The Catalytic Activity of Muscle Phosphoglucomutase in the Crystalline Phase

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A suspension of microcrystals of phosphoglucomutase in 60% ammonium sulfate exhibits a maximal catalytic activity in substrate-velocity studies that is about 0.2 of that obtained with the soluble enzyme under the same conditions. The apparent Michaelis constants for the reaction in the crystalline phase are altered to an even smaller extent, relative to that in solution, although the parameters for the monophosphate and bisphosphate are increased more than 3 and more than 5 orders of magnitude, respectively, by the sulfate present. The compatibility of larger crystals with a reaction that constitutes part of the catalytic process also is demonstrated.

Catalytic activity in the crystalline phase has been demonstrated for a substantial number of enzymes (Makinen and Fink, 1977; Westbrook and Sigler, 1984) since the pioneering work of Richards on crystalline ribonuclease (Richards, 1963). But not all enzymes crystallize in a catalytically active form. Hence, one criterion for evaluating the crystallographic structure of phosphoglucomutase (cf. the accompanying paper, Lin et al., 1986), or any other enzyme, is its activity in the crystalline phase. But an evaluation of enzymic activity can be particularly difficult for enzymes that are crystallized from ammonium sulfate solutions when, like phosphoglucomutase, substrate binding depends extensively on anionic interactions (Ray and Long, 1976). The present paper assesses the results of quantitative activity measurements on a microcrystalline form of phosphoglucomutase in substrate-velocity studies conducted at an ammonium sulfate concentration of 60% of saturation. The conversion of the phosphoenzyme to the dephospho form in millimeter-size crystals also shows the compatibility of the tetragonal crystal lattice of phosphoglucomutase with reactions that correspond with part of the catalytic cycle. A preliminary account of some of these results has been published (Wierenga et al., 1981).

EXPERIMENTAL PROCEDURES

Materials—Microcrystals of phosphoglucomutase were prepared via procedure (a) described in a paper submitted for publication. 1

Enzyme-grade ammonium sulfate was obtained from Schwarz-Mann; MES 2 was from Calbiochem-Behring; glucose-6-phosphate dehydrogenase and NADP from Boehringer Mannheim; α-D-glucose 1-phosphate from Sigma; and α-D-glucose, 1,6-bisphosphate was prepared as described previously (Ray and Roscelli, 1984). A buffer mixture which contains 50 mM MES, 15 mM MgSO 4, 1 mM MgEDTA, with an apparent pH of 6.5 in 50% (NH 4 ) 2SO 4 is referred to as "crystallization buffer."

Enzymic Activity of Phosphoglucomutase in Microcrystals—To 40 µl of an assay mixture containing 60% (NH 4 ) 2SO 4 crystallization buffer, 0.020-0.10 mM Glc-1-P, and Glc-P 2 at a constant ratio to Glc-1-P of 1:4, was added to 10 µl of a suspension of microcrystals (about 5 mg/ml) in the same solution except for the absence of Glc-1-P and Glc-P 2 . Just prior to the addition, the preparation of microcrystals was washed three times by suspension and subsequent centrifugation in about 20 volumes of 48% (NH 4 ) 2SO 4 plus crystallization buffer and transferred to 60% (NH 4 ) 2SO 4 (plus crystallization buffer) in the same way. After a reaction time at 25 °C of not more than 2 min, the reaction was stopped by adding 25 µl of 1 N HCl. After 5 min, 20 µl of 1 N NaOH was added to partially neutralize the acid and the samples were centrifuged. The Glc-6-P produced was measured by adding 40 µl of the centrifuged sample to 0.75 ml of an assay mixture containing 0.05 M Tris-chloride, pH 8.1, 1 mM EDTA, 0.5 mM NADP, and excess glucose-6-P dehydrogenase; the change in optical density produced at 340 nm was monitored as a function of time until no further change was observed. A blank value to assess the contribution of the measured activity by the soluble enzyme in the preparation of microcrystals was subtracted from each activity determination on the basis of an identical assay conducted with the supernatant of a centrifuged sample of microcrystals. An additional series of measurements was conducted with a sample of enzyme made up in 60% ammonium sulfate in such a way that all of the enzyme remained soluble. The activity of a dissolved sample of microcrystals at 25 °C and pH 7.5 in the absence of sulfate and at saturating concentrations of Glc-1-P and Glc-P 2 was measured in a continuous assay analogous to that described by Dawson and Mitchell (1989).

