Ray, W. J., Jr., & Mildvan, A. S. (1973) *Biochemistry 12*, 3733–3743.
Ray, W. J., Jr., & Puvathingal, J. M. (1984) *Anal. Biochem. 146*, 307–312.
Ray, W. J., Jr., & Puvathingal, J. M. (1986) *J. Biol. Chem. 261*, 11544–11549.
Ray, W. J., Jr., & Post, C. B. (1990) *Biochemistry 29*, 2779–2789.
Ray, W. J., Jr., & Puvathingal, J. M. (1990) *Biochemistry 29*, 2790–2801.
Ray, W. J., Jr., Puvathingal, J. M., & Liu, Y. (1991) *Biochemistry 30* (following paper in this issue).
Ray, W. J., Jr., Post, C. B., & Puvathingal, J. M. (1989) *Biochemistry 28*, 559–569.
Schreuder, H. A., Groendijk, H., van der Laan, J. M., & Wierenga, R. K. (1988) *J. Appl. Crystallogr. 21*, 426–429.
Walter, J., Steigemann, W., Singh, T. P., Bartunik, H. D., Bode, W., & Huber, R. (1982) *Acta Crystallogr. B38*, 1462–1472.
Weast, R. C. (1970) in *Handbook of Chemistry and Physics*, 50th ed., pp D-174 and D-205, CRC Press, Boca Raton, FL.

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

William J. Ray, Jr.,* Joseph M. Puvathingal, and Yiewi Liu
Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907
Received January 23, 1991; Revised Manuscript Received April 17, 1991

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

† This research was supported in part by Grant GM08963 from the U.S. Public Health Service.
* To whom correspondence should be addressed.

[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.

0006-2960/91/0430-6875$02.50/0

sulfate in this way allows the glucose phosphate substrates to bind to the active site of the enzyme after diffusing into the crystal via the 4_1 channel, which is some 40 Å in diameter (Ray et al., 1991). Exposure of desalted crystals of the phosphoenzyme even to very low concentrations of glucose phosphates, or crystals of the dephosphoenzyme to low concentrations of V_i in the presence of Glc-1-P, produces extensive fracturing. However, procedures were devised to allow the crystal-phase formation of an equilibrium mixture of substrate/intermediate/product complexes, as well as a substantial conversion to a mixture of transition-state analogue complexes [and later to a single complex of this type (see below)] in such a way that an apparent annealing of the crystal accompanies formation of the complexes. No fracturing was observed in the treated crystals, and the quality of the X-ray diffraction pattern was essentially unaltered. The principles that form the basis for this procedure are considered in some detail.

Although one expects treatment of a catalytically active crystalline enzyme with a substrate to produce an equilibrium mixture of substrate and product complexes, one of the primary reasons for employing a transition-state analogue inhibitor is to obtain a single complex. This objective causes problems in the case of the only known transition-state analogue inhibitors of phosphoglucomutase, the mixed diesters of glucose phosphate and vanadate, V-6-Glc-1-P and V-1-Glc-6-P (Percival et al., 1990). These mixed diesters bind to the enzyme in ways that are sufficiently dissimilar that distinctly different spectral changes in the near UV are produced (Ray et al., 1990). Thus, both bind essentially exclusively with their phosphate groups at the distal phosphate-binding subsite of the active site and the vanadate groups at the proximal phosphate-transfer subsite. The similarity between the binding modes for the two anionic groups of these mixed diesters requires that the glucose ring interact differently with the dephosphoenzyme in the E·V-6-Glc-1-P and E·V-1-Glc-6-P complexes, and it is these differences (which are reflected in the way that Glc-1-P and Glc-6-P normally bind to the phosphoenzyme) that produce the differing spectral changes (Ma & Ray, 1980; Ray et al., 1990). The vanadate-based inhibitor system is particularly complex because of the slow interconversion of the above two complexes in the presence of excess Glc-1-P and V_i. Part of this interconversion is catalyzed by traces of active enzyme, conversion of Glc-1-P to Glc-6-P after dissociation of the inhibitor, and part is spontaneous, formation of V-1-Glc-6-P from the Glc-6-P thus formed, plus V_i. The present paper describes procedures devised to deal with these complexities and to produce a steady-state system in the crystalline phase where the V-6-Glc-1-P complex heavily predominates, and accomplish this without damaging diffraction grade crystals; in fact, the treatment actually improved the X-ray diffraction pattern of such crystals. It seems likely that a number of these procedures, together with the considerations that led to their adoption, will be applicable to other crystalline enzyme systems, particularly systems where substrate, effector, or heavy atom binding produces conformational changes that fracture crystals or reduce the attainable resolution of the X-ray diffraction pattern.

Experimental Procedures

Materials and procedures not described below are described in the accompanying paper (Ray et al., 1990). As in that paper, "millimeter size" crystals or larger were used throughout. Studies involving activity assays were conducted on the same "batch" of crystals. Solutions that also contained V_i were made up from stock solutions just prior to use, by adding 2% v/v of a concentrated stock solution of NaH_2VO_4/Na_2HVO_4, 1:9, prepared by either dissolving V_2O_5 (Aldrich) in or heating $NaVO_3$ (Alpha) with aqueous NaOH.

A stock solution of 5 M $Na_{1.6}K_{0.4}$ malonate, 500 mL, was prepared after dissolving 64 g of 87% KOH in 85 mL of water, adding 105 mL of "50%" NaOH (about 18 M), and cooling in ice. Granular 99% malonic acid (Aldrich), 263 g, was added slowly with stirring, allowing the temperature to rise during the addition with only intermittent cooling. The pH was adjusted to 7.9 with 50% NaOH (so that a pH of about 7.6 was obtained on diluting to 2.5 M). The solution was passed through a column of Chelex resin (Bio-Rad), regenerated with 0.8 N NaOH/0.2 NKOH, and equilibrated with part of the solution. The effluent was stored at room temperature. A 4 M solution of disodium malonate was prepared similarly from 210 mL of 50% NaOH, 160 mL of H_2O, and 210 g of malonic acid. A 1 M solution of Na_2Glc-1-P was prepared from the solid material (Sigma) and stored at 4 °C. In special cases, Glc-P_2 was removed from the Glc-1-P according to Ray et al. (1990).

Enzyme activity was measured at 25 °C by means of the coupled Glc-6-P dehydrogenase reaction; the Glc-6-P produced was followed by the increase in optical density at 340 nm (Ray & Puvathingal, 1990). The protein concentration was measured either by optical density, $\epsilon_{278} = 4.3 \times 10^4$ M cm^{-1} [cf. Ray et al. (1983)] or by a modification of the Bio-Rad Coomassie blue assay that provided increased reproducibility (details available on request). Crystals of the ^{32}P-labeled phosphoenzyme, Mg^{2+} form, were produced by overnight equilibration of crystalline phosphoenzyme with trace amounts of [^{32}P]Glc-1-P (Ray et al., 1989) in a small volume of "storage solution" (2.3 M $(NH_4)_2SO_4$, 16 mM $MgSO_4$, 1 mM EDTA,[3] 50 mM MES, pH 6.5). The diffusible radioactivity subsequently was removed by successive washings with fresh storage solution. The crystalline *phospho*enzyme was obtained by treating crystals successively for two 2-h periods and then incubating them overnight with 2 mM Glc-P_2 in storage solution. The crystalline *dephospho*enzyme was produced similarly, but by using 20 mM Glc-1-P instead.

Solubilities of crystalline compounds were determined after stirring suspensions of crystals for up to a week to achieve equilibrium.

Assay for the Phospho and Dephospho Forms of Phosphoglucomutase Present in the Crystal Phase. Crystals to be assayed (two per assay) were washed with storage solution, blotted nearly dry with a paper wick, and rinsed into a 1.5-mL plastic centrifuge tube that contained 0.4 mL of 20 mM Tris-Cl, pH 7.5, a solution in which they rapidly dissolved. The concentration of soluble protein was determined by optical

[2] Abbreviations: E_P and E, the phospho and dephospho forms of phosphoglucomutase; Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, D-glucose 6-phosphate, equilibrium mixture of α- and β-anomers; Glc-1,6-P_2 or Glc-P_2, α-D-glucose 1,6-bisphosphate; Glc-P, or GP, equilibrium mixture of Glc-1-P and Glc-6-P, plus 0.5 equiv of Glc-P_2 (see footnote 4); (CC) the central complexes: (E_P·Glc-1-P + E·Glc-P_2 + E_P·Glc-6-P); E·V-6-Glc-1-P or E·V-6-Glc-1-P(Mg), the transition-state analogue complex produced by the binding of Glc-1-P and inorganic vanadate, V_i, to the Mg^{2+} complex of E; E·V-1-Glc-6-P or E·V-1-Glc-6-P(Mg), the corresponding complex involving Glc-6-P and V_i; DTE, dithioerythritol; PEG, polyoxyethylene, or poly(ethylene glycol); subscript ss, steady state; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; a(H_2O), activity of water; FWHM, full width at half maximum height.

