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# Crystallization of Coupling Factor 1 ($CF_1$) from Spinach Chloroplast

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Summary: Spinach chloroplast coupling factor ($CF_1$) was crystallized at 20°C from 0.05 M TRIS-$PO_4$, containing 4 mM ATP, 15 mM KCl, 1.0 mM EDTA and 1.80 M $(NH_4)_2SO_4$, at pH 7.8. Some unit cell parameters were determined by electron microscopy and by X-ray diffraction. The cube shaped crystals have a tetragonal lattice, a = b = 135 Å, c = 280 Å with eight molecules per unit cell; possible space group P422 or $P4_22_12$, hence half a molecule in the asymmetric unit. Crystals grown at pH 7.5 in the absence of ATP have an orthorhombic lattice, a = 125 Å, b = 145 Å, c = 169 Å ($C222_1$), eight molecules per unit cell.

Subchloroplast particles appear in electron micrographs as vesicular structures with 90-100 Å spheres on their surface which have been identified to be the $CF_1$ portion of the coupling factor complex from spinach chloroplasts protruding from the outer surface of the thylakoid membranes. The "spheres" can be removed by either EDTA (1) or silicotungstate treatment (2), but it could be shown that the above mentioned structures reappear after reconstitution with homogeneous $CF_1$.

Since homogeneous preparations of $CF_1$ are available (3, 4) extensive investigation of the characterization of this enzyme is possible, e.g. molecular weight, sedimentation coefficient by means of analytical ultracentrifugation (5, 6), elastic and inelastic light scattering, as well as small angle X-ray scattering in solution (6, 7). However, until now the

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Abbreviations used are: SDS = sodium dodecyl sulfate; $CF_1$ = chloroplast coupling factor; $F_1$ = coupling factor of beef heart; $\alpha,\beta,\gamma,\delta,\varepsilon$ = subunits of the $CF_1$ ATPases in order of decreasing molecular weight; EDTA = ethylene diamine tetraacetic acid, disodium salt.

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enzyme from spinach chloroplasts has not been crystallized. The preparation of crystalline $CF_1$ forms disclose the way for further investigation of the structure of this complex by X-ray diffraction in order to elucidate the three-dimensional structure of $CF_1$. This paper describes the successful crystallization of the coupling factor ($CF_1$) in the presence and in the absence of ATP at 20°C. Small angle X-ray scattering measurements reveal that the diameter of the $CF_1$ molecule without ATP is 110 Å and in the presence of 4 mM ATP is 98 Å. This indicates a swelling of the $CF_1$ molecule in the absence of ATP which is accompanied by a decrease of the sedimentation coefficient from 13.5S to 12.8S.

## MATERIALS AND METHODS

$CF_1$ from spinach chloroplast was prepared according to Lien and Racker (3) and further purified by agarose column chromatography 0.5 m (BioRad, California) in the presence of 4 mM ATP, 0.1 mM EDTA in 0.05 M TRIS-$PO_4$, pH 7.8. The $CF_1$ preparation had a specific activity of 26 µmol ATP/mg protein/min, and after the following blue dextran chromatography a specific activity of 31 µmol ATP/mg protein/min. The specific activity of $CF_1$ was determined with the pH-stat assay under the following conditions: 0.8 mM ATP disodium salt, 10 mM $CaCl_2$, and approximately 14-16 µg/ml of $CF_1$, pH 8.0, 25°C in 0.05 M TRIS-HCl.

Crystallization: Stock solutions of $CF_1$ in 0.05 M TRIS-$PO_4$, pH 7.8, contained 10 mg/ml enzyme in 10 mM KCl, 5 mM ATP disodium salt, 1 mM EDTA, and 0.08 M $(NH_4)_2SO_4$. The clear solution was placed in special crystallization chambers (8, 9) where the solution chamber containing the $CF_1$ sample had a volume of 50 µL and the surrounding outer chamber contained the buffer in a total volume of 100 µL. The concentration of $(NH_4)_2SO_4$ was raised gradually over a period of 14 days to 1.4 M $(NH_4)_2SO_4$ in the buffer chamber. Normally, a particulate precipitate developed in the concentration range between 1.4-2.0 M $(NH_4)_2SO_4$. Only the clear supernatant of the $CF_1$ solution was used for further crystallization by transferring it to another crystallization chamber after centrifugation at 10,000 xg. The $(NH_4)_2SO_4$ concentration was raised to 1.9 M in the buffer chamber over a period of three days where small cube-like crystals appeared after two or three weeks. The crystallization chambers were stored in a desiccator over the buffer used in the crystallization process at 20°C in order to prevent drying of the crystallization chambers.

