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Enhanced Diffractivity of Phosphoglucomutase Crystals. Use of an Alternative Cryocrystallographic Procedure†

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Abstract

A continuous procedure for replacing the 2 *M* $(NH_4)_2SO_4$ within crystals of rabbit muscle phosphoglucomutase by 55% polyethylene glycol 600 (PEG 600) is described. The success rate (absence of fracturing) approaches 100% in spite of the fragility of the crystals. The procedure is based on the use of a biphasic PEG/salt mixture in conjunction with a flow cell that allows several crystals to be treated at once. The holdup volume of the cell is small and its design minimizes concentration gradients. Cooling treated crystals to 253 K elicits a substantial increase in diffractivity that allows data collection to be extended from about 2.75 to about 2.35 Å. However, neither the dimensions of the unit cell nor the structure of the asymmetric unit are significantly altered. Comparisons are made between data sets collected at 289 K using crystals in 2.2 *M* $(NH_4)_2SO_4$ and at 253 K using crystals in 55% PEG 600. Models of the enzyme refined against one or the other data sets are also compared. These comparisons suggest that, at most, only a minor fraction of the increased diffractivity is caused by lowered atomic *B* values. The increased diffractivity also is not the result of a simple temperature effect since at high-salt concentration the same cooling protocol fails to significantly increase diffractivity. A decrease in mosaic spread is considered as a possible explanation for the increased diffractivity.

1. Introduction

Rabbit muscle phosphoglucomutase, PGM,‡ crystallizes from $(NH_4)_2SO_4$ at about 48% saturation in the presence of low concentrations of polyethylene glycol 400 (PEG 400): space group $P4_12_12$; unit cell, 174.4 × 174.4 × 101.1 Å. The asymmetric unit, a dimer (M_r = 61 500 per monomer), packs well around the 4_1 axis but relatively poorly in the *ab* plane. Reflections can be observed with an area detector to about 2.65 Å, but data that can be processed accurately does not extent beyond about 2.75 Å. On film, some of the diffraction spots exhibit halos or have an irregular shape, and mottling of the water ring is noticeable. Such observations indicate significant disorder within the lattice (Faure *et al.*, 1994, and references therein).

Crystals of phosphoglucomutase are exceedingly sensitive to small changes in the concentration of external solutes when these are made rapidly. Hence, the solute-replacement procedure used here, which requires large changes in both the type and concentration of the solute, uses a continuous concentration gradient applied over an interval of many hours. A flow cell was designed to facilitate treating several crystals at a time and harvesting the treated crystals. The flow cell and pumping circuit were designed to use a small volume of solution (4–8 ml total) so that an excessive amount of an exchangeable ligand would not be required when it was necessary to incorporate such a ligand into the procedure. (Ligands that induce conformational changes in PGM must be present continuously during the solute-replacement procedure to preserve the integrity of the lattice: Ray *et al.*, 1991.)

Unlike the step-wise solute-replacement procedure described earlier (Ray *et al.*, 1991), the present procedure uses a 'penetrating' polyethylene glycol, PEG 600, to replace both the $(NH_4)_2SO_4$ within the crystals and the non-penetrating PEG 3350 that remains outside the crystals during the early phases of the procedure (Fig. 1). This substitution eliminates concentration gradients within the treated crystals.

Crystals of PGM, treated as above, exhibit substantially enhanced diffractivity when cooled to 253 K for data collection. A molecular model of PGM (Liu, Ray & Baranidharan, 1997) was used in an attempt to deduce the origin of this increase.