[3] The Mg^{2+}/EDTA mixture that was used here 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)].

density, after centrifugation. Subsequently, the amount of dephosphoenzyme in the solution was measured via a modification of the procedure of Lowry and Passonneau (1969). After each successive addition of NAD, Glc-6-P dehydrogenase, and Glc-P_2, the solution was removed from the cuvette, centrifuged, and returned for an absorbance measurement. (A small fraction of the protein in solutions obtained by dissolving crystals sometimes exhibits an enhanced tendency to coagulate during mixing operations.)

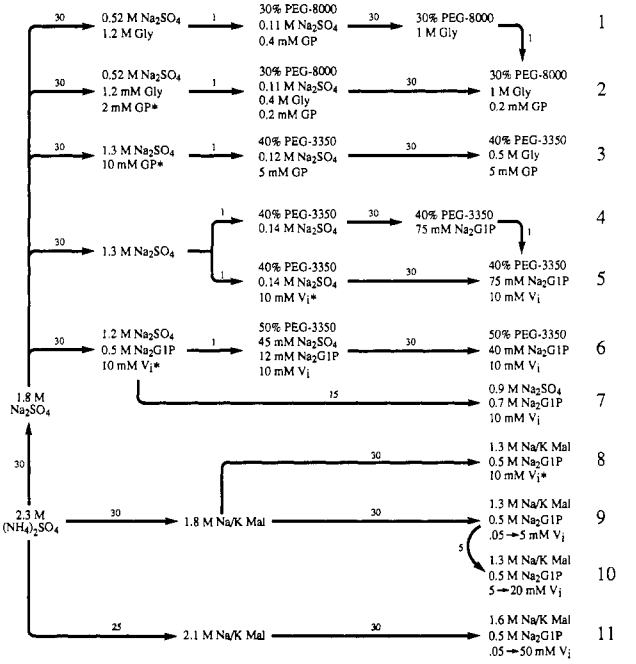
Assay for Vanadate-Based Inhibitor Complexes Present in the Crystal Phase. One crystal of the enzyme/enzyme-inhibitor complex, Mg^{2+} form, was rinsed directly into 4 mL of a stirred reaction mixture, the initial solution for a multiturn-over end-point assay. This solution contained 10 Glc-1-P and 25 μM Glc-P_2 (saturating concentrations), plus 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, and 0.2 mg/mL of serum albumin. After an interval of 20 s (to ensure dissolution of the crystal), a 10-μL sample was added to a spectrophotometer cell that contained 1 mL of final assay solutions, the initial solution plus 0.25 mM NAD and 50 μg of Glc-6-P dehydrogenase (Boehringer) treated in the manner described previously (Magneson et al., 1987). The Glc-6-P thus generated was measured (in terms of increased optical density at 340 nm) for 30–60 min, and the final optical density was obtained after 6–7 h. In other cases the crystal was crushed immediately before addition of the initial assay solution, and an aliquot of the solution thus produced was quickly diluted further with the final assay solution. In such cases, in order to obviate time lags due to the coupling enzyme, the dehydrogenase was omitted, time aliquots were quenched in acid, and the sample was brought close to pH 8 with excess Tris base, in the presence of 10 mM EDTA. Subsequently, the Glc-6-P that had been produced was quantified with the dehydrogenase assay (Michal, 1984). In addition, the enzyme–inhibitor complex in an aliquot from the initial solution sometimes was reactivated by dilution and overnight incubation in a solution containing 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2 mg/mL serum albumin, and 1 mM DTE. A subsequent activity assay (excess Mg^{2+}), initiated with a sample of the diluted solution, was used to quantify the enzyme present that had failed to react with the inhibitor plus that which could be reactivated. The activity obtained also was compared with the total enzyme present, as assessed by use of the Bio-Rad protein reagent.

Results

As in the accompanying paper (Ray et al., 1991), a number of procedures are described that produced unacceptable results, along with the changes in protocol that successfully addressed the pertinent problem, when these illustrate important aspects of the final procedures. A number of these procedures are summarized in Scheme I. The results of several solution studies to determine why poor conversions to the V-6-Glc-1-P complex initially were obtained in the crystal phase and how to increase those yields are described in the Appendix.

Formation of Substrate Complexes of Phosphoglucomutase in the Crystal Phase. When desalted crystals of phosphoglucomutase in 30% PEG-8000 plus 1 M glycine (Ray et al., 1991) were treated with glucose phosphates,[4] expansion cracking invariably occurred, whether the concentration of glucose phosphates was high, 5 mM monophosphates, intermediate, as in sequence 1, Scheme I, or low, 10 μM. However, such fracturing was entirely eliminated by using the same desalting procedure, but including the glucose phosphates in all transfer solutions, beginning with 1.8 M Na_2SO_4 (sequence 2, Scheme I) where the salt concentration is high enough to prevent significant binding. According to the partition studies in the accompanying paper, the effective concentrations of

Scheme I: Some of the Transfer Sequences Used in This Study[a]



[a] Only the major components of the various reaction mixtures are shown. The numbers of steps used to accomplish the indicated changes are shown above the respective arrows. Concentrations were changed linearly between adjacent solutions, except where indicated by an asterisk, in which case the concentration was increased to its *final* value in the *first* step of the sequnce. PEG solutions also contained 10 mM HEPES buffer, pH 7.5, (formation of glucose phosphate complexes, first three sequences) or pH 8.0 (formation of vanadate-based complexes, next four sequences), along with 2.5 mM $MgSO_4$/0.5 mM EDTA; Na_2SO_4 solutions contained 50 mM HEPES buffer with pH values parallel with those above, plus 15 mM $MgSO_4$/1 mM EDTA (first 7 sequences). The pH of malonate solutions was 7.6 (last four sequences) plus 20 mM $MgSO_4$/1 mM EDTA. In solutions that contained an equilibrium mixture of glucose monophosphates, GP (first three sequences), Glc-P_2 also was present at half the concentration indicated for the monophosphates. In this scheme, Gly, Mal, and G1P stand for glycine, malonate, and Glc-1-P, respectively. For other details, see Results. The following comments refer to the various transfer sequences. (1) Crystals fractured in the final step, even when GP was as low as 10 μM. (2) Crystals did not fracture, but liquefied over a period of several hours. (3) Crystals were unaltered visually; their diffraction of X-rays was not substantially altered relative to untreated crystals. (4) Crystals shattered in the final step. (5) Crystals did not fracture, but liquefied within a few hours; a substantial fraction of the enzyme was present in the uninhibited state. (6) Crystals neither fractured nor liquefied on standing, but a substantial fraction of the enzyme was present in the uninhibited state. Prior to the phase transfer step, the diffractivity of the crystals toward X-rays was marginally altered, if at all. (7) Crystals were unchanged visually, and conversion to the V-6-Glc-1-P complex was at least 98%; however, the crystals no longer diffracted X-rays. (8) Crystals fractured badly at about step 6 of the final phase of the transfer sequence. (9) Final phase of transfer conducted at 4 °C. Crystals were unaltered visually, and the enzyme was present almost entirely as inhibitor complexes; however, the ratio of the V-6-Glc-1-P and V-1-Glc-6-P complexes was close to the equilibrium ratio in solution. (10) Final two series of transfer steps conducted at 4 °C. Crystals were unchanged visually, and their X-ray diffraction pattern improved relative to untreated crystals. At least 98% of the enzyme was present as the V-6-Glc-1-P complex. (11) Same as in 10, but the crystals almost always fractured.

[4] The phosphoglucomutase reaction requires that saturating Glc-P_2 be present, along with glucose 1- and 6-phosphates, for complete conversion to the catalytically active complexes. Herein, a 2:1 molar ratio of *mono*phosphates to *bis*phosphate was used and the mixture referred to as "glucose phosphates", Glc-P.

glucose *mono*phosphates and glucose *bis*phosphate within the channels of crystals at the end of the transfer were about 10 mM, which is about 100-fold and 5×10^4-fold higher than the dissociation constants for their respective complexes with the soluble enzyme at low ionic strength (Ray & Long, 1976). Moreover, the final concentration to which the unfractured crystals were exposed was 20-fold higher than concentrations that produced fracturing when employed in a one-step procedure.

As opposed to crystals desalted in the absence of glucose phosphates, which were stable for at least a week, the crystals desalted in the presence of glucose phosphates at 30% PEG-8000, although unfractured, liquefied over a period of several hours after the transfer was complete, apparently due to a coupled breakdown in the crystalline lattice, stressed by ligand binding, and the sorption of water (see Discussion). (We distinguish between liquefaction and dissolution of crystals; in the former case, the droplets remaining where the crystals had rested showed no obvious tendency to dissolve in the surrounding solution.)