Flat cubical crystals could occasionally be grown at pH 7.5-7.8 using 10 mg/ml of $CF_1$ in the presence of 4 mM ATP at 20°C with 1.5 M $(NH_4)_2SO_4$. But more reproducible orthorhombic crystals were grown from solutions of 20 mg/ml of $CF_1$ at pH 8.0 (see Table I). At pH 7.8 the crystals were very thin (Fig. 1), but at a more basic pH the crystals of $CF_1$ became thicker and

were stable against temperature and X-ray exposure. Bragg maxima could be observed on Laue photographs to about 3.0 Å resolution using an Elliot rotating X-ray generator and perpendicular focussing mirrors (10). These crystals were very sensitive to radiation damage with a lifetime of 2 hours.

Crystallization of $CF_1$ at pH 7.0 with EDTA without ATP: Due to the dissociation of $CF_1$ in the cold without protection of ATP, attempts were made to modify the crystallization conditions in the presence of $(NH_4)_2SO_4$ or $MgSO_4$ at 20°C. However, these crystals of $CF_1$ can only be grown to useful sizes in the pH-range of 7.0-7.5, and modification of the pH causes them to dissolve. Furthermore, when increasing the EDTA concentration above 5 mM the crystals cracked. The trigonal crystals obtained had sizes of 0.3 x 0.5 x 0.1 mm and were very sensitive to X-rays, but the lifetime could be increased by taking X-ray photographs of the crystals under a stream of cold nitrogen (4°C).

Powder diagrams of crushed crystals of $CF_1$ of both types were studied to investigate the difference between these two crystal forms. Such photographs reveal resolved individual diffraction maxima from which tentative cell dimensions for the crystal forms of $CF_1$ could be obtained.

X-ray diffration: The single crystals of $CF_1$ were mounted in thin-walled glass capillaries (Ø 1 mm) with a droplet of mother liquor at both ends, and the capillaries were sealed with dental wax. X-ray diffraction photographs were taken on an Enraf-Nonius procession camera with a crystal-to-film distance of 10 cm. $CuK_\alpha$-radiation, monochromatized by a bent quartz monochromator, was used from a rotating anode X-ray generator (G x 13, Elliott, U.K.). Exposure times ranged from 5 to 6 hours (20°C).

Small angle X-ray scattering measurements on concentrated $CF_1$ solutions were carried out as described in (6, 11). Analytical ultracentrifugation was carried out as described in (6).

Determination of the crystal density was performed by using a calibrated water-saturated bromobenzene (Merck, Darmstadt, FRG) and xylene (Merck) mixture (12).

Electron microscopy and optical diffraction patterns, obtained from negatively stained single crystals, were performed as described in (13).

## RESULTS

After a period of four to five weeks the small $CF_1$ crystals grew to sizes of about 0.15 x 0.08 x 0.05 mm in the presence of ATP, but seldom as large as 0.15 m in all principal directions (Fig. 1) (see Table I). The relatively thin crystals show the characteristic structures seen in figure 2 when negatively stained and examined by electron microscopy. The optical diffraction pattern indicated a unit cell with dimensions of a = b = 150 Å, $\gamma = 90^\circ$ and c = 280 Å. The redissolved crystals of $CF_1$ sedimented as one band with 13.5S, whereas the crystals (seen in Fig. 1b) grown at pH 7.5 and 4.0 mM EDTA

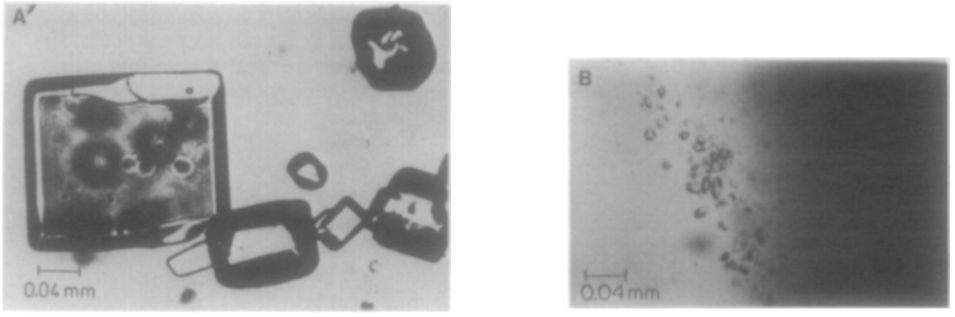


FIG. 1. (A) Single crystals of $CF_1$ grown in the presence of 5 mM ATP, pH 8.0.

(B) Single crystals of $CF_1$ grown at pH 7.5 in the presence of 4.0 mM EDTA.

