H-ATP synthase couples a transmembrane proton flow to ATP synthesis, and F, is the water-soluble part of this enzyme (1). F, has ATPase activity, and its M, is 380,000–400,000. The subunit structure of F, is αβγδε, and the catalytic sites reside most likely on the β subunits. However, the isolated β subunit by itself shows only 0.1% of ATPase activity. The ATPase-active F, complex appears to be an important contribution for studies of F,.

The 55-kDa Polypeptide Released from Spinach Thylakoid Membranes with 1 M LiCl Is Not the β Subunit of Chloroplast F1*

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Masa H. Sato, Toru Hisabori, and Masasuke Yoshida
From the Department of Life Science, Faculty of Science, Tokyo Institute of Technology, Nagatsuta, Yokohama 227 and the Department of Biology, Yokohama City University, Seto, Yokohama 226, Japan

It was reported by Frasch et al. (Frasch, W. D., Green, J., Caguiat, J., and Mejia, A. (1989) J. Biol. Chem. 264, 5064–5069) that washing spinach thylakoid membranes with 1 M LiCl caused the release of the β subunit of chloroplast F1 (CF1) which, existing as 180-kDa complexes of β, retained considerable ATPase activity. We repeated these procedures and confirmed that a CF1 β-like 55-kDa polypeptide was a major constituent of the 1 M LiCl-washed extract. However, the extract contained another polypeptide of which the M, was 14,000, and these two polypeptides comprised a complex with approximate M, 550,000 that had the same mobility in native polyacrylamide gel electrophoresis as that of ribulose-1,5-bisphosphate carboxylase. Only very low ATPase activity, less than 1% of the reported value, was detected for the extract and the purified complex. Antibody against the β subunit of F, from a thermophilic bacterium PS3 showed a clear cross-reactivity with the CF1 β subunit but not with the 55-kDa polypeptide. Analysis of the N-terminal amino acid sequences of the 55- and 14-kDa polypeptides and the whole complex revealed that the complex was ribulose-1,5-bisphosphate carboxylase and that the 55- and 14-kDa polypeptides were its large and small subunits, respectively.

EXPERIMENTAL PROCEDURES

Preparation of the Washed Extract and Purification of the Complex—We repeated the procedures described by Frasch et al. (5) for the preparation of thylakoids from market spinach, extraction of proteins from thylakoid membranes by washing with a solution containing 1 M LiCl, and purification of the released protein by gel permeation chromatography. However, we used a Sephacryl S-400 column (2.2 × 90 cm) for the purification of the released protein instead of Bio-Gel P-150, which was no longer commercially available. In brief, thylakoids were prepared from leaves of market spinach in 50 mM Tricine-NaOH buffer (pH 7.3) containing 10 mM NaCl, 10 mM MgCl2, 3 mM NaN3, and three proteolysis inhibitors (5). All the Tricine-NaOH buffers (50 mM, pH 8.0) used after this step contained proteolysis inhibitors and 4 mM MgATP. Thylakoids were incubated at 4 °C in the dark for 1 h with stirring in Tricine buffer containing 0.25 M sucrose and then incubated at 4 °C for 30 min with stirring in Tricine buffer containing 0.25 M sucrose and 1 M LiCl. Membranes were removed by centrifugation, and the supernatant was concentrated again and applied on a Sephacryl S-400 column. The column was equilibrated and eluted with Tricine buffer containing 20% glycerol at a flow rate of 1 ml/min, and each 5-ml fraction was collected. Some of the chromatographies were carried out using Tricine buffer without 20% glycerol. Protein peak fractions, usually three fractions around the peak, were combined, concentrated, and used for analysis as a purified complex.

Polyacylamide Gel Electrophoresis and Western Immunoblotting—Proteins were analyzed by polyacrylamide gel electrophoresis either on 15% polyacrylamide gels in the presence of SDS as described by Laemmli (10) or on 5% polyacrylamide gels by the Laemmli system without SDS (native polyacrylamide gel electrophoresis). Kits for molecular weights of 140,000–70,000 and 16,950–5,210 (Sigma) were used as Mr standards in SDS polyacrylamide gel electrophoresis. Gels were stained by Coomassie Brilliant Blue R-250. Western immunoblotting was carried out as described in Ref. 11. The antibody used was rabbit polyclonal antibody against the β subunit of F1 from a thermophilic bacterium PS3 (TF1) (12).

