Communications

Improving the Quality of Protein Crystals through Purification by Isoelectric Focusing*

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Preparative flat-bed gel isoelectric focusing has reduced or eliminated observed heterogeneity in three different proteins, thereby greatly improving the quality of crystals grown from each of them. This improvement is manifested both in crystal size and in increased measurability of diffraction data. Isoelectric focusing is a general and nondestructive technique that may facilitate the crystallographic study of proteins which are biologically interesting, but which, for reasons of microheterogeneity, do not yield diffraction quality crystals.

Biological relevance has largely superseded technical convenience as the principal reason for conducting a protein crystallographic study. As a result, protein crystallographers are addressing specific biochemical questions which require the study of specific proteins and present increasingly challenging crystalization problems. Two approaches to overcoming these problems are the development of more systematic means to find crystallization conditions and the implementation of methods to increase the homogeneity of the starting material.

Along the lines of the first approach, McPherson (1) and Blundell and Johnson (2) have summarized successful crystallization procedures up to 1976 and McPherson has demonstrated the merits of polyethylene glycols as precipitants (3). Carter and Carter (4) have described the use of incomplete factorial experiments for investigating crystallization conditions where time and materials are in limited supply. Kam, Shore and Fehér (5) have reported a light scattering method to predict whether small protein aggregates will grow to crystals or form amorphous precipitate. Finally, several workers have considered the mechanics of small scale crystallization experiments as well as techniques for coagulating nucleation, limiting nucleation, and increasing crystal size (6-14).

Apart from crystallization conditions, purity of the protein sample is intuitively a factor in the success of a crystallization experiment. Contamination by other species of macromolecules is the most obvious source of impurity, and one frequently expends much effort to eliminate these contaminants. However, heterogeneity in the protein of interest can also be a problem. We use the term microheterogeneity to describe this type of impurity. Sequence variation and incomplete or uneven proteolysis are sources of microheterogeneity in the polypeptide chain itself. In addition, post-translation modification of the protein, e.g. covalent attachment of saccharides, phosphorylation, and methylation, may not be complete and the moieties attached may be heterogeneous as well. Protein crystallization depends upon specific weak attractive interactions between protein molecules which are repeated over many thousands of molecules to form an ordered three-dimensional lattice. While most of the surface of a crystalline protein is in contact with only solvent, the interacting surfaces are quite specific. Microheterogeneity, if it occurs at or near the interaction surfaces, could easily terminate crystal growth at that point or cause serious lattice perturbations.

Microheterogeneity is usually undetected when only biochemical activity is monitored during protein isolation and is also difficult to detect when gel filtration and sodium doxycyl sulfate gel electrophoresis are used to determine purity. Isoelectric focusing involves the electrophoretic separation of protein species through a pH gradient to zones of zero net charge. It is one of the most sensitive nondestructive separation techniques, capable of resolving variants of a single protein. In this paper, we explore the utility of preparative flat-bed gel isoelectric focusing to achieve higher purity in the starting material and report the success of the technique in improving the quality of crystals of three separate proteins.

The three proteins investigated are trimeric hemerythrin (15), an invertebrate oxygen transport protein; carboxyl proteinase from Rhizopus chinensis (16); and the Fab fragment of J539 (17), a mouse myeloma immunoglobulin. The crystallization problems presented by these proteins are quite different. Hemerythrin crystals readily grew as clusters with generally too many crystal nuclei in an experimental set-up. Diffraction from carboxyl proteinase crystals extends beyond 2 Å spacings but data collection was hampered by very low intensity at high resolution and a short crystal lifetime in the x-ray beam. Crystals of J539 Fab large enough to diffract to 2.5 Å spacings grew only sporadically, so that the limited amount of starting material available made it impractical to consider data collection to that resolution.

MATERIALS AND METHODS

All preparative and analytical isoelectric focusing was performed on LKB Instruments Multiphor flat-bed apparatus using an LKB Model 2103 power supply.

Analytical Gel Isoelectric Focusing—50-μl aliquots of protein solutions (1-5 mg/ml) were applied to a commercially prepared polyacrylamide slab gel containing wide range ampholytes (pH 3.5-9.5, LKB Ampholine PAG plates). The protein was focused for 1.5 h at 4°C using a constant power setting of 30 watts and a maximum voltage of 1400 V. The gels were fixed, stained, and washed according to the manufacturer’s instructions.