Table I. Conditions for crystallization of $CF_1$ (20°C).

| pH | $CF_1$ (mg/ml) | $(NH_4)_2SO_4$ | crystal size (mm) |
|---|---|---|---|
| 7.0 | 20 mg/ml, 0.05 M TRIS-$PO_4$; 10 mM KCl, 4 mM EDTA | 1.85 M | 0.13 x 0.05 x 0.2 |
| 7.2 | 0.05 M TRIS-$PO_4$; 10 mM KCl, 4 mM EDTA | 1.65 M 1.0 M $MgSO_4$ | 0.20 x 0.1 x 0.05 |
| 7.5 | 0.05 M TRIS-$PO_4$ 10 mM KCl, 4 mM EDTA 4 mM ATP | 1.7 M 0.5 M $MgSO_4$ | 0.20 x 0.1 x 0.05 |
| 7.5 | 0.05 M TRIS-$PO_4$ 15 mM KCl, 5 mM EDTA | 1.95 M | 0.15 x 0.2 x 0.1 |
| 7.8 | 0.05 M TRIS-$PO_4$ 15 mM KCl, 5 mM EDTA 5 mM ATP | 1.80 M | 0.1 x 0.1 x 0.1 |
| 7.8 | 0.2 M $K_2HPO_4$, 5 mM ATP, 5 mM KCl | 2.0 M | 0.1 x 0.05 x 0.1 |

without ATP (Table I) yielded a sedimentation constant of 12.8S. Moreover, SDS-polyacrylamide gel electrophoresis of both crystal forms of $CF_1$ showed the five principal bands, although the band attributed to the δ-subunit was weak but clearly seen in the $CF_1$ crystal form without ATP. Laue and 3.5° precession photographs taken of the $CF_1$ crystals reveal tetragonal symmetry with a = b = 135 Å and c = 280 Å (Fig. 2) for the crystal form

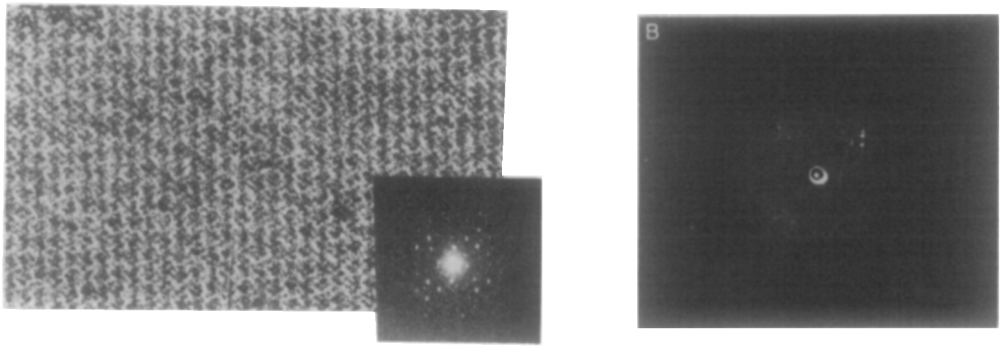


FIG. 2. (A) Electron micrograph of negatively stained crystals of $CF_1$ in the presence of ATP. Insert: Optical diffraction pattern. Magnification: 320.000 x.

(B) 3.5° precesion photograph of a $CF_1$ crystal grown in the presence of 5 mM ATP, pH 8.0. Film-to-specimen distance 100 mm, exposure time 12 h.

in the presence of ATP and orthorhombic symmetry for $CF_1$ crystals with a = 150 Å, b = 138 Å and c = 300 Å grown from solutions containing no ATP (Table I). From density measurements we calculated eight molecules of $CF_1$ in the unit cell for the tetragonal lattice, hence there is half of the $CF_1$ molecule in the asymmetric unit. The volume of unit cell per unit protein molecular weight (14) of $CF_1$ (330,000 (5, 6)) was found to be 3.16 $Å^3$/dalton, revealing a fractional volume occupied by $CF_1$ of 32.2%, and the fractional volume occupied by solvent of 67.8% The corresponding values for the $CF_1$ crystal form without ATP are a = 125 Å, b = 145 Å and c = 169 Å with eight molecules in the unit cell and one half in the asymmetric unit. Like almost all protein crystals with high solvent content, $CF_1$ did not give high resolution diffraction patterns. This is consistent with the concept that the stability is determined by the number and strength of the intermolecular interactions in the crystal rather than by the intrinsic stability of the protein molecule, $CF_1$.