To obviate liquefaction, the concentration of the "excluded" solute was increased from 30 to 40% (Ray et al., 1991). Because of this increase, the transfer between salt-rich and PEG-rich phases could be made at a higher concentration of Na_2SO_4, 1.3 M (sequence 3, Scheme I). PEG-3350 also was substituted for PEG-8000 to reduce the viscosity of the solutions employed. [In 40% solutions, PEG-3350 and PEG-8000 reduce $a(H_2O)$ similarly, primarily via a noncolligative effect.] The final concentration of glycine also was reduced but maintained at a value close to its solubility limit in 40% PEG-3350, 0.5 M, so that its concentration within the channels of the crystal was close to its solubility limit in water, 3 M [see Modus Operandi and Discussion of Ray et al. (1991)]. Crystals transferred through this sequence in the presence of glucose phosphates (sequence 3, Scheme I) showed no signs of either fracturing or subsequent liquefaction, even after standing in contact with the final solution for more than a week, although the concentration of glucose phosphates was 25-fold higher than that used at 30% PEG-8000, where the crystals liquefied, and 500-fold higher than that which caused fracturing in a single-step treatment.

Treated crystals were examined by X-ray diffraction, as in the accompanying paper (Ray et al., 1991). The joint estimate of full width at half-maximal height (FWHM) for a number of strong, well-defined reflections within the resolution shell, 3–3.5 Å, were not appreciably altered by the treatment; neither were unit cell dimensions substantially affected (<0.5% change).

Flash Cooling of Crystals after Conversion to Their Glucose Phosphate Complexes. Crystals converted to the glucose phosphate complexes (sequence 3, Scheme I) were flash cooled to −140 °C on three different occasions in the manner successfully used in the accompanying paper (Ray et al., 1991) for desalted native crystals. The only difference between the surrounding liquid in the two cases was the presence or absence of 5 mM glucose phosphates. Although the flash-cooling process did not substantially decrease the resolution of the observed X-ray diffraction pattern, individual reflections were substantially elongated along *c* [the direction perpendicular to the fracture patterns that are produced when glucose phosphates are introduced in a single-step procedure (see above)], so much so that data collection with such crystals was not feasible. One other example of the failure of stressed phosphoglucomutase crystals to undergo flash cooling also has been observed, and this type of behavior may be general.

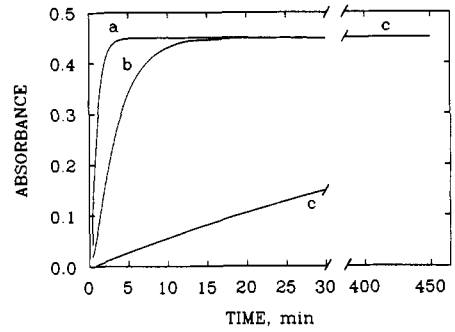
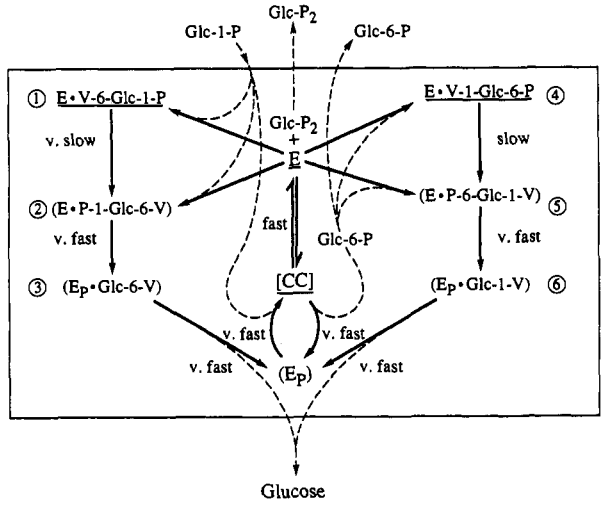


FIGURE 1: Comparison of product–time plots produced in enzymic assays conducted in the presence of excess EDTA and initiated with various complexes of the enzyme: (a) the $E_P(Mg)$ complex; (b) the complex involving V-1-Glc-6-P; and (c) the complex involving V-6-Glc-1-P. Plots were scaled on the basis of the maximal product obtained in each assay, P_{max}, which was proportional, within ±3%, to the amount of protein present in the assay. The times required to produce $0.5P_{max}$ were ~0.5, 2.0, and 52 min, respectively, at 25 °C. For additional details, see Experimental Procedures.

Formation of Complexes between the Dephosphoenzyme and the Vanadate Esters of Glucose Phosphate at High Salt and Low Enzyme Concentrations. Initial studies to define conditions for forming the complex of V-6-Glc-1-P and the enzyme in the crystal phase were conducted with a dilute suspension of an $(NH_4)_2SO_4$-induced precipitate of the dephosphoenzyme in storage solution. The suspension was freshly prepared at a higher salt concentration and was catalytically active under these conditions [cf. Ray (1986)]. Treatment of the suspension with 5 mM V_i and 20 mM Glc-1-P produced approximately 95% conversion to the slowly dissociating vanadate-based inhibitor complex, E·V-6-Glc-1-P [see Ray and Puvathingal (1990) for assay procedures]. The much higher concentrations of V_i and Glc-1-P required to produce the V-6-Glc-1-P complex under these conditions is caused partly by the competitive binding of the SO_4^{2-}, 2.3 M, in storage solution [cf. Ray (1986)] and partly by condensation reactions of V_i that are favored at high ionic strength [cf. Habayeb and Hileman, (1980) and Heath and Howarth (1981)]. But when millimeter-size crystals of the dephosphoenzyme were treated in a similar manner, only marginal conversions to the slowly dissociating inhibitor complex were observed. [To measure the fraction of enzyme present as the V-6-Glc-1-P complex, crystals were dissolved directly in an assay solution that contained excess EDTA to bind both the V_i initially present within the crystals and that formed subsequently via dissociation of the inhibitor complex [see Ray and Puvathingal (1990)].

Because the ratio of enzyme to Glc-1-P within the crystal is much larger than that used in previous solution studies (Ray et al., 1990; Ray & Puvathingal, 1990), the reaction of Glc-1-P and V_i with the dephosphoenzyme was examined at much higher concentrations of enzyme, first at low and later at high salt concentration. The Appendix describes these studies, which show that both the active enzyme·glucose phosphate complexes and the alternative E·V-1-Glc-6-P inhibitor complex become important when the above reaction is conducted at much higher ratios of enzyme/Glc-1-P but at ratios that still are lower than those that characterize the crystal phase process. The Appendix also describes an assay for the relative amount of both inhibitor complexes present in a mixture that also contains the reactive glucose phosphate complexes of the enzyme and provides a kinetic evaluation of the slow transformation of the V-1-Glc-6-P complex into active enzyme (see Figure 1). The Appendix also provides a value for the equilibrium ratio of E·V-1-Glc-6-P/E·V-6-Glc-1-P, about

Scheme II: Steady-State Reactions within a Crystal of Phosphoglucomutase Suspended in a Solution That Prevents Dissolution of the Crystal and also Contains High Concentrations of Glc-1-P and V_i[a]



[a] The solid rectangle represents the boundaries of the crystal. Processes involving diffusion across this boundary are represented by dashed arrows; steady-state reactions are shown with solid arrows. Species whose fractional abundance is marginal under these conditions are shown in parentheses; species that might predominate are underlined; CC represents the catalytically active central complexes. The Mg^{2+} that is bound to each enzymic species is omitted.

42/58, and describes thin-film dialysis studies designed to mimic some aspects of the steady-state process that characterizes the crystalline phosphoglucomutase system in the presence of Glc-1-P and V_i.

A Model for the Steady-State Formation of the Vanadate-Based Inhibitor Complex in the Crystal Phase. Scheme II shows the reactions (solid arrows) that are expected to occur when a crystal of phosphoglucomutase, dephospho form, is treated with Glc-1-P and V_i. This scheme is based both on the studies described in the Appendix and on previous work (Percival et al., 1990; Ray & Puvathingal, 1990) that provides support for the individual reactions shown in eqs 1–10.

$$\text{Glc-1-P} + V_i \rightleftharpoons \text{V-6-Glc-1-P} \quad (1)$$

$$\text{E} + \text{V-6-Glc-1-P} \rightleftharpoons \text{E•V-6-Glc-1-P} \quad (2)$$

$$\text{E•V-6-Glc-1-P} \rightleftharpoons \text{E•P-1-Glc-6-V} \quad (3)$$

$$\text{E•P-1-Glc-6-V} \longrightarrow E_P + \text{Glc} + V_i \quad (4)$$

$$E_P + \text{Glc-1-P} \rightleftharpoons (\text{CC}) \rightleftharpoons \text{E} + \text{Glc-1,6-P}_2 \quad (5)$$

$$(\text{CC}) \rightleftharpoons \text{V-1-Glc-6-P} \quad (6)$$

$$\text{Glc-6-P} + V_i \rightleftharpoons \text{V-1-Glc-6-P} \quad (7)$$

$$\text{E} + \text{V-1-Glc-6-P} \rightleftharpoons \text{E•V-1-Glc-6-P} \quad (8)$$

$$\text{E•V-1-Glc-6-P} \rightleftharpoons \text{E•P-6-Glc-1-V} \quad (9)$$

$$\text{E•P-6-Glc-1-V} \longrightarrow E_P + \text{Glc} + V_i \quad (10)$$

$$2\,\text{Glc-1-P} \xrightarrow{\text{E},V_i} \text{Glc} + \text{Glc-1,6-P}_2 \quad (11)$$

Note that eq 11 is the sum of eqs 1–5; there also is a group of corresponding equations involving Glc-6-P.