Preparative Flat-bed Gel Isoelectric Focusing—In general, the procedure is that presented in LKB Application Note 198 (18). A gel slurry was prepared using 4 g of dry, washed Sephadex G-75 (Ultronex, LKB) and a solution of 5.5 ml of the desired pH range carrier ampholytes (Pharmalyte, Pharmacia, or Ampholine) in 104.5 ml of deionized water. The gel was poured into a flat glass tray and allowed
to air-dry as described by the manufacturer. Protein samples were either mixed into a fraction of the gel slurry or added dropwise to the gel after drying. The proteins were focused overnight (12-18 h) at 8 watts constant power and a maximum voltage of 1500 V at 4 °C. Protein bands were visualized by staining and destaining a print of the gel, made by placing a sheet of filter paper directly on the gel for approximately 30 s. The gel was placed over this print on a light box using tick marks for alignment. Stained bands on the print were visible through the translucent gel, allowing the corresponding protein bands on the gel to be precisely scooped out with a spatula. Since preparatively focused bands are seldom straight, this method gave consistently better results than simply segmenting the gel with the fractionating grid provided by LKB. The scooped out gel fractions were placed in minicolumns (LKB) and eluted with at least 2 bed volumes of buffer. The effluent was then concentrated by ultrafiltration and at the same time extensively washed to remove contaminating ampholytes. If the wash is done with water or a suitable buffer, ampholyte concentration can be monitored by measuring the buffering capacity of aliquots of the eluant.

**Trimeric Hemerythrin—**Coelomic hemerythrin from *Siphonosoma funafuti* was generously supplied by Dr. A. W. Addison, Drexel University. Between 50 and 70 mg of protein in 20 mM cacodylate, 10 mM NaNO₃, pH 7.0, were applied to preparative electrofocusing gels in three separate experiments. The following mixture of Ampholine was used to establish an effective pH 3.5-7 gradient: 1 part pH 3.5-5.0, 1 part pH 4.0-6.0, and 2 parts pH 5.0-8.0. Filter paper prints were made of the focused gels to insure that there were no protein bands other than those of orange-colored metazidehemerythrin. The orange bands were carefully cut from the gel with a spatula and eluted as described above. Crystals were grown from 2.5-5.0 mg/ml of protein solutions in cacodylate buffer by vapor diffusion with polyethylene glycol 6000 as a precipitant. J539 Fab—A sample of J539 (85.2 mg) Fab was pooled from a number of separate preparations, all obtained from Dr. Stuart Rudikoff of the National Cancer Institute. This sample was concentrated to 4 ml and extensively dialyzed against distilled water by ultrafiltration. It was subsequently focused in a pH 4.0-6.5 gradient (Ampholine) and extracted as described above. Crystallization samples were concentrated to approximately 20 mg/ml and crystallized from 35% saturated ammonium sulfate, 30 mM zinc sulfate, 70 mM cacodylate, pH 6.5, as described (17).

**Rhizopus Carboxyl Proteinase—**Rhizopus carboxyl proteinase was purchased as a lyophilized powder from Miles/Seikagaku. A sample (50 mg) was dissolved in 3 ml of deionized water and applied dropwise to a gel containing Pharmalyte, pH 4.0-6.5. After isoelectric focusing, the gel was divided into two fractions separated midway between the two prominent bands (identified as described above) which were eluted by washing the gel with 3 bed volumes of crystallization buffer, 20 mM CaCl₂, 50 mM cacodylate, pH 6.0. The sample was washed and concentrated by pressure dialysis to 15 mg/ml using an Amicon YM 10 concentrator. For crystallization, 10-μl aliquots were placed in sealed chambers. Crystals would routinely appear within 1 week and continue to grow for a period of approximately 1 month.

**RESULTS**

**Trimeric Hemerythrin—**Four principal protein fractions were reproducibly obtained in three separate preparative isoelectric focusing experiments. Samples of these fractions were focused on an analytical gel about 1 week after fractionation; results are shown in Fig. 1. The observed banding pattern is stable; the same pattern was obtained from the same fractions after standing 15 months at 4 °C. Fractions 1 and 4 are relatively homogeneous, but fractions 2 and 3 have reached a stable state of heterogeneity. All four fractions readily crystallize under the same conditions as unfraccionated hemerythrin, but only from fraction 1 are markedly improved protein crystals obtained. These grow singly at times, with dimensions up to 0.1 × 0.2 × 0.5 mm and appear to be isomorphous with crystals from unfraccionated material (space group P₂₁, a = 80.31, b = 45.11, c = 62.58 Å, β = 104.8°). The structure has been solved to a resolution of 5.5 Å with data from a fraction 1 crystal.

1. M. A. Navia, unpublished results.
2. J. L. Smith and W. A. Hendrickson, unpublished results.

**FIG. 1.** Results of isoelectric focusing purification on crystallization of trimeric hemerythrin. *a,* analytical electrofocusing gel of hemerythrin fractions purified in a preparative isoelectric focusing experiment. Samples were applied in pairs at opposite poles of the gel. *Left to right:* unfraccionated hemerythrin, and preparative fractions 1-4, respectively. *b,* typical crystals grown from unfraccionated protein on the same scale as in c. *c,* crystal grown from preparative fraction 1 protein mounted in a 1-mm diameter capillary tube.

