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# Crystallization of Wild-Type and Mutant Ferricytochromes *c* at Low Ionic Strength: Seeding Technique and X-ray Diffraction Analysis*

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## Abstract

Ferricytochromes *c* were crystallized at low ionic strength by macroseeding techniques. Large crystals were grown by seed-induced self-nucleation which occurred anywhere in the drop, regardless of the location of the seed crystal. This unusual crystal-seeding method worked reproducibly in our hands, and X-ray quality crystals have been prepared of several ferricytochromes *c*: horse, rat (recombinant wild type), and two site-directed mutants of the latter, tyrosine 67 to phenylalanine (Y67F) and asparagine 52 to isoleucine (N52I). Crystals of any one of these four proteins could be used as seeds for the crystallization of any one of the others. All the crystals are of the same crystal form, with space group $P2_12_12_1$. There are two protein molecules per asymmetric unit. The crystals are stable in the X-ray beam and diffract to at least 2.0 Å resolution. Full crystallographic data sets have been collected from single crystals of all four proteins.

## Introduction

Eukaryotic cytochrome *c* is a small heme protein, present in the intermembrane space of mitochondria. Cytochrome *c* transports electrons between its physiological reductants (cytochrome *c* reductase, cytochrome $b_5$ and cytochrome $b_2$) and oxidants (cytochrome *c* oxidase and cytochrome *c* peroxidase) (Margoliash & Schejter, 1966; Pettigrew & Moore, 1987; Moore & Pettigrew, 1990). Since the 1960's, the crystal structures of the cytochromes *c* from a wide taxonomic range of eukaryotes have been solved and refined by X-ray diffraction methods. These include the proteins from horse (Dickerson *et al.*, 1971; Bushnell, Louie & Brayer, 1990), bonito (Tanaka, Yamane, Tsukihara, Ashida & Kakudo, 1975), tuna (Takano & Dickerson, 1981*a*,*b*), rice (Ochi, Hata, Tanaka & Kakudo, 1983), and yeast (Louie, Hutcheon & Brayer, 1988; Louie & Brayer, 1990; Berghuis & Brayer, 1992). All these structures were obtained from crystals grown at the extremely high salt concentrations of near-saturated ammonium sulfate.

There are numerous indications that the structural properties of cytochrome *c* differ at high and low ionic strengths. Such differences have been detected by visible (Goldkorn & Schejter, 1977; Osheroff, Borden, Koppenol & Margoliash, 1980), Raman (Liu, Grygon & Spiro, 1989) and NMR (Rush, Koppenol, Garber & Margoliash, 1988) spectroscopic studies, as well as by small-angle X-ray scattering (Trewhella, Carlson, Curtis & Heidorn, 1988). However, other investigators have reported little or no essential differences between the structures of proteins at low and high ionic strengths. These include, for example, the NMR study of horse cytochrome *c* (Feng & Englander, 1990) and the X-ray crystallographic study of T4 lysozyme at low, medium and high ionic strengths (Bell *et al.* 1991). Obviously, such conclusions depend on what structural features the investigator considers to be significant for the protein in question. In the case of cytochrome *c* for instance, the lower and upper