In Scheme II the steady state described by eqs 1–10 is represented in terms of fluxes (dashed arrows) across the boundary of a crystal (solid lines) when the external solution contains only Glc-1-P plus V_i (in addition to other nonreactive components such as Na_2SO_4). This scheme emphasizes a primary conclusion obtained from the studies in the Appendix: even when inhibition is "complete", i.e., when "all" of the crystalline enzyme is present as the inactive complexes, V-6-Glc-1-P (**1**) and V-1-Glc-6-P (**4**), the system still is in the steady state, due to the slow conversion of the V-6-Glc-1-P complex and the more rapid conversion of the V-1-Glc-6-P complex into complexes of the active enzyme **3** and **6**, via **2** and **5**, respectively, plus subsequent reactions (eqs 5 and 6). Thus, Glc-P_2 is continually produced via eq 5 and must be eliminated from the crystal by diffusion in order that the normal glucose phosphate complexes (CC) not accumulate. Since the V-1-Glc-6-P complex is converted into the active enzyme much more rapidly than the V-6-Glc-1-P complex (see Appendix), under otherwise identical conditions $[\text{Glc-P}_2]_{ss}$ will be lower if the fraction of the complexes present as the V-1-Glc-6-P complex is minimized by using high concentrations of Glc-1-P and by employing as high a concentration of V_i as possible, so that in the steady state V-6-Glc-1-P competes efficiently with Glc-P_2 for binding to the dephosphoenzyme that is continually produced via eq 5.[5] Another possibility for minimizing the underdesirable complexes, optimizing the depletion of Glc-6-P via diffusion by using small crystals, was not employed.

Attempts To Form the Enzymic Complex V-6-Glc-1-P in Sulfate-Free Crystals of Phosphoglucomutase. Replacement of the $(NH_4)_2SO_4$ in the crystalline dephosphoenzyme was accomplished by stepwise transfer to 40% PEG that contained Na_2Glc-1-P at close to saturation (sequence 4, Scheme I). Treatment of such crystals with V_i produced extensive fracturing, as in one-step attempts to form the glucose phosphate complexes (see above), even when very low concentrations of V_i were used. Inclusion of 10 mM V_i in the transfer protocol beginning with 1.3 M Na_2SO_4 (sequence 5, Scheme I) eliminated fracturing, but the treated crystals liquefied on standing, although the 40% PEG-3350 used here prevented liquefaction after formation of the glucose phosphate complexes. However, the use of 50% PEG-3350 (containing 10 mM V_i and Na_2Glc-1-P at close to saturation) eliminated both fracturing and liquefaction (sequence 6, Scheme I). [An essentially saturating concentration of 0.04 M Na_2Glc-1-P in 50% PEG-3350 produces a concentration of about 0.8 M Glc-1-P within the crystal (Ray et al., 1991)]. However, the treated crystals contained substantial amounts of uninhibited enzyme. The V-1-Glc-6-P complex also was present, in addition to the desired V-6-Glc-1-P complex. As noted above, both of the undesired glucose phosphate and alternative inhibitor complexes are present because of the slow diffusional escape of Glc-P_2 and Glc-6-P from the channels of the crystal. In the present case, escape is slowed by the unfavorable partition between the internal phase, which is largely aqueous, and the external solution of 50% PEG-3350 (see below). Changing the nature of the external solution proved to be the only feasible way to circumvent this problem.

Formation of the V-6-Glc-1-P Complex in Crystals of Phosphoglucomutase at High Salt Concentration. Because of the diffusional problems described above, subsequent attempts to form the inhibitor complex was conducted in aqueous media. Crystals brought to 1.2 M Na_2SO_4/0.5 M Na_2Glc-1-P/0.01 M V_i (early stage of sequence 6, Scheme I), were

[5] Obtaining a kinetic expression for the system in Scheme II is impractical. However, the much greater than 4-fold decrease observed in the fraction of enzyme present as the V-1-Glc-6-P complex, f_6, produced by a 4-fold increase in $[V_i]$ (sequence 10, Scheme I) can be rationalized qualitatively by considering only the most important of two competing reactions in this scheme when there is a choice. Thus, increasing concentrations of V_i decrease $[\text{Glc-6-P}]_{ss}$ by decreasing the fraction of E(Mg) that returns directly to [CC], which produces Glc-6-P. The lower the value of $[\text{Glc-6-P}]_{ss}$, the lower f_6 and the slower the generation of [CC] from the V-1-Glc-6-P complex. In fact, in such a system, the expression for f_6 is quadratic, so that it at least is feasible for a given change in $[V_i]$ to produce an even larger fractional change in f_6.

largely converted to the V-1-Glc-1-P complex without adversely affecting their diffractivity. Attempts to drive the equilibrium toward the desired product by utilizing somewhat lower sulfate and somewhat higher Glc-1-P concentrations (sequence 7, Scheme I) were unsuccessful from a crystallographic standpoint (see below), although crystals treated in this manner were unchanged visually and were converted completely (≥98%) to the V-6-Glc-1-P complex.

Subsequent studies were conducted with higher concentrations of an alternative protein precipitant, dianionic malonate, that does not bind to the dephosphoenzyme as efficiently as does SO_4^{2-} and is much more soluble, especially at reduced temperatures. (Because the dipotassium salt of malonic acid is more soluble than the disodium salt but the dipotassium salts of sulfate and Glc-1-P are less soluble than their disodium salts, most transfer sequences were conducted with $Na_{1.6}K_{0.4}$ malonate; however, some of the intermediate solutions used were substantially supersaturated with respect to K_2Glc-1-P; but this salt crystallizes from such solutions extremely slowly, even at 4 °C.) Crystals of phosphoglucomutase transferred stepwise from storage solution to 1.8 M sodium/potassium malonate, pH 7.6, were stable indefinitely. Such crystals were transferred to 4 °C, where chemical reactions should be slowed much more than diffusional processes, and later to 1.3 M sodium/potassium malonate/0.5 M Na_2Glc-1-P/20 mM V_i (sequence 10, Scheme I). Pilot studies (4 °C) at 0.5 M Glc-1-P, but at lower concentrations of V_i, showed that 0.5 mM V_i produced approximately a 50% conversion to a mixture of inhibitor complexes, with the remainder being accounted for by uninhibited enzyme i.e., by CC. On this basis, a transfer protocol was used where the concentration product [V_i][Glc-1-P] was increased in successive steps in such a way that the calculated fraction of inhibitor complexes present would increase monotonically in steps of about 0.03 (30-step procedure), if one assumes that the concentration of these complexes is proportional to the above concentration product and ignores the possibility of lattice-based binding cooperativity (see Discussion). This transfer produced no visual fracturing and provided conversions to the desired crystalline inhibitor complex equal to at least 95%.

The X-ray diffraction patterns obtained from the above crystals (at −5 °C) were improved relative to those from untreated crystals (at 16 °C) with regard to the Bragg spacing within which significant data could be collected and were at least as good in terms of the FWHM calculated jointly for individual reflections in the manner described above. When moist crystals of this type were stored in vapor-phase contact with the final solution at −5 °C (freezing point, obtained as the melting point of a frozen solution to avoid supercooling, −10 °C), the fraction of the enzyme present as the V-6-Glc-1-P complex remained above 0.95 for at least 48 h. None of the above operations produced a substantial change in unit cell parameters.