2. Materials and methods

2.1. *Crystal treatment*

Crystals of phosphoglucomutase were obtained by slow evaporation of water (about 1% per week) from previously 'demetallated' ammonium sulfate solutions (Ray *et al.*, 1991), about 1.9 *M*, containing 30 mg ml^{-1}

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‡ The following abbreviations are used: PGM, rabbit muscle phosphoglucomutase; HEPES, *N*-2-hydroxyethylpiperazine ethane sulfonate; Na_2Mal, disodium malonate; ID, inside diameter; $a(H_2O)$, activity of water.

of protein.* This solution also contained about 4.5% purified PEG 400 (Ray & Puvathingal, 1984) plus crystallization buffer (*cf.* Ray *et al.*, 1991). The PEG in the crystal growth solution was removed and the ammonium sulfate concentration in the surrounding medium increased to 2.2 *M* by placing the crystals in a flow cell, Fig. 2(*a*), and using a linear gradient (total volume = 16 ml) to achieve the final conditions during a 20 h time interval. A Buchler™ peristaltic pump with 0.15 mm ID tubing was used, together with an interval timer that activated the pump for 12 s every 2 min. The crystals subsequently were transferred to 1.8 *M* sodium malonate buffer, pH 7.6, containing 16 m*M* Mg^{2+}, 1 m*M* EDTA, and 50 m*M* HEPES, pH 7.5, by using the same flow cell (transfer time ≃12 h), and were stored therein until used.

Three to five selected crystals, treated as above, were cooled to 277 K and transferred to an equilibrating solution prior to the phase-transfer step. The equilibrating solution was prepared by thoroughly mixing (at 277 K) equal volumes of 40% purified PEG 3350 and 2 *M* sodium malonate containing 50 m*M* HEPES buffer, pH 7.5, 20 m*M* Mg^{2+} and 1 m*M* EDTA. The lower layer of the biphasic mixture thus produced served as the equilibrating solution. Crystals were transferred to this solution from the storage solution in five steps of equal increments over a period of about 30 min. Although the ionic strength of the storage and equilibrating solutions was essentially the same, premixed solutions were employed in the five-step transfer, as described previously (Ray *et al.*, 1991). (The equilibrating solution was centrifuged briefly before use to eliminate droplets of the upper phase.) The crystals were then transferred to the bottom of the flow cell in Fig. 2(*b*), which also contained equilibrating solution plus cotton fibers from a Q-Tip™ to prevent contact between crystals and to separate them from the lower support screen. After attaching the top section, the flow cell was completely filled with equilibrating solution *via* the lower I/O port. The flow was then reversed and the flow cell slowly flushed from top to bottom *via* the upper O/I port. About 0.5 ml of the upper PEG-rich layer from the above biphasic mixture was used: flushing time, about 3 min. (Again, brief centrifugation was used to remove droplets

* The dimeric asymmetric unit does not exist as such in solution, where only monomers are present, even at 30 mg ml^{-1} (WJR, unpublished quasi-elastic light-scattering studies).

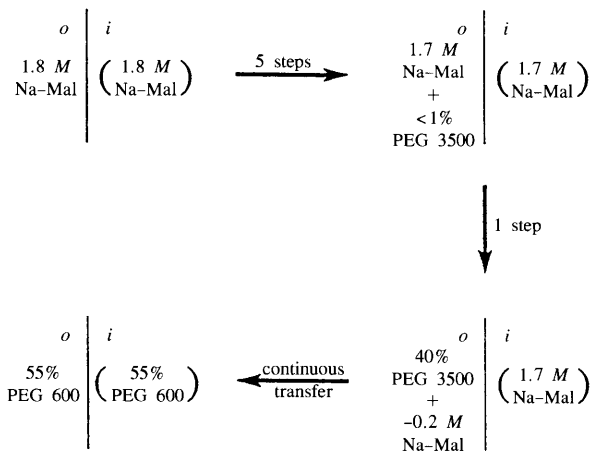


Fig. 1. The sequence of operations conducted in the PEG/salt replacement procedure. The vertical lines represent the surface of the crystal; *i* and *o* refer to the solution inside the pores of the crystal, and outside the crystal, respectively. The disodium malonate was at pH 7.6. Concentrations of solutes within the pores of the crystal, shown in parenthesis, are inferred, based on equal activities of solutes at equilibrium across a boundary permeable to Na_2Mal but not to PEG 3350. The lower right-hand representation shows how a permeable solute, Na_2Mal, that partitions unequally between outside and inside phases, 40% PEG 3350 and water, respectively, can largely eliminate the osmotic effect that would be produced in the absence of that solute, *viz*, if the outside solution were 40% PEG and the inside solution were water.