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heme-crevice bonds – the interactions between the side chain of Lys79 and the backbone of Thr47, and those between the side chains of Glu90 and Lys13 – are thought to be important for maintaining the native active conformation of the protein. When they are disrupted by chemical modification (Osheroff, Borden, Koppenol & Margoliash, 1980) or site-directed mutation (Crump, 1993), there are large decreases in the rates of reaction with physiological electron-exchange partners. Being on the protein surface, these heme-crevice bonds could easily be subjected to the influence of changes in the ionic strength of the solvent. Emphasizing the importance of salt bridges on the protein surface is the observation (Horovitz, Serrano, Avron, Bykroft & Fersht, 1990) that, in general, they act in a cooperative fashion, thus, like other types of interactions in proteins, playing an important role in structure stabilization. Since their strength will depend on the electrostatic field, their number and total energy are likely to differ at low and high ionic strengths. The electrostatic field of cytochrome *c* itself is important for its productive interaction with redox partners (Rush & Koppenol, 1988) since it orients the former with respect to the latter. Therefore, not only the existence of a given charge, but also its location on the surface of cytochrome *c* before interaction may have an impact on the activity of the protein. X-ray crystallographic structures in general show that some charged side chains on the protein surface are highly disordered, and could be mobile, while others are fixed well. For a small protein such as cytochrome *c* in which about a third of the residues are charged, it is likely to be important to distinguish between those which are mobile and those which are fixed and localize the latter side chains under conditions close to physiological ionic strength. For cytochrome *c*, it is important to know the conformation of residue side chains, particularly for those which define solvent accessibility to the heme, those which are involved in interaction with redox partners, and those which determine the electrostatic field of the protein.

Feng & Englander (1990) reported that the structure of horse cytochrome *c* is not affected by salt concentration except at amino-acid positions 61, 83, 87, 88 and 89, at which conformational changes at one or more backbone atoms are 'considered clearly significant.' It is interesting to note that in the recent crystal structures of cytochrome *c*–cytochrome *c* peroxidase (C*c*P) complexes (Pelletier & Kraut, 1992), determined at low ionic strength, many of the residues (Lys86, Lys87 and residues 81–83) found by Feng & Englander (1990) to have salt-dependent conformations, are observed in tight contact with the C*c*P molecule. Other residues of cytochrome *c* are observed with conformations in the complex that differ from those in the free form, crystallized at very high salt concentrations; among these Gln16, Lys13 and Glu90 show changes considered to be due to differences in ionic strength of the crystallization media. It is remarkable that Lys13 and Glu90 form the upper heme-crevice bond, and Gln16 and Lys13 are in the interface between the two proteins in the complex. Clearly, the structure of the cytochrome *c* at low ionic strength is required to determine whether the differences are due to ionic strength or to complexation.

Even the behaviour of some internal residues in cytochrome *c* appears to be affected by the ionic strength of the medium (Liu, Grygon & Spiro, 1989). Indeed, Tyr48 shows loss of hydrogen bonding to the heme in ferric horse cytochrome *c* at an ionic strength of 0.005 *M* as compared to one of 1.5 *M*, and the hydrogen/deuterium exchange rate for Trp59 increases for the ferric and ferrous proteins as the ionic strength increases from 0.005 to 1.5 *M*. These internal residues are most likely to contribute to the 'cytochrome-*c* fold' and both interact with the heme, emphasizing their importance.

Finally, the highest salt concentrations used in the above studies were much lower than those for the crystallization of cytochromes *c*. Thus, in the NMR study of Feng & Englander (1990), it was limited to 210 m*M*, and to $\sim 2.5$ *M* in the case of the X-ray study of T4 lysozyme at different ionic strengths (Bell *et al.*, 1991). However, cytochromes *c* have been crystallized in the presence of at least 90% saturated ammonium sulfate (5 *M*), which is 15 *M* in ionic strength, and containing 0.5–0.8 *M* additional salts. Rice cytochrome *c* was crystallized at somewhat lower ionic strength, $\sim 11$ *M* (Ochi, Hata, Tanaka & Kakudo, 1983). It is possible that at such high levels above physiological, and for a protein which is as highly charged as cytochrome *c*, even more differences between the structures at high and low ionic strength exist, than have been detected so far.

Up to the present work, only one example of the crystallization of a cytochrome *c* (tuna) at low ($\sim$45 m*M*) ionic strength has been reported (Walter, Westbrook, Tykodi, Uhm & Margoliash, 1990). We consider it important to develop a generally applicable procedure to crystallize cytochromes *c* at low ionic strength, since we presume that such structures are likely to resemble the functional protein, and be more closely related to the structure in complexes with Fab fragments, which are also crystallized at low ionic strength (Jemmerson *et al.*, 1994).