Sources of Failures in Producing the V-6-Glc-1-P Complex in the Crystal Phase. Some failures of alternative schemes for producing undamaged crystals of the V-6-Glc-1-P complex relate to deviations from the general approach to forming complexes in the crystalline phase that eventually evolved (see Discussion). (1) When the monotonic series of transfers ([sodium/potassium malonate] = 1.8 M) → ([sodium/potassium malonate] = 1.3 M/[Glc-1-P] = 0.5 M) was made at constant [V_i] = 10 mM (sequence 8, Scheme I), crystals fractured badly because this sequence does not increment [V-6-Glc-1-P] in a manner that produces gradual saturation of the crystal (see Discussion). (2) When the final concentration of V_i was 5 mM (sequence 9, Scheme I), conversion to a mixture of V-6-Glc-1-P and V-1-Glc-6-P complexes was essentially complete, but their relative concentrations could not be displaced very far from equilibrium (cf. Appendix), even by changing the external solution frequently enough that external [Glc-6-P] did not exceed 0.002[Glc-1-P]. Thus, the internal ratio [Glc-6-P]/[Glc-1-P] must have been much higher than the ratio in the external solution. However, when [V_i] was increased to 20 mM, the efflux of Glc-6-P from the crystal essentially ceased and the presence of the V-6-Glc-1-P complex increased to ≥95%.[5] (3) Complete conversion to the V-6-Glc-1-P complex could be achieved at a higher [sodium/potassium malonate] = 1.6 M (sequence 11, Scheme I). However, the combination of a strained lattice and an a(H_2O) *lower* than that at which the crystals grew produced crush cracking (Ray et al., 1991), although native crystals survived transfer to 3.5 M sodium/potassium malonate without damage to the lattice or significantly increasing the FWHM for individual reflections. (4) Lower concentrations of V_i could be used to achieve good conversions to the desired inhibitor complex, but lower concentrations of sodium/potassium malonate were required. The combination of a strained lattice and an a(H_2O) *greater* than that at which the crystals were grown produced the opposite type of effect, expansion cracking (Ray et al., 1991). In the case of sulfate, when the Na_2SO_4 concentration was lowered from 1.2 to 0.9 M, with a simultaneous increase in Na_2Glc-1-P concentration from 0.5 to 0.7 M (sequence 7, Scheme I), cracking was not observed, but the treated crystals failed to diffract.

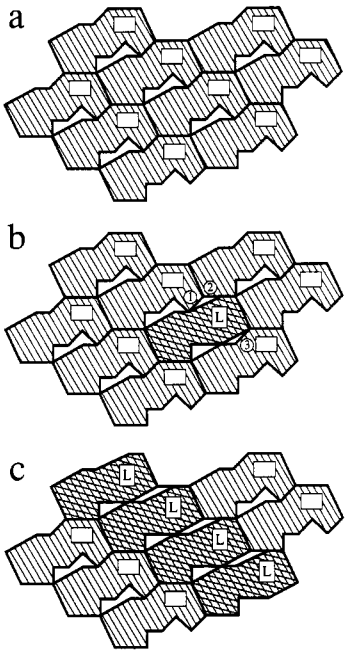
Discussion

A primary objective of this study was to devise procedures for producing protein–ligand complexes in the crystal phase when direct exposure of diffraction-quality crystals to saturating concentrations of the ligand damages the lattice and when suitable annealing of the partially saturated crystal can reduce or eliminate the damage. The protein crystal used in these studies was phosphoglucomutase, which is obtained from $(NH_4)_2SO_4$ (2.1 M), contains 60% by volume of the aqueous phase (Lin et al., 1986), and has a relatively fragile lattice. This fragility is caused in part by the large continuous channels that penetrate the crystal and the relatively limited contacts between the asymmetric units along a given channel and those along adjacent channels (Dai et al., 1991). The enzyme is quite active in the crystalline phase and appears to bind the substrate in a manner comparable to that observed in solution (Ray, 1986). The crystals used in the present study were millimeter size or larger (Ray et al., 1991). These diffract to a Bragg spacing of between 2.5 and 2.7 Å.

When ligand binding alters molecular contacts in a protein crystal, as it must when the crystalline lattice is damaged on formation of the ligand complex, it seems reasonable to consider the possibility that lattice-based binding cooperativity contribute to the damage, especially when unit cell dimensions are not significantly altered by the binding process. Thus, intermolecular interfaces that are unique to the crystal phase can modulate binding interactions in a manner formally analogous to the binding cooperativity observed in solution with many oligomeric proteins. In the simplest case, two monomers related by a local 2-fold symmetry axis constitute the asymmetric unit; the binding of a ligand alters this monomer–monomer contact *but no other*. In such a case, any binding cooperativity will be localized within the asymmetric unit and will not cause crystals to fracture on ligand binding.

However, binding cooperativity that affects contacts where the above symmetry relationship is crystallographic can pro-

Scheme III: Two-Dimensional Representation of Lattice-Based Binding Cooperativity and Its Effects on the Crystalline Lattice[a]



[a] (a) Lattice of the native enzyme; (b) lattice of the enzyme where a ligand L has altered intermolecular contacts 1–3; and (c) a ligand-binding pattern that would be favored by lattice-based binding cooperativity of the type represented in panel b.

duce conformational changes in molecules that tend to propagate through the crystal, even when cell dimensions are unaltered by the binding process. The cartoons in Scheme III illustrate this possibility with a two-dimensional lattice. A ligand-free lattice is represented by Scheme IIIa; a possible effect on molecular contacts produced by the binding of one ligand molecule within this region of the lattice is shown in IIIb, where three lattice contacts are altered without changing cell dimensions. The ligand-induced changes in contacts 1 and 3 are related by crystallographic symmetry and can be propagated to adjacent molecules via increased affinity for the ligand, while the alteration in contact 2 cannot be propagated.

When ligand-binding sites are fractionally saturated, lattice-based binding cooperativity that is propagated through a crystal will produce *extended patterns* of molecules with altered contacts, as in Scheme IIIc, as opposed to *random arrangements* of such contacts. Whereas lattices may fracture for other reasons, it seems reasonable to suggest that *patterns* of altered contacts such as those illustrated in Scheme IIIc are particularly likely to irreversibly damage a crystalline lattice if the crystal is subject to osmotic stress.

In the case of oligomeric proteins, nature has devised subunit contacts that ensure a fixed degree of cooperativity regardless of how the ligand-binding process is conducted. But it is less likely that the protein–protein contacts found in crystals have been subjected to similar evolutionary pressure. Hence, it seemed possible that a ligand-binding procedure in which some type of annealing process was given an opportunity to operate on stressed/weakened molecular contacts in partially saturated crystals might substantially reduce the degree of lattice-based cooperativity associated with ligand-binding, at least in some cases, by allowing protein molecules to partially compensate for weakening of the original molecular contacts via changes in their structure elsewhere.

Regardless of how annealing is achieved, a primary objective in attempting to moderate ligand-induced damage to a crystalline lattice is to ensure that ligand-binding occurs more or less *uniformly* throughout the crystal. The desirability of such an objective is supported by observations made with crystals that were suspended in PEG-3350 where sulfate had been replaced by glycine [sequence 1, Scheme I, and Ray et al. (1991)] in order to facilitate the binding of glucose phosphates at the active site of the enzyme [cf. Ray (1986)]. Exposure of millimeter-size desalted crystals even to very low concentrations (as low as 10 μM) of glucose phosphates, which are known to induce a conformational change in the solution enzyme on binding (Ma & Ray, 1980), produces extensive fracturing of the crystalline lattice. Moreover, the fracturing occurs perpendicular to the major channels of the crystal; it occurs first near the surface of the crystal and is propagated inward. Apparently, this fracturing is produced by preferential ligand binding to and saturation of protein molecules that line the channels of the crystal close to the surface, before a significant amount of ligand penetrates farther into the crystal. Since the binding process alters cell dimensions insignificantly, if at all, it appears that the desalted crystals are somewhat stressed osmotically. By contrast, inorganic phosphate, which does not produce a substantial change, if any, on binding [cf. Ma and Ray (1980)] also does not produce fracturing of the same type of crystals, even at a concentration of 1 mM, which is 100-fold higher than that of the glucose phosphates used in the crystal-binding study described above. But more important are the results obtained by using glucose phosphates in a multistep saturation procedure that provides both a relatively uniform binding throughout the crystal as sites are gradually saturated and sufficient time intervals between steps for annealing of partially saturated crystals. With such a procedure, much higher concentrations (500-fold higher) of glucose phosphates can be introduced into crystals without producing visible damage and without a significant effect on the regularity of the lattice, as deduced via X-ray diffraction measurements.

The success of the gradual saturation protocol vis-ã-vis the failure of a one-step procedure with the same final endpoint in itself strongly suggests that an annealing of the partially saturated crystal is a sine qua non for the successful binding of glucose phosphates to inhibitor-free crystals of phosphoglucomutase. In addition, the following observations support the suggestion that some type of time-dependent annealing of protein crystals is possible, at least with those used in this study. Thus, in one 20-step solute replacement scheme, a protocol that involved 4-min or 6-min intervals between steps invariably produced fracturing of the crystals. But when the same protocol was used with the same solutions and the same batch of crystals, but employing a 15-min interval between steps, this not only produced unfractured crystals but crystals with improved lattice characteristics, as verified by X-ray diffraction measurements (W.J.R., to be published elsewhere). Whether this type of approach will prove useful with protein crystals where binding produces fracturing even when no osmotic effects are present, viz., because of changes in cell dimensions, is moot, but seems worth considering.