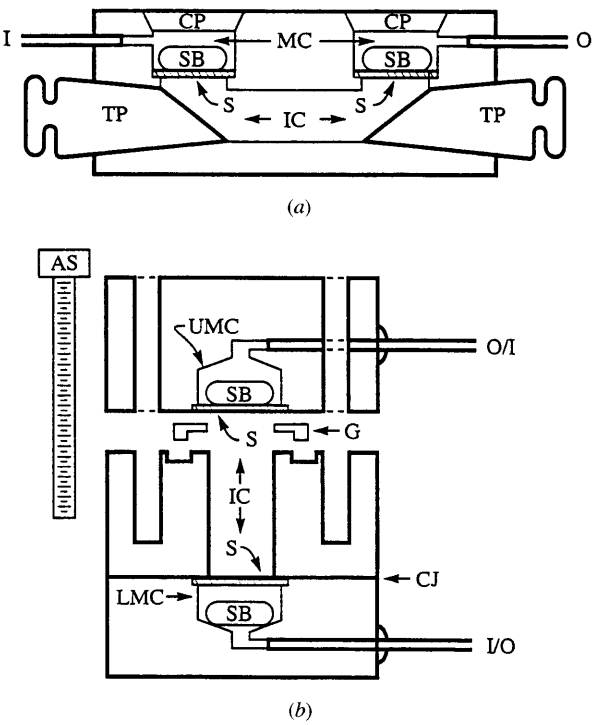


Fig. 2. Flow cells. (*a*) The flow cell for solute replacement when aqueous salt solutions were used at room temperature. Constructed from 1.9 cm^2 Plexiglass; CP, construction plug, glued in place; *I* and O, 1.7 mm OD (15 gauge) stainless steel inlet and outlet tubes, respectively; SB, Teflon covered stirring bar; MC, mixing chamber; S, Nitrex™ nylon screen; TP, tapered Teflon plug, cut at an angle; IC, flow cell chamber. (*b*) The irrigator for solute replacement at 277 K when 55% PEG 600 was used. Constructed from 1.9 cm^2 Plexiglass; AS, assembly screw (four required); O/I and I/O, 0.38 cm ID peristaltic pump tubing attached to the flow cell with silicone glue to serve as inlet/outlet tubes; UMC and LMC, upper and lower mixing chambers, respectively; G, Teflon gasket; SC, S, and IC, see (*a*); CJ, construction joint.

of the other layer.) If conducted slowly enough, this operation, in which a syringe was used as a manual pump, removed most of the lower layer from the crystals and the walls of the flow cell. The flow was then reversed again and a linear gradient pumped into the lower I/O port. The initial and final solutions in the gradient maker were, respectively, the PEG-rich upper layer from the above biphasic mixture and 55% PEG 600 that contained 10 m*M* HEPES, pH 7.5, 1 m*M* Mg^{2+} and 0.05 m*M* EDTA. (The reservoir and mixing chamber of a small-volume gradient maker were fabricated from the barrels of plastic syringes by using Tygon™ tubing and silicone glue.) Three channels of a peristaltic pump with the same size tubing were used to produce and deliver the gradient: one channel pumped from the reservoir to the mixer while two channels pumped from the mixer to the flow cell *via* a Y-joint. (When changes in the pumping protocol were required, tubing connections were made and broken at previously positioned blunt connectors, fashioned from No. 20 hypodermic needles, in order to avoid introducing air bubbles into pump lines.) The pumping rate was 0.5 ml h^{-1} and the total volume of the gradient was 8 ml, although half that rate and volume could be used. To achieve such rates, a Manostat™ cassette pump, equipped with 0.05 cm (ID) Manosil™ silicone rubber tubing, was employed, after adding a one-turn 100 Ω potentiometer in series with the manufacturer's speed control for fine adjustment of the pumping rate. Smooth operation required the replacement of cassette tubing after each run. To prevent bubble formation in the flow cell while pumping the gradient, the distal end of the tube attached to the exit port was kept several inches above the flow cell. The reservoir and mixer also were kept in an elevated position relative to the pump. When all of the gradient solution had been pumped from the mixer, the flow cell was pumped for one more hour from the reservoir. Subsequently all but 1.5 ml of solution was removed from the reservoir, a magnetic stirring bar added, and the solution in the flow cell recycled through the reservoir for a further 2 h (to ensure complete equilibration). To monitor crystals without interrupting the transfer, the housing was removed from a magnetic stirrer and the motor plus magnet mounted beneath the observation stage of a dissecting microscope, so that the crystals could be observed, either directly, or by tilting the irrigator. (For smooth operation at slow speeds, it was necessary to carefully balance the stirrer magnet.) All of the above operations were conducted at 277 K. The transfer sequence is summarized in Fig. 1. A diagram of the apparatus is shown in Fig. 3.

