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Removal of Salt from a Salt-Induced Protein Crystal without Cross-Linking. Preliminary Examination of "Desalted" Crystals of Phosphoglucomutase by X-ray Crystallography at Low Temperature†

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ABSTRACT: A model procedure for removing salt from relatively fragile salt-induced protein crystals is proposed. The procedure is based on physical principles and is validated by using millimeter-size crystals of rabbit muscle phosphoglucomutase grown from a 2.1 M solution of ammonium sulfate. Three types of operations are included in the procedure: initial transfer to salt solutions of reduced concentration; transfer to the organic-rich phase of an equilibrium biphasic mixture obtained with aqueous solutions of polyoxyethylene and the salt; and addition of various replacement cosolutes in aqueous solutions of polyoxyethylene to reduce osmotic stress on the crystal as the remaining salt is removed. A critical feature of the overall procedure is maintenance of near equilibrium throughout by using a large number of steps involving small changes in solute concentration. The conditions used in the actual transfer were adjusted to eliminate the fracturing of crystals by visually distinguishing between two opposing types of fracture patterns: those produced by osmotic crushing as opposed to osmotic expansion. Basic requirements for a successful procedure with other protein crystals are a high permeability toward small solutes and a relatively slow dissolution rate at salt concentrations for which biphasic mixtures can be obtained. Desalted crystals of phosphoglucomutase have no visible fractures, are stable in the final solution for at least a week, and exhibit no noticeable change in the resolution of their X-ray diffraction pattern. In fact, desalted crystals can be rapidly cooled to 160 K, whereas untreated crystals are almost completely disordered by the same cooling procedure. The component of the desalting mixture whose presence is crucial to the success of the cooling process is polyoxyethylene, which apparently impedes the formation of ice within the protein crystal. Diffraction data obtained with an area-detector diffractometer did not differ significantly, either in terms of quality or resolution range, between crystals in 2.3 M ammonium sulfate at room temperature and crystals at 160 K in which ammonium sulfate had been replaced by glycine. The successful use of the following replacement solutes, instead of glycine, also is documented: sucrose, glycerol, and a low molecular weight poly(ethylene glycol) (PEG-400).

In tetragonal crystals of rabbit muscle phosphoglucomutase grown from 2.1 M $(NH_4)_2SO_4$ (space group $P4_12_12$; a = 174 Å; c = 101 Å), the asymmetric unit is a dimer (M_r = 122000) that packs into a helical array around the unique crystallographic 4_1 screw axis (Lin et al., 1986). The helical array surrounds a channel with a diameter of about 40 Å centered on the 4_1 axis. A second channel, similar in average cross section, but with a wider variation in diameter, is centered on the parallel crystallographic 2_1 screw axis. The liquid phase, which accounts for approximately 60% of the volume of the crystal, occupies these channels and provides access to the active site of each enzyme molecule from the crystal surface through the 4_1 channel (Dai et al., to be published). The accessibility of the active site is sufficient to allow evaluation

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of the catalytic activity of the enzyme in microcrystals suspended in 2.5 M $(NH_4)_2SO_4$ (Ray, 1986). Although the crystalline enzyme is at least 0.2 times as active as the solution enzyme under the same conditions, and although the respective Michaelis constants for Glc-1-P and Glc-P_2[1] are essentially the same in both environments, the study of crystal-phase activity demonstrated the impracticality of attempting to saturate the enzyme with glucose phosphates in 2.5 M $(NH_4)_2SO_4$. Thus, SO_4^{2-}, an inhibitor of the enzyme competitive with both Glc-1-P and Glc-P_2, increases K_m for Glc-1-P by about 2000-fold and K_m for Glc-P_2 by about 5×10^5-fold under these conditions and thus precludes a structural study of the catalytically active complexes by X-ray crystallography. To remove SO_4^{2-} from the crystals, and thereby facilitate binding of Glc-1-P/Glc-P_2, it was necessary to devise a "desalting" procedure.[2]

While it is possible to briefly expose phosphoglucomutase crystals to glutaraldehyde and subsequently to remove salt from the cross-linked crystals by diffusion (W.J.R., unpublished results), the possibility of using a procedure that obviates chemical modification is more attractive. In fact, a procedure that avoided cross-linking was required during development of a binding protocol for the transition-state analogue inhibitor that is described in the accompanying paper (Ray et al., 1991), because the success of trial procedures was monitored by measuring enzymic activity after dissolving treated crystals. The present study describes a procedure for eliminating sulfate from unmodified millimeter-size crystals of phosphoglucomutase without substantially altering their suitability for X-ray diffraction studies. In fact, treated crystals, but not untreated ones, can be flash cooled to 160 K without adversely affecting their X-ray diffraction pattern. Factors responsible for this differing response to flash cooling are described.

Because of the generality of the principles on which the desalting procedure is based, analogous procedures should be feasible for other protein crystals. Although there are a number of references in the literature to the desalting of robust protein crystals (Schreuder et al., 1988; Bode & Huber, 1978; Fink & Petsko, 1981; Walter et al., 1982; Douzou & Petsko, 1984), the studies described here apparently represent the first systematic procedure for desalting a large relatively fragile protein crystal without cross-linking.

Experimental Procedures

Crystals of rabbit muscle phosphoglucomutase were grown in the manner described previously (Lin et al., 1986) by vapor diffusion from a solution of the enzyme in aqueous 45–48% saturated $(NH_4)_2SO_4$, 4.2–4.5% (w/v) PEG-400, and 15 mM Mg^{2+}, buffered at an apparent pH[3] of 6.5 with 50 mM MES. After being harvested, the crystals were transferred in 30 steps of equal increments (see below) from mother liquor to "storage solution": 2.3 M $(NH_4)_2SO_4$, 50 mM MES, apparent pH 6.5, 16 mM Mg^{2+}, and 1 mM EDTA.[3] Unless otherwise stated, the crystals used for all studies were "millimeter-size" (see Results).

Heavy metal contaminants in stock solutions of 3.9 M $(NH_4)_2SO_4$, 2.4 M Na_2SO_4, and 2 M glycine were eliminated by passing them through columns of Chelex resin (Bio-Rad) before use. The Na_2SO_4 solution was stored at 35 °C to prevent crystallization. Polyoxyethylenes were purified according to procedure b of Ray and Puvanthingal (1984), except that the final column step was conducted at 25% PEG. The product subsequently was isolated by lyophilization in plastic bottles and stored at −20 °C. Stock solutions that contained PEG were stored in the dark and discarded after about 2 weeks. Other reagents and procedures have been described (Ray et al., 1989; Ray & Puvathingal, 1990).

The relative activity of water in various solutions was assessed by vapor-phase equilibration of 50-μL droplets versus 10 mL of external solution in a vapor-tight chamber; a siliconized 12-depression agglutination slide was used to support the droplets. In the case of salt solutions, final concentrations were assessed by conductivity. A Radiometer CDM2e meter with a water-jacketed flow-through cell was used; assay solutions were pumped at a constant rate, first through a stainless steel temperature-equilibration coil and then through the conductivity cell. In the case of solutions containing PEG, relatively low concentrations of Cibacron Blue (Pharmacia) were added to droplets prior to the equilibration step, and final concentrations were assessed spectrophotometrically.