**FIG. 2.** Results of isoelectric focusing purification on crystallization of *Rhizopus chinensis* carboxyl proteinase. *a,* analytical electrofocusing gel of the commercial protein (band 1) and two fractions isolated from a preparative isoelectric focusing experiment (bands 2 and 3). *b,* typical crystal grown from the pl 5.2 isozone (band 2). *c,* typical crystals grown from the commercial sample (band 1) on the same scale as *b.*

**Carboxyl Proteinase—**The two isozymes of carboxyl proteinase (19) known to be present in the commercially prepared material were separated in a preparative isoelectric focusing experiment (Fig. 2). The crystals of the unfraccionated material grow as cube-shaped blocks with a maximum dimension of 0.6 mm, while the crystals of the pl 5.2 fraction have bricklike morphology and grow larger with a maximum dimension of over 1 mm (Fig. 2). However, the two crystal forms are isomorphous having the space group P₂₁, with a = 60.33, b = 60.66, and c = 107.0 Å. The pl 6.0 isozone has not crystallized under any conditions. The crystals grown from
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the pl 5.2 isozyme reach a volume up to 3.6 times that of crystals from unfracionated carboxyl proteinase and typically decay at a slower rate in the x-ray beam. The improvement in crystals from unfractionated carboxyl proteinase and typically increase in the ratio of length to cross-section.

2.0 to 1.8 s

Several protein fractions were subsequently purified in a preparative experiment. These were labeled the p, q, r, and s fractions. Crystals were grown from all four of these fractions and typical specimens of each are shown in Fig. 3 (b-e). A progressive increase in the ratio of length to cross-section is observed going from fraction p (quite usable blocks) to fraction s (essentially useless clusters of rods).

DISCUSSION

The heterogeneity of these three proteins is manifested in three separate ways. In the case of hemerythin, we suspect sequence microheterogeneity and formation of heterogeneous trimers. Sequence variants of hemerythins are commonly found and subunit mixing usually results (20, 21). Trimeric hemerythin does not easily dissociate, so mixed subunit trimers can probably be isolated over the time course of a preparative isoelectric focusing experiment. We speculate that hemerythrin fractions 2 and 3 are mixed subunit trimers and typical specimens of each are shown in Fig. 3 (b-e). A progressive increase in the ratio of length to cross-section is observed going from fraction p (quite usable blocks) to fraction s (essentially useless clusters of rods).

The preliminary nature of these crystallographic studies prevents us from explaining the specific observed crystallization improvements in molecular terms. A clear example, however, of how subtle structure variations can affect the crystallization of a protein is provided in the study of the octomeric hemerythrin from Phascolosopis gouldii. Two variants of this hemerythrin have been sequenced and differ at 5 of 113 amino acid positions (20). While the mixture of the two variants gave only small clusters of crystals, both of the purified variants form large, single crystals in a variety of modifications. None of the crystal forms of one variant are isomorphous with those of the other, however.

The three-dimensional structure of one of these has been determined, by molecular repositioning, at 5.5 Å resolution (23). We find all the variable residues on the surface of the protein subunit and on the surface of the octameric aggregate as well. Such substitutions are least likely to affect the activity and quaternary structure of the protein and thus may occur more frequently than internal changes. They are also the very substitutions that would be expected to affect the specific intermolecular interactions necessary for successful crystal growth. Indeed, we observe this effect in octameric hemerythin. Three of the five variable residues occur in a lattice contact region in the known crystal structure. The sites of the other two substitutions are exposed to solvent in this crystal. The lattice contact sequence is Asp-Gly-Phe-Ile-Asn in the crystal structure and Glu-Gly/Thr-Phe-Ile-His in the other variant. At a 5.5 Å resolution, it is impossible to determine in atomic detail how these substitutions would perturb the lattice contact. However, this is one of but two lattice contact regions in the crystal, and small deformations here could easily disrupt crystal growth.

We have shown that flat-bed gel isoelectric focusing is a convenient means for reducing protein microheterogeneity and that such purification has produced improved crystals of three proteins. This technique should be applicable to a wide range of proteins. There are two principal limitations to the method. The experiments must be run at low ionic strength (100 mm) (26), and it is difficult to deal with proteins having extreme isoelectric points (pl < 3.0, pl > 9.5). Despite these limitations, isoelectric focusing should be considered in cases where one has a particularly interesting protein which does not yield to the standard crystallization techniques to give suitable crystals.

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