Molecular Weight and Subunit Stoichiometry of the Chloroplast Coupling Factor 1 from Chlamydomonas reinhardtii

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The molecular weight of the Chlamydomonas reinhardtii coupling factor 1 (CF1) is 4.2 x 10^6 as determined by gel exclusion chromatography and sedimentation equilibrium. In addition, a measured sedimentation coefficient of 12.9 S results in a calculated molecular weight of 3.9 x 10^6. These molecular weight estimates are too high to support an αβγε-type subunit stoichiometry and are suggestive of an αβγα-type enzyme. The subunit stoichiometry of the C. reinhardtii CF1 was determined from the distribution of label into the subunits of uniformly labeled CF1. An αβγε ratio of 2.9:2.9:1:1 was obtained.

The energy transducing membranes of bacteria, mitochondria, and chloroplasts contain a proton-translocating ATP synthetase. This ATP synthetase complex is structurally similar in all three membrane systems and is comprised of an extrinsic membrane protein, called coupling factor 1 or F1, and an intrinsic membrane protein, Fo (1-4). The F0 consists of three to five polypeptide chains (1, 5) which form a proton channel across the membrane (1, 6), while the F1 sector demonstrates ATPase activity and is thought to contain the catalytic site(s) for ATP synthesis during oxidative and photophosphorylation (7, 8). The F1 protein is easily removed from the membrane in soluble form and has been purified to homogeneity. Purified F1 proteins from a wide variety of sources, including Escherichia coli, the thermophilic bacterium FS3, yeast and mammalian mitochondria, and algal and plant chloroplasts, contain five different subunits designated α, β, γ, δ, and ε in order of decreasing molecular weight (1, 4, 9-11, 45). The α and/or β subunits probably contain the active site for ATP synthesis, γ is thought to be involved in proton flow through the membrane, δ is required for the binding of F0 to F1, and ε is suggested to play both a structural role in the binding of F1 to F0 and a possible regulatory role in the expression of ATPase activity by the F1 complex (1, 9). Relevant to the determination of the function of these various subunits is the elucidation of the structure of the complex. The determination of the subunit stoichiometry is basic to the understanding of structure. However, this stoichiometry has not yet been rigorously determined for all of the above mentioned classes of coupling factors (2, 12).

The bacterial and yeast mitochondrial coupling factors are found to consist of αβγδε-2 complexes. This stoichiometry is supported by several lines of experimental evidence, the most convincing of which involves the quantitation of subunits/complex using preparations of F1 isolated from cells uniformly labeled with 14C precursors (5, 13-15). In the case of the mammalian mitochondrial enzyme, suggestions for αβγδε complexes as well as for αβγδε-2 complexes have been made (12, 16). The former stoichiometry is based on the titration of specific tyrosine residues by radioactively labeled chemical modifiers (17), densitometry of stained SDS-polyacrylamide gels (18), and the number of nucleotide binding sites on the enzyme (19). The αβγδε-2 stoichiometry is supported by the results of Senior (20), who quantitated the number of subunits α, γ, and ε/b by titration of sulphydryl groups on the enzyme with radioactive N-ethylmaleimide. However, estimates of the molecular weight of the mammalian mitochondrial F1 complex range from 360,000 to 384,000 (4, 12) and, therefore, based on the calculated molecular weights of the subunits, favour an αβγδε stoichiometry.

In the case of the plant chloroplast coupling factor 1, however, the widely accepted subunit stoichiometry is αβγδε-2 (2). Baird and Hammes (21) proposed a minimum subunit stoichiometry of αβγδε/CF1 complex based on the results of subunit cross-linking studies. Other laboratories later suggested an αβγδε-2 stoichiometry on the bases of the relative staining intensity of the subunits of spinach CF1 separated by SDS-polyacrylamide gel electrophoresis (22) and the relative incorporation of 14C into the subunits of CF1 isolated from pea plants grown under a [14C]CO2 atmosphere (23). In addition, the molecular weight of CF1, as measured by analytical centrifugation (24) and small angle x-ray scattering (25) ranges from 325,000 to 335,000, which does not allow for an αβγδε stoichiometry. Although a higher molecular weight of 417,000 was determined by Yoshida and co-workers (26) by analytical centrifugation in 10% methanol, it was later suggested by Paradies and Kagawa (27) that this number might be an overestimate since the thermodynamic preferential interaction parameters were not taken into consideration. The resulting lower estimate of the molecular weight might still exclude an αβγδε-2 stoichiometry.

