SHORT COMMUNICATIONS

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Characterization of two crystal forms of cytochrome c from *Valida membranaefaciens*.

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Abstract

Two different crystal forms of cytochrome c from the yeast *Valida membranaefaciens* have been grown by vapor diffusion from polyethylene glycol 4000. Both have been characterized by X-ray diffraction. The first crystal form was grown at 277 K but proved unstable and dissolved when the temperature was increased above 288 K. This crystal was of triclinic space group $P1$ and had unit-cell dimensions of $a = 68{\cdot}4$, $b = 35{\cdot}7$, $c = 33{\cdot}24$ Å, $\alpha = 116{\cdot}9$, $\beta = 114{\cdot}9$, $\gamma = 75{\cdot}0°$. Following dissolution of the triclinic crystals, the second crystal form also grew at 277 K but remained stable even at room temperature. It was of orthorhombic space group $P2_12_12_1$ with cell dimensions $a = 27{\cdot}95$, $b = 63{\cdot}68$, and $c = 64{\cdot}28$ Å. Although rather small in size, both crystal forms diffracted X-rays well and the resolution of the patterns extended beyond 3 Å.

Introduction

Cytochrome c is a ubiquitous electron-transport protein of about $M_r = 14\,500$, existing as monomers of 80 to 135 amino-acid residues and containing a covalently bound protoporphyrin IX heme prosthetic group. The amino-acid sequences have been obtained for the protein from a vast number of organisms and the rigorous conservation of nearly 50% of its residues has made it a model for molecular evolutionary studies (Dickerson, 1980). The structures of cytochrome c molecules from a number of organisms have also been determined by X-ray diffraction analyses and these demonstrate the structural conservation of its characteristic heme-binding pocket as well as a number of other features. Currently, the protein is under study as a model for directed modification of sequence-structure elements (Hampsey, Das & Sherman, 1986; Louie, Hutcheon & Brayer, 1988).

We have crystallized the cytochrome c from *Valida membranaefaciens* which is an ethanol utilizing yeast of molecular weight $M_r = 12\,500$ bearing many similarities to mammalian cytochrome c. We have characterized those crystals, which we describe below, and are now proceeding with a molecular-replacement solution of the crystal structure. Such a study should contribute further to our understanding of this unique class of protein molecules.

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Experimental

Lyophilized protein was dissolved in distilled water to a concentration of 20 mg ml^{-1} and stored at 253 K. This stock solution was used for all screening and optimization experiments. Crystallization trials designed to identify conditions that would yield crystals of the cytochrome c were carried out according to the procedures described by McPherson (1990) with initial attention focused on precipitant type and concentration, pH, and temperature. Nine-well glass depression plates were used for vapor diffusion trials in sealed plastic boxes. In each well of the depression plates, 16 μl droplets of protein solution were equilibrated against 25 ml reservoir solutions. Parallel experiments were initially conducted at 277 and 295 K. During the course of the experiments, it became evident that 277 K was far more productive than 295 K and the warmer temperatures were ultimately abandoned. No crystals were ever obtained using any salt as a precipitating agent, no crystals were ever obtained when any but the lowest ionic strength was employed.

All crystal forms were obtained under essentially the same conditions. The optimal crystallization trials were comprised of 7 μl of stock protein mixed with 2 μl of 0·1 M Tris-HCl buffer at pH 8·0 to 8·5 and 7 μl of reservoir solution which was 25% polyethylene glycol 4000 in H_2O with no buffer added. Crystals generally appeared only after 2–4 weeks.

For preliminary characterization, crystals were mounted and sealed in quartz capillaries along with a small amount of mother liquor. Photographs of the reciprocal lattice were recorded on a Buerger precession camera using nickel-filtered Cu $K\alpha$ radiation produced by an Enraf Nonius generator fitted with a fine-focus tube operated at 45 kV and 32 mA. Three-dimensional X-ray diffraction data were collected on a San Diego Area Detector Systems two panel multiwire detector with monochromated Cu $K\alpha$ radiation produced by a Rigaku RU-200 rotating-anode generator operated at 45 kV and 150 mA.

Results

In virtually every case where crystals were obtained, the first, and sometimes only, crystals observed in the samples were thin, apparently rectangular plates or vast arrays of these plates, like those seen in Fig. 1(*a*), growing as thin

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scales. The largest of these occasionally had dimensions as large as 0·5 × 0·3 × 0·2 mm but were generally smaller. An example is seen in Fig. 1(*b*). After a period of time these crystals were occasionally observed to redissolve over a period of a few days and a second crystal form to appear. The second crystal habit was that of a rectangular prism. These seldom grew large and were typically 0·4 × 0·2 × 0·2 mm in size or less. The crystals were, however, stable for a long period of time and never exhibited morphological transformation.