In the current studies, two somewhat different procedures were used to attain the above objectives during formation of two different types of ligand complexes of crystalline phosphoglucomutase. Before discussing these, it seems reasonable to suggest that if a one-step ligand-binding procedure damages a protein crystal, the possibility of success with a multistep saturation sequence will be optimized for a sequence with a fixed number of steps if each step increases the fractional saturation, f_L, by equal amounts. Unfortunately, designing such a saturation sequence may require a knowledge of binding

parameters that is difficult or impossible to obtain. One of these parameters is K_L, the concentration of ligand that half-saturates the sites in the crystalline phase, although solution studies may establish the approximate range of K_L. In the absence of binding cooperativity, achieving a stepwise increment in f_L, or $[L]/(K_L + [L])$ if a Michaelis–Menten binding pattern applies, requires that [L] be substantially less than K_L, initially, and that [L] be increased in steps of continually increasing size until $[L] \gg K_L$. In such a case, the required concentration sequence is given by

$$[L]_i = iS_fK_L/(N_f - S_f) \quad (12)$$

where i designates the ith step, n_t is the total number of steps, and S_f is the limiting fractional saturation. A different protocol is required if binding is cooperative: the higher the cooperativity, the more closely spaced should be the increments of L in the region where $[L] \approx K_L$. Because of these differences in strategy, a trial-and-error approach employing the guidelines described later may provide the most efficient way of designing a satisfactory gradual-saturation sequence.

In the present study, the complexes involving glucose phosphates were obtained by introducing these at a stage of the desalting process where binding is minimal (at 1.8 M SO_4^{2-}) and maintaining these at a constant concentration as the competitive inhibitor, SO_4^{2-}, first is reduced in concentration and then is entirely replaced by glycine, which binds much less tenaciously, if at all, to the anion-binding subsite of phosphoglucomutase (sequence 3, Scheme I). To describe how fractional saturation might change during such a procedure, let I represent sulfate and K_I^2 the product of the dissociation constants for the two sulfates that bind to the dephosphoenzyme competitively with Glc-P_2 [cf. Ray (1986)]. If binding-induced changes fail to propagate through the crystals and if at the midpoint of the transfer sequence the crystal is half-saturated, the function $[L]/([L] + [I]^2K_L/K_I^2)$ would change in a manner that should produce a more or less monotonic increase in f_L for the present system when [L] is constant throughout at a value much greater than K_L. If the half-saturation point were attained either much earlier or much later in the saturation sequence, more abrupt changes in f_L would occur. Cooperative binding also would produce more abrupt change in f_L, although with a 30-step protocol matching the midpoint of the transfer sequence with the half-saturation point seems to be more important than a closer spacing of steps near the midpoint, at least in the present system.

In the case of the vanadate-based inhibitor complex, E·V-1-Glc-6-P(Mg), the concentration of the eventual cosolute that was used to prevent dissolution of the crystals, malonate dianion, was not changed markedly during the formation of the complex (1.8 M → 1.3 M), so that a saturation protocol analogous to that above could not be used. However, an assay for the fraction of this complex present in a crystal was devised so that an estimate of the half-saturation point could be obtained. On this basis, the concentration product [Glc-1-P][V_i] was increased stepwise according to eq 12 (sequence 9, Scheme I) since [V-6-Glc-1-P] should be proportional to this product. Whether or not this saturation sequence was optimal, millimeter-size crystals of phosphoglucomutase survived the overall transfer without fracturing or sustaining significant damage to the crystalline lattice while being converted essentially quantitatively to the V-6-Glc-1-P complex. By contrast, an earlier saturation protocol with the same starting point and the same number of steps (sequence 8, Scheme I) produced extensive fracturing, apparently because the midpoint of the transfer sequence failed to adequately match the half-saturation point (see Results).

On the basis of the above observations, we suggest the following trial-and-error approach for designing multistep saturation sequences to minimize the damage to protein crystals that can accompany ligand binding. This approach assumes that K_L is not known, that L is the only species whose concentration is varied, and that a relatively large number of steps will be used, e.g., 20–30. If fracturing that is independent of the time interval between successive steps occurs relatively early in a stepwise saturation sequence described by eq 12, it is likely that the sequences were initiated at too high a ligand concentration; if fracturing occurs relatively late in the sequence, that sequence likely was initiated at too low a ligand concentration. If neither increases in the delay time between steps nor the indicated shifts in the midpoint of the saturation sequence significantly alter the concentration at which fracturing occurs, binding cooperativity may be indicated. In such a case, clustering the majority of the transfer steps within this concentration range would be in order. However, the above procedures will be no more effective than a single-step procedure (where no osmotic effects are produced) unless some sort of annealing accompanies the saturation process.

A problem with the gradual-saturation approach, in the case of very tightly bound ligands, is one of transport. If the effective concentration of a protein molecule along the channels of a crystal is as high as in the present case,[6] about 50 mM (it may well be higher in other crystals), and if the dissociation constant for the ligand in the crystalline lattice were nanomolar, to achieve 3% saturation of the molecules in the crystal in the first step of the transfer sequence would require that the crystals be suspended in 0.03 nM ligand. But such a concentration also would require that the ligand in 3×10^7 volumes of solution enter the crystal, which clearly is unreasonable. In such cases, the use of a relatively high concentration of a more weakly bound competitive inhibitor, particularly one that does not produce a conformational change in the protein on binding, would allow a much higher concentration of tightly bound ligand to be employed in the first step without increasing the fractional saturation produced by this step. [In the present system, the inhibitor, V-6-Glc-1-P, is bound exceedingly tenaciously (Ray & Puvathingal, 1990); however, because of an unfavorable equilibrium constant, a relatively low concentration of the inhibitor is produced in solution by the much higher concentrations of V_i and Glc-1-P used [cf. Percival et al. (1990)].] In addition, both the dianionic cosolute and the lattice-based cooperativity substantially decrease the effective binding of V-6-Glc-1-P in the crystal phase, so that the concentrations of Glc-1-P and V_i employed are not excessively low, even when the fractional saturation with V-6-Glc-1-P is low.

A related but different type of problem was encountered during initial attempts to form the glucose phosphate and inhibitor complexes with desalted crystals of phosphoglucomutase suspended in a concentrated solution of PEG-3350 (30–40%): liquefaction of the crystals after forming ligand complexes via the binding/annealing procedure described above (see Results). This liquefaction stands in contrast with the behavior of crystals treated similarly but in the absence of an active-site ligand. This behavioral difference suggests that whatever annealing was obtained via the gradual-satu-

[6] The "concentration" or activity of a crystalline compound usually is taken as 1. However, for some purposes, e.g., binding studies, "crystalline" proteins are best considered, instead, as ordered gels (Fink & Petsko, 1981), because their composition is altered by concentration changes in the surrounding solution. In such cases, it is appropriate to specify the concentration of active sites within the gel.

ration approach did not entirely relieve the stress induced by ligand binding. Because of this stress, a reduced water activity is required to prevent liquefaction. In other words, since crystal formation is accompanied by the elimination of protein-interface water, strain-weakened crystals should absorb water and liquefy more readily than their unstrained counterparts. This rationale also is in accord with the relative success of flash cooling native crystals (40% PEG-3350/1 M glycine) and crystals converted to the glucose phosphate complexes (same solution plus 5 mM glucose phosphates) (see Results). In fact, the increased tendency of crystals to liquefy on conversion to the transition-state analogue complexes, as opposed to the substrate/product complexes, may well be due to more extensive structural changes on formation of the analogue complex (see below).

Related effects also were observed when attempts were made to convert the crystalline enzyme to the V-6-Glc-1-P complex in salt solutions. Here, the combination of strained molecular contacts and low external $a(H_2O)$ produced fracturing of the crystal in a manner previously identified as crush cracking (Ray et al., 1991), while similarly strained contacts and high external $a(H_2O)$ produced expansion cracking in one case, and a total elimination of diffractivity without visible cracking in another (see Results). In the latter case, where loss of diffractivity occurred without visible fracturing, it seems worth noting that conducting the same modification of the crystal at a somewhat higher concentration of the solubility-determining salt prevented the loss of diffractivity.

In order to obtain the optimal $a(H_2O)$ for producing the V-6-Glc-1-P complex in the crystal phase without the competitive binding effects of sulfate (because of its structural similarity to phosphate), malonate dianion was used, instead of sulfate, as the solubility determinant, along with a reactant, Na_2Glc-1-P. (The alkali metal salts of oxalate and succinate are too insoluble for convenient use in this manner.) In addition, the increased effect of malonate on $a(H_2O)$, relative to sulfate (see below) allowed the crystalline V-6-Glc-1-P complex thus formed to be cooled to −5 °C for data collection, in order to preserve its chemical stability. (Scheme II shows the reactions that this complex undergoes at higher temperatures.)

