Coupling Factor ATPase Complex of *Rhodospirillum rubrum*

PURIFICATION AND PROPERTIES OF A RECONSTITUTIVELY ACTIVE SINGLE SUBUNIT*

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The role of coupling factors in the mechanism of energy conversion has been extensively investigated in respiratory as well as in photosynthetic systems. Evidence concerning the action of these proteins has been gained through combined use of isolation and reconstitution techniques. The solubilized coupling factors from mitochondria (1), chloroplasts (2), Escherichia coli vesicles (3), and *Rhodospirillum rubrum* chromatophores (4) have ATPase activity, as well as the ability to activate ATP synthesis/or hydrolysis (or both) when recoupled to depleted membranes. All these coupling factors have a similar molecular weight and were reported to dissociate into five nonidentical subunits (5, 6).

The best defined soluble coupling factor from photosynthetic bacteria was purified from an acetone powder extract of *R. rubrum* chromatophores (7). It was very similar to the coupling factors isolated from other sources (5), showing a molecular weight of 350,000 and dissociating into five different subunits (6). Its recoupling activity was, however, tested on EDTA-depleted particles since no reconstitutable membrane could be obtained after acetone treatment.

In an alternative method (8), *R. rubrum* chromatophores lost 95% of their photophosphorylation activity but retained their capacity for energy storage when extracted with 2 mM LiCl. The photophosphorylation activity of the depleted particles could be reconstituted up to 90% by recombination with their LiCl extract, provided that the LiCl extraction was carried out in the presence of ATP (9).

This work describes the purification and characterization of the soluble factor present in the LiCl-ATP extract from *R. rubrum* chromatophores. It was found to contain only one subunit out of the five subunits demonstrated in the *R. rubrum* acetone coupling factor (6). This subunit, which by itself did not possess catalytic activity, was able to restore both ATP synthesis and hydrolysis upon recoupling to the LiCl-depleted membranes. Thus, the LiCl-ATP extraction provides in *R. rubrum* chromatophores a method for removing a single subunit and enables an investigation of its function in these chromatophores.

EXPERIMENTAL PROCEDURES

**Preparation of Chromatophores—Rhodospirillum rubrum** cells (strain S1) were grown as previously described (10). For large scale preparation, an aliquot of exponentially growing cells served as an inoculum for a fresh liquid medium. This procedure was repeated several times.

Chromatophores were isolated from harvested cells according to published procedures (9, 11, 12). Such preparations were designated as coupled chromatophores.

**LiCl Extraction—*R. rubrum*-depleted chromatophores** were prepared from coupled chromatophores by treatment with 2 mM LiCl in the presence of 4 mM ATP, as previously described (8, 9). Coupled chromatophores which underwent the same procedure, but without LiCl and ATP, were designated as control chromatophores. Both depleted and control chromatophores were suspended in 10 mM

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Tricine/NaOH, pH 7.3, glycerol (1:1, v/v), and 3 mM MgCl₂, to a concentration of 1 mg of bacteriochlorophyll per ml, and stored at −20°C.

The supernatant of the LiCl-depleted *R. rubrum* chromatophores, containing the crude LiCl factor, was precipitated with 60% saturated ammonium sulfate for storage and further purification. These purification steps are described under "Results." In order to measure activity in the crude extract, LiCl was removed from a sample of the crude LiCl factor by dialysis as described by Binder and Gromet-Elihan (9). This solution was designated dialyzed LiCl factor and was stored at −20°C. Fig. 1 summarizes the extraction procedure.

Recoupled chromatophores were obtained by incubating depleted chromatophores (10 μg of bacteriochlorophyll) with the required amount of LiCl factor as previously described (8, 9). Control chromatophores were incubated under the same reconstitution conditions.

**Preparation of Acetone Coupling Factor**—The coupling factor complex, containing all the five subunits, was extracted from an acetone powder of coupled *R. rubrum* chromatophores according to Melandri and Baccarini-Melandri (14), by a modification described by Gromet-Elihan (8). The coupling factor so obtained was designated acetone coupling factor and was stored at −20°C with no further purification. In a similar way, a four-subunit coupling factor complex was extracted from an acetone powder of LiCl-depleted *R. rubrum* chromatophores and was stored at −20°C.