Crystal-Treatment Apparatus. Crystals to be treated were placed in the well of a single-depression slide that previously had been coated with epoxy resin and scored, while the resin still was tacky, to produce an uneven surface. This surface prevented crystals from resting squarely on one face and thus essentially eliminating the accessibility of that face to the bulk solution; it also facilitated the transfer of treated crystals, which were manipulated by sucking them into the cut-off plastic tip of a mechanical pipette. The treated slide was supported in a vapor chamber equipped with a sealable port. When crystals were stored for more than an hour or so, between or after treatments, the solution used in the chamber (10 mL) was the same as that in which the crystals were suspended.

In rate studies with crystals, a micro-stirring bar was positioned at one edge of a 0.4-mL droplet held in the well of an untreated depression slide. A vapor chamber containing the depression slide plus 10 mL of the same solution as in the droplet was supported above a magnetic stirring motor regulated by an external rheostat.

X-Ray Diffraction Data. X-ray diffraction patterns were obtained by use of a Siemens X-100 area-detector system in conjunction with an Elliot GX-20 rotating anode X-ray generator equipped with a Cu anode and operated at 35 kV and 40 mA; a pyrolytic graphite monochromator was used to select Cu K-α radiation. All data were collected with the detector positioned 200 mm from the crystal and at a 2Θ setting of 20°. To reduce absorption of the diffracted X-rays by air, a helium-filled cone equipped with 0.5 mil mylar windows was placed between the crystal and the detector.

Crystals were mounted for flash-cooling studies as follows. A 0.3-mm o.d. glass X-ray capillary was prepared for crystal mounting by removing the tip with a hot wire, placing a bead of sealing wax about 0.5 cm from the cut end, and coating that end with a thick layer of hydrocarbon grease (Glisseal, Borer Chemie AG, FDR). Just prior to mounting, a crystal plus a small droplet of surrounding liquid were suspended in immersion oil (type A, Cargille Laboratories) that previously had been equilibrated with the same liquid and separated from it

[1] Abbreviations: Glc-1-P, α-D-glucose 1-phosphate; Glc-P_2, α-D-glucose 1,6-bisphosphate; PEG, polyoxyethylene, or poly(ethylene glycol); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; a, activity, FWHM, full width at half-maximum height.

[2] For brevity, the term "desalting" is used to describe processes in which the $(NH_4)_2SO_4$ used as a crystallizing agent is replaced by another solute, whether that solute is a relatively nonpolar solute such as PEG-400, a polar nonionic solute such as sucrose, an internal salt such as glycine, or another salt such as the substrate, Na_2Glc-1-P.

[3] Apparent pH values are measured at high salt and are uncorrected. The Mg^{2+}/EDTA mixture used in the storage solution and in various desalting procedures maintained the enzyme in its Mg^{2+} form and protected against the binding of adventitious Zn^{2+} [cf. Ray (1967)].

by centrifugation. The opened tip of a 0.2-mm X-ray capillary was positioned parallel and close to one face of the crystal, and the aqueous phase surrounding the suspended crystal was carefully removed by aspiration, along with a much larger volume of the immersion fluid. Gentle suction was then used to pull the "dried" crystal onto the grease and affix it at the tip of the mounting capillary. Once the crystal was affixed, the capillary was cut about 1 cm below the wax bead and the free end of the resulting 1.5-cm capillary with its attached crystal was slipped into a hollow brass rod attached in the usual way to a goniometer head. To stabilize the assembly, the wax bead and the end of the brass rod were covered with Plasticine. The mounted crystal was cooled rapidly to 160 K in a stream of nitrogen (flow rate 6 L/min) supplied by an Oxford Cryostream cooler (Stoe Diffraction Systems, U.K.; Cosier & Glazer, 1986) and was maintained at this temperature during data collection.

Modus Operandi. The paradigm used to develop a procedure for desalting phosphoglucomutase crystals, with the specific goal of removing sulfate, is described below. It depends on several observations about the properties of these crystals in particular, and protein crystals in general, as rationalized in terms of physical principles that generally must be important in maintaining crystal integrity, i.e., in avoiding both fracturing and dissolution of crystals.

First we consider what may be a crystal-specific response to a general effect: osmotic shock-induced fracturing of protein crystals as the result of a change in the concentration of an external solute. In general, if osmotic shock is produced by *increasing* the activity of water in the surrounding solution (i.e., by lowering the concentration of an external solute), protein crystals likely will fracture because initially water will diffuse into the crystal more rapidly than solute diffuses out. In the case of phosphoglucomutase, even a small increase in the external water activity can produce cleavage planes perpendicular to the parallel 4_1- and 2_1-channels of the crystal. If osmotic shock is produced by *lowering* the activity of water in the surrounding solution, the difference in diffusion rates for water and solute again likely will produce osmotic fracturing. In the case of phosphoglucomutase, fractures thus produced proceed irregularly through the crystal, as if the crystal were being crushed by a temporary decrease in the amount of water within the channels. An assessment of fracturing patterns in phosphoglucomutase crystals was used to adjust procedures for altering solute concentrations so that no visible cracks were produced in the process. In describing specific cases below, fractures of the first type are referred to as "expansion cracks", those of the second type are called "crush cracks".

Many protein crystals likely will withstand substantial osmotic stress without fracturing, so that complete elimination of osmotic stress is only an idealized goal. However, in seeking to avoid osmotic fracture, it remains prudent to avoid large differences in water activity between the crystal and the surrounding solution. In practice, relatively large concentration changes that fractured phosphoglucomutase crystals when made suddenly, could be made without causing visible damage if the overall change was made in a series of small steps; thus, the use of a large number of small steps was adopted as a general procedure. In these steps the activities of internal and external water never were far from equilibrium, and sufficient time for re-equilibration, based on actual measurements, was allowed between steps.

A second important principle is that in an equilibrium biphasic mixture the activity of each component is the same in both phases. Because there is no difference in the activity of water between two phases in equilibrium, no osmotic effects will accompany the transfer of a crystal from one phase to the other. Hence, by using suitable biphasic mixtures, one can make drastic changes in the composition of the solution *surrounding* a crystal in a single step without risking osmotic fracture. As an example, the concentrations in one biphasic mixture that was used are as follows: in the lower, salt-rich phase, $[Na_2SO_4] = 0.5$ M and [PEG-8000] < 0.1% (w/v); in the upper, PEG-rich phase, [PEG-8000] = 30% and $[Na_2SO_4] = 0.14$ M. The activity of water, $a(H_2O)$, is equal in the two phases because the concentrated Na_2SO_4 in the lower phase reduces $a(H_2O)$ to the same extent as does the PEG plus dilute salt in the upper phase, i.e., the equal reduction of $a(H_2O)$ in the two phases depends on a partition of Na_2SO_4 that greatly favors the salt layer over the PEG layer, plus a partition of PEG that is in the opposite direction.

