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Crystallization of proteins from soluble cytochrome P-450cam to membrane protein cytochrome bc_1 complex

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Introduction

In the 1960s, Gunny's laboratory was divided into two research arms: one focusing on the bacterial genetics of *Pseudomonas* and the other on the enzymology of the catabolic pathway of camphor in *Pseudomonas*. The enzymology group was dedicated to investigating two enzymes involved in the initial steps of the camphor degradation pathway, ketolactonase and 5-exo-hydroxylase. These two enzymes catalyze the incorporation of one atom of molecular oxygen into the camphor molecule and the reduction of the other oxygen atom to water. The enzymes are both monoxygenases or mixed function oxidases [1]. I am proud to have been a member of Gunny's group during this exciting time and to have been involved in research on both enzyme complexes as a graduate student and later as a postdoctoral associate between 1964 and 1969. The research strategies and techniques I learned or developed under Gunny's guidance during my stay at the University of Illinois laid a solid foundation for my research career in the field of bioenergetics and complicated membrane protein complexes. I thank Gunny for his continued mentorship and encouragement.

Ketolactonase, a multicomponent FMN–protein complex

The presence of ketolactonase in the camphor degradation pathway was first demonstrated by Conrad, Dubus, and Gunsalus in 1961 [2]. This enzyme, which oxygenates (+)-camphor or its metabolites, is inducible to a high level in *Pseudomonas putida* strain C1 grown on (+)-camphor and had been separated into two components, termed E1 and E2, by Conrad [3] before I joined Gunny's laboratory in 1964. E1 is an FMN-dependent NADH dehydrogenase and E2 is a substrate-specific ketolactonase. E1 was purified and characterized by Trudgill [4]. However, attempts at purification of E2 were complicated by loss of activity. After a short period of "apprenticeship" with Dr. Trudgill to familiarize myself with the laboratory's routine protocols and instruments, I set out to purify the E2 component of ketolactonase. I tried all the protein purification methods then available, including all types of electrophoresis, but with no success. Finally a breakthrough came when I attempted to purify the enzyme using DEAE-cellulose column chromatography under anaerobic conditions. Since the glove box was the only anaerobic device in the laboratory at that time, I was forced to use a self-made flow-over fraction collector to collect samples, as the space in the box was so limited that no automatic fraction collector was small enough to fit in it. With this device, fractions with a large volume were collected during the entire chromatographic process. Surprisingly, when large volume fractions were collected, the total activity recovered was much higher than with small volume fractions. This inspired me to explore the possibility of the involvement of more than one component in E2. The E2 fraction was eventually separated into two components (E2 and E3) and each was purified to homogeneity by procedures involving ammonium sulfate precipitation, gel filtration, DEAE-cellulose, and calcium phosphate column chromatography [5]. The apparent molecular weights of E1, E2, and E3 are 37, 80, and 120 kDa, respectively. E3 can be further dissociated to a protein of 60 kDa (monomer) without loss of activity. Although pure E1 and E2 are colorless, containing no cofactor, they have high affinity for

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FMN. E3 is yellowish and contains 2 mol of FMN per mole protein (120 K). A hypothetical architectural assembly of these three protein components and a reaction mechanism involving a FMN biradical complex were proposed based on this early work on ketolactonase. Although this FMN-containing monooxygenase system was very interesting and merited further investigation, the work was overshadowed by the excitement of the discovery of cytochrome P-450cam [6] in 5-exo-hydroxylase [7].

Witness the discovery of cytochrome P-450cam

5-Exo-hydroxylase was initially separated into two components: an iron protein called fraction B, and an FAD-containing flavoprotein called fraction A. Fraction B was purified to homogeneity and shown to be a non-heme iron protein termed putidaredoxin [7]. However, attempts to further purify fraction A were unsuccessful, as increases in protein purity were accompanied by decreases in enzymatic activity. Although fraction A is red and the involvement of hemoprotein in other hydroxylation systems was reported, little attention was given to the role of hemoprotein, as we more or less assumed an association of flavin and iron in this fraction until Dr. Katagiri joined Gunny's group in 1967. I recall that when we showed Dr. Katagiri fraction A, he was astonished that we did not consider the fraction as a hemoprotein, cytochrome P-450. When he reduced fraction A with sodium dithionite and bubbled the reduced sample with CO, a beautiful cytochrome P-450 spectrum was observed and termed cytochrome P-450cam [6]. Fraction A was subsequently separated into cytochrome P-450cam and an FAD-containing flavoprotein termed putidaredoxin reductase [7].