**Analytical Methods**—Bacteriochlorophyll was determined using the absorption coefficient in vivo given by Clayton (15). Protein was measured according to the method of Lowry et al. (16). Photophosphorylation activity was assayed as outlined by Gromet-Elihan (8), but at 35°C and with 66 mM N-methyl phenazonium methosulfate (9). ATP formation was measured according to Avron (17). ATPase activity was determined according to the radiochemical method (18) as modified by Oren and Gromet-Elihan (19), employing [γ-32P]ATP as substrate. The assay was performed in the presence of 4 mM ATP, with either 2 mM MgCl₂ or 4 mM CaCl₂ (19). Trypsin activation was carried out according to Gepshtein and Carmeli (20). SDS-polycrylamide gel electrophoresis was carried out according to the procedure described by Weber and Osborn (21), except that the preparation of samples was performed by heating the protein mixture (with 0.1% SDS and 0.1% β-mercaptoethanol) at 90°C for 15 min. Samples were applied on a horizontal slab gel apparatus (14 × 15 cm), Yeda Scientific Instruments, Rehovot. Scanning of the gel pictures was carried out on a Gilford Instrument at A = 600 nm and the enzymatic activity of the ammonium sulfate precipitate.

**RESULTS**

**Purification of LiCl Factor**

About 400 g wet weight of *Rhodospirillum rubrum* packed cells gave 120 mg protein in the crude LiCl factor (see Fig. 1). This crude LiCl factor has been purified in the following steps, all carried out at 4°C.

**Step 1: Ammonium Sulfate Precipitation**—The LiCl factor was precipitated by adding solid ammonium sulfate to 60% saturation at 4°C. The precipitate was collected by a 10-min centrifugation at 10,000 × g and resuspended in a small volume of Buffer A to give a concentration of about 10 mg of protein per ml. The protein so obtained was designated AS-LiCl factor (see Fig. 1). A sample of this solution (0.5 ml) was desalted on a Sephadex G-25 column (20 × 1 cm), equilibrated with 60 ml of Buffer A at room temperature, for checking the enzymatic activity of the ammonium sulfate precipitate.

**Step 2: Sephadex G-200 Gel Filtration**—The protein solution from Step 1 was loaded on a Sephadex-200 Pharmacia Laboratories glass column (2.6 × 70 cm), with an internal support of siliconized glass beads in order to improve the flow rate (23). Fig. 2 shows a typical elution profile of such a column. The enzymatic activity of the LiCl factor is coincident with the second protein peak, eluted at about 1½ times the void volume of the column.

**Step 3: Rechromatography of Sephadex G-200**—The active fractions of three columns of Step 2 were precipitated in 60% ammonium sulfate and centrifuged, and the pellet was resuspended in a small volume of Buffer A as previously described. This protein solution was loaded again on a Sephadex G-200 column and eluted as described in Step 2. In the elution profile of the second Sephadex G-200 column (Fig. 3), the active LiCl factor is eluted parallel to the main protein peak.
It can be seen that the high molecular weight impurities are largely reduced in this step. The activity appears in a single symmetrical peak. The active fractions were pooled and stored at -20°C.

Table I summarizes typical yields and specific recoupling activities obtained by the purification steps. Step 1 (60% ammonium sulfate) gives a 7-fold purification over the dialyzed LiCl factor, which had a specific recoupling activity of 3.3. Thus, an overall purification of 31-fold was achieved, which is greater than the purification obtained with acetone powder extracts of either R. rubrum (7) or Rhodopseudomonas capsulata (24).

Fig. 4 illustrates the relative recoupling activities of the LiCl factor preparations at the different purification steps. The degree of reconstitution increases as a function of the amount of protein present in all the LiCl factor preparations. However, with the purest LiCl factor, the recoupling activity increases linearly with protein concentration up to 60% reconstitution (Fig. 4A), whereas with the LiCl factor of Step 1, it remains linear up to only 40% of reconstitution, and then drastically decreases (Fig. 4B). This decrease probably can be due to the presence of impurities or inhibitor in this LiCl factor preparation. With the original crude extract dialyzed LiCl factor (Fig. 4C), the curve is parallel from the beginning to the second decreased part observed in Fig. 4B. According to these curves, the value of 30% reconstitution was chosen for the definition of the recoupling unit in Table I, since up to this percentage all the different LiCl factor preparations show a linear correlation between amount of protein and reconstitution. Thus, one recoupling unit consists of 300 μg of protein from the original crude extract, 50 μg of protein from Step 1, and 10 μg of protein from Step 3 (Fig. 4). For 60% reconstitution, much larger differences would be recorded between the various purification steps: 250 μg of protein from Step 1 will be required (Fig. 4B) as compared to only 25 μg of protein from Step 3 (Fig. 4A). With the purified LiCl factor, complete reconstitution can be obtained (Fig. 4A).