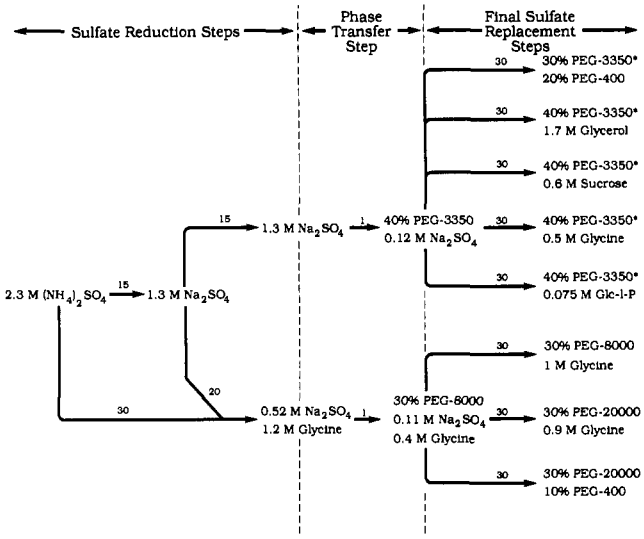
Biphasic mixtures also can be prepared with various salts and low molecular weight organic solutes, e.g., methylpentanediol, which can permeate the pores of most protein crystals. Such systems are not considered here, since only when the phase transfer step does not change the composition of the solution within the pores of crystals can one be certain that the phase transfer will succeed.

Since the transfer of a protein crystal between the phases of a biphasic mixture produced with one permeating and one nonpermeating solute does not alter the composition of the solution within the channels of the crystal, the surface of the crystal becomes, in effect, a new phase boundary. Although the *activity* of the permeating solute (e.g., Na_2SO_4) within the channels of the crystal and its activity in the external solution must be the same both before and after the transfer, the *concentration* of this solute within the crystal and without may differ somewhat at these points, because activity coefficients are environmentally sensitive. Nevertheless, *for simplicity, the concentration of permeating solute within the crystal, both before and after the transfer, will be taken as equal to its concentration in the external solution prior to the transfer.*

When one solute in the biphasic mixture is excluded from the channels of the crystal, the surface of the crystal acts as a semipermeable membrane relative to the excluded solute. Hence, in the above example, $[Na_2SO_4]$ within the channels of the crystal can be lowered essentially to zero in steps subsequent to phase transfer by lowering $[Na_2SO_4]$ in the surrounding solution of PEG. But, as in the case of a semipermeable membrane, this can be accomplished without generating substantial osmotic pressure (and in the present case fracturing crystals), only by adding to the external solution of PEG a permeating "replacement solute" that will enter the crystal as Na_2SO_4 is removed. The replacement solute serves to maintain a water activity inside the crystal that is approximately the same as the activity outside. Thus, it is important in selecting a replacement solute to match the partition of the original permeating solute between the two phases relatively closely. In the above example, osmotic pressure differences will be minimized if the replacement solute partitions between the internal aqueous phase and the external PEG phase precisely as does Na_2SO_4, which substantially prefers the internal aqueous phase.

A different criterion related to dissolution of the crystal was used in choosing the excluded solute. The primary function of the excluded solute is to achieve a satisfactory reduction in $a(H_2O)$ so that the crystal does not either dissolve or liquefy by sorption of water from the surrounding medium; the permeating solute present in the external phase can assist in this

Scheme I: Outline of Various Transfer Sequences Used in This Study[a]



[a] Only the major components of the various mixtures are shown. The number above each arrow shows the number of steps used for that transfer. In each case, 10 mM HEPES (PEG solutions) or 50 mM HEPES (salt solutions), pH 7.5, also was present along with 2 mM $MgSO_4$/0.5 mM EDTA (PEG solutions) or 16 mM $MgSO_4$/1 mM EDTA (salt solutions). X-ray diffraction rocking curves were obtained as described in the text for those cases where the final solution is flagged by "*"; the diffraction pattern was examined without analysis of the rocking curve for all other cases.

respect (see below). At all stages, and especially in the final solution, the closer $a(H_2O)$ remains to its value where the crystals were grown, the better the chance of a successful desalting operation. On the other hand, kinetic factors may significantly slow dissolution rates for the protein and lessen the practical significance of modest increases in $a(H_2O)$. Thus, experience with phosphoglucomutase has shown that an exact identity between $a(H_2O)$ in the crystal growth medium and the final desalting solution is not required to stabilize crystals for substantial periods of time.

Additional requirements for a workable desalting procedure are related to the rate at which the system approaches equilibrium. A primary factor is the rate at which the permeating solute equilibrates between the channels of the crystal and the surrounding solution since, in a series of transfer steps, stress related to deviations from solute equilibrium in individual steps can be cumulative. The Results show how the rate of equilibration was estimated in the present system. As implied above, the other key rate is that at which protein in the crystal phase comes to equilibrium with dissolved protein in the surrounding solution. This rate limits the extent to which *temporary* increases in $a(H_2O)$ at an intermediate state of the desalting process, as well as permanent increases in the final solution, can be tolerated. Rate considerations are treated further under Discussion.

Successful procedures for desalting phosphoglucomutase crystals incorporate the main aspects of the paradigm outlined above: to minimize osmotic shock, changes in solute concentration are made in small steps or by moving crystals across a phase boundary; the holding time between individual steps is balanced between the time needed for permeating solutes to reach quasi-equilibrium and the rate of crystal dissolution; and the final removal of sulfate involves the choice of a replacement solute on the basis of the criteria described. Successful desalting schemes are summarized in Scheme I. Several trial procedures that yielded unacceptable results are also noted under Results, along with modifications that eliminated the cause of failure. We hope that these will provide guidelines for devising related procedures in other systems, with fewer failures.

Results

Studies using crystalline enzyme were conducted with millimeter-size crystals of relatively consistent habit that can be described as a bisected tetragonal bipyramid with the nontetrad vertices truncated to show slight development of the form {110}. For typical crystals three out of the four planes of {110} are visible, and the bisecting plane is nearly parallel to some member of {110} (within 15°). Given this habit and the consistent dimensions of the crystals used in this study (ca. 0.5 mm along [001] and 0.9–1.2 mm for the longest dimension perpendicular to [001]), we estimate that the maximum distance from the surface to the center of any crystal along a 4_1 channel is in the range 0.22–0.29 mm. Moreover, it can be shown that 90% of the molecules in such crystals are within 0.12–0.16 mm of the surface.

Diffusion Rates within the Channels of the Crystal. Enzyme crystals in storage solution were treated for several hours with trace amounts of $[^{32}P]HPO_4^{2-}$. A single crystal was removed from the radioactive solution, blotted, and suspended in 0.2 mL of fresh storage solution for a "stirred-droplet" rate study. The amount of radioactivity in time aliquots (10 μL) taken from the stirred droplet was measured and, after 0.5 h, the crystal was removed, blotted, and dissolved in order to measure the remaining radioactivity. More than 90% of the label diffused out of the crystals within 3 min. Since the treatment with $[^{32}P]HPO_4^{2-}$ did not produce covalent labeling, and since phosphate does not bind significantly to the enzyme at 2.3 M $(NH_4)_2SO_4$, the time required for 90% of the label to clear the crystal was taken as the time required for an external salt of comparable size to nearly equilibrate with the solution in the channels of crystals.

