The M_r-value of chloroplast coupling factor 1

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The M_r of spinach chloroplast coupling factor 1 has been determined by sedimentation equilibrium and by light scattering to be 400 000 ± 24 600 and 407 000 ± 20 000, respectively. These values differ substantially from that obtained previously (325 000) and are consistent with an $\alpha_3\beta_3\gamma\delta\epsilon$ subunit stoichiometry.

Coupling factor 1 *M_r-Value* *Light-scattering* *Sedimentation equilibrium* *Subunit stoichiometry* *Chloroplast*

1. INTRODUCTION

Coupling factor 1 from chloroplasts (CF_1) is an oligometric, hydrophilic protein that contains the catalytic sites of the H^+-ATPase (ATP synthase). Like its close relatives from the other coupling membranes, CF_1 contains 5 distinct subunits, labeled α–ϵ in order of decreasing M_r-value (cf. [1,2]). Several lines of evidence, including the sulfhydryl content of the subunits [3,4], labeling in vivo with $^{14}CO_2$ [5], dye binding (3) and cross-linking studies [6] are consistent with an $\alpha_2\beta_2\gamma\delta\epsilon_2$ stoichiometry for these subunits. This stoichiometry is based on M_r 325 000 for CF_1 [7,8]. A higher M_r was reported [9], but this was ascribed to methanol-induced aggregation [10].

In contrast, the M_r of the mammalian mitochondrial enzyme is 360 000–384 000 [11] and that of the *Escherichia coli* and thermophilic bacterial lusymes is 360 000–370 000 [12] and 380 000 [13], respectively. A convincing case has been established for a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ for the bacterial enzymes [12,13]. In view of the discrepancy between these results and those for the chloroplast coupling factor, we have redetermined the M_r of CF_1. While this work was in progress, the M_r of CF_1 from *Chlamydomonas reinhardii* was determined to be 420 000 [13].

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Abbreviations: CF_1, chloroplast coupling factor 1; ECF_1, *E. coli,* coupling factor 1; SDS, sodium dodecylsulphate

2. MATERIALS AND METHODS

The ammonium sulfate used was enzyme ultra pure grade from Schwarz/Mann. All other chemicals were high purity commercial products, and all solutions were made with deionized distilled water.

The CF_1 was prepared from fresh market spinach using a combination of the procedures in [3,15]. Enzyme, having a 305 : 340 nm fluorescence ratio (excitation at 280 nm) <1.5, was collected and stored in 2 M ammonium sulfate, 10 mM Tris–SO_4 (pH 7.1), 1 mM EDTA, 0.5 mM ATP at 4°C. Its purity was checked by SDS–polyacrylamide gel electrophoresis. The CF_1 was activated by heating at 63–64°C for 5 min in 40 mM Tris–HCl, 2 mM EDTA, 40 mM ATP, 10 mM dithiothreitol (pH 8.0). The specific activity at 37°C in 5 mM ATP, 5 mM $CaCl_2$, 40 mM Tris–HCl, 2 mM EDTA (pH 8.0), immediately after activation, was at least 12 μmol . mg^{-1} . min^{-1}, as determined by measurement of released phosphate with ammonium molybdate [16]. An extinction coefficient of 0.482 cm^2/mg at 277 nm [17] was used for determining concentrations of CF_1.

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For the light scattering experiments and determination of the refractive increment, the ammonium sulfate precipitate of CF_1 was pelleted at 12 100 × g for 20 min at 4°C. The pellet was dissolved in 10 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA and passed through two 3 ml G-50 fine Sephadex centrifuge columns [18] equilibrated in the same buffer. The CF_1 collected from the centrifuge columns was dialyzed against the same buffer for 3 h. The dialysis buffer was then deaerated and used to dilute the stock enzyme solution to the desired concentrations; the dialysis buffer was used as a reference blank when required.