After I finished my dissertation research on ketolactonase in 1968, I turned my efforts to cytochrome P-450cam. A large-scale purification procedure was developed involving autolysis, step-wise DEAE-cellulose column chromatography, ammonium sulfate fractionation, gel filtration, calcium phosphate column chromatography, and a second DEAE-cellulose column chromatography. The procedure yielded 600 mg of purified cytochrome P-450cam from 1 kg of frozen *P. putida* cell paste. This large quantity of purified protein enabled me to try crystallizing this cytochrome using ammonium sulfate as the precipitating agent.

The crystallization of cytochrome P-450cam free of P-420 from ammonium sulfate under reduced pressure

Fifty mg of purified P-450cam in 2.5 mL of 50 mM phosphate buffer, pH 7.0, was treated with 2.5 mL of 10% neutralized cysteine solution in the same buffer for 30 min at room temperature to convert the trace amount of P-420 in the sample to active P-450cam. The solution was cooled to 0 °C and brought to 60% ammonium sulfate saturation by the slow addition of solid, powdered salt. After incubation for 15 min, the precipitate was collected and redissolved in a minimal volume (~0.4 mL) of 5 mM phosphate buffer containing 100 μM camphor. The precipitate, if any, was removed by centrifugation at 26,000*g* for 10 min and the clear supernatant solution was adjusted to about 30% ammonium sulfate saturation. The sample was then placed in a 10-mL beaker and transferred to a precooled desiccator containing a few mL of the same buffer. The desiccator was evacuated to 8 mm Hg at 4 °C and left undisturbed in the cold room. Red crystals formed within 10 days in the presence of amorphous material. To separate the amorphous material from the crystal, the mixture was stirred gently to make a suspension and then allowed to settle for a short time. Since the crystals settled faster than the amorphous material, the upper layer containing mostly amorphous material was removed and centrifuged. The clear supernatant mother liquor was used to wash the settled crystals. This process was repeated until the crystals were completely free of contamination by the amorphous material. The washed crystals were dissolved in a minimal volume of 5 mM phosphate buffer containing 100 μM camphor and 300 μM mercaptosuccinate and bought to 30% ammonium sulfate saturation by the slow addition of solid, powdered salt. A small amount of precipitate formed was removed by centrifugation. The supernatant solution was then seeded with a few crystals prepared earlier and allowed to stand undisturbed until crystallization was completed. Fig. 1 shows cytochrome P-450cam crystals obtained in 1970 [8,9]. Although these crystals might be large enough for today's X-ray diffractometer, they were not suitable for structural work in the 1960s. Fortunately, subsequent

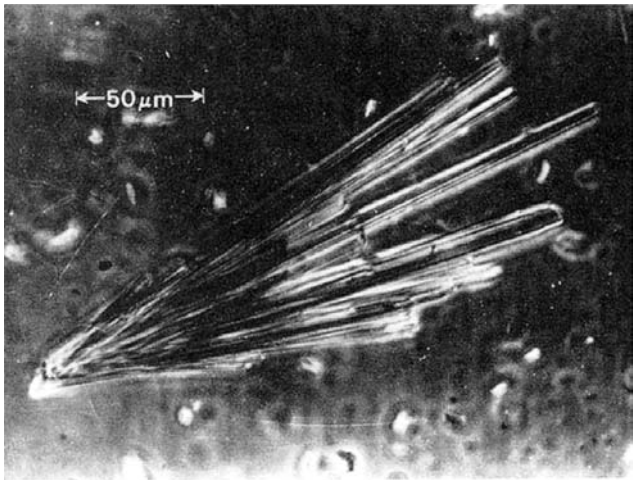

Fig. 1. First crystals of cytochrome P-450cam.

investigators in Gunny's group were able to continue the crystallization work on cytochrome P-450cam [10] and improve the quality of crystals, making them suitable for structural determination 15 years later [11].