Such a time interval is approximately what is expected for free diffusion in a channel open at both ends [cf. Bishop and Richards (1968)]. Thus, if a channel initially contains a uniform concentration of solute, S, and if $[S] \rightarrow 0$ at the ends of the channel, the time t required for a solute with a diffusion coefficient D to decrease to 0.1 of its original value at the center of a channel l cm long is approximately $1.2\ l^2/D$ [cf. Figure 4.1 of Crank (1956) and Figure 7 of Fink and Petsko (1981)]. If D is taken as 10^{-5} cm s^{-1}, the calculated t would be about 1 min for channels 0.05 cm long, as opposed to the measured value of 3 min. Hence, in subsequent studies involving transfer of crystals from one solution to another, a transfer step usually was made every 3 min. However, with large crystals such as those subsequently used for data collection (up to 1.5 mm for the largest dimension perpendicular to [001]) 4-min intervals were required, and, with the solute PEG-400, 5-min intervals were used.

Serial Transfer of Crystals from High-Sulfate Solutions to Solutions Containing Polyoxyethylene at Low Sulfate Concentration. In initial studies, crystals of phosphoglucomutase, in either the phospho or the dephospho form [see the accompanying paper (Ray et al., 1991)], were successfully transferred in 30 steps from a storage solution containing 2.3 M $(NH_4)_2SO_4$ to the lower salt-rich phase of the biphasic mixture obtained by equilibrating 30% PEG-8000 with an equal volume of a 0.66 M Na_2SO_4 solution containing 1.5 M glycine, 50 mM HEPES, pH 7.5, 16 mM Mg^{2+}, and 1 mM EDTA. (Such biphasic mixtures usually were clarified prior to use by standing overnight; otherwise they were centrifuged after standing for several hours). In later experiments, an artificial lower phase was used, i.e., one that was not actually

equilibrated with the PEG-rich phase (see below). In all cases, the series of transfer solutions was prepared by mixing the initial and final solutions in various proportions so that the change in concentration of the various components between each step was the same.

The large number of steps proved to be absolutely necessary. Attempts to use 10 transfer steps in an analogous procedure produced expansion cracking, even when the equilibration time between steps was increased 3-fold so that the total elapsed time was the same as for successful transfers. Moreover, use of a series of premixed solutions also was required, i.e., mixing a different solution into a droplet that contained the crystal frequently caused fracturing. Replacement of NH_4^+ by Na^+ over the course of the transfer was not necessary but was beneficial because it reduced pH-drift due to loss of NH_3 from small volumes of stock transfer solutions and because phosphoglucomutase crystals appear to be less soluble in Na_2SO_4 than at the same concentration of $(NH_4)_2SO_4$, as is generally the case [cf. Arakawa and Timasheff (1985)].

At the end of the above serial transfer, the salt concentration in the solution surrounding the crystals was low enough to allow detectable dissolution over a period of several hours. Hence, the following phase-transfer step usually was conducted immediately. In initial studies, several crystals at a time, along with a small volume of the lower salt-rich phase, were transferred to a freshly centrifuged sample of the PEG-rich phase by using a mechanical pipette. Essentially all of the lower phase subsequently was removed with a fine-tip mechanical pipette. (Removal of *all* visible droplets of the lower phase from the surface of the crystals in this step was important; otherwise, in later steps crystals adhered to one another or to the surface of the depression slide in a manner that made detachment difficult.) When an artificial upper phase was used, 30% PEG, 0.13 M Na_2SO_4, 0.35 M glycine, 10 mM HEPES, pH 7.5, 5 mM Mg, and 1 mM EDTA, a 15–30-min equilibration period was allowed after crystal transfer before removing the last of the encapsulating lower phase droplet. It should be noted that crystals *never* fractured during phase transfer, whereas numerous failures occurred in prior or subsequent steps.

At this stage of the transfer, the concentration of Na_2SO_4 in the channels of the crystal was taken as 0.52 M (see Modus Operandi). As a guide in preparing solutions for the third and final stage of the desalting procedure, the partition of the following substances between the above salt-rich and PEG-rich phases was evaluated: glycine, 3:1 (from the approximate molar ratio of solubility in the two solutions); inorganic vanadate, dianion, 2.0:1 [optical density measurement at 265 nm, pH 10.5 (Ray & Post, 1990)]; glucose, 2.4:1 [neocuproin assay (Dygert et al., 1965)]; Glc-6-P, 5.4:1 [dehydrogenase assay (Michal, 1984)]; $MgSO_4$, 6:1 [8-hydroxyquinoline assay, cf. Ray and Roscelli (1964)]; Glc-P_2, 14:1 (dehydrogenase assay after hydrolysis of acid-labile phosphate).

The above partition studies were conducted because crystals transferred to the PEG-rich phase (30% PEG; 0.13 M Na_2SO_4) could not be transferred subsequently to sulfate-free 30% PEG without crush-cracking, even when the equilibration time between steps was doubled, unless a permeating solute was used to replace the Na_2SO_4. But when glycine, for example, was used as a replacement solute at the maximum concentration obtainable in 30% PEG-8000, a 30-step transfer of crystals from the PEG-rich phase to a solution of 30% PEG-8000 1 M in glycine (with 10 mM HEPES, pH 7.5, 1 mM $MgSO_4$, and 0.1 mM EDTA) proceeded without any evidence of cracking. Moreover, transfer from PEG/sulfate to PEG/glycine had no noticeable effect on the resolution of the X-ray diffraction pattern from these crystals (see below) and storage for 1 week in the final solution produced no noticeable surface erosion, although $a(H_2O)$ in this solution is somewhat greater than that at which the crystals were grown. [The activity of water in 30% PEG-8000 is equivalent to that in 0.60 M $(NH_4)_2SO_4$; in 30% PEG-8000 plus 1 M glycine it is equivalent to that in 1.6 M $(NH_4)_2SO_4$, in contrast with the crystal growth solution, which contains 2.1 M $(NH_4)_2SO_4$].

The above and other successful transfer protocols are summarized in Scheme I. For reasons noted under Discussion, desalting operations also were conducted at 40% polyoxyethylene, using PEG-3350 instead of PEG-8000. At 40% PEG, sulfate could be replaced by either glycine or Glc-1-P, present at close to saturating concentration in the final solution, or by 0.6 M sucrose or 1.7 M glycerol. In desalting operations conducted at 30% PEG-3350, the sulfate also could be replaced by 20% PEG-400, which permeates the crystal.

In each of the above cases, X-ray diffraction studies were conducted to show that the integrity of the crystal had not been adversely affected by the desalting procedure. In all cases outlined in Scheme I, the resolution of the diffraction pattern extended at least as far as for untreated crystals, where a large majority of the possible reflections with interplanar spacings of approximately 2.7 Å are measurable, and almost none with spacings smaller than 2.5 Å (Lin et al., 1986). For some crystals, a series of diffraction patterns consisting of 10–30 adjacent "frames" were measured with a Siemens X-ray area-detector system and an oscillation angle of 0.05–0.15° per frame. These measurements were used in conjunction with the interactive computer graphics program XELOP (Minor & Bolin, to be published) to examine the three-dimensional profiles of a number of strong well-resolved reflections with interplanar spacings in the range 3.5–3 Å. In addition, a least-squares fitting procedure with $2n + 1$ free parameters was used to determine a common full width at half maximum (FWHM) for n reflections from the same crystal. A single crystal was examined for each of the desalting procedures marked with an asterisk in Scheme I; data from several untreated crystals were subjected to the same analysis. For each of the desalted crystals, the FWHM is within the range 0.16–0.19°, whereas for untreated crystals the FWHM values are in the range 0.16–0.27°. Thus, the diffraction patterns obtained from desalted crystals were of a quality similar to those from the best of the untreated crystals, both in terms of resolution and in terms of the angular width of typical reflections.