We present evidence here supporting an αβγε stoichiometry for a four-subunit chloroplast coupling factor. This stoichiometry is suggested by the distribution of 14C label in the subunits of CF1 purified from uniformly labeled cells of the green alga Chlamydomonas reinhardtii. In addition, we have estimated a molecular weight for this CF1 complex by analyti-
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The observed range of 390,000 to 420,000 is supportive of an $a_2$/$b_2$ stoichiometry.

**MATERIALS AND METHODS**

**Analytical Gel Filtration**—Gel filtration experiments were performed on a Sepharose CL-4B column (1.3 cm) equilibrated in 20 mM Na-Tricine, pH 8.0, and 1 mM EDTA. One hundred-microliter samples of *C. reinhardi* CF (final concentration, 0.5 mg/ml) were applied in three separate runs to the column with each of the following molecular weight standards: thyroglobulin (50 mg/ml), ferritin (50 mg/ml), catalase (40 mg/ml), alkaline phosphatase (10 mg/ml), and aldolase (50 mg/ml). The proteins were eluted in the above buffer at a flow rate determined by a hydrostatic pressure of about 125 cm and collected as 0.78-ml fractions. The elution volumes ($V_e$) were determined at the peak maximum. The void ($V_v$) and included ($V_i$) volumes were determined by the elution volumes of blue dextran and ferricyanide, respectively. Thyroglobulin, catalase, pyruvate kinase, and aldolase were detected in fractions by a calorimetric protein assay.

**Distribution Coefficient ($K_d$)**—The distribution coefficient ($K_d$) for each sample was calculated from the equation:

$$K_d = V_e - V_v / V_i - V_v$$

A plot of the distribution coefficient for each sample versus the logarithm of its molecular weight yielded a calibration curve for the determination of the molecular weight of the *C. reinhardi* CF.

**Sedimentation Velocity Experiments**—Sedimentation velocity experiments were performed on a Beckman Model E analytical ultracentrifuge equipped with an electronic speed controller, RTIC unit, monochromator, and photoelectric scanner with multiplexer. Double sector 12-mm aluminium-filled Epon centerpieces were used. Sedimentation at 36,000 rpm was monitored by UV absorbance at 280 nm and was performed at either 12 or 20°C. The midpoint of the sedimenting boundary was used to analyze the data. Observed sedimentation coefficients were corrected to standard conditions (water at 20°C) using the measured relative densities and viscosities. Samples were run either in 100 mM Tris/Cl, pH 8.0, and 1 mM EDTA or 100 mM Na-Tricine, pH 8.0, and 1 mM EDTA at concentrations of CF ranging from 0.15 to 0.50 mg/ml. Relative densities of sample solutions were determined at room temperature to ±0.4% precision by weighing aliquots in 100-μl Dade micropipettes. The micropipettes were calibrated with water at the same temperature. An Umbelodisc-style viscometer was used to measure the relative viscosities of the solvent at 24.80 ± 0.03°C.

**High Speed Sedimentation Equilibrium Measurements**—CF was performed at 20.0 ± 0.5°C in 100 mM Na-Tricine, pH 8.0, and 1 mM EDTA at concentrations of CF, ranging from 0.15 to 0.50 mg/ml. The sample channels of a 12-mm six-channel Yphantis centerpiece with quartz windows were filled to a column height of approximately 2.5 mm (100). The samples were initially overspeeded at 20,000 rpm for 3 h. The speed was then reduced to 10,000 rpm. Scans of the equilibrium distribution of protein absorbance at 280 nm were recorded on the photoelectric scanner after 13 h of sedimentation at the lower speed. The speed was reduced to 8,000 rpm; additional scans were taken after equilibrium was re-established (6 h). In order to record the base-line absorbance measurement, the column was cleared of protein by sedimenting the samples for 2 h at 30,000 rpm.

**Immunoprecipitation**—CF was precipitated from the crude chloroform extract with antibodies raised against the purified *C. reinhardi* CF, in buffer containing 20 mM sodium phosphate, pH 7.5, 140 mM sodium chloride, 10 mM EDTA, 25 mM sodium azide and 4 mg/ml of bovine serum albumin. The reaction was incubated for 36 h at 4°C. The immunoprecipitate was pelleted at 10,000 x $g$ for 10 min, washed two times in 20 mM sodium phosphate, pH 7.5, 140 mM sodium chloride.

