronicled the preparation, reconstitutive activity and nucleotide binding properties of purified β subunit, from an LiCl extract of *R. rubrum* chromatophores [11,13,41,42]. The $\alpha\beta$ heterodimer was not reported in these papers, probably because the prolonged exposure to LiCl employed in the extraction led to its presence as only a minor component (Fig. 2). In support of this explanation, a recent LiCl extract from chloroplast thylakoids – whose isolation involved a relatively short exposure to LiCl (2.5 h) before $(NH_4)_2SO_4$ precipitation – has been shown to contain an $\alpha\beta$ complex (of unspecified stoichiometry) [34].

ATPase activity in a β subunit preparation was reported by Harris et al. [14]. The K_m and inhibitor sensitivities of this activity are similar to those reported here for the $\alpha\beta$ heterodimer (Fig. 7 and Table IV), although the V_{max} for MgATPase activity of the β

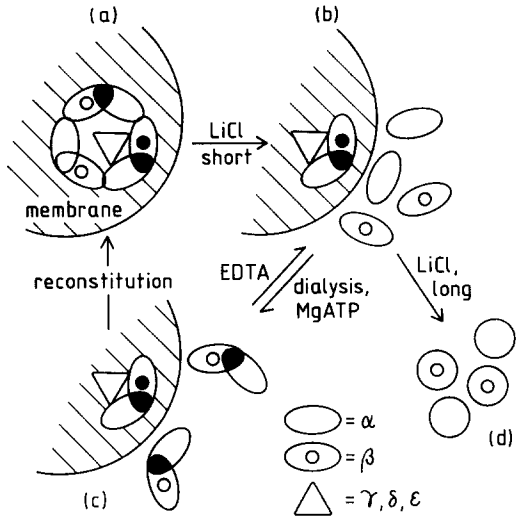

Fig. 8. Model for dissociation and reconstitution of membrane bound RF_1. (a) RF_1 ($\alpha_3\beta_3\gamma\delta\epsilon$) bound to chromatophore membrane, showing asymmetrical position of ($\gamma\delta\epsilon$) subunits. Two non-exchangeable nucleotide binding sites ($\alpha\beta$ interface) and one catalytic site (on β) are shown filled (black). (b) α and β subunits dissociate from the membrane in LiCl, leaving 1 heterodimer attached. (c) $\alpha\beta$ heterodimers (reconstitutively active) reform in MgATP, on removal of LiCl. Each has one non-exchangeable site filled. (d) Irreversible changes of α and/or β to inactive form by prolonged LiCl exposure (depicted by transition from ovals to circles). Note the non-equivalence of the $\alpha\beta$ and $\alpha\beta'$ interfaces in holo-RF_1.

preparation was about 13 nmol min^{-1} (mg protein)$^{-1}$ [14] as compared to 110 nmol min^{-1} (mg protein)$^{-1}$ determined here for the $\alpha\beta$ heterodimer. We have argued, therefore, that most of the ATPase activity observed in that β preparation was due to a small fraction of contaminating $\alpha\beta$ heterodimer [12]. As reported above, however, the purified β subunit appears to have a residual ATPase activity (approx. 0.6 nmol min^{-1} (mg protein)$^{-1}$), comparable with reported values for hydrolysis by purified β subunits from *E. coli* and CF_1 [9,10]. Clearly, association with the α subunit strongly stimulates this activity.

Reconstitution of ATP synthesis (and hydrolysis) in stripped chromatophores by β subunit preparations of *R. rubrum* have been extensively studied [11,13]. The experiments shown in Fig. 1 (above) and elsewhere [12], however, indicate that reconstitutive activity purifies with the $\alpha\beta$ heterodimer and away from the β subunit. Furthermore, we have shown (Fig. 6) that both the α and β subunits are bound to the membrane on reconstitution. We conclude, therefore, that an $\alpha\beta$ heterodimer, and not the β subunit alone, is needed for reconstitution.

The discrepancy between this conclusion and that of Gromet-Elhanan and co-workers [11,13] might be reconciled in terms of unknown variations in strain, growth conditions and/or chromatophore preparation procedures between the two laboratories. Thus, the chromatophores of Gromet-Elhanan and coworkers may relinquish only the β subunit on LiCl treatment and, hence, would require only β for reconstitution.

However, there is a large difference between the potency of our $\alpha\beta$ preparation and the β preparation of Gromet-Elhanan and coworkers. This may be expressed either as a specific reconstitutive activity, or as the amount of protein needed for maximal reconstitution. For the $\alpha\beta$ preparation used in Fig. 5, the specific reconstitutive activity was about 40 U (mg protein)$^{-1}$. After conversion to the same scale, activities of between 1.5 [43] and 6.5 U (mg protein)$^{-1}$ [21] for β preparations can be calculated from the data of Gromet-Elhanan and coworkers. Furthermore, using our preparation, the amount of $\alpha\beta$ needed for maximal reconstitution is 0.15 μg (μg BChl)$^{-1}$, i.e. 1.4 mmol $\alpha\beta$ (mol BChl)$^{-1}$ (Fig. 5); Gromet-Elhanan et al. report at least 6 μg (μg BChl)$^{-1}$, i.e. 118 mmol β (mol BChl)$^{-1}$ (assuming a molecular weight of 51 kDa for β [19]). Thus, using either criterion for activity, their results might be explained by the presence of small quantities of a highly active $\alpha\beta$ preparation. As little as 4% by weight (which in terms of detection by, for example, electrophoresis would mean only 2% α subunit) could account for their results.

Of possible significance is the observation by Avni et al. [23], that reconstitution of ATPase activity in LiCl-treated chromatophores by chloroplast β subunit preparations is facilitated by the addition of 'trace levels' of an $\alpha\beta$ mixture (derived from CF_1). However, the levels of $\alpha\beta$ added (Fig. 3 of ref. [23]) amounted to 0.1 μg (μg BChl)$^{-1}$ (equivalent to 1 mmol $\alpha\beta$ (mol BChl)$^{-1}$, if their $\alpha\beta$ preparation is assumed to be a heterodimer) which would be close to saturating in our system. Again, therefore, reconstitution by $\alpha\beta$ is a strong possibility.

Finally, the requirement for an $\alpha\beta$ heterodimer may explain earlier observations [14] that reconstitutive activity was diminished by incubation of 'β preparations' at 30°C in dilute solutions in the absence of ATP – conditions shown to promote dissociation of the $\alpha\beta$ complex [12]. It seems likely, therefore, that reconstitution of ATP hydrolysis and synthesis in LiCl-treated chromatophores by previous 'β preparations' was caused by traces of $\alpha\beta$, and not by the isolated β subunit.

Whatever the relationship between our current observations and previous work, the present data clearly establishes the $\alpha\beta$ heterodimer of *R. rubrum* as a highly-active reconstitutive element, which stoichiometrically restores the ability of LiCl treated chromatophores to synthesise and hydrolyse ATP. In addition, it is very stable (retaining catalytic activity after size exclusion HPLC, as described under Methods, and showing no loss of activity after 24 hours at 25°C) and relatively easy to prepare. Moreover, its high intrinsic ATPase activity (being several orders of magnitude

higher than that of F_1-ATPases under unisite conditions), its relative simplicity (possessing 1 non-exchangeable and 1 catalytic nucleotide binding site, as opposed to as many as 6 nucleotide binding sites in RF_1) and its freedom from the (cooperative) effects of other F_1 components, all suit it for future investigations into the fundamental properties of catalysis by F_1-ATPases.

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