Crystals of PGM so treated were mounted in 1 m*M* glass capillaries and attached to a goniometer head. The attached capillary plus crystal were transported to the diffractometer in a Dewar flask, precooled to 273 K. The diffractometer was equipped with a variable-temperature dry air source (FTS Air-JetTM, model XRII 851A0) plus a diffuser that directed a column of chilled air toward the goniometer head from the opposite side of the χ circle. The mounted crystal was protected from ambient air by a removable plastic cover while the goniometer head was being attached to the diffractometer. After centering the crystal, the temperature of the air, monitored at the surface of the capillary close to the crystal, was lowered to 253 K over a time interval of 45 min to 1 h.

2.2. *Data collection*

A data set for native crystals, suspended in 55% $(NH_4)_2SO_4$, was collected in the same way as the low-temperature data set described by Liu *et al.* (1997), but at 293 K.

2.3. *Freezing points*

To avoid supercooling, the freezing point of 3.4 *M* Na_2malonate and 55% PEG 600 was estimated by a procedure where ice crystals in a siliconized glass capillary, 0.5 mm OD, were introduced into a column of the solution in the sealed end of a 2.2 mm siliconized glass capillary, after equilibrating both crystals and solution to a predetermined temperature. Subsequent growth of ice crystals or the lack thereof was monitored through a 5× lens. Temperature was measured with a thermocouple (see above) before and after introducing the ice crystals.

3. Results

3.1. *Factors limiting the design of the solute-exchange flow cell*

The design of the flow cell used to replace the 1.8 *M* Na_2malonate in PGM crystals with 55% PEG 600 in the solute-exchange procedure was dictated by several considerations. [The 55% $(NH_4)_2SO_4$ in the native crystals

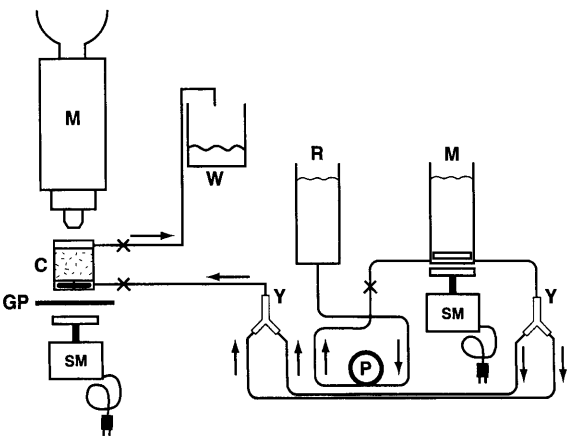


Fig. 3. Diagram of apparatus showing flow during solute replacement. M, microscope; C, flow cell; GP, glass plate; SM, magnetic stirrer; P, pump; R, reservoir; M, mixer; W, waste reservoir; x, connectors; Y, Y joints.

had been replaced previously with 1.8 *M* Na_2malonate to facilitate the subsequent salt/PEG replacement.] One consideration was minimizing the volume of solution required (see *Introduction*). The capacity to treat several crystals at once in a cell where they could be removed readily for storage also was considered, since the treatment requires 12–16 h. More than a dozen different flow cells that fulfilled these criteria to varying extents were fabricated and tested. In all but one case the success rate (percentage of unfractured crystals at the end of the treatment) was unacceptably low – much lower than with the more tedious and somewhat different step-wise procedure described earlier (Ray *et al.*, 1991). The main problem was the tendency toward streaming when a viscous solution with a different density is pumped from a small-bore tube (to minimize the volume in the system) into an unstirred chamber with a significantly larger cross section. Although such streaming, due to laminar flow, was not always visible, over a period of time concentration gradients developed in all but one flow cell tested. In some cases, merely changing the orientation of the cell exposed the partially treated PGM crystals to these gradients and caused immediate fracturing.