**Isolation of $^{14}C$-labeled CF**—*C. reinhardi* cells were grown in Tris/acetate/phosphate medium (31) at 1 mCi of [2-$^{14}$C]acetate/liter. By late log to stationary phase, approximately 80 to 90% of the label was incorporated into the cell. One-liter cultures were routinely used for preparations of CF. CF was isolated from cells equivalent to 17 to 20 mg of chlorophyll by a scaled-down version of the chloroform extraction procedure developed earlier (11). A few modifications included three washes of the harvested cells with cold Tris/acetate/phosphate medium and an increase to five washes of the thylakoid membranes with 10 mM sodium pyrophosphate, pH 7.8. When the chloroform extract was to be used directly for gel electrophoresis or for immunoprecipitation, it was centrifuged for an additional 30 min at 100,000 x $g$. The CF, from the chloroform extract was purified on DEAE-Sephadex A-50 as described earlier (11). The purified protein was concentrated by ultrafiltration on Amicon PM-10 membranes, and samples were either used directly for SDS-gel electrophoresis or stored as precipitates in 50% saturated ammonium sulfate. Approximately 2,000 to 3,000 cpm/μg of purified protein were routinely obtained. The specific activity of the purified protein was around 30 μmol of ATP hydrolyzed/μg of protein/min when assayed in 2% ethanol (29).

**Fractionated Sedimentation**—The subunits of $^{14}C$-labeled CF, were separated on SDS-polyacrylamide gels (30). The bands corresponding to the α, β, γ, and ε subunits were sliced out, dried at 80°C to remove water and burned in a Packard Tri-Carb Sample Oxidizer (Model B 306), and the label associated with each band was recovered as [14C]CO$_2$. The CO$_2$ was trapped in 6 ml of Carbosorb and counted in 12 ml of Permafluor V. Between 95 and 100% of the label was routinely recovered. The molar ratio of label associated with each band was calculated from the counts recovered from each band/g of molecular weight. Background counts in the nonstaining region of the gel were negligible (less than 10% of the counts in the smallest subunit) and were ignored.

**Immunoprecipitation**—CF was precipitated from the crude chloroform extract with antibodies raised against the purified *C. reinhardi* CF, in buffer containing 20 mM sodium phosphate, pH 7.5, 140 mM sodium chloride, 10 mM EDTA, 25 mM sodium azide and 4 mg/ml of bovine serum albumin. The reaction was incubated for 36 h at 4°C. The immunoprecipitate was pelleted at 10,000 × $g$ for 10 min, washed two times in 20 mM sodium phosphate, pH 7.5, 140 mM sodium chloride.

![Figure 1](image1.png) **Figure 1.** Sepharose CL-4B gel filtration of CF. The column was run as described under "Materials and Methods." The molecular weights of the standards were obtained from Worthington Enzymes. The standard line was fit to the experimental points by linear regression. •, molecular weight (MW) standards; x, *C. reinhardi* CF.

![Figure 2](image2.png) **Figure 2.** Sedimentation velocity boundaries of CF, in 100 mM Na-Tricine, pH 8.0, as a function of time sedimented. The protein concentration was 0.5 mg/ml; centrifugation was performed at 36,000 rpm and 12.6°C.
FIG. 3. Sedimentation equilibrium data for CF, in 100 mM Na-Tricine, pH 8.0, and 1 mM EDTA. The rotor speed was 8,000 rpm and the temperature was 20.0 ± 0.5°C. The initial protein concentrations used were 0.16 mg/ml (A), 0.35 mg/ml (B), and 0.54 mg/ml (C). The coordinates on data sets 1 and 3 are offset by a constant, a, in order to present the data for all three channels in the Yphantis centerpiece in one figure. a = -7 cm² (data set 1) or -13 cm² (data set 3). The molecular weight was determined from the slope of these lines.

Fig. 4. Determination of the molecular weight of the subunits of CF₁ separated by SDS-polyacrylamide gel electrophoresis. The samples were prepared and run on 10% acrylamide gels, and the relative mobilities (Rₑ) were determined as described under "Materials and Methods." The standards used for the calibration curve include phosphorylase b (92,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400) (all purchased from Bio-Rad Laboratories), pepsin (34,700), trypsinogen (24,000), and β-lactoglobulin (18,400) (all purchased from Sigma; E. coli F₁, 55,400, 50,400, 31,500, 19,300, and 14,300; Ref. 13), and spinach CF₁ (61,000, 57,000, 34,500, 20,800, and 15,700; Ref. 22). The calibration curve was fit to the experimental points by linear regression.

