A PRELIMINARY X-RAY CRYSTALLOGRAPHIC STUDY OF SPINACH CYTOCHROME *c*

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1. Introduction

Recent studies on plant cytochrome *c* have shown some characteristic features in the primary structures and properties in comparison with those of animal and microbial origins [1–3]. A further understanding of these characteristics is expected by the elucidation of the three-dimensional structure with X-ray crystallography. The three-dimensional structures of horse and bonito cytochrome *c* were presented by Dickerson et al. [4] in their X-ray crystallographic studies at 2.8 Å resolution, while, on plant cytochrome *c* the first preliminary study was reported by Morita and Ida [5] on the protein from rice embryos, and the structural study is now under way. Recently Asada and Takahashi [6] purified cytochrome *c* from spinach leaves in the course of the simultaneous purification of peroxidases, ferredoxin, ferredoxin-NADP reductase and sulfite reductase, and they obtained the protein in a crystalline form, which is the fourth crystalline cytochrome *c* following after the proteins from wheat germ, corn pollen and rice embryo or bran [7–10]. The crystals of spinach cytochrome *c* have grown to the size suitable for the X-ray crystallographic analysis. The present paper describes a preliminary X-ray analysis of spinach cytochrome *c*.

2. Materials and methods

Spinach cytochrome *c* was isolated and purified by Asada and Takahashi [6]. The purification was attained by fractional precipitations with acetone and with ammonium sulfate, ion-exchange chromatography on DEAE-Sephadex A-50 and on CM-Sephadex C-50, gel filtration chromatography on Sephadex G-75 and on Sephadex G-100, and crystallization from ammonium sulfate solution. The recrystallized preparation showed the absorbance ratio, A_{410}/A_{278}, of more than 4.0. The crystalline cytochrome *c* was dissolved in 0.01 M phosphate buffer, pH 7.0, at the concentration of about 0.3% (w/w) and the solution was saturated with ammonium sulfate. Large crystals (0.4 × 0.5 × 0.8 mm) were formed in a cold room after a month. Alternatively, the protein was dissolved in water (about 1% concentration) and dialyzed against 3.2 M ammonium sulfate solution containing 0.78 M sodium chloride by means of a Zeppezauer's dialysis apparatus (3 mm in inner-diameter) [11]. Large crystals appeared at 4° after a week, and they exhibited the same crystal habits as those of the above crystals though the long axis of the former was elongated more than the latter.

The crystals were once transferred into 95% saturated ammonium sulfate solution containing 0.01 M sodium phosphate buffer, pH 7.0, and were mounted in thin-walled glass capillaries in the usual manner. The X-ray photographs were taken by a Buerger precession camera using Ni-filtered CuKα radiation at room temp.

3. Results and discussion

The microphotograph of octahedral crystals of spinach cytochrome *c* is shown in fig. 1. The size is

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Fig. 1. Microphotograph of crystals of spinach cytochrome *c*.

more than 0.3 X 0.4 X 0.8 mm, and the faces of {011} and {110} groups are well developed, the schematic crystal habits being shown in fig. 2. Reflections were observed out to at least 1.8 Å but with apparent change in intensities at the outer spacing after

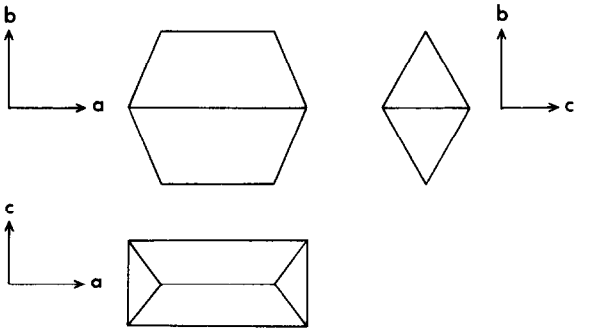

Fig. 2. Orthorhombic crystal of spinach cytochrome *c*.