To eliminate concentration gradients, a vertical flow cell with a magnetic stirring bar in the bottom of the cell was used. The stirring bar was separated from the crystals by a plastic screen (Fig. 2*b*). The crystals almost always fractured when this stirring bar was omitted even though a thoroughly mixed solution of PEG 3350/PEG 600 from a gradient device was pumped through the flow cell. However, the exit stirrer shown in the flow cells of Fig. 2 is probably unnecessary.

The flow cell in Fig. 2(*a*) that accommodates 50–100 crystals is more convenient to use and sufficed for solute-replacement procedures involving aqueous solutions at room temperature. Thus, it was used routinely to transfer crystals from 55% $(NH_4)_2SO_4$ to 1.8 *M* Na_2malonate prior to the salt/PEG replacement step. But, it does not entirely eliminate streaming of concentrated PEG solutions at 277 K and could not be used successfully for the salt/PEG procedure.

3.2. *The effect of temperature on the diffractivity of PEG-treated crystals*

On the basis of qualitative observations, the salt/PEG exchange, per se, produced only a marginal enhancement in the diffractivity of PGM crystals at the temperature of the exchange: 277 K. The major enhancement occurred during cooling to 253 K. An additional increase also occurred during the time required to index the crystal (at 253 K), as though the crystal were undergoing self annealing.

3.3. *The 253 K/high-PEG and 289 K/high-salt data sets*

A merging *R* factor of 5.4% (13–2.4 Å resolution) was obtained for data collected at 253 K with five PEG-treated crystals (Table 1 of the accompanying paper, Liu *et al.*, 1997). A second data set was collected in a similar manner on the same instrument with three PGM crystals of comparable size that were grown under the same conditions and mounted in the same way, but suspended, instead, in 2.2 *M* $(NH_4)_2SO_4$ at 289 K. The merging *R* factor (13–2.7 Å) for this and other comparable data sets (not described) was about 15% (Table 1).

Table 1. *Characteristics of the merged data in the 289 K high-salt data set within shells of approximately equal volume in reciprocal space*

Only reflections with $I/\sigma \geq 1$, where σ is obtained from processing statistics, are included. In the resolution range used for refinement, 6–2.7 Å, $\sigma \geq 1$ for 38 174 unique reflections (97% of theory).

Resolution range (Å)	% of theory	Cumulative %
5.63–20.00	99.8	99.8
4.49–5.63	99.8	99.8
3.93–4.49	99.5	99.7
3.57–3.93	99.0	99.5
3.31–3.57	98.6	99.3
3.12–3.31	97.9	99.1
2.96–3.12	97.0	98.8
2.83–2.96	94.9	98.3
2.73–2.83	93.5	97.8
2.63–2.73	89.3	97.0
2.55–2.63	22.6	90.3

3.4. *Data comparison: 289 K/high-salt data versus 253 K/high-PEG data*

Data obtained under the above two conditions were compared in three different ways. Two of these involved constructing plots of of $\log(I_i/\sigma_i)$ *versus* resolution and of expected coordinate error (Luzzati, 1952) for a model refined against both data sets (see below): Fig. 4, inset, and Fig. 4, respectively. Fig. 4, inset, shows that the number of significant reflections observed at 289 K and high salt decreases rapidly below about 2.65 whereas significant reflections obtained at 253 K and high PEG extend to about 2.35 Å. The Luzzati plot shows that the number of reliable reflections in the 253 K/high-PEG data set exceeds that in the 289 K/high-salt data set by about 50%, based on the resolution range where the crystallographic *R* factor = 0.3.