**Subunit Structure of the Purified LiCl Factor**

The band pattern of the LiCl factor preparation on a SDS-gel as compared to that of acetone coupling factor preparations is illustrated in Fig. 5. The crude extract of R. rubrum acetone coupling factor, isolated from coupled chromatophores (Fig. 5A), gave a general pattern of five subunits, confirming the observations of Johansson and Baltscheffsky (6). The acetone coupling factor, prepared from LiCl-depleted R. rubrum chromatophores retained 2% of this activity. A, purified active LiCl factor, eluted from the second Sephadex G-200 column in Step 3; B, LiCl factor precipitated with 60% ammonium sulfate and desalted on Sephadex G-25 column (Step 1); C, dialyzed LiCl factor obtained as described under “Experimental Procedures” (see Fig. 1).
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rubrum acetone coupling factor (Fig. 5A). The other visible bands in the purified LiCl factor (Fig. 5C) do not migrate parallel to any of the identified subunits of the R. rubrum acetone coupling factor (Fig. 5A). Some remaining traces of material appear in the general area of the presumed β subunit, but they do not look like a definite single band. They seem, therefore, to be contaminations which accompanied the β-band.

These observations suggest that the enzymatic activity of the LiCl factor, when recoupled to depleted membranes is correlated with the presence of the β subunit.

Molecular Weight Determination of LiCl Factor

The molecular weight of the purified LiCl factor (obtained after Step 3, see Table I) was determined by two different methods: SDS-gel electrophoresis (Fig. 6) and equilibrium sedimentation in the analytical ultracentrifuge (Fig. 7). In the SDS-gel electrophoresis method (Fig. 6), known protein markers with \( M_r \) between 17,000 and 68,000 were used, including the four visible CF, subunits. From the electrophoretic mobilities of the purified LiCl factor a \( M_r = 55,000 \) is found for the β-band (Fig. 5C).

Sedimentation and diffusion coefficients of the same purified LiCl factor were obtained from ultracentrifugation runs (Fig. 7), as described under "Experimental Procedures." The sedimentation coefficient was found to be \( s_{20 \text{w}} = 4.39 \pm 3\% \) and the diffusion coefficient \( D_{20 \text{w}} = 7.55 \). The value of \( \bar{c} = 0.749 \text{ ml/g} \) for the partial specific volume was used (7). This value is in good agreement with the experimental value of \( \bar{c} = 0.737 \text{ ml/g} \), found for CF, (26). From these data, a value of 55,500 ± 6% was calculated according to the Svedberg equation.
ATP is essential for preservation of recoupling activity of the coupling factor. 0.610 mg of protein of crude LiCl factor was somewhat less sensitive to changes in ATP concentration than the purified LiCl factor. At 1 mM ATP, the purified LiCl factor was almost completely inactive (Fig. 8B), while the crude LiCl factor was able to reconstitute up to 27% of the photophosphorylation activity (Fig. 8A).

Extraction of coupled chromatophores with 2 mM LiCl in the presence of ATP results in 98% inhibition of photophosphorylation and ATPase activities (Table II). As has been reported earlier (8, 9), the complete loss of photophosphorylation activity was not accompanied by any change in the light-induced energy-linked reactions. The R. rubrum acetone coupling factor, containing all five subunits (6), was found to be a very active soluble ATPase (7). However, the purified β subunit had neither Mg2+- nor Ca2+-ATPase activity by itself, even after trypsin activation (Table II). Nevertheless, its recoupling to the LiCl-depleted chromatophores led to restoration of both ATPase and ATP synthesis activities to about the same extent. At a ratio of 90 µg of protein to 10 µg of bacteriochlorophyll, the β subunit restored 62% of the original ATPase activity and 71% of the original photophosphorylation activity (Table II). These data indicate that the purified β subunit is absolutely required for both ATP synthesis and hydrolysis by the chromatophores since its removal abolishes both activities, while its reattachment restores them.

The presence of a specific low affinity binding site for ATP in the soluble β subunit is indicated by the finding that ATP is absolutely necessary to preserve its enzymatic activity when reattached to LiCl-depleted membranes (Fig. 8B). ATP could not be replaced by either ADP or PPi, as reported by Binder and Gromet-Elhanan (9).