Conversion of the Phosphoenzyme to the Dephospho Form in Large Crystals—Crystals of phosphoglucomutase, phospho form, with a long axis of about 1 mm were obtained in the manner described in a paper submitted for publication. 3 Conversion to the dephospho form was accomplished by soaking in an ammonium sulfate solution, 53%, that contained crystallization buffer and 20 mM Glc-1-P. The solution was replaced every hour for a total of five treatments. Subsequently, the Glc-1-P was removed by soaking in the same solution lacking Glc-1-P for six half-hour intervals. The amount of the dephospho enzyme in a sample was determined by treating with excess Glc-P 2 and measuring the Glc-6-P formed (Ma and Ray, 1980).

RESULTS

Enzymic Activity of Phosphoglucomutase in Microcrystals—Activity measurements were conducted by using freshly washed suspensions of microcrystals prepared by a temperature-induced phase separation in the presence of 2.5% polyethylene glycol. 4 In spite of substantial clumping of the crystals, most of the bipyramids prepared in this manner had a

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1 W. J. Ray, Jr., and C. E. Bracker, Jr., manuscript submitted for publication.

2 The abbreviations used are: MES, 4-morpholineethanesulfonic acid; Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, D-glucose 6-phosphate; Glc-P 2, α-D-glucose 1,6-bisphosphate; K M, the observed value of the Michaelis constant for A; K Mα, the Michaelis constant for α

3 When both the alternate substrate and the metal ion activator are saturating, but in the presence of a competitive inhibitor, K m, corrected for competitive inhibition (an analogous symbolism is used for k cat, the catalytic constant).

4 W. J. Ray, Jr., and J. M. Puvathingal, manuscript submitted for publication.
maximal thickness no greater than 3 microns. Because of the relatively high catalytic efficiency of the enzyme, only a very small amount of the crystalline suspension could be used in an assay mixture. This required that the ammonium sulfate concentration in the assay be maintained at a rather high level (50–60%) to minimize the amount of soluble protein present. Under these conditions the soluble enzyme accounted for less than 5% of the measured activity. Because the slurry of enzyme crystals was diluted by 5-fold in the assay, a short assay interval (2 min) was used so that any increase in soluble enzyme during the assay was minimized. Product-time plots (not shown) were linear over this time interval. Because of the high sulfate concentration, it was difficult to saturate the enzyme with either Glc-1-P or Glc-P2. Hence, since the reaction is ping-pong (Ray and Peck, 1972), both phosphates were varied at a constant ratio (Plowman, 1972) in presence of saturating concentration of Mg2+ (see below) in order to evaluate Vmax by extrapolation of the linear double reciprocal plots that were observed—cf. Wierenga et al. (1981). Velocity measurements extended from about 0.2 to 0.6 Vmax in each case. These data show that the specific activities of the soluble enzyme in the supernatant from a centrifuged sample of microcrystals and a soluble enzyme fraction prepared separately in 60% (NH4)2SO4 are the same. The results also show that at 60% (NH4)2SO4 and at the pH of the crystal growth (6.5, see Footnote 3) soluble phosphoglucomutase exhibits only about 0.3 of the activity obtained at a low salt concentration and at a somewhat higher pH. No attempt was made to determine the relative sizes of the effects on enzymic activity produced by these differences in assay conditions. In turn, Vmax of the enzyme in microcrystals was about 0.2 that of the soluble enzyme under the same conditions, although the apparent Michaelis constant was similar for both solution and crystal phases. The results of these assays are summarized in Table I.