The third type of comparison involved the atomic *B* values for a molecular model refined against the 253 K/high-PEG data set (Liu *et al.*, 1997) that includes 494 water molecules [crystallographic *R* factor = 0.163 (6–2.4 Å); r.m.s. deviation of bonds and angles from ideal values: 0.018 Å and 3.2°, respectively]. This model also was refined against the 289 K/high-salt data set by simulated annealing, as described in the above reference, except that TBATH was set at 2000 K instead of 5000 K. All water molecules in the 253 K/high-PEG model were retained in the 289 K/high-salt model: *R* factor = 0.162 (6–2.7 Å); r.m.s. deviation of bonds and angles from ideal values: 0.020 Å and 3.8°, respectively. The r.m.s.

coordinate difference after least-squares superposition of corresponding C^{α} atoms in 301 selected core residues in monomer (2) of the two models (Table 3 of Liu *et al.*, 1997) is 0.11 Å. Although several differences of 1 Å or more in C^{α} positions of non-core residues occur in surface features of domain IV in monomer (1), the overall similarity between the two models indicates that the basic crystal structure is not significantly altered by the PEG treatment or by the subsequent cooling.

After refinement, the ratio of diffuse scattering to Bragg scattering was approximated as the average of the individual values of $[1-\exp(-B_i\sin^2\theta/\lambda^2)]$ (Clarage, Clarage, Phillips, Sweet & Caspar, 1992) for all protein atoms in both models. Since at 2.7 Å this average was 0.83 and 0.805 for the 289 K/high-salt and 253 K/high-PEG models, respectively, B-value differences do not appear to be large enough to account for the observed differences in I_i/σ_i (Fig. 4).

3.5. *Comparison of water activities in solutions to which PGM crystals were exposed*

The activity of water in a 2.2 M $(NH_4)_2SO_4$ solution (close to where PGM crystals were grown), in 55% PEG 600, and in 3.4 M Na_2malonate (where diffractivities of PGM crystals were examined) is compared on the basis of freezing-point depression. In this comparison, pure liquid water at the freezing point of the above solutions is used as the reference, *i.e.* the state where $a(H_2O)$ is taken as 1. The conventional assumption (Eisenberg & Crowthers, 1979) that ΔH_{fus} for the melting of ice in contact with such solutions is approximately equal to that of ice in equilibrium with water at 273 K is also made. [Although this is not a good assumption for accurate measurements of $a(H_2O)$ in solutions that freeze well below 273 K, it is reasonable for qualitative comparisons.] Thus,

$$\ln[a(H_2O)] = (\Delta H_{fus}/R)(273.3^{-1} - T_f^{-1}), \qquad (1)$$

where T_f is the freezing temperature.

Values of $a(H_2O)$ for the above three solutions, together with values for T_f are recorded in Table 2. To the first approximation, these results show that $a(H_2O)$ decreases more or less regularly from H_2O, to 2 M $(NH_4)_2SO_4$, to 55% PEG 600, to 3.4 M Na_2malonate.