Amino Acid Sequencing—Amino acid sequences were analyzed by
reaction mixtures for ATPase assays contained 50 mM Tricine-NaOH (pH 8.0), 5 mM ATP, 5 mM MgCl₂, and 40 mM octyl glucoside. The reaction mixture was preincubated for 3 min at 37 °C, and the reaction was initiated by addition of the enzyme. After 30 min of incubation at 37 °C, the reaction was terminated by the addition of 0.25 ml of 2% perchloric acid, and the amount of Pi produced was measured as described previously (15). One unit of activity is defined as the activity liberating 1 pmol of P₁/min. Protein concentrations were assayed by the Bradford procedure (16). Sninach CF₁ was purified as described in Ref. 17. Ten microliters of each fraction of chromatography shown in Fig. 2 were loaded on a gel.

RESULTS

Sephacryl S-400 Chromatography of the 1 M LiCl-washed Extract—In order to obtain the β₃ complex we carried out the procedures reported by Frasch et al. (5). We confirmed that washing thylakoid membranes with 1 M LiCl caused the specific release of a protein complex which contained a polypeptide with M₉₅, 55,000 (Fig. 1a, left two lanes). As shown in Fig. 2, when the washed extract was applied to Sephacryl S-400 chromatography, most of the protein in the washed extract was eluted as a single peak. The constituents of the fractions around the protein peak were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1a). It was evident that the protein complex eluted at around fraction 39 contained a polypeptide of which the M₉ was estimated to be 55,000. The electroforetic mobility of this 55-kDa polypeptide in SDS-polyacrylamide gel was the same as or very similar to that of the CF₁ β subunit. As shown already by Frasch et al. in Fig. 2 of their paper (5), another two polypeptides, one below the band of the CF₁ ε subunit and the other at almost the same position of the CF₁ γ subunit, were also seen in the gels (Fig. 1a). These results are essentially the same as those reported by Frasch et al. (5).

Native Polyacrylamide Gel Electrophoresis of the Washed Extract and Fractions of Chromatography—It is evident from Fig. 1b that a protein complex moving slower than CF₁ in the native polyacrylamide gel electrophoresis is almost the only constituent of the washed extract (left two lanes). Consequently, it is also the case for the eluted fractions from Sephacryl S-400 chromatography (right lanes). It is noteworthy that the electrophoretic mobility of the protein complex contained in the washed extract and eluted fractions is the same as that of ribulose-P₁ carboxylase which is contained in our preparation of CF₁ (Fig. 1b, left two lanes). Since the report by Frasch et al. (5) did not include results of native polyacrylamide gel electrophoresis of their complex, we cannot compare our results with theirs.

Gel Permeation HPLC of the Purified Complex—The molecular weight of the protein complex was estimated by gel permeation HPLC using a G4000SW column. As shown in Fig. 2 (inset), the elution of the complex was earlier than that of CF₁ and the M₉ of the complex was estimated to be 550,000. This value is much larger than the reported value, 180,000 (5).

Subunits of the Protein Complex—As described above, there is a protein band running faster than the CF₁ ε subunit in the SDS-polyacrylamide gel electrophoresis. The molecular weight of this small polypeptide was estimated to be 14,000 from another SDS-polyacrylamide gel electrophoresis with M₉ standards. Judging from the relative staining intensity of this 14-kDa band to the 55-kDa band and from the fact that the 14-kDa polypeptide was always copurified with the 55-kDa polypeptide, we concluded that the 14-kDa polypeptide, as well as the 55-kDa polypeptide, was also a subunit of the protein complex. The faint band running at almost the same mobility as the CF₁ γ subunit was eluted only at the latter part of the protein peak of Sephacryl S-400 chromatography (Fig. 1a) and may not be an intrinsic subunit of the complex.

Western Immunoblotting—Western immunoblotting of the...
were separated on 12.5% SDS-polyacrylamide gels, and protein bands were stained with Coomassie dye, and the right two lanes (2, a and b) show the immunoreaction with anti-TF\(_1\)\(\beta\) antibody. Approximately 10 pg of proteins were blotted to polyvinylidene difluoride membranes. The blotted membranes were incubated with the blocking medium and then with the solution of the anti-TF\(_1\)\(\beta\) antibody. After washing the membranes, the second antibody, goat anti-rabbit IgG antibody conjugated to alkaline phosphatase, was allowed to bind the primary antibodies on the membranes. Localization of bound antibodies were visualized by the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Identical amino acids between the two sequences designated by asterisks are also members of the conserved amino acids for almost all ribulose-P\(_2\) carboxylase small subunits from various sources (22). Therefore, we concluded that the 14-kDa polypeptide was the ribulose-P\(_2\) carboxylase small subunit. Disagreement at positions 6, 7, 8, 9, 11, and 12 can be explained by the possible presence of multiple genes of ribulose-P\(_2\) carboxylase small subunits in spinach chromosomes as established in other plants (23, 24) or by the difference of strains of market spinach.