In this paper we report the crystallization of four cytochromes *c*, the natural horse protein, recombinant rat wild-type cytochrome *c* and two site-directed mutants of the latter – Asn52 to Ile (N52I) and Tyr67 to Phe (Y67F). The horse and rat protein structures are required for studies of their interaction

with Fab fragments of monoclonal antibodies. Even though these cytochromes differ by only five residues, one of these changes is on an extremely immunogenic surface, and appears to have a significant effect on the antigen–antibody interaction (Jemmerson, Mueller & Flaa, 1993). The rat cytochrome *c* and its mutants are being studied in connection with some interesting thermodynamic properties (Luntz, Schejter, Garber & Margoliash, 1989; Hickey *et al.*, 1991; Schejter *et al.*, 1994) and their relation to a buried water molecule held next to the heme crevice by hydrogen bonds to the side chains of Asn52, Tyr67 and Thr78 (Takano & Dickerson, 1981*b*; Louie & Brayer, 1990; Bushnell, Louie & Brayer, 1990).

## Experimental

Commercial horse cytochrome *c* (Sigma), purified by cation-exchange chromatography in the ferric form on CM-cellulose (Brautigan, Ferguson-Miller & Margoliash, 1978), and stored for several years at 253 K, was repurified in the ferrous form by HPLC (Waters). A 21.5 × 150 mm cation-exchange column (Waters SP 5PW) and a gradient of 10–250 m*M* of potassium phosphate pH 7.0, containing 2 m*M* dithiothreitol were employed. As in other cation-exchange chromatography of cytochrome *c* (Margoliash & Walasek, 1967; Brautigan, Ferguson-Miller & Margoliash, 1978) the trace of deamidated protein separated in front of the native monomeric major component, which was followed by small amounts of dimeric, trimeric and succeeding polymerized forms, in that order. Recombinant rat wild-type cytochrome *c* was prepared and purified as described previously (Koshy, Luntz, Garber & Margoliash, 1992) and the mutant rat proteins according to Luntz, Schejter, Garber & Margoliash (1989) and Schejter *et al.* (1994). For crystallization of the recombinant proteins, fraction II was utilized. This chromatographic fraction amounts to about 75% of the whole preparation and contains the protein which is not acetylated at its N terminus (Koshy, Luntz, Garber & Margoliash, 1992). The purified protein was dialyzed against 10 m*M* phosphate buffer pH 7.0, concentrated by adsorption onto a 7 × 35 mm column of carboxymethylcellulose, and oxidized on the column with minimal ferricyanide. The column was then washed thoroughly with 10 m*M* phosphate pH 7.0 and the protein eluted in a concentrated solution with the same buffer at 250 m*M*. Final concentration or dilution, as required for crystallization and equilibration to the required ionic strength, was performed by centrifugation, using Centricon 10 microconcentrator units (Amicon). The concentrations of ferricytochrome *c* were determined on a Hitachi 557 spectrophotometer at 410 nm ($\varepsilon_{mM}$ = 106.1). PEG's of different molecular weights (1, 4, 6, 10 kDa Fluka, Sigma) were tested as precipitants. 24-well tissue-culture plates with cell capacities of 3.5 ml (Linbro) and 3.2 ml (Corning), were used as crystallization chambers and covered with microscope coverslips (Fisher Scientific Company). Stopcock grease silicone lubricant (Dow Corning) and/or petroleum jelly were used for sealing the crystallization units. All crystal handling, including photography, was carried out under a dissecting microscope with polarizing equipment (Olympus).

A preliminary X-ray study of the horse cytochrome *c* crystals was carried out on a precession camera mounted on an X-ray generator with a 1.5 kW copper sealed tube (Enraf–Nonius). The beam was nickel filtered to obtain Cu $K\alpha$ radiation, and data were recorded on Kodak direct-exposure Scientific Imaging Film (Charles Supper Company).