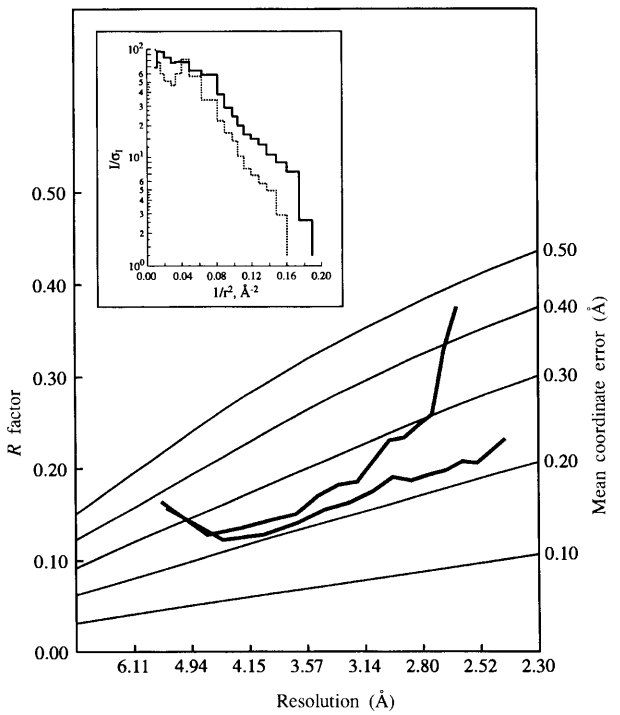


Fig. 4. Comparison of data obtained at 289 K and high salt with that obtained at 253 K and high PEG. Luzzati plots of expected coordinate error for the 289 K/high-salt model, upper plot, and 253 K/high-PEG model, lower plot. Inset: semilog plot of average values for $I/\sigma(I)$, from the *XDS* data-processing program (Kabsch, 1988) in various resolution shells, *versus* $1/r^2$, where r is resolution: — data obtained at 253 K and high PEG; · · · data obtained at 289 K and high salt.

Table 2. *Comparison of water activity in three solutions used in the current studies*

Solution	Approximate freezing point* (K)	$a(H_2O)$†
2 M $(NH_4)_2SO_4$	266	0.93
55% PEG 600	256‡	0.85
3.4 M Na_2Mal	243	0.72

*Measured as described in *Materials and methods*. †Calculated according to equation (1). ‡When nucleated, ice crystals grow at 253 K, but did not appear to grow at 255 K; the Union Carbide Chemicals Company brochure on Carbowax indicates that the freezing point of this solution is about 256 K.

4. Discussion

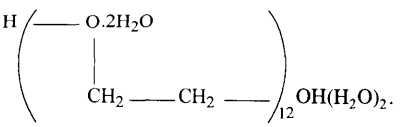
The use of polyethylene glycol as a cryoprotectant for phosphoglucomutase crystals during rapid cooling to liquid nitrogen temperatures has been described (Ray, Post, Liu & Rhyu, 1993). When used in this manner, cryoprotectants function by decreasing the rate at which potentially damaging ice crystals grow until the temperature becomes too low and the solution too vitreous to support efficient crystallization. In contrast, the present usage of PEG depends to a large extent on a different effect: freezing-point depression, *i.e.*, an equilibrium effect. Thus, in the procedure described, the formation of ice crystals in the surrounding solution was suppressed during intervals of many hours at temperatures well below 277 K. In general, freezing-point depression depends both on ideal and on non-ideal properties of the solute (Eisenberg & Crowthers, 1979). But in a 55% solution of PEG 600 the primary effect is non-ideal,

since the depression based on ideal properties is less than 2 K. However, a minor kinetic effect also operates, since data collection was conducted 2–3 K below the equilibrium freezing point of the surrounding solution of PEG. (Although once nucleated, ice crystals will grow in 55% PEG at 253 K; such solutions can be maintained for many hours in capillaries at that temperature without ice formation.)

Changes in the atomic motion within a molecular lattice that reduce the diffuse scattering of X-rays, or changes in the regularity of the lattice that reduce the mosaic spread, or both, would improve the signal/noise ratio in a derived data set. Hence, an attempt was made to determine which type of change is more likely or more important in the present case.

Under specified conditions, the total scattering from a given crystal is equal to the sum of the diffuse scattering and the Bragg scattering. Moreover, the ratio of the two is given by $[1-\exp(-B_i\sin^2\theta/\lambda^2)]$ (Clarage *et al.*, 1992). Hence, an approximation of the fall-off in diffraction intensity with resolution was made by using isotropic atomic temperature factors for refined models of treated and untreated crystals. Thus, the same atomic model was refined by simulated annealing against these two data sets. An average value of the above expression was then calculated for all non-H atoms plus 494 water O atoms in the asymmetric unit. (The r.m.s. coordinate difference between the two models was 0.11 for 301 core C^α atoms: *cf.* Liu *et al.*, 1997). According to this approach, at 2.7 Å, for example, diffuse scattering resulting from thermal motion exceeds Bragg-type scattering by several fold and is even larger at 2.4 Å. In such a case, a small fractional decrease in diffuse scattering would produce a much larger fractional increase in Bragg-type scattering. However, the difference between the average value of $[1-\exp(-B_i\sin^2\theta/\lambda^2)]$ for the two models appears to be too small to account for the observed difference in diffractivity produced by the salt/PEG replacement procedure (*Results*). This conclusion is in accord with another observation described herein: at high-salt concentration, cooling PGM crystals from 289 to 253 K produces no significant increase in signal/noise ratio and no increase in resolution.