When measuring the diameter with small angle X-ray scattering methods or from powder photographs a mean radius can be obtained. In contrast, overall dimensional changes were observed in the crystalline preparations of $CF_1$ in the absence of ATP, but at high EDTA concentrations, giving the maximum diameter of

Table II. $CF_1$ diameter determined from small angle X-ray scattering.

| pH | EDTA (mM) | $CF_1$ conc. [a] (mg/ml) | diameter (Å) [b] |
|---|---|---|---|
| 7.0 | 1.0 + 5 M ATP | 25 | 98.5 |
| 7.0 | 2.0 | 25 | 110.0 |
| 7.0 | 4.0 | 25 | 108.5 |
| 7.5 | 4.0 | 20 | 108.3 |
| 7.5 | 4.0 + 5 mM ATP | 20 | 98.3 |
| 7.8 | 2.0 + 5 mM ATP | 20 | 98.0 |
| 7.8 | 2.0 + 5 mM ATP | 25 | 97.1 |
| 7.8 | 4.0 + 5 mM ATP | 25 | 97.8 |
| 8.0 | 5.0 + 5 mM ATP | 25 | 97.8 |

a) = determined from the absorbtion at 278 nm using an extinction coefficient of

$$\varepsilon_{1\ mg/ml}^{1\ cm} = 0.45 \quad (6)$$

b) = obtained according to

$$D(R) = \left(\frac{1}{2\pi R}\right) \int_0^\infty I(h_1\rho_o)h \sin h \cdot dh \quad (6)$$

115 Å of $CF_1$ (see Table II). Since the latter is changed by only 2.5%, it must be concluded that the $CF_1$ becomes almost spherical on the addition of ATP and high salt, consistent with the results obtained previously. Such a change is supported by the intensity changes of the single crystal diffraction pattern as well as by the powder pattern. Of course, it is possible that size changes are due in part to alteration in the electron density of the solvent corresponding to the "salt effect" (15) (Table II).

## DISCUSSION

No changes, e.g. decrease in the activity of the $CF_1$ during crystallization and of redissolved crystalline $CF_1$ crystals, were observed. From this and from the latent $Ca^{2+}$-activated ATPase activity of the redissolved $CF_1$ crystals we conclude that the $CF_1$ is crystallized in the biologically active, or a closely related conformation. Since the space group of the $CF_1$ crystals grown in the presence of ATP at pH 8.0 is either P422 or $P4_22_12$, it seems to indicate that the molecular 2-fold

symmetry axis coindides with the crystallographic 2-fold axis of point group 422.

The crystal property of $CF_1$ with respect to resolution extends the diffraction spots to 15 Å, only, and is not of the same quality as that of the single crystals of $F_1$ obtained from rat liver (16). Furthermore, crystallization of the $CF_1$ molecule at 4°C without protection of ATP yielded single crystals of a completely different shape, revealing only three bands on SDS-polyacrylamide gel electrophoresis, namely α, β and γ. This crystalline preparation showed an average molecular weight of 250,000, whereas the crystalline preparation at 20°C revealed a weight-average molecular weight of 330,000, determined by small angle light scattering techniques (17).

Considering the volume fraction of $CF_1$ within the unit cell, the hydrodynamic volume of one $CF_1$ molecule is calculated to be $4.11 \times 10^5$ Å$^3$ from which a hydrodynamic radius of R = 46 Å is determined in the presence of ATP, and R = 51.5 Å for the $CF_1$ crystal form in the absence of ATP at pH 7.5. This value is smaller than the one determined by small angle X-ray scattering of $CF_1$ in solution and by inelastic light scattering, assuming a globular molecule. Since the dry volume of $CF_1$ was calculated with $\bar{\nu}_2 = 0.745$ (6) to be $4.02 \times 10^5$ Å$^3$, a degree of hydration of 0.25 g $H_2O$/g $CF_1$ was calculated. This is considerably smaller than 0.54 g $H_2O$/g $CF_1$ obtained from hydrodynamic methods in the absence of ATP (6), but comparable with the value of 0.14 g $H_2O$/g $CF_1$ measured by NMR-techniques (18). However, the crystalline form of $CF_1$ without ATP is significantly more hydrated (0.45 ml/g) and comparable with the small angle X-ray scattering data (6). Since the swelling process is reversible (17) on addition of 4 mM ATP, and full ATPase activity is retained, we can conclude that the multimeric enzyme $CF_1$ is stabilized by ATP and divalent cations, e.g. $Mg^{2+}$, pH-dependent subunit-subunit interactions, and possible salt links between the subunits and subunit and ATP. Furthermore, the swollen $CF_1$ looses essentially all traces of metal ions in the presence of EDTA and becomes sensitive to protease attack (Paradies, unpublished results).

Furthermore, the crystal form of $CF_1$ reported here is apparently different from the crystals obtained from $F_1$ from beef heart (19) since the optical diffraction pattern together

with the additional X-ray diffraction pattern (19) show the presence of odd order equatorial reflections in contrast to the proposed symmetry obtained by Spitzberg and Haworth (19) from optical diffraction patterns alone, as well as the crystalline preparation from the $F_1$ portion of the equivalent enzyme system from the thermophilic bacterium TS 3 (20).

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