RESULTS

Molecular Weight of CF₁—C. reinhardi CF₁, isolated and purified as described above, is a four-subunit enzyme containing α, β, γ, and ε subunits (see Fig. 5). The association of the δ subunit with the C. reinhardi CF₁ complex is not stable, resulting in a loss of the δ subunit upon storage. The four-subunit enzyme therefore provided us with a more homo-
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neous preparation and has been used in this study. The apparent molecular weight of the C. reinhardi CF1 was estimated on a gel filtration column by calibration with proteins of known molecular weight ranging from 161,000 to 667,000 (Fig. 1). CF1 was run on the column three times along with at least one of the standard proteins and eluted as a single symmetrical peak each time. A molecular weight of 420,000 ± 15,000 was estimated from the calibration curve in Fig. 1. Although this value agrees well with the apparent molecular weight of 380,000 for the yeast mitochondrial F1, as determined by gel filtration (5), the molecular weight estimate is high compared to those made for other F1 proteins using different analytical techniques, e.g. centrifugation or small angle light scattering (25, 34).

We therefore calculated a molecular weight for CF1 based on its sedimentation coefficient as measured by analytical ultracentrifugation. Representative sedimenting boundaries shown in Fig. 2 are symmetrical and show no indication of protein aggregation during the course of the measurement. Sedimentation measurements on CF1 were made in 100 mM Na-Tricine, pH 8.0, or 100 mM Tris/Cl, pH 8.0, at 20 °C. Sedimentation coefficient values of 12.9 ± 0.4 and 12.8 S were calculated on the samples run in 100 mM Na-Tricine and 100 mM tris/Cl, respectively. These values are independent of protein concentration, based on measurements made on three samples varying in concentration from 0.5 to 2.0 mg/ml, and compare well with measurements of sedimentation coefficients of bacterial and mitochondrial F1 proteins (12). Based on the assumption that the sedimentation coefficient of a protein is proportional to the % power of its molecular weight (35), an estimate of the molecular weight of the C. reinhardi CF1 can be obtained from its sedimentation coefficient using a suitable reference protein with a known sedimentation coefficient and molecular weight. This relationship holds true for a series of solid particles of identical f/f0 (where f/f0 is the ratio of the frictional coefficient of the actual particle to that of an unhydrated sphere with the same partial specific volume and molecular weight). Since F1 ATPases are highly conserved proteins (1, 2), the choice of an F1 ATPase from a different source as a reference is reasonable. Unfortunately, it has been difficult to accurately establish the molecular weight of F1 proteins. We have chosen, the rat liver mitochondrial F1, as our reference standard since the s20,w (determined by analytical centrifugation) and molecular weight appear to be reliably determined in two different laboratories (36, 37). The molecular weight of the C. reinhardi CF1 thus estimated from the % power of its molecular weight dependence on S is 390,000. Since the above estimations of molecular weight rely on the use of calibration standards, the molecular weight of CF1 was also directly determined by sedimentation equilibrium to be (4.2 ± 0.5) x 10^6. The measurements were performed at rotor speeds of 8,000 and 10,000 rpm at three different protein concentrations. The results are independent of either protein concentration or rotor speed. In addition, the plots of ln (A20,w) versus r^2 (Fig. 3) are linear and show no indication of sample heterogeneity during the run.

Our estimates of the molecular weight of the C. reinhardi CF1 are incompatible with the αβ2γε2 stoichiometry suggested for chloroplast coupling factors (21–23). The apparent molecular weight of the subunits of the C. reinhardi CF1 are 57,000 (β), 53,000 (α), 38,000 (γ), and 15,000 (ε). These subunit molecular weights have been calculated from a calibration curve (Fig. 4) constructed by the method of Weber and Osborn (30). If spinach CF1 (22), E. coli F1 (13), or commercially available standards are used independently to construct standard curves, the average calculated molecular weights are 56,000 ± 2,000 (β), 52,000 ± 2,000 (α), 37,000 ± 1,000 (γ), and 15,000 (ε). Using either set of numbers, the αβ2γε2 stoichiometry predicts a molecular weight of between 283,000 to 288,000 for the CF1 complex. This number is much lower than any estimate that we have made for the molecular weight of the CF1 complex, thus suggesting that the accepted subunit stoichiometry of plant chloroplast coupling factors is likely not to be the correct one in the case of the C. reinhardi CF1.