For equilibrium ultracentrifugation, the ammonium sulfate precipitate of either *E. coli* F_1 or CF_1 was pelleted as above and the pellet dissolved in 0.1 M sodium phosphate buffer (pH 8.0), 0.1 mM EDTA. This solution was passed through a 1 × 10 cm column of Sephadex G-50 medium equilibrated with the phosphate–EDTA buffer. The CF_1 used in the light scattering experiments was a different preparation from that used in the ultracentrifuge experiments.

2.1. *Measurement of refractive increment*

Both the refractive index of the dialysis solvent and the refractive increment of CF_1 were measured using a Model BP-200-V Brice Phoenix Differential Refractometer. A Ditric interference filter was used to obtain light at 632.8 nm from a Type AH-3 mercury vapor lamp; this is the same wavelength of light used for the light scattering measurements. The refractometer cell was thermostated at 21°C for all measurements except the instrument calibration which was done at 25°C.

2.2. *Calibration of the refractometer was with NaCl solutions*

The NaCl was dried in an oven at 125°C for at least 5 days. The values of $\Delta n/\Delta c$, (difference in refractive index/difference in concentration), at 589 nm, 578 nm, 546 nm, and 436 nm [19] were extrapolated to 632.8 nm. After measuring the total slit image displacement, Δd, between distilled deionized water and the salt solutions, the calibration constant, k, was calculated with the relationship:

$$\Delta n = k\Delta d$$

From the same relationship, Δn was calculated for CF_1 (1.48–4.58 mg/ml) in 10 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA (pH 8.0), and the refractive index increment, $(dn/dc)_{c=o'}$ was obtained by extrapolation of $\Delta n/\Delta c$ to zero enzyme concentration through a linear least squares analysis.

2.3. *Light scattering measurements*

Measurements of the Rayleigh scattering of the protein solutions minus that of the buffer, $\bar{R}_\theta$, were obtained with a Chromatix KMX-6 low angle laser light scattering spectrophotometer which uses a 2 mV HeNe laser as the light source. Samples were flowed through a 0.1 μm Millipore filter to remove dust and then into the sample cell by means of a syringe drive. Rayleigh scattering of the dialysis buffer was measured between each sample of enzyme. The sample cell was thermostated at 21°C, and the enzyme (0.087–0.622 mg/ml) was in 10 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, (pH 8.0) for all measurements.

2.4. *Ultracentrifugation*

A Beckman model E analytical ultracentrifuge equipped with scanning optics and an on-line computer system [20,21] was used. Each compartment of Yphantis cell was loaded with 0.10 ml either sample or reference buffer to give a column height of about 2.5 mm. The samples were allowed to centrifuge for 16–24 h at 8000–10 000 rev./min and at a rotor temperature of 20°C. Using the computer/scanner system, about 100 absorbance (at 277 nm) and radial distance values were determined for each scan. Each absorbance value was the average of about 100 digitized sample and reference photomultiplier pulses. One pulse was collected in each rotor revolution. The collection program calculated the radial position and the standard deviation for each absorbance value. The data were plotted as absorbance (A) *vs* radius (R) or as ln A *vs* R^2 on a Textronix 4014 graphics terminal. The useful data range and approximate baseline values were determined with the aid of the cursor mode of the 4014 terminal. Sedimentation equilibrium was achieved in each run. M_r-Values were calculated from the slopes of plots of ln A *vs* R^2 according to the equation:

$$M_r = \frac{2RT}{(1-\bar{\nu}\varrho)\omega^2} \cdot \frac{d(\ln A)}{dR^2}$$

A value of 0.741, an average of that calculated in [7,8], was used for the $\bar{\nu}$ of CF_1 and the density of the buffer, ϱ, was determined to be 1.012 at 20°C.

Exact baseline values were determined by an incrementing procedure that minimized the standard deviation of ln A *vs* R^2 plots from linearity. This deviation was determined from the sigma B value in [22]. Baseline values were typically $<0.05A$ and the non-sedimenting nature of these baselines was confirmed by running the rotor at high speed for 1–2 h after equilibrium was achieved.