Although rocking curves were not constructed, the reflections produced by treated crystals were substantially sharper than those of untreated crystals. This difference is reflected in the merging R factor for data sets obtained with five treated and three untreated crystals: 5.4 and 15.2%, respectively. On this basis we suggest that an effective narrowing of mosaic spread is a more likely rationale for the present results than a reduction in diffuse scattering. However, what changes at the molecular level might be responsible for a reduced mosaic spread is not obvious. On the other hand, changing the internal solvent (61% of the crystal by volume) from 55% or 2.2 M $(NH_4)_2SO_4$ to 55% PEG 600 represents an enormous change in the properties of the solvent. The composition of the PEG solution is actually close to that of a dihydrate of the oxyethylene unit,



Hence, an effective increase in the strength of lattice forces would not be surprising.

Two reports have appeared in which increased diffractivity is produced by a solute-replacement protocol (Yennawar, Yennawar & Farber, 1994; Schick & Jurnak, 1994). A common denominator of these and the present report is a decrease in the activity of water in the external solution and thus within the crystal. In both previous cases, increased lattice interactions are indicated by a decreased size of the unit cell. In the present case, where no such change was detected, altered lattice interactions are suggested mainly by default (see above).

Although reducing the activity of water is one way to strengthen intermolecular interactions, it is clear that the treatment described depends on the identity of the solute employed, since a high concentration of a salt that reduces $a(H_2O)$ even further than 55% PEG 600 does not produce enhanced diffractivity. In fact, it may be a combination of reduced water activity and the lack of access to intermolecular contacts, because of both the size and nature of the solute, that is important in the present system (*cf.* Sousa & Lafer, 1990). Thus, PEG 200 could not be used as a replacement solute because of the solubility of PGM crystals at high concentrations of this polyethylene glycol. (Increased solubility in the presence of PEG 200 suggests an increased access to intermolecular contacts.)

Our inability to provide a definitive rationale for the observed increase in the diffractivity of treated PGM crystals raises the question of whether this effect depends primarily on idiosyncracies of the PGM lattice. However, the above references plus observations with rubisco crystals (Zhang & Eisenberg, 1994) suggests that effects of the type reported here may be attainable with a variety of protein crystals. In the case of rubisco, when the concentration of PEG is kept high enough to prevent dissolution of the crystals, lattice disorder produced by a change in pH slowly disappears due to self annealing (Zhang & Eisenberg, 1994). A reduced mosaic spread produced by self annealing may provide a rationale for the results reported here.

In spite of the increased diffractivity of PGM produced by the PEG/salt replacement, the transfer of a fragile PGM crystal from high-salt to high-PEG concentrations is not without its problems. Thus, the transfer cannot be conducted directly or rapidly (*cf.* Ray *et al.*, 1991) and in the present case cannot be conducted at room temperature without adversely affecting diffrac-

tivity.* In addition, the quality of crystals subjected to salt/PEG replacement at 277 K begins to deteriorate when the crystals are stored at 273 K for more than a day or two, although sensitivity to X-ray damage at 253 K is not adversely affected to a significant extent, if at all. In spite of these problems, the improved diffractivity realized by the transfer process was well worth the relatively minor inconvenience of conducting the process itself, once the major problems associated with devising a successful transfer procedure were solved.

* Recent studies show that a modified solute-replacement protocol similar to that described herein, but conducted at 296 K, can directly improve the diffractivity of PGM crystals to an even greater extent, *i.e.*, without a cooling step (W. J. Ray Jr & S. Baranidharan, unpublished results).

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