Subunit Stoichiometry of the C. reinhardi CF1—CF1 isolated from cells grown on [2-14C]acetate was used to calculate the stoichiometry of the four subunits in the purified complex. Since the cells were grown continuously on the labeled carbon source, the question of differential utilization of amino acid pools within the chloroplast and cytosol does not arise. The subunits should, therefore, be uniformly labeled with carbon-
14. The per cent carbon by weight in most polypeptide chains is quite constant (13), and the measurement of label in each subunit can therefore be used as an estimate of the relative amount of protein in each subunit. The subunits were separated by SDS-polyacrylamide gel electrophoresis (Fig. 5), and the molar ratio of label incorporated into each subunit relative to the $\gamma$ subunit was calculated as shown in Table I. This ratio is based on the apparent subunit molecular weights as calculated from the standard curve in Fig. 4. However, other estimates of the subunit molecular weights do not significantly change the resulting ratio. For example, those based on the use of the subunits of E. coli F$_1$ as the sole standard resulted in subunit molecular weights of 55,300, 49,600, 35,500, and 14,500 and a subunit ratio of 2.9:3:1:0.1:1.0. The labeled protein was prepared four times from three different strains of C. reinhardtii, and the subunit ratio, relative to the $\gamma$ subunit, was determined on crude chloroform extracts of thylakoid membranes, on immunoprecipitates of these chloroform extracts, and on the purified protein. These results are summarized in Table II. The average subunit ratio/ $\gamma$ subunit predicts an $\alpha\beta\gamma\epsilon$ subunit stoichiometry for the CF$_1$ complex from C. reinhardtii.

It is possible that polypeptides contaminating the CF$_1$ preparation might co-migrate with the subunits of CF$_1$ and might result in an overestimate of the label associated with any one band. However, the purified protein showed no contaminating polypeptides in the same molecular weight range as $\alpha$, $\beta$, $\gamma$, or $\epsilon$ when analyzed by the two-dimensional gel electrophoretic system of O'Farrell (32) (data not shown). In addition, the subunit ratios of CF$_1$ estimated from crude chloroform extracts of the thylakoid membrane of strain 21gr mt+ do not differ from the estimates on the purified CF$_1$ (Table II, lines 1 and 2).

**DISCUSSION**

The calculated molecular weight of the C. reinhardtii CF$_1$ complex based on the $\alpha\beta\gamma\epsilon$ stoichiometry suggested by the labeling experiments and the estimated subunit molecular weights is 383,000. Our value of 420,000 (sedimentation equilibrium) supports the above stoichiometry. The estimate of 380,000 (sedimentation velocity) was obtained from our measured sedimentation coefficient of 12.9 S and the use of the rat liver mitochondrial F$_1$ as a standard of known molecular weight and sedimentation coefficient. The use of other standards, however, e.g. beef heart mitochondrial F$_1$ (38, 39), yeast mitochondrial F$_1$ (40, 41), or bacterial F$_1$ (34, 42), does not change the calculated estimate of the molecular weight of the C. reinhardtii CF$_1$. We calculate molecular weights of 360,000, 409,000, and 377,000, respectively. These values are all close to the molecular weight predicted by our measured subunit stoichiometry and far exceed the value predicted by an $\alpha\beta\epsilon$ stoichiometry. These data directly support an $\alpha\beta\gamma\epsilon$-type stoichiometry for the isolated chloroplast coupling factor ATPase and are in keeping with the subunit stoichiometries determined for the E. coli F$_1$, the thermophilic bacterial F$_1$, the Salmonella typhimurium F$_1$, and the yeast mitochondrial F$_1$ (5, 13-15). The C. reinhardtii CF$_1$ is an unusually stable enzyme (in comparison with the higher plant CF$_1$s) (11, 29) and therefore lends itself to studies on conformational characterization. We see no evidence for dissociation or aggregation of the enzyme during the course of our molecular weight measurements. This appears to be a problem in the case of the higher plant CF$_1$s (24, 26) and might, therefore, result in erroneous measurements of molecular weight and subunit stoichiometry.

Bearing in mind the conservation of structure and function of energy transducing complexes, it is possible that the subunit stoichiometry is similar in prokaryotic membranes as well as in eukaryotic organelle membranes. The 6-fold axis of symmetry observed for wheat CF$_1$ as viewed in the electron microscope is suggestive of an $\alpha\beta\gamma\epsilon$-type structure for this higher plant enzyme as well (43). More recently, immunoprecipitates of the CF$_1$-CF$_2$ complex from bean and oat thylakoid membrane extracts have been shown to contain $\alpha\beta\gamma\epsilon$-type complexes (44). It is likely that a determination of the CF$_1$ subunit stoichiometry from various higher plant sources might result in the discovery of more $\alpha\beta\gamma\epsilon$-type enzymes.

**REFERENCES**

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