The key to the success of P-450cam crystallization is the conversion of the trace amount of P-420 [12] present in the purified sample to active P-450cam by treatment with a mercapto-compound. The inclusion of substrate, camphor, also significantly increased the stability of the sample. Gunny threw a party for the laboratory to celebrate our success in crystallization of cytochrome P-450cam. At that party, he presented me with an envelope with part of a 100-dollar bill showing through the window. He told me that if I could identify the face on the bill, I could have it; otherwise, the money would go to my wife, Linda. Obviously, Linda got the money, since I was only familiar with bills bearing pictures of Washington or Lincoln.

Crystallization of multisubunit membrane protein complex–mitochondrial cytochrome bc_1 complex

Interest in protein crystallization and atomic structure determination by X-ray crystallography has grown exponentially since the 1990s with technological advances in diffractometer and computation devices. A great majority of determined protein structures are of soluble proteins of relatively small molecular mass. Despite the successful Nobel Prize-winning work on the bacterial reaction center reported in 1985 [13], investigators generally shy away from membrane protein crystallization, especially of proteins with multitransmembrane helices. Quite often investigators studying membrane proteins ignore crystallization, thinking that it is too difficult. For example, the multisubunit cytochrome bc_1 complex, which plays a key role in biological energy conservation, was purified from submitochondrial particles in the early 1960s, but crystals suitable for X-ray crystallographic work were not obtained until 1990 [14], even though evidence showing that the purified cytochrome bc_1 complex is crystallizable was reported as early as 1983 [15].

Membrane protein crystallization requires no fancy apparatus

Although a large quantity of purified protein of cytochrome bc_1 complex was available in our laboratory, crystallization of such a protein complex was not a major focus for us until 1989. Under an unusual circumstance, Dr.Yue, an established geologist from China, came to our laboratory as a visiting scholar. Since the closest tie between his expertise and ours was crystallization and structure, I directed him to work on the crystallization of the cytochrome bc_1 complex. Not expecting much in the way of significant results, I asked him to be frugal in using laboratory resources. Dr. Yue was very resourceful: he sealed the tips of Pasteur pipets and used them as a crystallization apparatus and polyethylene glycol as a precipitating agent [14]. Frozen cytochrome bc_1 complex, 22 mg/mL in 50 mM Tris–HCl buffer, pH 8.0, containing 1 mM EDTA and 0.66 M sucrose, was thawed and mixed with an equal volume of precipitating solution, containing 12% polyethylene glycol 4000, 0.5 M sodium chloride, 2.6% heptanetriol, 0.08% decanoyl-*N*-methylglucamide, 0.1% sodium azide, 1 mM EDTA, and 50 mM Tris–HCl buffer, pH 8.0. The mixture was incubated at 0 °C for 10 min before centrifugation to remove any precipitate. Fifty-μL aliquots of clear solution were placed in the tip-sealed Pasteur pipets. Air bubbles, if present on the top of the solution, were removed and the wall of the pipet above the solution was dried before the pipet was filled with equilibrating mixture (Fig. 2A). Care was taken to ensure that an air space approximately 3 mm in length was formed between the two solutions. The equilibrating solution was the same as the precipitating solution except 1 M sodium chloride and 22% PEG were used. The pipets were sealed with Parafilm paper and placed in a shock-free chamber at 0 °C. Good-sized crystals formed within 2–4 weeks depending on the volume of protein solution used. By this method a crystal as large as $4 \times 2 \times 1$ mm was obtained. These crystals diffract X-rays to low resolution. When they were redissolved in buffer containing detergent, the specific activity, based on cytochrome *b*, is the same as that observed before crystallization. The ratio of cytochromes *b* to c_1 is 2, and the excess cytochrome c_1 present in the purified enzyme was retained in the mother liquor.