In fact, several properties of the malonate dianion recommend it for use in crystallizing proteins. The room temperature solubility of its dipotassium salt (which is greater than 5 M)[7] is greater than that of $(NH_4)_2SO_4$, and the disodium salt is nearly as soluble (>4 M). In addition, malonate appears to be at least as powerful a precipitant as sulfate, while Na_2SO_4 is a better precipitant than $(NH_4)_2SO_4$ (Arakawa & Trimasheff, 1985). Moreover, small volumes of malonate solutions are not subject to pH changes due to loss of NH_3, and disodium malonate decreases water activity to a substantially greater extent than does $(NH_4)_2SO_4$.[8] [2.5 M $(NH_4)_2SO_4$ freezes at −11 to −12 °C while 2.5 M dipotassium malonate freezes at −17 to −18 °C.] In fact, 4 M dipotassium malonate freezes only with great difficulty (below −30 °C), tending to form a glass, instead. On this basis, it would be surprising if crystals transferred to concentrated dipotassium malonate solutions were not more amenable to flash cooling [cf. Ray et al. (1991)] than crystals in $(NH_4)_2SO_4$ solutions.

It should be emphasized that the feasibility of the operations described herein depended critically on the quality of the crystals used, particularly in the case of large crystals. Thus, crystals with a longest dimension of 0.3 mm could be transferred successfully between the solutions in Scheme I by using only a few steps. By contrast, crystals for which the longest dimension is 2 mm generally did not survive these transfers, although crystals with a longest dimension of 1.5 mm usually did. But somewhat smaller crystals that exhibited noticeable surface imperfections almost always fractured. Another critical aspect of these operations was the manipulation of crystals during treatment so that the face on which the crystals rested did not long remain occluded from the surrounding solution. In addition to the precautions described previously [see the accompanying paper, Ray et al. (1991)], care was taken to shift the position of each crystal during each addition of fresh solution.

Finally, from a mechanistic standpoint, the possibility that the transition-state analogue complex E·V-6-Glc-1-P(Mg), differs structurally from the apoenzyme at positions *remote* from the active site, and differs to a larger extent than does the mixture of catalytically active substrate/intermediate/product complexes, is intriguing. The remoteness of the differences in question is suggested by the unusual depth of the active-site cleft in phosphoglucomutase, which by itself places the binding site at a position relatively distant from intermolecular contacts in the crystal. In fact, the distance between the α-carbon of the active-site serine phosphate and the *nearest* α-carbon involved in an intermolecular contact with an adjacent molecule is 15 Å (Dai et al., 1991), and most contact residues are considerably farther away. On the other hand, it is not clear whether the fracturing of crystals produced by Glc-1-P + V_i is due solely to the binding of V-6-Glc-1-P or is caused by this binding in conjunction with the nonspecific binding/reaction of inorganic vanadate with protein side chains. (By itself, 20 mM V_i does not alter the phosphoglucomutase lattice.) Hopefully crystallographic studies now in progress will provide a distinction between these possibilities.

Acknowledgments

We are grateful to Dr. Jeffrey Bolin for helpful discussions and suggestions throughout this study.

Appendix

Solution Studies of the Reaction of Glucose 1-Phosphate and Vanadate with the Dephosphoenzyme. Because of the initial failure to convert the crystalline dephosphoenzyme to the V-6-Glc-1-P complex, in spite of the extensive conversion of dilute suspensions of the enzyme to this complex under the same conditions (see Results), the reaction was examined in solutions containing 1 mM enzyme (60 mg/mL). Under these conditions, not only was conversion to the V-6-Glc-1-P complex incomplete, but the fraction of the enzyme present as this complex slowly decreased with time. Subsequent studies with [^{32}P]Glc-1-P, followed by chromatographic separation of the products, showed that on mixing under these conditions, Glc-1,6-P_2 was produced in an amount equivalent to approximately 0.3 of the enzyme present. Subsequently, the amount of Glc-1,6-P_2 present slowly increased with time in a linear manner, although the increase was equal only to about 0.02 equiv/h. Other studies showed that under these conditions equilibrium between Glc-1-P and Glc-6-P is achieved immediately. Subsequently, the Glc-6-P thus produced reacts with V_i to form V-1-Glc-6-P, which binds to the enzyme to produce

[7] A stock solution of 5 M dipotassium malonate, can be prepared in a manner analogous to that described for sodium/potassium malonate by substituting 320 g of 87% KOH pellets plus 163 mL of water, for the basic solution described under Experimental Procedures.

[8] Although the relative values of $a(H_2O)$ in salt solutions are concentration dependent, some idea of the relative effect of these salts on $a(H_2O)$ is provided by the observation that $a(H_2O)$ is the same in 2 M $(NH_4)_2SO_4$, 1.9 M Na_2SO_4, and 1.6 M disodium malonate.

the alternative transition-state analogue complex, E·V-1-Glc-6-P. These observations, together with previous studies (Percival et al., 1990; Ray et al., 1990) provide support for the description of the reaction in question that appears as eqs 1–10 under Results. Although all of these reactions, except for those in eqs 3 and 8, are rapid at ratios of Glc-1-P/enzyme such as those above, the relative importance of eqs 5 and 6 during a short time interval are greatly reduced at low enzyme concentration where much higher ratios of Glc-1-P/enzyme can be achieved. Thus, under the same conditions but at 1 μM enzyme, very little Glc-6-P or Glc-P_2 is produced during a short time interval during which essentially complete conversion to the (inactive) V-6-Glc-1-P complex is achieved (Ray & Puvathingal, 1990).

Characterization of the Complex between the Dephosphoenzyme and the Mixed 1-Vanadate 6-Phosphate Diester of Glucose. On the basis of the above studies, formation of complexes involving both V-6-Glc-1-P and V-1-Glc-6-P is expected on treatment of the crystalline enzyme with Glc-1-P plus V_i. Hence, the kinetic properties of latter complex, obtained by treating *dilute* solutions of the dephosphoenzyme with excess Glc-6-P plus V_i, were examined, to supplement those of the alternative complex involving V-6-Glc-1-P, described earlier (Ray & Puvathingal, 1990). Figure 1, plot a, shows a product–time plot obtained by initiating an enzymic assay with the active E_P(Mg) or E(Mg) complexes or, plot b, with the inactive V-1-Glc-6-P complex. Excess EDTA was present in this assay for reasons noted in the main body of this paper [cf., Ray and Puvathingal (1990)]. The excess EDTA also acts as a scavenger for the free Mg^{2+} initially present plus that which dissociates from the catalytically active central complexes [cf. Magneson et al. (1987)] to yield inactive complexes of the enzyme. The product produced in this assay, Glc-6-P, reaches a limiting value when all of the Mg^{2+} has dissociated from the enzyme. The limiting product is obtained in essentially equal amounts both from the inhibited and uninhibited forms of the Mg^{2+} enzyme, but at much different rates: rapidly from the uninhibited enzyme, E(Mg) and E_P(Mg) plus substrate/intermediate/product complexes thereof, and more slowly from the E·V-1-Glc-6-P(Mg) complex as it dissociates essentially exclusively to one of the above complexes, E(Mg), which is rapidly converted to the catalytically active E·Glc-P_2(Mg) complex in the assay mixture [cf. Ray and Puvathingal (1990)]. Because of the substantial difference in the Mg^{2+} dissociation rates for the V-6-Glc-1-P complex and the catalytically active complexes, the half-time for dissociation of E·V-1-Glc-6-P(Mg) to give E(Mg) in such an assay is approximately equal to the time required to produce half of the maximal amount of product, P_{max} (Ray & Puvathingal, 1990).

Although the above assay can be used with ease to quantify a mixture of complexes involving V-1-Glc-6-P (plot b) (half-time for Mg^{2+} dissociation, about 2 min), and V-6-Glc-1-P (plot c) [half-time for Mg^{2+} dissociation, about 52 min, cf. Ray and Puvathingal (1990)], it is more difficult to quantify species in an analogous mixture that also contains the uninhibited forms of the enzyme, E(Mg), E_P(Mg), and substrate/intermediate/product complexes thereof, for which half-maximal product is obtained at 27 s (plot a). The difficulty is exacerbated by the initial lag in the assay produced by the coupling enzyme. However, by omitting the coupling enzyme and using time aliquots taken from that portion of the product–time plot subsequent to the 2-min point (when the activity of essentially all of the uninhibited enzyme had disappeared due to loss of Mg^{2+}), reasonable and reproducible estimates of the relative concentrations of all three species in a mixture could be obtained, since the rate constant for approach to P_{max} is known for each species.