When the initial crystal form was investigated with X-rays, an extensive exploration of its reciprocal lattice failed to reveal the presence of any symmetry save that relating Friedel pairs. The observed reflections could only be indexed on a triclinic lattice consistent with space group $P1$ and this had corresponding real unit-cell dimensions of $a = 68{\cdot}4$, $b = 35{\cdot}7$, $c = 33{\cdot}2$ Å, $\alpha = 116{\cdot}9$, $\beta = 114{\cdot}9$, $\gamma = 75{\cdot}0°$. The volume of the unit cell is $V = 6{\cdot}8 \times 10^4$ Å^3. If two molecules of cytochrome c of 14 500 daltons each are assumed as the asymmetric unit then the volume to mass ratio for the unit cell is $V_m = 2{\cdot}34$ Å^3 dalton^{-1}. This is near the center of the range observed for most protein crystals (Matthews, 1968) and is most probably correct.

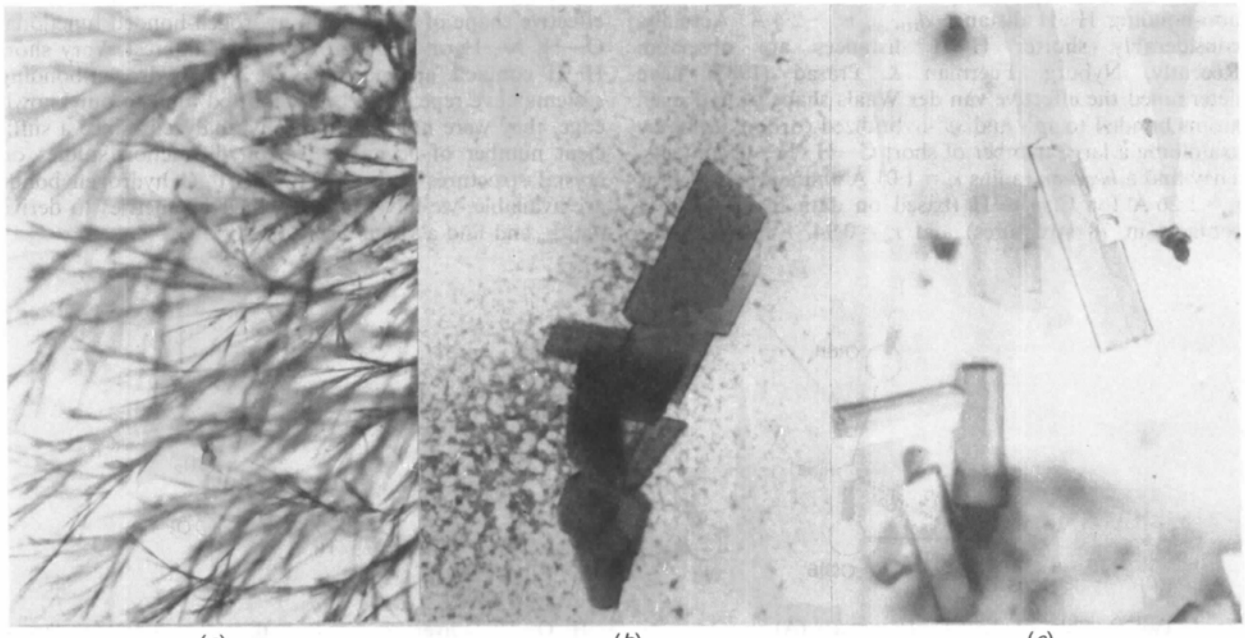
These triclinic crystals diffracted strongly and beyond 2·8 Å resolution but were physically unstable. Mechanical manipulation or warmer temperatures caused them to rapidly deteriorate in the X-ray capillary. In addition, they decayed far more in the X-ray beam than might be expected for most protein crystals.

When the triclinic crystals dissolved spontaneously at 277 K, rectangular prismatic crystals, like those seen in Fig. 1(*c*), subsequently formed. These crystals were also investigated by X-ray photography and proved stable at room temperature, and also quite resistant to X-ray exposure over several days. Exploration of the reciprocal lattice of these crystals demonstrated it to contain three perpendicular mirror planes and the reflections to fall on an orthogonal net. The reciprocal lattice exhibited *mmm* symmetry. Axial reflections were systematically absent when $h00$, $0k0$ or $00l \not\parallel 2n$. There were no other systematic absences. The orthorhombic space group is, therefore, $P2_12_12_1$ and the unit-cell dimensions are $a = 27{\cdot}95$, $b = 63{\cdot}68$, $c = 64{\cdot}28$ Å.

The volume of the orthorhombic unit cell is $V = 1{\cdot}14 \times 10^5$ Å^3 and by the same arguments presented above for the triclinic crystals, we conclude that there is one molecule of cytochrome c in the asymmetric unit. This yields a value for the volume to mass ratio of $V_m = 2{\cdot}4$ Å^3 dalton^{-1}. These crystals diffract to beyond 3 Å but suffer from their small size. Nevertheless, using the area detector system, a three-dimensional diffraction data set of 3200 reflections to about 3 Å resolution has been recorded from the crystals.