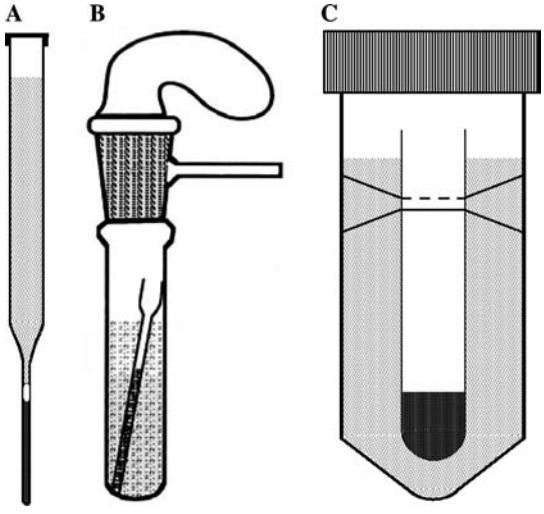

Fig. 2. Simple apparatus for growing protein crystals: (A) Pasteur pipet. (B) Thunberg tube good for anaerobic conditions or reduced pressure. (C) Small test tube for growing large quantity of crystals.

When crystals were grown in X-ray capillary tubes in 50 mM Mes buffer, pH 7.0, instead of 50 mM Tris–HCl buffer, pH 8.0, they diffract X-rays to 7 Å resolution in the presence of mother liquor. In order to grow crystals under inert atmosphere or reduced pressure conditions, aliquots of crystallizing protein solution were placed in capillary tubes and inserted into a Thunberg tube filled with equilibrating solution (Fig. 2B). Under anaerobic conditions, the setup was repeatedly evacuated and flushed with argon. Under reduced pressure conditions, the setup was evacuated until the desired pressure was achieved.

For preparation of large amounts of crystals, 0.5 mL of protein solution was mixed with 0.5 mL of precipitating solution, incubated for 10 min, and centrifuged. The clear solution was placed in a small test tube, which was mounted in a 50-mL conical centrifuge tube filled with 40 mL of equilibrating solution (Fig. 2C) [14]. Thin square crystals formed in 2 weeks with some amorphous precipitates, which were removed by repeated washing with mother liquor as described before [8].

Cytochrome bc_1 complex can be crystallized in the gel state

Since membrane protein crystals are grown in buffer containing detergents, it is often difficult to keep the crystals steady when mounted in a capillary tube for diffraction data collection. This is especially true for thin crystals, which require the presence of some mother liquor to keep the crystal from bending. To circumvent these difficulties, the cytochrome bc_1 complex was crystallized in the gel state. Purified cytochrome bc_1 complex, 20 mg/mL, in 50 mM Mes buffer, pH 7.0, containing 0.67 M sucrose was mixed, at 18 °C, with an equal volume of precipitating solution containing 0.08% decanoyl-*N*-methylglucamide, 3.6% heptanetriol, 0.5 M sodium chloride, 12% polyethylene glycol, and 0.8–0.4% low-gelling-temperature agarose. The mixture was placed in the capillary tubes, cooled to 4 °C, overlaid with equilibrating solution, and incubated in a shock-free environment at 4 °C. Crystals formed within 2–4 weeks [16]. The size, shape, and diffraction quality of these crystals were similar to those obtained in the liquid state.

Detergent plays a key role in membrane protein crystallization and quality of crystals

To grow cytochrome bc_1 complex crystals suitable for high-resolution structural analysis, sample preparation was improved and crystallization conditions fine-tuned. The detergents used in protein purification, such as sodium cholate and sodium deoxycholate, were recrystallized. Ammonium acetate fractionation was increased to 15 steps, as small incremental changes in salt concentration produced a better preparation. The purified cytochrome bc_1 complex, 20 mg/mL, in Tris–HCl buffer, pH 8.0, containing 0.66 M sucrose, was diluted with an equal volume of 0.5 M sucrose solution. The diluted solution was mixed with 0.3 vol of 50% saturated ammonium acetate solution. The precipitates were collected by centrifugation and redissolved in 50 mM Mops buffer, pH 7.8, containing 20% glycerol and 0.16% sucrose monolurate (or 0.1% decanoyl-*N*-methylglucamide or diheptanoyl phosphatidylcholine), to a final protein concentration of 20 mg/mL. After incubation at 0 °C for 2 h, the solution was mixed with 4/7 vol of precipitating solution, 50 mM Mops buffer, pH 7.8, containing 12% PEG-3000, 0.5 M KCl, and 20% glycerol. The mixture was incubated overnight and then centrifuged at 40,000*g* for 20 min to remove any trace amounts of precipitate. The clear solution was collected and used for crystallization by the vapor diffusion sitting-drop method. Good-shaped crystals appeared in 1 to 2 weeks (Fig. 3). These crystals diffract X-rays to 2.9 Å resolution [17]. For a quick check of the ability of the cytochrome bc_1 complex to be crystallized, 3-μL aliquots of the clear solution were used for vapor diffusion crystallization against 200 μL of equilibrating solution, using the hanging-drop method. Small crystals appeared in 1 to 2 days depending on the volume ratio of protein solution and equilibrating solution. Crystals should appear in the absence of amorphous precipitates; appearance of some amorphous precipitates would indicate that the amount of detergent used is insufficient, and failure of crystals to form within 2 days would indicate that either too much detergent or too little PEG was used.

