Communication

Crystallization of Tuna Ferricytochrome c at Low Ionic Strength*

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Mark H. Walter, Edwin M. Westbrook, Scott Tykodi, Andrew M. Uhm, and Emanuel Margoliash

From the Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208 and the Argonne National Laboratory, Biological and Medical Research Division, Argonne, Illinois 60439

Previous crystallographic studies of tuna ferricytochrome c have employed crystals grown from solutions of ammonium sulfate, corresponding to an ionic strength of 9.5 M (Takano, T., and Dickerson, R. E. (1981) J. Mol. Biol. 153, 95–115). To obtain a structure at a lower ionic strength, the ferric tuna protein was crystallized at neutral pH with polyethylene glycol at an ionic strength of 45 mM. These crystals (space group P21, a = 37.11 Å, b = 107.66 Å, c = 55.75 Å, β = 105.3°) contain four molecules/asymmetric unit and grow to dimensions of 0.2 × 0.4 × 1.0 mm in 2–4 weeks. They diffract to beyond 1.8 Å and are stable in the x-ray beam. We have recorded 28,198 unique Bragg reflections (83% of those possible) to a resolution of 1.89 Å from a native crystal. We are undertaking a solution of this structure by the molecular replacement method.

Eukaryotic cytochrome c is a small highly charged water-soluble protein of the mitochondrial intermembrane space. A major function of this protein is to transport electrons between cytochrome c reductase and cytochrome c oxidase, integral proteins of the mitochondrial inner membrane (1). Three-dimensional crystallographic studies have been completed for cytochrome c from horse (2–4), bonito (5–9), tuna (10–17), rice (18), and bakers' yeast (19). While these studies have been conducted at physiological temperatures and pH values, the ionic strengths employed ranged from 7.5 M (20) to 12.5 M (2). The ionic strength for the tuna ferricytochrome c structure determination was 9.5 M (13). These values are far removed from both the physiological ionic strengths of approximately 50 mM and from ionic strengths of 25–100 mM, which are commonly used for most of the experiments which have characterized the function of the protein (1, 21, 22).

The results of visible (23–29), Raman (30), and NMR (31–33) spectroscopy and small angle x-ray scattering (34) suggest that the structures of cytochrome c at high and low ionic strength are not identical. For example, Goldkorn and Schejter (27) studied the reduction of horse heart cytochrome c by ascorbate and reported biphasic kinetics at an ionic strength of 4 mM and monophasic kinetics at an ionic strength of 104 mM. From this result and the observed influence of ionic strength upon the Soret band, the authors postulated an ionic strength-dependent equilibrium between conformers which differ by small structural rearrangements. Similarly Osheroff et al. (28) found that the pK of the 695-nm band of ferricytochrome c increased from less than 9 to greater than 9.4 as the ionic strength increased from 10 to 500 mM. This 695-nm band is representative of a structure present in the normal low spin functional conformation of cytochrome c in which the sulfur atom of methionine 80 coordinates to the central heme iron atom (35). From this result, these authors argued that the stability of the heme crevice increased as the ionic strength increased, as would be expected from the protein's high net positive charge. Wand et al. (32), measuring the hydrogen-deuterium exchange rate for cytochrome c by NMR found the carboxyl end of the N-terminal α-helix to be partially unfolded at an ionic strength of approximately 75 mM. Liu et al. (30), applying ultraviolet resonance Raman spectroscopy to ferricytochrome c in neutral solutions at ionic strengths of 5 mM and 1.5 M, found that the α-helical content remains essentially the same but that the hydrogen bonding environment of a tyrosine, tentatively assigned as tyrosine 48, becomes weaker at the lower ionic strength. Liu et al. (30) also studied the hydrogen-deuterium exchange rate of tryptophan 59 at ionic strengths of 5 and 900 mM and found the rate of exchange for ferricytochrome c to decrease by a factor of 7.5 as the ionic strength was increased. Trewhella et al. (34), employing small angle x-ray scattering, observed that the radius of gyration of ferricytochrome c decreased by 8% when the ionic strength was increased from approximately 10 mM to approximately 210 mM.

To examine the possible effects of ionic strength on the structure of the protein, we have crystallized cytochrome c with PEG1 in a neutral phosphate solution at an approximate ionic strength of 45 mM. Analysis of these crystals will provide a structure for cytochrome c under conditions commonly used to characterize its properties. This paper presents a procedure for low ionic strength crystallization, the characterization of these crystals, and the collection of x-ray diffraction data. Preliminary accounts of this work have been presented (36–38).

EXPERIMENTAL PROCEDURES

Protein Purification and Crystallization—Cytochrome c was extracted from tuna hearts, purified by the method of Brautigan et al. (39), and crystallized by the hanging drop method (40, 41). A systematic examination was conducted of the influence on crystallization behavior of protein concentration, buffer, pH, nature and molecular

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One of us (M. H. W.) dedicates this work to Matilda Wyscarver.

§ To whom correspondence should be addressed: Dept. of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, 2153 Sheridan Rd., Evanston, IL 60208.
weight of precipitant, temperature, counterion, and species of cytochrome c. We found that a 35 mg/ml solution of tuna ferricytochrome c in 30 mM pH 6.8 sodium phosphate buffer containing 30% (w/v) PEG 1000 at 18 °C yielded crystals suitable for high resolution x-ray diffraction analysis.