Three-dimensional X-ray data sets were collected on a Siemens/Xentronics multiwire area detector (Durbin *et al.*, 1986), mounted on a Huber four-circle goniostat. The X-ray source was a Rigaku RU200 rotating copper anode generator working at 5 kW. Cu $K\alpha$ radiation was selected with a graphite monochromator. Crystallographic data were reduced with the program package *XENGEN* (Howard *et al.*, 1987). For horse, rat wild-type, and rat Y67F and N52I mutant proteins the crystal-to-detector distances were 13.5, 13.5, 14.0 and 13.5 cm, respectively, and the swing angles were 25, 20, 17 and 30°, respectively. These settings resulted in nominal resolution limits of 2.06, 2.22, 2.48 and 1.86 Å (Table 1). All data were collected with a 0.3 mm collimator. Each frame width was 0.2°. The collection time per frame was 120 s for the horse, 200 s for the rat wild-type, and 180 s for the Y67F and N52I mutant proteins. Data sets were collected in multiple crystal settings, which were then merged to produce the final three-dimensional data sets for each protein.

## Results

Crystallization attempts were carried out using the vapor-diffusion method with both hanging- and sitting-drop techniques using PEG as precipitant (McPherson, 1976, 1985, 1989). Initially, drops contained 40 mg ml$^{-1}$ protein, 20–25%(*w*/*v*) PEG (molecular weight 1000 Da), and 0.05 *M* potassium phosphate buffer pH 6.8. These conditions are similar to those used by Walter, Westbrook, Tykodi, Uhm & Margoliash (1990). The reservoir wells contained PEG with concentrations ranging between 28 and 32% in the same buffer. Crystals appeared in 2–3 weeks after setup and continued to grow for 3–4 weeks, achieving their maximal dimensions of 0.75 ×

Table 1. *Summary of crystallographic data sets for natural horse, recombinant rat wild-type, Y67F and N52I cytochromes c*

| Source | Unit-cell parameters (Å, °) | Nominal resolution* (Å) | No. of unique reflections | Completeness of data (%) | $R_{sym}$ (%)† | Data redundancy‡ $\langle N_o/N_R \rangle$ | $\langle I/\sigma(I) \rangle$§ |
|---|---|---|---|---|---|---|---|
| Horse | $a = 56.25$ | 2.06 | 8803 | ∞–2.06 Å | ∞–2.06 Å | 4 | ∞–2.06 Å |
| | $b = 105.71$ | | | 65% | 4.0% | | 22.03 |
| | $c = 35.29$ | | | ∞–2.36 Å | 2.19–2.06 Å | | 2.14–2.06 Å |
| | $\alpha = \beta = \gamma = 90$ | | | 80% | 17.3% | | 1.89 |
| Rat wild type | $a = 55.59$ | 2.22 | 9938 | ∞–2.22 Å | ∞–2.22 Å | 5 | ∞–2.22 Å |
| | $b = 105.84$ | | | 93% | 3.4% | | 26.81 |
| | $c = 34.67$ | | | ∞–2.36 Å | 2.36–2.22 Å | | 2.24–2.22 Å |
| | $\alpha = \beta = \gamma = 90$ | | | 99% | 16.3% | | 1.90 |
| Rat mutant Y67F | $a = 56.38$ | 2.48 | 5090 | ∞–2.48 Å | ∞–2.48 Å | 4 | ∞–2.48 Å |
| | $b = 105.66$ | | | 64% | 4.6% | | 21.77 |
| | $c = 35.46$ | | | ∞–2.63 Å | 2.64–2.48 Å | | 2.53–2.48 Å |
| | $\alpha = \beta = \gamma = 90$ | | | 73% | 22.6% | | 0.99 |
| Rat mutant N52I | $a = 56.92$ | 1.86 | 13494 | ∞–1.86 Å | ∞–1.86 Å | 9 | ∞–1.86 Å |
| | $b = 107.14$ | | | 74% | 3.2% | | 40.67 |
| | $c = 34.20$ | | | ∞–1.97 Å | 1.97–1.86 Å | | 1.88–1.86 Å |
| | $\alpha = \beta = \gamma = 90$ | | | 83% | 15.4% | | 2.69 |

* Nominal resolution is defined by the location of the outer edge of the detector face. All crystals examined diffracted beyond the detector edge.