**DISCUSSION**

We have earlier reported that extraction of Rhodospirillum rubrum chromatophores by 2 mM LiCl (8) in the presence of ATP (9) yielded not only particles unable to photophosphorylate, but a supernatant containing a protein that restored this activity to the LiCl-depleted chromatophores. The present communication deals with characterization of both the active LiCl factor and the LiCl-depleted chromatophores. The active protein factor has been purified (Table I) and found to contain one polypeptide subunit with a molecular weight of 55,000 (Figs. 6 and 7), most likely the β subunit of the coupling factor of R. rubrum (Fig. 5C). This subunit has no ATPase activity by its own right either before or after purification, but its reattachment to the LiCl-depleted chromatophores leads to the complete restoration of their ATP-linked activities (Table II). This contrasts with the soluble protein, obtained by acetone extraction of coupled R. rubrum chromatophores (6), which is a normal five-subunit coupling factor, showing a Ca2+-dependent ATPase activity by itself (7) and able to recouple both EDTA and LiCl-depleted particles (1, 8).

The LiCl-depleted particles, from which the β subunit has been removed, lost all their ATP synthesis and hydrolysis activities (Table II), but retained the other four subunits of the coupling factor (Fig. 5D). These findings indicate that the LiCl-ATP treatment releases only the β subunit from R. rubrum chromatophores, leaving all the other four coupling factor subunits still attached to the LiCl-depleted membranes (Fig. 9).

The fact that the other four subunits remain attached to the depleted membranes explains a number of observations which are specific to the LiCl-depleted chromatophores.

1. Electron micrographs of negatively stained preparations

**Table II**

**Photophosphorylation and ATPase activity of Rhodospirillum rubrum chromatophores and soluble LiCl factor**

<table>
<thead>
<tr>
<th>Test system</th>
<th>ATP synthesis (µmol/mg of protein/h)</th>
<th>ATP hydrolysis (µmol/mg of bacteriochlorophyll/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coupled chromatophores</td>
<td>762 (100)</td>
<td>69 (100)</td>
</tr>
<tr>
<td>Depleted chromatophores</td>
<td>19 (2)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Soluble LiCl factor</td>
<td>19 (2)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Trypsin activated LiCl factor</td>
<td>0 *</td>
<td>0 *</td>
</tr>
<tr>
<td>Recoupled chromatophores</td>
<td>541 (71)</td>
<td>44 (62)</td>
</tr>
</tbody>
</table>

* ATPase activity of the soluble LiCl factor is given by micromoles of ATP/mg of protein/h.
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have not revealed any major morphological difference between coupled and LiCl-depleted R. rubrum chromatophores (30). On the other hand, removal of the five-subunit F1 from mitochondria (5) or CF2 from chloroplasts (31) resulted in loss of the protruding spherical knobs observed in electron micrographs, and these were shown to reappear after reconstitution (5,31).

2. Addition of the purified β subunit is enough to restore completely both ATP synthesis and hydrolysis activities to LiCl-depleted R. rubrum chromatophores (Table II, Fig. 4), whereas a preparation of the single β subunit from Escherichia coli (32) or the α + β subunits from chloroplasts (33) failed to bind to their respective EDTA-depleted particles. These particles, unlike the LiCl-depleted chromatophores, lost all five subunits of the coupling factor. Our results support the suggestion (33) that some of the other subunits (which remain attached to the LiCl-depleted chromatophores, see Fig. 9) are required for binding of the α or β (or both) subunits to fully depleted particles. Recent findings obtained in E. coli (34), mitochondria (35), and chloroplasts (36) indicate that either the γ or the δ subunits, or both, are necessary for attachment of the other subunits to the membrane.

3. Removal of the β subunit from R. rubrum chromatophores by LiCl treatment does not increase the leakiness of their membrane to protons, since the light-induced H+ uptake as well as the quenching of atebrin fluorescence are not affected (6,9). On the other hand, removal of the five-subunit coupling factor (CF2) from chloroplasts results in loss of proton uptake, and its recoupling leads to reconstitution of the light-induced pH changes (37). It is therefore concluded that the removed β subunit is not involved in proton translocation across the membrane. Hence, this process probably requires one or more of the other four coupling factor subunits which remain attached to the LiCl-depleted chromatophores (Fig. 9).

In light of the above findings it is obvious that the β subunit of R. rubrum coupling factor is functioning specifically in ATP-linked reactions and does not have any additional structural role in the membrane.

This work demonstrates for the first time a specific depletion of one subunit out of the whole coupling factor complex in a system that enables us to check its function in vivo.

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