A less extensive activity study subsequently was conducted in 50% ammonium sulfate and at essentially saturating substrates (data not shown). Under these conditions, the soluble enzyme was 0.5 as active as at low salt and, therefore, the crystalline enzyme was about 0.2 as active as the soluble enzyme. Although from a practical standpoint the crystalline enzyme could not be studied at ammonium sulfate concentrations much less than 50%, if the above trend is extrapolated linearly to low salt concentration, the crystalline form of the enzyme still would be 20% as active as the soluble enzyme.

Although a comprehensive examination of the activity requirement of Mg2+ in 60% (NH4)2SO4 containing crystallization buffer was not conducted, at a concentration of Glc-1-P, KmGlc-1-P for the soluble enzyme is about 4 mM. A value of about 5 mM for KmGlc-1-P at saturating Glc-1-P and about 25 mM for KmGlc-1-P at saturating Glc-P2 can be calculated by using two simultaneous equations. The value of K95Glc1p (1981). Velocity

| Table I |
|---|---|
| Form assayed | kmax * | Kapp |
| Soluble enzyme | 140 | 48 |
| Supernatant enzyme | 147 | 53 |
| Microcrystalline enzyme | 28 | 66 |

* The value of kmax at the same temperature, but at pH 7.5 and in the absence of ammonium sulfate, is about 480 units/mg at 25 °C.

Activity of Crystalline Phosphoglucomutase

A rough estimate of values for the apparent Michaelis constants of Glc-1-P and Glc-P2 in 60% (NH4)2SO4 at essentially saturating Mg2+, and at saturating concentrations of the other sugar phosphate, can be obtained from the data in Table I, plus the additional observation that at 90 mM Glc-1-P, KmGlc-1-P for the soluble enzyme is about 4 mM. A value of about 5 mM for KmGlc-1-P at saturating Glc-1-P and about 25 mM for KmGlc1-p at saturating Glc-P2 can be calculated by using two simultaneous equations. The value of K95Glc1p (1981) is 3 × 10^-5-fold larger than that obtained under normal assay conditions in the absence of sulfate and that for KmGlc-1-P is about 5 × 10^-5-fold larger (Ray and Peck, 1972). This difference arises from the competitive binding of two sulfates by the dephosphoenzyme (which affects KmGlc1p) as opposed to one sulfate by the phosphoenzyme (which affects KmGlc-1-P). By assuming that the binding of the first sulfate is about the same for the phospho- and dephosphoenzyme, the second sulfate is bound to the dephosphoenzyme about 0.01 as tightly as the first, which is in accord with the relative binding of diaminophosphonate to the two sites in the dephosphoenzyme (Ray and Middavan, 1973). Since K95 for a constant ratio of Glc-P2 to Glc-1-P is approximately the same for the soluble and crystalline forms of phosphoglucomutase (Table I), a value of 25 mM for K95Glc-1-P at saturating Glc-P2 is used in the subsequent section to approximate the critical thickness of crystalline phosphoglucomutase under the above assay conditions.

Approximation of the Critical Thickness of Phosphoglucomutase Crystals for Activity Measurements—An approximation of the critical thickness, λ, for crystalline phosphoglucomutase under the steady state assay conditions used in the previous section can be made according to Hoogenstraaten and Sluytermann (1969):

\[
\lambda = \frac{[F(P)]D_{h}}{k_{m}(E)}
\]