† $R_{sym} = \sum|I_{obs} - I_{avg}|/\sum I_{avg}$.

‡ Where $N_o$ is the number of observations and $N_R$ is number of reflections, not merging Bijvoets.

§ Reported by the *XENGEN* statistics package. For all resolution shells, except the outer one shown here, $I/\sigma(I)$ was greater than 2.

0.5 × 0.2 mm. Crystals occasionally grew at pH 6.2 and 7.5 and, in time, attained the same size as the crystals grown at neutral pH.

Attempts were made to grow crystals using macroseeding techniques for two reasons. First, we hoped for a more dependable yield of crystals. Second and more important, we were trying to induce crystallization of mutant proteins and cytochromes *c* from different species in an isomorphous crystal form, to simplify structure analyses and to avoid ambiguous conclusions resulting from conformation changes due to different surface interactions in different packings. After a convenient technique for such crystallization was developed for horse heart ferricytochrome *c*, it was used to crystallize rat recombinant wild-type cytochrome *c* and its two mutants, Y67F and N52I.

Originally, small crystals of horse heart cytochrome *c* were used as seeds for crystallization of the same protein. Several different approaches, described by Thaler *et al.* (1981, 1985) and by McPherson (1989), were used in attempts to enlarge the seeds, but all of them failed. Washing seed crystals with buffer containing less precipitant than would be present at its final concentration, in order to initiate their dissolution before placing them into the droplet with higher precipitant and protein concentrations, was not successful – in our best cases crystals remained the same size as at the time of seeding. A gradual increase in protein concentration was attempted by periodically adding 1 μl of protein in the corresponding solution of PEG in buffer. This resulted in slow dissolution of the seed crystals in 4–6 weeks. Another method attempted for enlarging small crystals was to lower periodically the protein and precipitant concentration in the drop above the well. For this purpose the bottom parts of the culture plates were warmed up for various periods of time, leaving hanging drops with small crystals at normal temperature. Water vapor condensed on crystal-containing drops and diluted them. By varying the degree of evaporation, different rates of dissolution were chosen, ranging from (1) initiation of dissolution, (2) dissolution of only the smallest crystals, to (3) complete dissolution of all crystals. Crystals regrew regardless of their original sizes and the rate of their dissolution, but always grew up to sizes smaller than the original ones. In some exceptional cases after complete dissolution, crystals reappeared and continued to grow for several months beyond their original sizes. These latter experiments were poorly reproducible and required too much time to be considered reliable.

In the successful experiments, droplets, supersaturated but containing no crystals, were seeded with crystals too small for use in data collection (0.15 × 0.05 × 0.025 mm to 0.2 × 0.08 × 0.03 mm). Particular attention was paid to the habit of seed crystals. Only those well developed in all three dimensions were employed. The seeds were washed twice with PEG solution in buffer at a concentration that would not initiate dissolution. In these attempts, the seeds

remained intact, but new, separate crystals appeared within 10–12 h. Two rates of growth were observed: after the first 2–3 weeks they attained sizes large enough for X-ray data collection (up to $\sim 0.4 \times 0.2 \times 0.15$ mm), and then continued to grow more slowly for at least 6–8 weeks. The number of new crystals appeared to be roughly dependent on the number of seeds and on which protein was crystallizing, decreasing in the following order: horse > rat > Y67F > N52I. In the example shown in Fig. 1, two seed crystals produced many more crystals of horse cytochrome *c* (Fig. 1*a*) than a single seed crystal (Fig. 1*b*). In routine setups half the drops were seeded with one crystal and half with two crystals.