Discussion

While the triclinic crystals appear ill suited for three-dimensional analysis owing to their instabilities, the orthorhombic crystals are adequate. The data set currently in hand provides us with the substance of a molecular-replacement search. We are proceeding along these lines using the known structure of yeast cytochrome c_5 as a search model. At the same time we will continue efforts to grow larger crystals of the othorhombic form in order to extend the analysis to a higher solution.



(*a*) (*b*) (*c*)

Fig. 1. (*a*) An example of the extensive tree-like arrays of small triclinic scales. (*b*) A group of triclinic single crystals. The triclinic crystals are frequently seen to dissolve over time and the orthorhombic prisms seen in (*c*) appear in their place.

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H···H van der Waals distance in cooperative O—H···O—H···O hydrogen bonds determined from neutron diffraction data.

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Abstract

The shortest possible H···H distance in hydrogen bonds of the cooperative type O—H···O—H···O has been determined from 23 neutron diffraction crystal structures of carbohydrates. The value of $d_{HH,min}$ ~2·05 Å is the same for all combinations of hydroxyl and water donors and acceptors and for all hydrogen-bonding geometries observed, and significantly shorter than the 2·4 Å based on classical van der Waals radii.

Indroduction

In classical and still widely accepted concepts, the shape of a bonded hydrogen atom is that of a sphere with radius r = 1·2 Å, see, for example, Bondi (1964). This, however, implies that in crystal structures, the shortest possible non-bonding H···H distance $d_{HH,min}$ is ~2·4 Å. Actually, considerably shorter H···H distances are observed. Recently, Nyburg, Faerman & Prasad (1987) have determined the effective van der Waals shape of hydrogen atoms bonded to sp^3- and sp^2-hybridized carbon atoms by examining a large number of short C—H···H—C contacts. They find a *head-on* radius r_h = 1·01 Å and a *side-on* radius r_s = 1·26 Å' for C(sp^3)—H (based on data for 500 H···H contacts in 78 structures) and r_h = 0·94, r_s = 1·32 Å for C(sp^2)—H (from 418 H···H contacts in 88 structures) ('*polar flattening*'). As a consequence, $d_{HH,min}$ depends on the orientation of the C—H groups: for C(sp^3)—H it is about 2·0 Å for head-to-head and over 2·5 Å for side-to-side contacts. The method for data evaluation is described in Nyburg & Faerman (1985). More recently, Ikuta, Ishikawa, Katada & Sano (1990) have theoretically studied the polar flattening of the H atom in several small molecules using Hartree Fock-type methods. They predict a linear relation of r_h and r_s with the Mulliken partial charge on the H atom: if the partial (positive) charge increases, both radii decrease and the 'flattening' $r_s - r_h$ increases. For the extreme case of H—F, theoretical values of r_h = 0·75 and r_s = 1·08 Å were determined.

These studies are restricted to H atoms *not* involved in hydrogen bonding. As van der Waals radii are an important factor in the characterization of a hydrogen bond, the effective shape of H atoms in hydrogen-bonded functional O—H, N—H or S—H groups are of interest. Very short H···H contacts around d_{HH}~2·1 Å in hydrogen-bonding systems have repeatedly been reported, but, to our knowledge, they were not systematically analyzed. Since a sufficient number of accurate neutron diffraction studies on crystal structures with O—H···O—H···O hydrogen bonds are available, we have analyzed their geometries to derive $d_{HH,min}$ and find a value of ~2·05 Å.

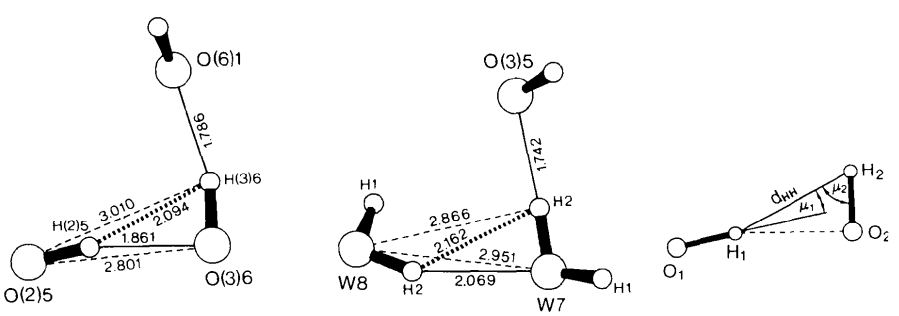

Fig. 1. Two examples of short H···H contacts (Å) in cooperative O—H···O—H···O hydrogen bonds, both in β-cyclodextrin–ethanol octahydrate, neutron diffraction at T = 15 K, Steiner *et al.* (1990). Water molecules are labelled as W, hydroxyl groups as O(m)n, H(m)n, where n is the number of the glucose residue in the cyclodextrin molecule and m the number of the hydroxyl group in the given residue. Standard deviations are between 0·006 and 0·009 Å.

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