Here, [F(P)] is the apparent Michaelis constant for Glc-1-P in 60% (NH4)2SO4 at saturating Glc-P2, about 25 mM (see previous section); k_{m} (Table I) is about 30 s⁻¹; (E) is 9 mM (as calculated from data in the accompanying paper, Lin et al., 1986); and λ is the distance from the surface in a thin, plate-like crystal at which the substrate concentration had decreased to half its value in the bulk solution because of binding to and reaction with the enzyme. Product inhibition is ignored in the derivation of Equation 1 (Hoogenstraaten and Sluytermann, 1969). In view of the fact that at low salt concentration, product accumulation does not significantly reduce the velocity of the phosphoglucomutase reaction at 50% conversion of substrate (Ray and Roscelli, 1966), ignoring product inhibition seems reasonable here. In Equation 1 the value of D_{h}, the diffusion constant for the substrate, is taken as 10⁻⁶ cm² s⁻¹, which is somewhat larger than that used in the above reference, because both channels and the fractional volume of solvent in crystalline phosphoglucomutase are much larger than for most protein crystals. Thus, channels have diameters of at least 60 A in many places (see the accompanying paper, Lin et al., 1986). Hence, a should vary from about 3–6 microns, respectively, at the lowest and highest substrate concentrations used (16 and 64 mM). Since the crystals employed in

* To compare the Michaelis constant for Mg2+ under these conditions with that observed at low ionic strength one must know to what extent the enzyme is saturated with Glc-1-P and Glc-P2 (Ray and Peck, 1972). Based on the estimates for KmGlc1-p and KmGlc-1-P given below, K95Glc1p increases by less than 3-fold in 60% (NH4)2SO4.
these studies had average thicknesses somewhat smaller than these values, diffusional limitations probably were not critical, and another of the major assumptions in derivation of Equation 1, viz. that the concentration of "free" enzyme does not vary substantially with distance from the surface, probably is reasonable. Of course, in the present system, the free enzyme is that to which sulfate instead of Glc-1-P is bound at the active site. On this basis, the approximately 5-fold reduction in enzyme activity observed in the crystalline relative to the solution phase would arise from factors other than diffusional limitations. But in view of the many assumptions involved in the approximation of $\lambda$, the only firm conclusion that can be drawn is that diffusional effects are unlikely to reduce activity by more than an order of magnitude. Hence, diffusional effects could conceivably account for all of the reduced activity observed in the crystalline state.

Conversion of the Phosphoenzyme to the Dephospho Form in Large Crystals of Phosphoglucomutase—The phospho form of phosphoglucomutase is the form that is used for the growth of millimeter-size crystals of the enzyme. To measure the dephosphoenzyme in the presence of the phospho form, the mixture of enzymes is treated with Glc-P$_2$ and the stoichiometric amount of Glc-6-P produced by reaction with the dephosphoenzyme (the phosphoenzyme does not react) is measured in terms of the NADPH generated during an oxidation catalyzed by the appropriate dehydrogenase (Lowry and Passonneau, 1969). The base-line trace in Fig. 1 was produced when a solution obtained from large crystals of the enzyme was assayed in this manner. Thus, such crystals contain very little of the dephosphoenzyme.

The treatment of the phosphoenzyme with Glc-1-P at low ionic strength does not produce significant amounts of the free dephospho form except at exceedingly low enzyme concentrations where the equilibrium, $E_{0} \cdot \text{Glc-P}_2 = E_{0} + \text{Glc-P}_2$, is shifted to the right (Peck et al., 1968; Ray and Peck, 1972). But sulfate binds competitively with Glc-P$_2$ (Ray and Roscelli, 1966) and also tends to drive this equilibrium to the right. In fact, at a 60% ammonium sulfate concentration, the above equilibrium is far enough to the right that two successive treatments of a 0.25 mM suspension of the precipitated enzyme produces 98% conversion to the free dephospho form. (See also the somewhat similar preparative procedure used by Ma and Ray, 1980.) The results obtained with a solution of enzyme prepared from crystals treated in a similar manner (see "Experimental Procedures") show that the same strategy also is successful in the crystalline phase (upper trace, Fig. 1). On the basis of the concentration of enzyme in the test solution and the optical density change at 340 nm produced on addition of Glc-P$_2$ (arrow), approximately 95% of the enzyme was converted to the dephospho form by the above treatment. (The absence of a change in optical density when no Glc-P$_2$ was added to the otherwise complete assay mixture shows that essentially none of the reactant Glc-1-P remained in the crystal after the washing procedure.) No visible cracks were produced in the crystals by their conversion to the dephospho form, and the crystalline state thus is compatible with this aspect of the enzymic reaction.