only 40 hr irradiation unlike the case of hexagonal crystals of rice cytochrome *c*. On the latter protein no apparent change in intensities was observed over 150 hr irradiation. A typical 17° precession photograph with the X-ray beam parallel with the *c* axis is shown in fig. 3, giving *hk*0 zone. Similar photographs were taken with the X-ray beam parallel with the other two axes, *b* and *a*, giving *h*0*l* and 0*kl* zones, respectively, and the former is shown also in fig. 3. The photographs of these principal zones and upper layers showed *mmm* symmetry indicating an orthorhombic unit cell. There were no systematic absences, and along two principal axes, *h* and *k*, only even-orders of

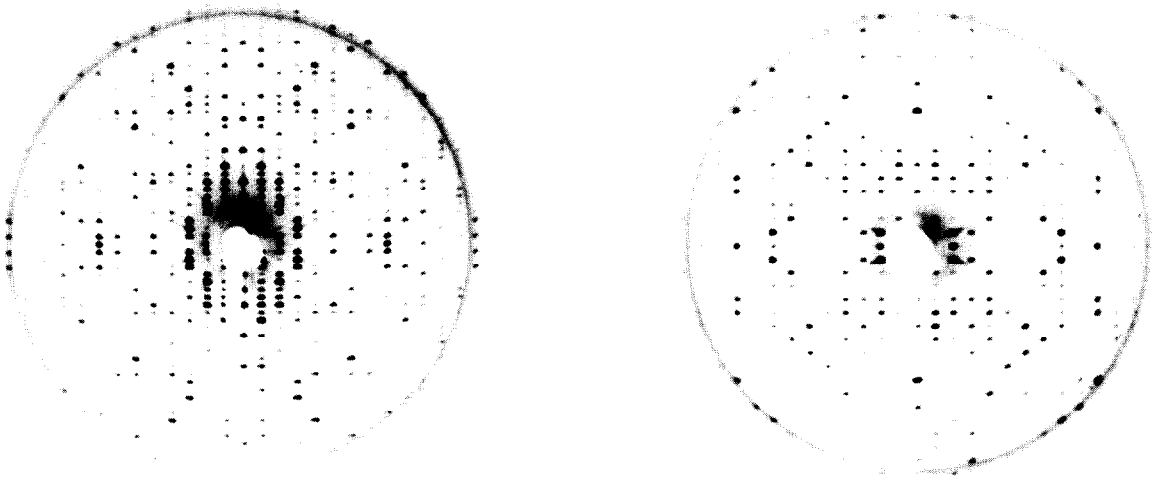

Fig. 3. Precession photographs (17°) of (a) *hk*0 and (b) *h*0*l* zones of spinach cytochrome *c*. The photographs were exposed for 20 hr.

reflections were observed, so that the space group is unambiguously identified as $P2_12_12$. Cell dimensions are a = 34.9 Å, b = 80.7 Å and c = 46.1 Å, and the volume of the unit cell is 130,000 Å^3. The density of the crystals measured at 20° by the density gradient method was 1.26 g/cm^3. These values give the asymmetric unit weight of 24,700 daltons. Therefore, it is concluded that one asymmetric unit contains one molecule of cytochrome c and the percentage of the solvent in the crystal is 48.6% (w/w), assuming a molecular weight of 12,700 daltons.

The crystals were able to be transferred from 95% saturated ammonium sulfate solution into 4.5 M phosphate, pH 6.8, without breaking crystals as in the cases of horse and rice cytochrome c [4, 5]. However, on the contrary to the latter two cases, the exchange of mother liquor for spinach cytochrome c crystals caused considerable changes of the diffraction patterns. Thus, although the symmetry of the crystals, $P2_12_12$, did not change, the cell shrank along two axes. The unit cell dimensions are a = 34.7 Å, b = 79.7 Å, c = 46.1 Å and V = 127,500 Å^3.

Although we failed to make derivatives with $PtCl_4^{2-}$ and mersalyl in both ammonium sulfate and phosphate solutions, a considerable change in intensities of diffraction spots was observed by soaking the crystals in 95% saturated ammonium sulfate solution containing 8 mM $K_2UO_2F_5$ for a week. We are continuing the analysis to determine the structure of the protein at high resolution.

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