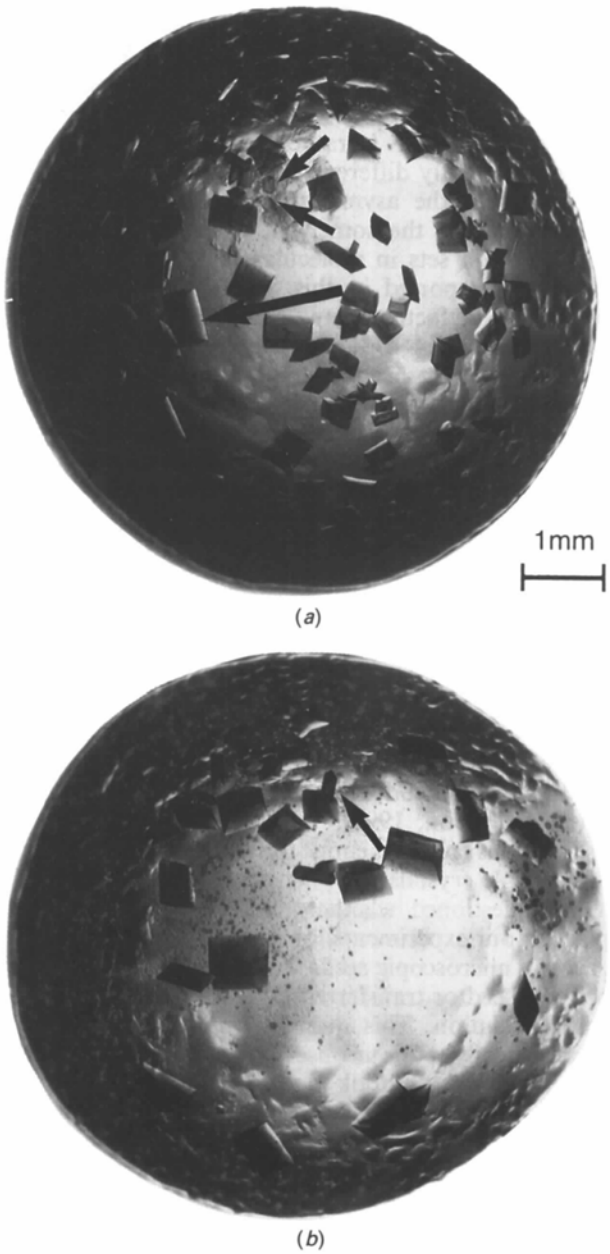



Fig. 1. Crystals of horse heart cytochrome *c* grown at low ionic strength from solutions of PEG by macroseeding. (*a*) The larger arrow points to the crystal employed in three-dimensional data collection, reported in this paper. The two smaller arrows show the two seeds, which in this case remained intact. As can be seen, the distribution of newly formed crystals did not depend on the location of the seeds. (*b*) The arrow indicates the approximate site of seeding. The seed crystal itself is not visible either because it dissolved or because it is underneath the two new crystals located at the tip of the arrow.

The starting concentrations of protein were tested in the range 5–55 mg ml$^{-1}$. Crystals appeared in the 10–45 mg ml$^{-1}$ range, the optimum being at approximately 30 mg ml$^{-1}$ for all four cytochromes *c* crystallized. Below 20 mg ml$^{-1}$ crystals appeared but seldom grew to sizes suitable for diffraction studies. At protein concentrations much higher than 30 mg ml$^{-1}$ new crystals formed, but did not grow to a large size because the protein gelled in less than one week. For all four proteins, the optimum pH for crystallization was 6.8–7.1. The temperatures tested were 285, 291 and 300 K. The crystals grew at all these temperatures and routinely we employed 291 or 300 K. 285 K was not used in order to avoid moisture condensation during crystal handling at room temperature. The concentrations of PEG used – 28 to 32%(*w*/*v*) – depended on the different solubilities of the four cytochromes *c* used in this work, in the following order: N52I < horse ≤ Y67F < rat wild type.