Does the Surface of a Phosphoglucomutase Crystal Really Behave Toward Polyoxyethylene as a Semipermeable Membrane? If PEG is entirely excluded from the channels of phosphoglucomutase crystals, the measured partition of a permeating solute between an external PEG solution and the channels should approach the ratio of its solubility in the PEG solution versus its solubility in water (subject to the approximation highlighted in Modus Operandi). To test exclusion of PEG by this approach, external conditions were chosen that maximized the discrimination in favor of the interior of the crystal, consistent with reasonable convenience: 50% PEG-3350 containing 0.040 M Na_2Glc-1-P, a saturating concentration. Since the solubility of Na_2Glc-1-P in water is approximately 1.1 M, the partition of Glc-1-P across a semipermeable membrane with water on one side and 50% PEG-3350 on the other would favor the PEG-free solution by some 27-fold.

To utilize this approach, crystals were transferred to the above PEG/Glc-1-P solution and allowed to equilibrate for several hours. Individual crystals were then blotted dry and dissolved in 0.2 mL of Tris-Cl, pH 7.5; both the protein and the Glc-1-P concentrations in this solution were measured. On the basis of these measurements and an estimate of 61% for the nonprotein volume of the crystals, the Glc-1-P concentration in the channels of the crystals was found to be 0.85 ± 0.01 M. Thus, the inside/outside partition was 21 instead of the expected value (27). By contrast, the internal concentration of Glc-1-P in equilibrium with external 0.5 M Glc-1-P/1.2 M Na_2SO_4 was 0.55 ± 0.05 M when estimated as above; hence, its partition is approximately equal to the expected 1:1 ratio, within the error of the experiment. In these calculations, the estimate for the nonprotein volume was obtained by the procedure of Matthews (1968) based on a value of 3.12 for V_M [cf. Lin et al. (1986)]. Furthermore, we assumed that Glc-1-P was uniformly distributed throughout the nonprotein volume and that this volume is entirely accessible, as implied by the second experiment.

In view of the number of approximations used in the above comparison, it is reasonable to suggest that the surface of a phosphoglucomutase crystal acts *more or less* like a semipermeable membrane, even toward PEG-3350, and should act more like such a membrane toward polyoxyethylenes of higher average M_r. Nevertheless, the apparent reduction in partition coefficient for Glc-1-P involving crystals suspended in the PEG-3350 solution, relative to a semipermeable membrane that completely excludes PEG-3350, is in the direction expected if PEG-3350 actually enters the channels of the crystal to some extent (see Discussion).

Preliminary X-Ray Diffraction Studies with Desalted Crystals. Although the X-ray diffraction characteristics and the unit cell dimensions of untreated crystals of phosphoglucomutase and crystals transferred to a PEG/glycine solution were similar at room temperature, the diffraction patterns obtained after rapid cooling to 160 K in a stream of dry nitrogen were vastly different. Untreated crystals immediately became opaque and diffracted only weakly at low resolution, if at all, although no fracturing was apparent in these crystals either at 160 K or after they were warmed to room temperature. Similar results were obtained with crystals cooled more rapidly by plunging directly into liquid methylcyclopentane at 160 K. By contrast, "desalted" crystals remained transparent after cooling and produced X-ray diffraction patterns comparable in resolution and quality to those obtained at room temperature from untreated crystals. Initial comparisons of reflections from untreated crystals at room temperature and from desalted flash frozen crystals at 160 K indicated no major difference in the width of their respective rocking curves, although on close inspection it was apparent that the half-width of these curves had increased somewhat, but not enough to adversely affect data collection.

Studies To Determine Why Desalted Crystals Survive Flash Cooling to Sub-Zero Temperatures. To identify the factors that account for the different response of untreated versus desalted phosphoglucomutase crystals toward flash cooling, crystals were examined at various stages of the desalting process: at 1.8 M Na_2SO_4; at 1.3 M Na_2SO_4; at 40% PEG-3350/0.12 M Na_2SO_4 (the PEG-rich phase in equilibrium with 1.3 M Na_2SO_4); and at 40% PEG-3350/0.08 M Na_2SO_4/0.125 M glycine [after completion of 1:4 of the sulfate/glycine replacement steps (see Scheme I)]. All of the partially desalted crystals became opaque on flash cooling to 160 K and failed to diffract to a significant extent.

To determine whether it was the removal of Na_2SO_4, the presence of glycine, or the presence of low molecular weight polyoxyethylenes (from the PEG-3350) that allowed the crystal lattice to survive the flash-cooling process, two sets of crystals were desalted in 30% PEG-20000. In one case, the final solution contained an essentially saturating concentration of glycine, as when PEG-3350 was used; in the other, 10% PEG-400 was used instead of glycine (see Scheme I). Although crystals in the PEG-20000/PEG-400 mixture survived the cooling,[4] those in the PEG-20000/glycine mixture became opaque and failed to diffract.

Studies of flash cooling with a stream of nitrogen gas also were conducted on small droplets or thin films of water or aqueous solutions containing PEG-400 and PEG-8000. Droplets were supported by a U-shaped platinum wire, and thin films were supported on a mesh of glass wool. While rapid cooling of thin films of water produced relatively few isolated ice crystals, as indicated by the number of Bragg reflections seen in X-ray diffraction patterns from different parts of the cooled films, cooling of 1.5-μL water droplets always produced polycrystalline specimen. However, increasing concentrations of PEG-400 in the droplets decreased the grain size of the ice, as judged by the diffraction patterns, until at 30% PEG only sharp Debye rings resembling a powder pattern were observed. [Usually this implies a grain size in the range of 1–10 μm (Cullity, 1978)]. Finally, at 40% PEG only a weak, diffuse "liquid-water" ring was obtained. Parallel experiments using droplets containing PEG-8000 at the same series of weight/volume concentrations produced essentially the same results.

Discussion

Although cross-linking the molecules of protein in a crystal by using a reagent such as glutaraldehyde has been the traditional prelude to removing salts from crystals grown at high salt concentration (Quiocho et al., 1967; Haas, 1968), previous studies with phosphoglucomutase suggested that a gentler approach, based on a gradual reduction in salt concentration followed by a phase-transfer step, might suffice for this and other protein crystals. One such observation is that phosphoglucomutase crystals, which grow quite slowly in 2.1 M $(NH_4)_2SO_4$, also dissolve exceedingly slowly at "zero" protein concentration under the same conditions. In fact, it can be shown that the product of the net growth rate per unit area of any crystal and the fractional supersaturation at which the growth rate is observed is equal to the dissolution rate (at zero protein concentration) under the same conditions [cf. Appendix in Ray and Puvathingal (1986)]. Since most protein crystals are grown at fractional supersaturations of less than 10 (fractional supersaturation is the ratio of the concentration of crystallizable protein to its equilibrium solubility), in general a slow-growing crystal will dissolve slowly at zero protein concentration under otherwise identical conditions and may dissolve slowly at zero protein concentration when the concentration of precipitant is much lower. In the case of phosphoglucomutase, where growth of millimeter-size crystals