**DISCUSSION**

As a catalyst, phosphoglucomutase is more efficient than the average enzyme, with a $k_{cat}/K_{m}$ value slightly larger than $10^{2}$ M$^{-1}$ s$^{-1}$ (Ray and Peck, 1972). Normally, an enzyme with such large efficiency exhibits a low activity in the crystal phase relative to that in solution because of diffusional limitations (cf. Makinen and Fink, 1977; Westbrook and Sigler, 1984). The present observation of a maximum velocity in the crystal phase approximately 0.2 of that in solution (in the presence of 60% ammonium sulfate) is related to a number of factors including a reduced value of $k_{cat}$ for the soluble enzyme (about 3-fold) at high salt, a somewhat lower concentration of enzyme and much larger channels in the crystal phase (see the accompanying paper, Lin et al., 1986) than for a number of other proteins, and a relatively small substrate. But by far the most important factor was the use of high substrate concentrations, which increases the distance to which the substrate diffuses into the crystal before its concentrations is appreciably reduced by binding to and reaction with the enzyme. In the present case, concentrations of up to about 10,000 $K_m$ were required because of the high concentration of sulfate, a competitive inhibitor (Ray and Roscelli, 1966) that also was present in the assay to reduce the concentration of soluble enzyme. Whether or not diffusional limitations actually contribute to the above 0.2-fold change in rate (see "Results"), the present data indicate that phosphoglucomutase is quite active in the crystalline phase. It thus joins the growing number of crystalline enzymes that have been shown to exhibit catalytic activity (Makinen and Fink, 1977; Westbrook and Sigler, 1984).

A second indication of the compatibility of the crystalline phase with the enzymic reaction is the conversion of the phosphoenzyme to the dephospho form without producing visible cracking, even to millimeter-size crystals (see "Results"). Although the free dephosphoenzyme is not an intermediate in the normal enzymic reaction (Ray and Peck, 1972), conversion of the phosphoenzyme to the dephospho form involves treatment with substrate under conditions where the enzyme is active. At low ionic strength, substrate binding produces a change in the ultraviolet spectrum of the enzyme that is large enough to suggest that a substantial conformational change accompanies the binding process (Yankeelov and Roshland, 1965; Ma and Ray, 1980). Whatever the nature of the change, it apparently is compatible with the integrity of the tetragonal crystals of the enzyme. This observation

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**Fig. 1.** Assay of phosphoglucomutase after conversion of the crystalline enzyme to the dephospho form. The recorder trace obtained when an aliquot of a solution prepared from either untreated crystals (lower trace) or treated crystals (upper trace) was added to an assay mixture that contained glucose-6-phosphate dehydrogenase (cf., Ma and Ray, 1980) so that the NADPH generated was equivalent to the Glc-6-P produced. The arrow indicates when Glc-P$_2$ subsequently was added (upper trace). The treatment of crystals is described under "Experimental Procedures."
Activity of Crystalline Phosphoglucomutase

provides support for the expectation that the binding of 6-deoxyglucose 1-phosphate and 1-deoxyglucose 6-phosphate to the crystalline enzyme will provide suitable substrate analog complexes for x-ray diffraction studies. In fact, millimeter-size tetragonal crystals can be treated with 0.1 M concentrations of the later deoxysugar phosphate without producing visible cracks although the diffraction properties of such crystals as yet have not been studied.

REFERENCES