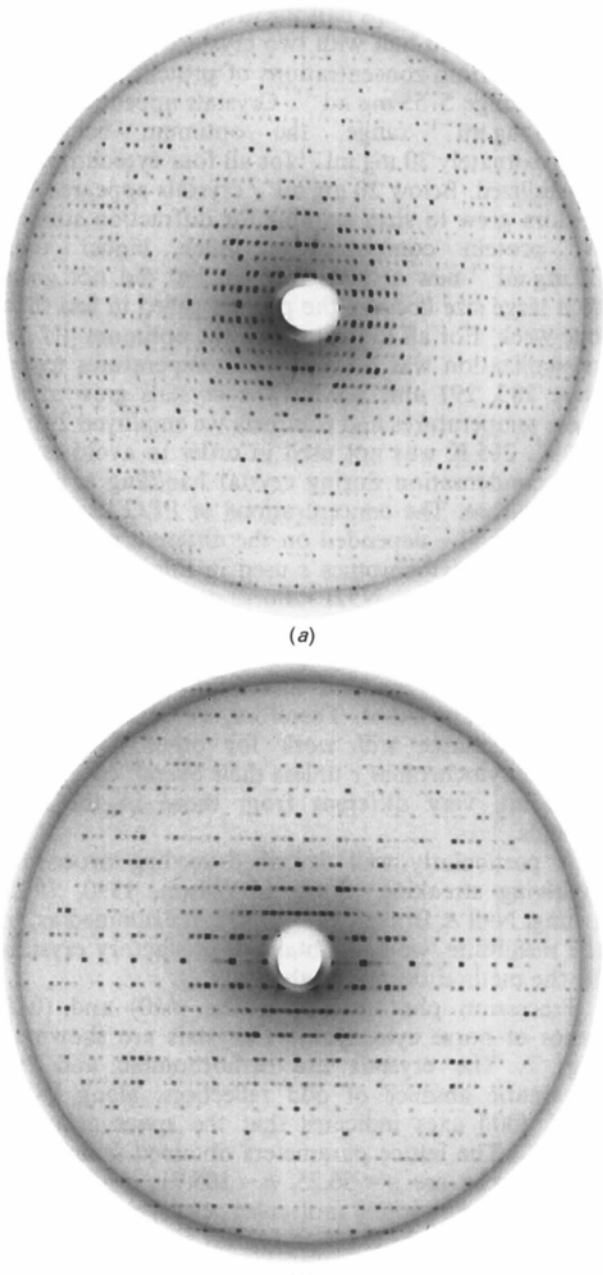
Seed crystals of any one of the four types of cytochrome *c* were used successfully in the crystallization of any other. Therefore, we expect that the same technique will work for other natural or mutant cytochromes *c* unless their overall conformations are very different from those in the seed crystals.

A particularly well described seeding procedure involving streaking (Stura & Wilson, 1990, 1991; Leung, Nall & Brayer, 1989) was not examined as, in the meantime, we had obtained satisfactory crystals by the method described above.

Precession photographs of the (*hk*0) and (0*kl*) zones of horse cytochrome *c* crystals are shown in Fig. 2. The crystals are orthorhombic and the systematic absence of odd reflections along [*h*00], [0*k*0], [00*l*] axes indicates that the space group is $P2_12_12_1$. The lattice parameters obtained from these photographs are $a = 56.25$, $b = 105.71$, $c = 35.29$ Å. Assuming two protein molecules per asymmetric unit gives $V_m = 2.13$ Å$^3$ Da$^{-1}$, a typical value for protein crystals (Matthews, 1968), corresponding to a solvent content of 42%. Although bonito and tuna cytochromes *c* have been crystallized in space group $P2_12_12_1$ (Tanaka, Yamane, Tsukihara, Ashida & Kakudo, 1975; Swanson, Trus, Mandel, Kallai &

Dickerson, 1977), the lattice parameters of these previous crystal forms were very different from those of our present crystals.