Relative Binding of V-6-Glc-1-P and V-1-Glc-6-P to the Dephosphoenzyme in Solution. To achieve equilibrium between the V-6-Glc-1-P and V-1-Glc-6-P complexes of the enzyme, dilute solutions of dephosphoenzyme were treated with V_i plus a large excess of an equilibrium mixture of Glc-6-P and purified Glc-1-P (no Glc-P_2 present). Initially, the product was almost entirely the V-1-Glc-6-P complex. Because several hours were required for the system to reach an equilibrium between the above two complexes, the reaction was conducted in a small volume (50 μL) in a dialysis thimble (Schleicher & Schuell) so that any Glc-P_2 produced during this time would not accumulate in the sample. At equilibrium, the ratio of the V-6-Glc-1-P and V-1-Glc-6-P complexes was 0.58/0.42. Since the concentration of α-V-1-Glc-6-P is estimated as about 7-fold greater than that of α-V-6-Glc-1-P [data from Tracey et al. (1988) and Ray et al. (1989)], the relative binding of V-6-Glc-1-P and V-1-Glc-6-P to the enzyme is about 10:1, while the relative dissociation rates of the respective complexes with E(Mg) is about 26:1, as was noted above.

A Solution Model for the Steady-State System Involving Crystalline Phosphoglucomutase Glucose 1-Phosphate and Inorganic Vanadate. Some of the properties of a crystalline enzymic system, where continuous treatment with a slowly reacting substrate produces a steady-state conversion to product, can be mimicked by thin-film dialysis involving concentrated solutions of the enzyme. Accordingly, several of the characteristics of the present system were examined by means of the dialysis procedure described in the previous section, although the average thickness of the dializing film was substantially greater than the length of one-half of the average channel (which is open at both ends) in a typical crystal of phosphoglucomutase. In addition, the concentration of enzyme in the dialysis thimble, about 1 mM, was only 2% of the effective concentration of the enzyme along the channels of the crystal (see Discussion). (Changes in the volume of the sample inside the dialysis thimble were opposed by the application of pressure equal to 10–15 torr). Such studies with 0.5 M Glc-6-P showed that 1 mM dephosphoenzyme could be converted almost completely to the V-1-Glc-6-P complex and maintained in this form by continuous dialysis against 10 mM Glc-6-P/1 mM V_i at low ionic strength. By contrast, it was not possible to produce the V-6-Glc-1-P complex, exclusively, under analogous conditions (even with 0.5 M purified Glc-1-P), because of continuous conversion of Glc-1-P to Glc-6-P and because entry into the right-hand cycle of Scheme II, involving the V-1-Glc-6-P complex, is substantially easier than entry into the left-hand cycle that involves the alternative complex (see above). But it was possible to maintain a nonequilibrium steady-state mixture of the complexes involving V-6-Glc-1-P and V-1-Glc-6-P, e.g., 0.65/0.35 instead of 0.58/0.42, by frequently replacing the solution in which the crystals were suspended, to prevent the accumulation of Glc-6-P in this solution.

References

Arakawa, T., & Timasheff, S. N. (1985) *Arch. Biochem. Biophys. 224*, 169–177.

Dai, J.-B., Liu, Y., Ray, W. J., Jr., Konno, M., Post, C. B., & Rossmann, M. G. (1991) (manuscript submitted).

Fink, A. L., & Petsko, G. A. (1981) *Adv. Enzymol. Relat. Areas Mol. Biol. 52*, 177–246.

Habayeb, M. A., & Hileman, O. E., Jr. (1980) *Can. J. Chem. 58*, 2255–2261.

Heath, E., & Howarth, O. W. (1981) *J. Chem. Soc., Dalton Trans.*, 1105-1110.
Lin, Z.-J., Konno, M., Abad-Zapatero, C., Wierenga, R., Murthy, M. N. R., Ray, W. J., Jr., & Rossmann, M. G. (1986) *J. Biol. Chem. 261*, 264-274.
Lowry, O. H., & Passonneau, J. V. (1969) *J. Biol. Chem. 244*, 910-916.
Ma, C., & Ray, W. J., Jr. (1980) *Biochemistry 19*, 751-795.
Magneson, G. R., Puvathingal, J. M., & Ray, W. J., Jr. (1987) *J. Biol. Chem. 262*, 11140-11148.
Michal, G. (1984) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) 3rd ed., pp 191-198, Verlag, Basel.
Percival, M. D., Doherty, K., & Gresser, M. J. (1990) *Biochemistry 29*, 2764-2769.
Ray, W. J., Jr. (1967) *J. Biol. Chem. 242*, 3737-3744.
Ray, W. J., Jr. (1986) *J. Biol. Chem. 261*, 275-278.
Ray, W. J., Jr., & Long, J. W. (1976) *Biochemistry 15*, 3993-4006.
Ray, W. J., Jr., & Puvathingal, J. M. (1990) *Biochemistry 29*, 2790-2801.
Ray, W. J., Jr., Hermodson, M. A., Puvathingal, J. M., & Mahoney, W. C. (1983) *J. Biol. Chem. 258*, 9166-9174.
Ray, W. J., Jr., Post, C. B., & Puvathingal, J. M. (1989) *Biochemistry 28*, 559-569.
Ray, W. J., Jr., Post, C. B., & Burgner, J. W., II (1990) *Biochemistry 29*, 2770-2778.
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).
Tracey, A. S., Galeffi, B., & Soroush, M. (1988) *Can. J. Chem. 66*, 2294-2298.

Systematic Comparison of Statistical Analyses of Electronic and Vibrational Circular Dichroism for Secondary Structure Prediction of Selected Proteins†

Petr Pancoska‡ and Timothy A. Keiderling*
Department of Chemistry, University of Illinois at Chicago, Box 4348, Chicago, Illinois 60680
Received September 26, 1990; Revised Manuscript Received April 19, 1991

ABSTRACT: The electronic (ultraviolet) circular dichroism (UVCD) and vibrational circular dichroism (VCD) of 20 proteins are systematically compared as to their relationship to the secondary structures of these proteins. The UVCD spectra are statistically treated by use of the same factor analysis methods used previously for VCD. The UVCD spectra can be reproduced as linear combinations of five subspectra. The first subspectrum reflected the expected α-helical UVCD shape, particularly at longer wavelengths, while the higher order ones had less obvious similarity to standard bandshapes. Cluster analysis on the UVCD factor analysis coefficients reflected the clustering on the basis of the fractional secondary structure parameters (from X-ray) but was less clear than VCD. Qualitative complementarity of protein VCD and UVCD spectra was demonstrated by combined cluster analysis of their respective factor analysis coefficients. Quantitative relationships between spectral coefficients and fractional secondary structure were determined by multiple regression analyses using only statistically important coefficients. These resulted in an ability to reproduce four of the structural parameters with errors for individual proteins comparable to the VCD result. In UVCD, the standard deviations of the regression fit for β-sheet were worse and for the undefined part of the structure were better than in VCD. Parallel analyses using the partial least-squares method showed UVCD in that case to have more error than VCD in reproducing the training set structural parameters. Comparison of the regression and partial least-squares methods illustrated limitations of total back-transformation of the UVCD spectra into structural parameters.

In the previous paper in this series (Pancoska et al., 1991), an analysis of the vibrational circular dichroism (VCD)[1] of proteins, which will here be referred to as paper II, we presented VCD data for 20 proteins in the amide I′ region of the infrared. Statistical analyses of these spectra using the principal component method of factor analysis (PC/FA) (Pancoska et al., 1979; Malinowski & Howery, 1980) gave us a simple way of categorizing the VCD spectra in terms of the coefficients of six subspectra into which all of the protein VCD spectra could be linearly decomposed. Since the PC/FA method sorts out correlated intensity changes in order of importance, this leads to a weighting of the importance of the characteristic coefficients for each protein. Cluster analysis (CA) (Sharaf et al., 1986) of these VCD coefficients indicated a common topology with that found from cluster analysis of

†This research was supported by NIH Grant GM30147. Partial support of the purchase of the UVCD spectrometer was provided by the National Science Foundation and the University of Illinois.
*To whom correspondence should be addressed at UIC.
‡Permanent address: Department of Chemical Physics, Charles University, Prague 2, Czechoslovakia.

[1] Abbreviations: CA, cluster analysis; FC, fractional coefficient (of secondary structure); FTIR, Fourier transform infrared (spectroscopy); KS, Kabsch and Sander (1983) protein X-ray crystal structure analysis; LG, Levitt and Greer (1977) protein X-ray crystal structure analysis; paper I, Pancoska et al. (1989); paper II, Pancoska et al. (1991); PC/FA, principal component method of factor analysis; RDI, relative dissimilarity index (in cluster analysis); S/N, signal to noise ratio; UVCD, ultraviolet circular dichroism (of electronic transitions); VCD, vibrational circular dichroism (in the infrared).

0006-2960/91/0430-6885$02.50/0