3. RESULTS

3.1. *Light scattering measurements*

The refractive index of 10 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA (pH 8.0) was found to be 1.328 and the refractive index increment of CF_1, $(dn/dc)_{c=o}$, to be 0.176 (±0.003) ml/g.

At low angles and low concentrations, the M_r of a particle is related to the excess Rayleigh scattering factor, $\bar{R}_\theta$, by:

$$\frac{Kc}{\bar{R}_\theta} = \frac{1}{M_r} + 2A_2c$$

where c is the concentration in g/ml, A_2 is a virial coefficient and:

$$K = \frac{2\pi n^2}{\lambda^4 N}\frac{dn}{dc}(1 + \cos^2\theta)$$

Here, n is the refractive index of the solvent, λ is the wavelength of the light, N is Avogadro's number, and $(1 + \cos^2\theta)$ is a geometrical factor which is a known property of the instrument. A plot of $Kc/\bar{R}_\theta$ *vs* $[CF_1]$ is shown in fig.1. Linear regression analysis gives an intercept of 2.46×10^{-6} which corresponds to M_r 407 000 (±7000). The maximum uncertainty in the M_r can be estimated to be ±5% from the standard error of the refractive index increment (±1.7%) and the intercept in fig.1 (±1.7%). The slope of the line is 7.74×10^{-5} ml/g. This small second virial coefficient indicates weak interactions between protein molecules and, in particular, the absence of protein aggregation.

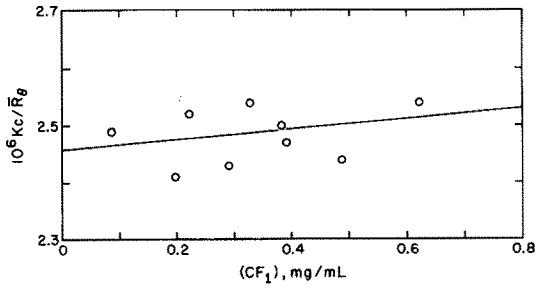

Fig.1. Dependence of $Kc/\bar{R}_\theta$ on $[CF_1]$. Here K is a constant, c is the protein concentration and $\bar{R}_\theta$ is the Rayleigh light scattering. Measurements were carried out as in section 2.

3.2. *Sedimentation equilibrium*

The M_r of CF_1 was also determined by equilibrium ultracentrifugation. Plots of A *vs* R and ln A *vs* R^2 for one run are shown in fig.2 and 3, respec-

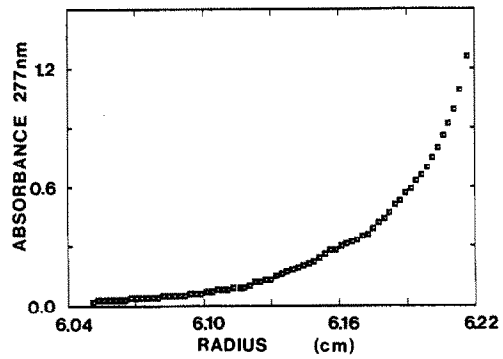

Fig.2. Example of a graph of A_{277} *vs* the radial distance from the center of the rotor for an ultracentrifuge run with CF_1. A background absorbance of 0.021 was subtracted from each point.

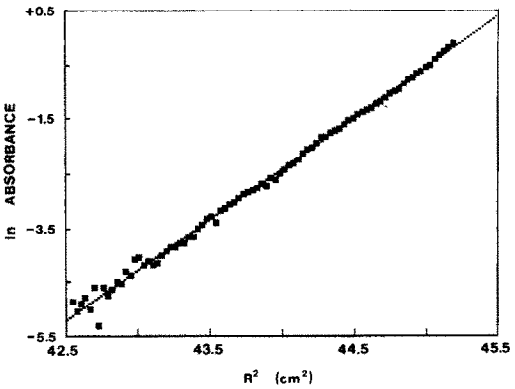

Fig.3. Example of a graph of ln A at 277 nm *vs* R^2 of the square of the radial distance. The data are from fig.2.