Full three-dimensional data sets have been collected and processed from single crystals for each protein. The approximate size of the crystals employed for the horse, rat wild-type, Y67F and N52I cytochromes *c* were 0.7 × 0.35 × 0.2 mm, 0.63 × 0.5 × 0.3 mm, 0.37 × 0.2 × 0.1 mm and 2.0 × 0.45 × 0.25 mm, respectively. The final statistics for these four data sets are summarized in Table 1. *R* factors between different data sets were calculated with the program package *XTAL* (Hall & Stewart, 1988). A value of 10% between rat wild-type and Y67F crystals shows that they are well isomorphous, while ~24% between horse cytochrome *c* and either rat wild-type or Y67F is rather high. This could be a result of slightly different configurations of the two molecules in the asymmetric unit. Supporting this suggestion was the somewhat different behavior of the two data sets in molecular replacement calculations (not reported in this article). Also, unexpectedly high *R* factors between the N52I and all the other crystals (24–41%) is likely to be a result of the mutation, which occurs in the interface of the two molecules in the asymmetric unit (as shown by preliminary molecular-replacement solution) and, therefore, could easily change their configuration.



(*a*)

(*b*)

Fig. 2. X-ray precession photographs taken from horse heart cytochrome *c* crystals at a crystal-to-film distance of 75 mm, an exposure time of 12 h and a precession angle $\mu = 15°$. (*a*) (*hk*0) and (*b*) (0*kl*) zones are shown.

## Discussion

The self-nucleation event used for the crystallization described in this paper is familiar to most investigators who utilize seeding techniques for crystallizing biological macromolecules. Spontaneous self-nucleation is generally considered to be a deleterious side effect of seeding, and special precautions are usually recommended to avoid it (see for example, Stura & Wilson, 1991). What is unique here is the use of this phenomenon for successfully growing large single crystals.

We questioned whether real self-nucleation took place in our experiments, or whether the new crystals grew on microscopic seeds, either separated from the original seed or transferred together with it from the source solution. This question cannot be answered unequivocally, but our observations suggest that self-nucleation actually took place. Indeed, single crystals were chosen as seeds and washed twice prior to transferring them into the drop of supersaturated protein solution. Thus, it would seem that the capture of additional seeds is unlikely. If disintegration of the seed crystals occurred, a higher concentration of newly grown crystals close to the parent seed would be expected. However, seeds were always introduced at the edge of the drop, with no mixing, and we always observed an isotropic distribution of new crystals (Fig. 1). In less than 12 h, new crystals attain a significant size, and it is difficult to imagine that in this short period all new seeds had diffused isotropically. Indeed, Sousa, Lager & Wang (1991) found that when seeds are introduced into a crystal-

lization drop of a low-viscosity medium, the seeds are distributed throughout the drop, leading to numerous small crystals. High viscosity of the medium resulted in very many small crystals near the seed, decreasing in number and increasing in size as the distance to the seed increased. In our experiments the viscosity was quite high because of the PEG, but the size and number of new crystals were independent of the distance to the seeds (see Fig. 1*a*).

How such seed-induced self-nucleation occurs is not clear (as is the case for any crystallization). Mock seeding, in which the same solution without seeds was introduced into the drops of supersaturated protein, showed that the seed crystals are the cause of this effect. No crystallization was detected in such mock-seeding experiments. An essential feature, which made self-nucleation following macroseeding useful for crystal growth in this case, may be the extremely high solubility of the protein. Rat cytochrome *c*, for example, does not precipitate even in 100% saturated ammonium sulfate. The solutions obtained from the concentration columns were generally about 100 mg of cytochrome *c* per ml, and after spinning in Centricon 10 tubes were above 200 mg $ml^{-1}$ and the protein remained in solution. At higher concentrations of PEG, the protein does not precipitate but instead forms a gel which, after further dehydration by PEG, hardens and becomes fragile. It is generally considered that self-nucleation during seeding is disastrous, because new nuclei are formed in large quantities and they compete with the seeds for protein, so that crystals of sufficient size cannot be obtained. It could be the case that for proteins as soluble as cytochrome *c*, nucleation is too difficult, and self-nucleation during seeding gives only a few nuclei, thus allowing them to grow to large sizes.

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