**DISCUSSION**

We tried to isolate the \(\beta_3\) complex from spinach thylakoid membranes by the procedures reported by Frasch et al. (5) and confirmed that the protein complex containing a CF\(_1\) \(\beta\)-like polypeptide was a main constituent of the 1 M LiCl-
washed extract. However, the properties of the complex we purified are significantly different from those reported by Frasch et al. as follows. Our complex has a $M_e$ of 550,000 rather than 180,000, comprises two kinds of polypeptides of $M_e$ 55,000 and 14,000, has extremely low ATPase activity, if any, and shows the same mobility as that of ribulose-P$_2$ carboxylase in native polyacrylamide gel electrophoresis. Antibody against the TF$_1$ $\beta$ subunit reacted clearly with the $\beta$ subunit of authentic CF$_1$ but not with any polypeptides contained in our complex. Analysis of the N-terminal amino acid sequences of the complex and the 55- and 14-kDa polypeptides proved that the complex is ribulose-P$_2$ carboxylase and that the 55- and 14-kDa polypeptides are the large and small subunits of ribulose-P$_2$ carboxylase, respectively. Ribulose-P$_2$ carboxylase is composed of eight large and eight small subunits. The molecular weights of spinach ribulose-P$_2$ carboxylase and its large and small subunits are 532,000, 52,600, and 13,900, respectively (19, 25), showing good agreement with the values of the complex we obtained. It is known that spinach ribulose-P$_2$ carboxylase is a major contaminant of the CF$_1$ preparation (27), and the ribulose-P$_2$ carboxylase large subunit and CF$_1$ $\beta$ subunit are practically indistinguishable from each other in SDS-polyacrylamide gel electrophoresis. Thus, it is obvious that we obtained ribulose-P$_2$ carboxylase instead of trimers of the CF$_1$ $\beta$ subunit, even though we followed the procedures described in Ref. 5. We could not confirm the presence of the CF$_1$ $\beta$ subunit in our preparation by Western immunoblotting or by N-terminal analysis. These results are reproducible in our laboratory since we repeated the experiments five times and obtained essentially the same results each time. The reason for the discrepancy between Frasch’s results and ours is not known. However the following points should be considered.

Patterns of SDS-polyacrylamide gel electrophoresis of the 1 M LiCl-washed extract and fractions of Sephacryl S-400 column chromatography (Fig. 1a) were very similar to those presented in Fig. 2, A and B, of Ref. 5, indicating we obtained a very similar preparation to that which Frasch et al. (5) obtained. Their gels clearly show the presence of a protein band at the position below the CF$_1$ $\epsilon$ subunit, and they estimated the $M_e$ of this band to be 9,000. We measured the electrophoretic mobility of the band in Fig. 2, A and B, of their report and re-estimated its $M_e$ using the $\beta$ (55,000), $\gamma$ (37,000), $\delta$ (20,000), and $\epsilon$ (15,000) subunits of CF$_1$ in the same figures as $M_e$ standards. The values thus obtained from Fig. 2, A and B, were 13,800 and 13,500, respectively. Therefore, the band found in their gels just below the position of the CF$_1$ $\epsilon$ subunit, which the major 55-kDa polypeptide always accompanied, is probably the same polypeptide as the 14-kDa polypeptide of our preparation, that is the small subunit of ribulose-P$_2$ carboxylase.

The $M_e$ of our complex was estimated to be about 550,000 using a HPLC gel permeation column, G4000SWx10, by which large proteins and protein complexes up to $M_e$ 700,000 can be analyzed (28). Frasch et al. (5) estimated the $M_e$ of their complex to be 180,000 from Bio-Gel P-150 chromatography. However, in general, proteins having a $M_e$ of more than 150,000 are eluted out at void volume from a Bio Gel P-150 column and hence cannot be analyzed (29). Therefore, their value could be an underestimation, and the real $M_e$ of the complex may be larger than 180,000.

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