[4] PEG-400 partitions between the 30% external PEG-20000 and the channels of the crystal in such a way that osmotic stress is produced by this desalting procedure during the final, sulfate replacement stage (cf. Modus Operandi). In fact, X-ray diffraction patterns obtained at room temperature from crystals desalted in a PEG-20000/PEG-400 mixture were somewhat less satisfactory than those from crystals desalted in 40% PEG-3350/glycine, where osmotic effects more or less balanced. Thus, the diffraction pattern of PEG-20000/PEG-400 crystals did not extend to Bragg spacings smaller than 3 Å, and the crystal seemed to have a larger mosaic spread than crystals desalted in PEG-3350/glycine. However, the contrast in behavior on cooling between crystals desalted in PEG-20000/PEG-400 and in PEG-20000/glycine was striking.

requires 6–8 weeks at a protein concentration of 30 mg/mL in 2.1 M $(NH_4)_2SO_4$, the surfaces of crystals are scarcely eroded after 2 h in 1 M $(NH_4)_2SO_4$. Moreover, substituting Na_2SO_4 for $(NH_4)_2SO_4$ further decreases the dissolution rate so that crystals can be kept for a day at 1.0 M Na_2SO_4, even though the activity of water is somewhat higher in 1.0 M Na_2SO_4 than in 1.0 M $(NH_4)_2SO_4$ (Weast, 1970).[5] The ability to temporarily withstand a reduction in salt concentration can be important in facilitating the design of desalting procedures that involve a biphasic mixture (see below).

A second observation that suggested the possibility of a gentler desalting operation is that crystals of phosphoglucomutase frequently fracture immediately when a small volume of a more concentrated or a less concentrated solution is added to a droplet in which crystals are suspended. Since the severity of the visible fracture frequently abates with time, and since crystals that fail to crack immediately do not crack on further standing, it is a *transient* aspect of the change that produces the damage. The present study suggests that transient osmotic shock produces the fracturing and that such shock is exacerbated by uneven mixing in the case of additions to a droplet containing crystals. This observation, together with a recognition of the rapidity in which small solutes penetrate the channels of crystals (see Results) suggested that relatively large changes in solute concentration might be made by using many small steps, whereas the use of a few larger steps would damage the same crystals. Hence, to reduce osmotic shock to an acceptable level during changes in the concentration of solutes, even with changes as gradual as 2.3 M to salt → 1.3 M salt in 30 steps, a series of premixed solutions was used. Although the above step size might seem tediously small, since the salts in question are 2:1 electrolytes the transient osmotic pressure generated close to the entrance of channels in the crystal by steps of this size is expected to exceed 1 atm, even when a generous allowance is made for the effects of nonideality.

The stepwise desalting procedure developed on the basis of these and related observations is a variation of that proposed by Fink and Petsko (1981) but involves a series of gradual changes rather than more abrupt ones, which fragile crystals are not likely to survive (Douzou & Petsko, 1984; Petsko et al., 1984). An added advantage of the present procedure is its greater amenability for modification to suit the characteristics of different crystals.

Clearly, phase transfer is the critical step in the proposed desalting procedure. Thus, almost by definition, the gentlest way to replace most of the salt surrounding a protein crystal by an organic solute is to employ a solute that forms a biphasic mixture either with the salt solution in which the crystal is grown or stored or with a salt solution to which the crystal can be transferred. Although the organic solute need not be a polymer, a polymeric solute that is too large to enter the pores of the crystal is the only sure choice[6] (see Modus Operandi).

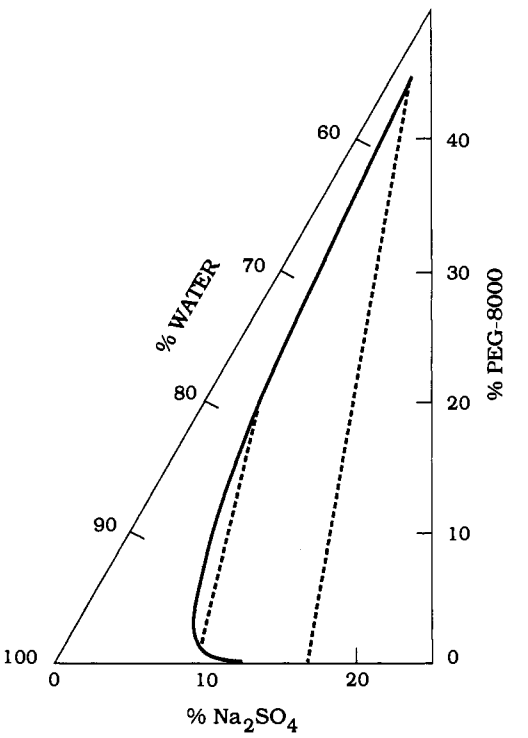


FIGURE 1: Partial phase diagram for Na_2SO_4/PEG-8000/H_2O mixtures. Composition of the liquid/liquid biphasic mixture (curved line) is shown, where concentrations are expressed as weight percent. In calculating these concentrations, the density of the solution was assumed to vary linearly with concentration from 0 to 30% PEG-8000 (d = 1.00 to 1.06 g/mL) and from 0 to 2.4 M Na_2SO_4 (1.00 and 1.26 g/mL). Salt concentrations in the separated phases of a biphasic mixture were determined by conductivity; the PEG concentration was estimated by a trial-and-error procedure in which the amount of PEG required to produce turbidity at a known Na_2SO_4 concentration was evaluated. Concentrations are expressed in terms of weight percentage of the anhydrous salt, where 10% = 0.77 M. Two tie lines (dashed lines) are shown.

The higher the concentration of salt at which the protein crystallizes, the higher the concentration of aqueous organic solute that likely will be required to stabilize the desalted crystal, and the less likely a suitable biphasic mixture can be found. In fact, it is probable that crystals grown in $(NH_4)_2SO_4$ solutions at concentrations higher than about 60% will be difficult to stabilize in an aqueous solution of polyoxyethylene unless the pores of the crystal are relatively small, so that a low molecular weight PEG can be employed. Thus, Figure 1 shows a partial 3-component phase diagram for PEG-8000, Na_2SO_4, and water; a phase diagram for mixtures involving $(NH_4)_2SO_4$ in place of Na_2SO_4 is similar. Although only two tie lines are represented (the bottom ends are at 0.7 and 1.3 M Na_2SO_4), the requirement for relatively high PEG concentrations to balance $a(H_2O)$ in solutions of Na_2SO_4 more than about 1 M (13% w/v) Na_2SO_4 is obvious. However, possible problems in crystal transfer and mounting related to the high viscosity of PEG solutions at such concentrations are greatly reduced by the crystal-mounting/data-collection procedure employed here.

Initially, 30% PEG-8000 was employed as the nonionic solute, since its reported size suggests that it would not readily enter the channels of the phosphoglucomutase crystal. Thus, the RMS radius of gyration of PEG-8000, on the basis of viscosity studies [cf. Knoll and Hermans (1983)], is about 30 Å, whereas the radius of the 4_1 channel is about 20 Å. Later, when it became necessary to further decrease the activity of water in the "excluded" solute to counteract the effect of substrate binding [see the accompanying paper (Ray et al.,

[5] A number of proteins are less soluble in concentrated aqueous solutions of Na_2SO_4 than in comparable solutions of $(NH_4)_2SO_4$ [cf. Arakawa and Timasheff (1985)]. Since $a(H_2O)$ actually is greater in concentrated Na_2SO_4 solutions than in $(NH_4)_2SO_4$ solutions of equal concentration [cf. Weast (1970)], it seems likely that the NH_4^+ ion is less excluded from the hydration sphere of proteins than is Na^+. In fact, NH_4^+ may preferentially enter some regions of the hydration sphere because of interactions analogous to those identified by Burley and Petsko (1986).