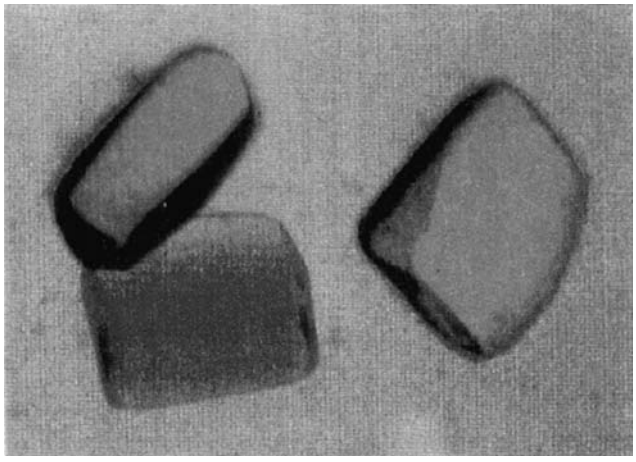

Fig. 3. Crystals of cytochrome bc_1 complex.

Crystals of cytochrome bc_1 complex can be frozen for storage or X-ray diffraction data collection

The cytochrome bc_1 crystals were grown in 20% glycerol. When crystals were mature, the concentration of glycerol in the mother liquor was gradually raised to 40% or higher by a process designed to protect the crystals from damage upon freezing, such as storage in liquid nitrogen or during data collection at 100 K. The mother liquors in each sitting drop were collected and the concentrations of glycerol were raised by an increment of 4% using an 80% glycerol solution in the same buffer system. The mixtures were centrifuged to remove any precipitate, and the clear solutions were placed back in the wells and incubated for 4 h to equilibrate with the crystals before the next increase in glycerol concentration. It took 5 cycles to raise the concentration of glycerol to 40%. The crystals were frozen in liquid propane and stored in liquid nitrogen before data collection. The crystals were found to diffract X-rays to 2.4 Å resolution using synchrotron radiation sources [18].

Protein crystallization is more art than science

Detergents play a vital role in membrane protein solubilization and crystallization. For crystallization, the purity of the detergents is critical, especially for obtaining high-resolution crystals. Using recrystallized sodium cholate in the initial steps of cytochrome bc_1 complex preparation improved the quality of the crystals significantly, with diffraction resolution improving from 2.9 [17] to 2.4 Å [18]. Protein–detergent interactions depend not only on the volume ratio between them but also on the absolute concentrations of both parts. In protein crystallization, the amount of detergent needed for one protein concentration may not be suitable for another protein concentration. Also, the protein–detergent interaction is affected by the presence of salts. When a membrane protein in detergent solution is mixed with precipitating solution, it takes time for the detergents bound on protein and those in solution to reach equilibrium. Therefore, it would not be surprising if a less efficient technician grows better membrane protein crystals than an efficient worker. We have observed that an investigator's personality and research style have significant effects on his success with membrane protein crystallization. And in our opinion, if an individual has attempted to crystallize a purified, homogeneous membrane protein for more than a year without success, the likelihood of future success is low, and a change of personnel may be beneficial. Membrane protein crystallization is more art than science; the intelligence and effectiveness of the investigator are rarely useful predictors of success.

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