Table 1

Comparison of the M_r of CF_1 and ECF_1

Enzyme	Mean M_r	Range
CF_1	406000	398000–411000
ECF_1	371000	364000–381000

M_r-values of 3 CF_1 aliquots and 3 ECF_1 aliquots in 0.1 M sodium phosphate buffer (pH 8.0), 0.1 mM EDTA, 0.05 mM ATP and 0.1 mM dithiothreitol were determined by sedimentation equilibrium in the same run

tively. The absence of upward or downward curvature in the ln A *vs* R^2 plot indicates that subunit dissociation or aggregation of CF_1 did not occur to a significant extent. Moreover, the ATPase activity of a CF_1 sample recovered from the ultracentrifuge cell after a run was identical to that of an aliquot of the same sample of CF_1 that had been stored at room temperature during the run. SDS–polyacrylamide gel electrophoresis of CF_1 recovered from the cell showed that all 5 subunits were present and that no detectable proteolysis had occurred. No significant changes in M_r values were obtained over 0.3–1.2 mg/ml CF_1 or when either 0.05 mM ATP or 0.1 mM dithiothreitol was present during ultracentrifugation. The mean M_r for CF_1, determined from 15 runs, was 400000 ± 25000, in agreement with the value obtained from light scattering measurements.

The M_r of *E. coli* F_1 of (ECF_1) determined by sedimentation equilibrium [23] is 360000–370000 and from amino acid composition deduced from the nucleic acid sequence of the genes encoding for ECF_1 subunits (assuming an $\alpha_3\beta_3\gamma\delta\epsilon$ composition) is 381000 [24,25]. ECF_1 is a well characterized protein to use as a standard. The M_r of ECF_1 (371000) is in agreement with that determined previously and the M_r of CF_1, determined in the same run averaged 406000 (table 1).

4. DISCUSSION

Our results, obtained by two independent methods and with different CF_1 preparations, indicate that CF_1 is M_r 400000. This value is significantly higher than that determined previously. In [7], CF_1 extracted from acetone-precipitated thylakoids and a lengthy procedure to purify the enzyme to homogeneity, as judged by polyacrylamide gel electrophoresis under non-denaturing conditions, were used. CF_1 from acetone-precipitated thylakoids often lacks the δ-subunit and is subject to attack by endogenous proteases during storage at room temperature. However, the study in [7] was done before it was realized that CF_1 contains 5 subunits. On the other hand, we have no explanation for the disagreement with the results in [8] where M_r 325000–330000 was obtained by light scattering and sedimentation.

The subunit stoichiometry of CF_1 is, in view of this revised M_r-value, likely to be $\alpha_3\beta_3\gamma\delta\epsilon$. Using M_r-values for the α and γ subunits derived from SDS–polyacrylamide gel electrophoresis, for the β and ϵ subunits from the amino acid sequence [26], and for δ from the amino acid composition [3], the calculated M_r of CF_1 for an $\alpha_2\beta_2\gamma\delta\epsilon_2$ stoichiometry is ~311000, whereas for an $\alpha_3\beta_3\gamma\delta\epsilon$ stoichiometry, the M_r is 408000. Since the M_r of the β and ϵ subunits of CF_1 calculated from the amino acid sequence data agree well with those obtained by SDS gel electrophoresis, it is unlikely that the M_r values for the α and γ subunits of CF_1 are in error by more than a few percent. The stoichiometry of the δ and ϵ subunits cannot be unambiguously assigned on the basis of the revised M_r. However, both the revised M_r and suggested stoichiometry are consistent with those of F_1 from other sources [11–13].

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