[6] The properties of a variety of water-soluble synthetic polymers, some of which might be useful in such operations, are discussed in a monograph by Molyneaux (1983).

1991)], 40% PEG-3350 was used instead. This substitution reduced the viscosity of the solution, relative to a solution of 40% PEG-8000, without substantially changing the effect of the solute on $a(H_2O)$. Again, because of the crystal-mounting procedure that eventually was employed, viscosity reduction now is considered more a convenience than a necessity.

Because of the reduced size of PEG-3350 [calculated RMS radius of gyration: 20 Å (Knoll & Hermans, 1983)], penetration of its major components into the 4_1 channel of the crystal is more likely. In fact, problems encountered in attempting to remove Glc-P_2 from such crystals during formation of a vanadate-based inhibitor complex [described in the accompanying paper (Ray et al., 1991)], as well as the results of flash-cooling studies with desalted crystals, are most readily rationalized in terms of partial penetration of PEG-3350 or low molecular weight components thereof.[7] In the case of flash cooling, partial penetration would increase in the volume of the crystal exposed to the excluded solute, which may well act as an inhibitor of ice crystal nucleation. In fact, we suggest that protein crystals grown from high molecular weight PEGs might be made more resistant to flash-cooling by the deliberate addition of PEG-200 or PEG-400. The partition of Glc-1-P between 50% PEG-3350 and the interior of crystals suspended in a mixture of the two also suggests that the interior contains some polyoxyethylenes. Nevertheless, the results of this study indicate that the semipermeable membrane model of the crystal surface, in which PEG-3350 is considered to be completely excluded from the interior, holds reasonably well.

When the excluded solute used in the desalting process is truly excluded, relief of osmotic stress by a permeating solute requires that it partition between the interior and exterior of the desalted crystal in such a way that the interior solution is substantially favored. Only in this way can it reduce water activity within the crystal without making a substantial contribution to reduce water activity in the external solution. In the present case, glycine initially was chosen as the permeating solute because it does not bind to the anion-binding sites of phosphoglucomutase and because its effect on nonpolar groups of proteins places it in a salt-like category (Arakawa & Timasheff, 1983). Later Na_2Glc-1-P, sucrose, glycerol, and even PEG-400 were used as permeating solutes (Scheme I).

The expected effect that a reduced dielectric constant would have on the pK_a values of amino acid side chains, and previous emphasis on avoiding a substantial reduction in dielectric constant during the infusion of crystals with cryoprotectants (Douzou & Petsko, 1984), argue that the electrostatic charge on adjacent molecules within the lattice should be maintained constant, or nearly so, to maximize the stability of crystals during changes in the permeating solutes. Thus, the ability of the relatively fragile phosphoglucomutase crystals to withstand the decreased charge–charge shielding that must accompany transfer from 2.3 M $(NH_4)_2SO_4$, pH 6.5, to 30% PEG-8000/1 M glycine, pH 7.5, is surprising, although the glycine solution that permeates the channels of the crystals at the end of this transfer should be close to saturation (about 3 M) and would have a dielectric constant in excess of 135 [cf. Douzou and Petsko (1984)]. But more surprising is the fact that 40% PEG-3350/0.6 M sucrose, 40% PEG-3350/1.7 M glycerol, and even 30% PEG-3350/20% PEG-400 can be used to replace essentially all of the salt in these crystals: surprising since, for example, the dielectric constant of 50% PEG at room temperature is only about 35 (Kaatze et al., 1978). Our results thus raise questions about whether the relative success of a previously reported multistep procedure for infusing cryoprotectants into protein crystals (Fink & Petsko, 1981), as compared to earlier one-step procedures, owed its success to maintenance of a more or less constant dielectric constant, as claimed, or simply to reduced osmotic shock. In fact, the current studies suggest that, at least in the case of protein crystals with large channels and relatively larger center-to-center molecule–molecule distances, *minimizing osmotic shock is more important than maintaining a constant dielectric constant in solute-replacement procedures.* Schreuder et al. (1988) also have considered the importance of $a(H_2O)$ and osmotic effects in crystal transfer protocols and have described a procedure for transferring protein crystals from the original mother liquor to solutions containing different precipitants. However, the procedure used by these authors fails to take into account a number of other critical features that should be incorporated into a general desalting procedure, and their procedure fails rather spectacularly with our crystals: they essentially explode over a period of several minutes when subjected to this desalting procedure.[8]

Choosing the identity and concentrations of the permeating and excluded solutes for a desalting procedure involves a complex set of interdependent goals. First, the concentration of excluded solute plus permeating solute in the external phase must be high enough to prevent dissolution or liquefaction of the crystal for a reasonable period of time. Second, the concentration of permeating solute must reduce $a(H_2O)$ within the channels of the crystal to the point where internal and external water activities are balanced within the capacity of the crystal to withstand osmotic stress, and do so without interfering with protein–protein contacts. Finally, the partition of the permeating solute, which can depend quite strongly on the choices made for the excluded solute, must be such that both of the above goals can be either realized or approximated. In fact, it is unlikely that all will be achieved simultaneously by any feasible set of choices.

In practice, the choice of identity and possible concentrations for the excluded solute was narrowed on the basis of whether or not a suitable biphasic mixture could be devised and what solute concentration was required to produce it. The suitability of the mixture was evaluated in terms of the concentration of salt in the salt-rich phase: how close it was to that of the

[7] The following considerations relate to the possible penetration of PEG-3350 into the channels of phosphoglucomutase crystals. (a) According to theory (Casassa & Tagami, 1969), the partition coefficient for a random coil polymer between a solution in which it is present at high dilution and a long inert cylindrical cavity, filled with the same solvent, is about 0.1 when the RMS radius of gyration, R, of the polymer is equal to the radius of the cylinder, a, and when partition is governed entirely by differences in conformational freedom. (b) Given $R = a$, the time required for a polymer such as PEG-3350 to reach equilibrium within a channel some 10^5-fold longer than R could well be much longer than the time periods for which crystals were suspended in PEG solutions during desalting operations: 1–2 h. (This aspect of the diffusional problem does not seem to have been treated in the literature, even on a theoretical basis.) (c) PEG-3350 is supplied as a heterogeneous population that includes a significant fraction of molecules with molecular weights lower than the average; this fraction would exhibit an enhanced tendency to penetrate the crystals that is difficult to deduce. In view of the uncertain application of these considerations to the present system, we refer to PEG-3350 as an excluded solute primarily for simplicity, but also because we observed, in comparable experiments, no qualitative differences between PEG-3350 and PEG-8000, for which partial penetration should be substantially decreased.