Calculations—Calculations of ionic strength, I, were performed using

\[ I = \frac{1}{2} \sum c_i (Z_i)^2 \]  

where \( c_i \) and \( Z_i \) represent the concentration and charge of the \( i \)th ionic species, respectively. For ionic strength calculations, ammonium sulfate, ascorbic acid, and sodium nitrate were assumed to be completely ionized, and the contribution of the phosphate ion (42) was included, while the contribution from the cytochrome c remaining in solution after crystallization, which may be significant (43), was not included in the calculations. In addition, the low ionic strength calculations included the use of activity coefficients to determine the activity of the phosphate ions (44). The molecular weight, \( M_c \), of tuna cytochrome c was calculated from the amino acid sequence (45) and assumed neutral amino acid side chains, a protonated carboxyl terminus, and protonated heme propionic acid side chains.

Crystal Characterization—Density measurements were performed in both a benzene/1,2-dichloroethane gradient (46, 47) and in an aqueous Ficoll gradient (48, 49). The crystal volume fraction occupied by protein and the number of molecules/unit cell were determined by Equation 1 of Westbrook (48), employing a unit cell volume of 2.15 \( \times 10^9 \) \( \text{Å}^3 \), a \( \rho_p \) = 0.715 cm\(^3\)/g (for horse heart cytochrome c) (50), an \( M_c = 12,030 \), and assuming the density of solvent was 1.00 for the density determined in the Ficoll gradient. For storage, crystals were transferred to a 40 mM pH 6.8 sodium phosphate buffer containing 30% (w/v) PEG 1000. Precession photographs (51) were recorded of the (hk0) and (0kl) zones, with precession angle of 16°, a film distance of 75 mm, and an exposure time of 36 h with graphite monochromated CuK\( \alpha \) x-rays from a rotating anode x-ray generator operating at 1.6 kilowatts. Three-dimensional diffraction data were recorded at the Midwest Area Detector Facility of Argonne National Laboratory to 1.89-Å resolution with a Nicolet/Xentronics multwire electronic area detector (52) at a crystal to detector distance of 14.0 cm. Two orientations of a single crystal were used to record data frames of 0.2° rotation about the vertical axis. These data were processed by the Xegen package (53) of computer programs.

RESULTS AND DISCUSSION

Shown in Fig. 1 are the monoclinic low ionic strength crystals of tuna ferricytochrome c, which grew to a size of 0.2 \( \times \) 0.4 \( \times \) 1.0 mm within 2–4 weeks, were stable in the x-ray beam, and diffracted to beyond 1.8 Å. X-ray precession photographs (Fig. 2) demonstrated that the crystals belonged to the Laue group 2/m. Systematic absences of all (0kl) reflections for which \( h + k = \text{odd} \) established the space group as P2\( _1 \). The monoclinic unit cell dimensions and angles were \( a = 37.11 \) Å, \( b = 107.66 \) Å, \( c = 55.75 \) Å, and \( \beta = 105.3° \).

The crystal and solvent densities as determined in the organic gradient were 1.173 and 1.07 g/ml, respectively, and implied that protein occupied 31% of the crystal volume and that 4.7 molecules occupied each unit cell. If one assumes four molecules/unit cell, the value of \( V_m \), 4.50 \( \text{Å}^3/\text{dalton} \), is clearly an unusual value, beyond the range commonly observed for protein crystals (54). In contrast, the crystal density as determined in the aqueous gradient was 1.219 g/ml and implied a protein volume fraction of 55% and 8.3 molecules/unit cell.

### Summary of diffraction data

| Parameters                  | Minimum \( d \) spacing (Å) of shell |
|-----------------------------|-------------------------------------|
|                            | 3.43  | 2.72  | 2.38  | 2.16  | 2.00  | 1.89  | Total |
| Reflections possible       | 5,575 | 5,709 | 5,680 | 5,653 | 5,683 | 5,694 | 34,174 |
| Reflections collected      | 5,273 | 5,564 | 5,286 | 5,088 | 4,632 | 2,355 | 28,198 |
| Reflections collected, %   | 91.6  | 97.5  | 93.1  | 90.0  | 81.5  | 41.4  | 82.5  |
| Reflections multiply observed, % | 60.8  | 59.6  | 48.1  | 40.6  | 30.0  | 17.5  | 45.9  |
| Mean intensity (counts)    | 3,609 | 1,272 | 498   | 285   | 175   | 92    | 1,107 |
| Mean \( f_0 \)             | 81.46 | 33.13 | 14.88 | 8.46  | 5.00  | 2.65  | 27.12 |
| \( R_{sym} \)              | 3.03  | 5.66  | 9.70  | 12.25 | 17.48 | 24.54 | 4.69  |
Assuming eight molecules/unit cell, the percent protein and $V_m$, 2.25 Å²/dalton, values fall within the ranges previously observed for most protein crystals (54).

Table I summarizes the data collection statistics. From a single crystal we have recorded 28,198 unique Bragg reflections, 83% of those possible within the 1.89 Å resolution sphere. The $R$-sym was 4.69% on intensity.

As shown in Table II, with the exception of precipitant, similar conditions were employed for the low and high ionic strength crystallizations. Also similar were the portion of the crystal volume occupied by protein and the packing of eight molecules into the unit cell.

We have also crystallized tuna ferrocytochrome c as well as fungal and mammalian cytochromes c under low ionic strength conditions. Analyses of these crystals are also in progress.

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