[8] Part of the problem with the procedure of Schreuder et al. (1988) is that the concentrations of solutes in two different solutions that reach identical values of $a(H_2O)$ via *vapor phase equilibration* will not be the same as those that produce identical values of $a(H_2O)$ when the two solutions are in contact across a semipermeable membrane, unless the membrane is impermeable to *both* solutes.

crystallizing solution and whether crystals transferred stepwise to that phase were reasonably stable. Next, the partition of possible permeating solutes between the two phases of the mixture was measured. These measurements plus vapor-phase equilibration studies were used to provide an initial estimate of the concentration of permeating solute in water that would at least partially offset $a(H_2O)$ in a solution that contained the excluded solute plus whatever permeating solute was expected in the external solution. After reasonable concentration adjustments, usually involving both solutes, final adjustments were made by observing the behavior of crystals in a provisional procedure based on the above estimates. Although it is too early to provide firm guidelines, procedures based on Na_2SO_4 [or Na_2malonate, see the accompanying paper (Ray et al. (1991)], PEG, and glycerol, sucrose, glycine, or an entity that will form a desired complex in the crystal phase (which may be a salt, as in the case of Na_2Glc-1-P) appear to be the best initial choices.

While the desalting protocol described here was the result of deliberately planned operations, our observations about the effect of polyoxyethylene on crystals of phosphoglucomutase subjected to flash cooling were the result of pseudoserendipity: the chance availability of desalted crystals after the failure of untreated crystals to survive the cooling process. While the cause of such failures usually is attributed to the formation of "ice" around or within the protein crystal (Douzou & Petsko, 1984), a general approach to circumventing this problem has not been established, although the procedure of Petsko and co-workers [cf. Fink and Petsko (1981)] has been employed in a few cases. Since the procedure described here seems to provide a basis for altering the character of the internal solution in protein crystals in a relatively gentle and systematic manner, the development of general guidelines for minimizing the damage that frequently accompanies flash cooling may be possible. While the outcome of such attempts is difficult to predict, the potential returns can be quite large (Fink & Petsko, 1981; Hope et al., 1989).

The problem of avoiding ice formation in or around protein crystals during rapid cooling is particularly challenging because nonequilibrium thermodynamics in a poorly defined system is at the heart of the problem. The nonequilibrium nature of such changes is suggested by the observation that sufficiently small samples of water can be cooled rapidly enough to largely circumvent formation of ice crystals, even in the absence of nucleation-inhibiting solutes. But there are practical limitations to the rate at which protein crystals suitable for X-ray diffraction studies can be cooled.[9] In addition, the shape and size of some of the solvent channels and/or cul-de-sacs within a crystal may well affect the tendency toward formation of ice crystals during rapid cooling [cf. Douzou and Petsko (1984)], and the formation of ice at a relatively few critical points may lead to unacceptable overall results. Although antinucleators other than the polyoxyethylenes that were used here may prove more effective in other systems, the results obtained with these materials in the present system and the general compatibility of polyoxyethylenes with protein crystals (McPherson, 1982) recommend them for studies in other systems. However, as noted under Modus Operandi, the procedures employed here naturally are restricted to systems where a reasonable diffusional flux within the protein crystal can be achieved readily [cf. Bishop and Richards (1968)], and the choice of the best molecular weight range of polyoxyethylenes for use in other protein systems is open. In fact we do not yet understand how polyoxyethylenes produce the effects observed in the present system: Are they related to the presence of major components of the "excluded" PEG in low concentrations throughout the crystal[7] or to the penetration of lower molecular weight contaminants (possibly increased by our purification procedure); in addition, are the effects produced throughout the crystal, close to the surface of the crystal, or primarily at crystal imperfections? We hope it will be possible, eventually, to answer such questions; but the utility of the approach described here may not depend on immediate answers.

[9] Crystals can be cooled more rapidly by plunging into liquid methylcyclopentane at 130 K than by bathing in a jet of nitrogen gas at 160 K, as was done for most of the rapid cooling studies described herein. But several untreated crystals seemed to be damaged as extensively by the liquid-hydrocarbon procedure as those subjected to the chilled-gas procedure, so that a very rapid decrease in temperature is not a panacea for eliminating temperature-induced structural changes in protein crystals.

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Formation of Substrate and Transition-State Analogue Complexes in Crystals of Phosphoglucomutase after Removing the Crystallization Salt†

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ABSTRACT: Crystals of phosphoglucomutase, grown in 2.1 M ammonium sulfate, "desalted", and suspended in a 30% polyoxyethylene-8000/1 M glycine solution as described in the accompanying paper [Ray, W. J., Jr., Puvathingal, J. M., Bolin, J. T., Minor, W., Liu, Y., & Muchmore, S. W. (1991) *Biochemistry 30* (preceding paper in this issue)], were treated with glucose phosphates to form an equilibrium mixture of the catalytically active substrate/product complexes. However, this treatment extensively fractured the crystals, even when very dilute solutions of glucose phosphates were used. But formation of the desired complexes was achieved, without fracturing, by introducing the glucose phosphates at high salt concentration, where they do not bind significantly to the enzyme, and maintaining their presence during subsequent sulfate-removal steps, in order to obtain essentially uniform binding throughout the crystal at all times. Although this procedure produced unfractured crystals of the catalytically active complexes, an adjustment in water activity was required to prevent the crystals from slowly liquefying in the presence of the added glucose phosphates. After this adjustment, the quality of diffraction-grade crystals subjected to this treatment was not significantly altered. An even larger adjustment in water activity was required to stabilize crystals that had been largely converted into a mixture of vanadate-based transition-state analogue complexes [cf. Ray, W. J., Jr., & Puvathingal, J. M. (1990) *Biochemistry 29*, 2790–2801] by means of an analogous procedure. The rationale for, and the implications of, this adjustment of water activity are discussed. The phenomenon of lattice-based binding cooperativity also is discussed together with a possible role for such cooperativity in the fracturing of protein crystals during formation of ligand complexes and possible ways to circumvent such fracturing based on the annealing of crystals at fractional saturation. An assay for quantifying the extent of formation of the vanadate-based transition-state analogue complexes in crystals of phosphoglucomutase is described. A solution to problems associated with producing and maintaining a steady-state in treated crystals is discussed within the context of maximizing the fraction of the crystalline enzyme present as a complex with one such inhibitor, glucose α-1-phosphate-6-vanadate. One of these problems, achieving a substantial reduction in sulfate concentration, could not be successfully addressed by employing the desalting procedure used to produce the substrate/product complexes, because of reduced diffusional rates in the final solution. Instead, the ammonium sulfate was replaced, stepwise, by sodium/potassium malonate, and an alternative procedure was used to insure essentially uniform binding throughout the crystal as the enzyme was converted into the desired complex. When diffraction-grade crystals were subjected to this treatment, the quality of the diffraction pattern actually was improved. The possibility of using both approaches in treating protein crystals with other reagents is discussed. Possible advantages of using solutions of sodium and/or potassium malonate for the growth of protein crystals, as opposed to ammonium sulfate, also are noted.

Crystals of phosphoglucomutase grown in 2.1 M $(NH_4)_2SO_4$ can be completely "desalted"[1] during transfer to a concentrated solution of a nonpermeating polyoxyethylene, or PEG[2] [see the accompanying paper, Ray et al. (1991)]. The removal of

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[1] For brevity, the term desalting is used to describe processes in which the $(NH_4)_2SO_4$ used as a crystallizing agent is replaced by another solute, even when that solute is an internal salt such as glycine.

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