Catalytic Reactions of Phosphoglucone Isomerase with Cyclic Forms of Glucose 6-Phosphate and Fructose 6-Phosphate*

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SUMMARY

Yeast phosphoglucone isomerase is shown to use and produce both the α and the β anomic forms of fructose-6-phosphate. Rapid quench techniques show a 20-fold preference in the utilization of the α anomer over the β anomic form. The previous report that the α anomer of glucopyranose-6-P is the preferred substrate form is confirmed. However the β anomer is also shown to react at a slower rate. Studies using active site labeled phosphoglucone isomerase suggest that both anomers of each substrate react at the same enzyme site. A reaction mechanism consistent with these observations involves opening of both α and β ring forms to the corresponding acyclic forms followed by isomerization via an identical enzyme-bound cis-enediol. In order to accommodate both the α and the β anomers, torsion around C—C bonds is envisioned for the β anomers to arrive at the same cis-enediol intermediates.

In studies by Salas et al. (1), yeast phosphoglucone isomerase was shown to act preferentially or exclusively upon the α-pyranose form of D-glucose 6-phosphate in the formation of D-fructose 6-phosphate. Subsequently, Wurster and Hess (2) have shown that yeast isomerase converts fructose-6-P to glucose-6-P that is largely or exclusively in the α-pyranose form. The present study concerns the form of fructose-6-P that is used and produced catalytically by action of isomerases of yeast and muscle.

Salas et al. also observed that the time lag in the coupling of yeast hexokinase, acting on α-glucose, and yeast glucose-6-P dehydrogenase, which is specific for β-glucose-6-P was largely eliminated by the addition of phosphoglucone isomerase (1). It was concluded that isomerase catalyzed the interconversion of α-glucose-6-P and the acyclic form of glucose-6-P which then rapidly cyclized in a random fashion presumably after leaving the enzyme surface. Information on the 1-epimerase activity of isomerase acting on both β-glucose-6-P and β-fructose-6-P is also provided in the present study.

EXPERIMENTAL PROCEDURE

Methods—Two solutions of equal volume containing either phosphoglucone isomerase or substrate, glucose-6-P or fructose-6-P, were mixed in a Ballou rapid mixing cell. The reaction was terminated by injection of a volume of 7% perehloric acid through a second mixing cell after a period of reaction that was adjusted by altering the distance between the cells and the rates of flow. The rapid quench apparatus, designed by D. P. Ballou (3), was standardized according to Barman and Gutfeuld using the alkaline hydrolysis of p-nitrophenylacetate (4). After centrifugation of the quenched reaction mixture, p-nitrophenol in acid was assayed using 50% KOH precipitating KClO₄ which was centrifuged. Glucose-6-P and fructose-6-P in this supernatant were determined with glucose-6-P dehydrogenase by NADP reduction measured at 340 nm, before and after addition of phosphoglucone isomerase, respectively.

Yeast phosphoglucone isomerase (500 units per mg) and glucose-6-P dehydrogenase were commercial products of Sigma Chemical Company. The activity of the isomerase preparation was determined under the following conditions: triethanolamine-HCl, pH 8.0, 100 mM; fructose-6-P, 5 mM; EDTA, 2 mM; NADP, 0.2 mM; 25°; glucose-6-P dehydrogenase, 0.2 units per ml. A unit is defined as the rate of glucose-6-P, micromoles per min, formed in the above assay. Epoxide-inactivated phosphoglucone isomerase was prepared by reaction with 1,2-anhydro-L-mannitol-6-P according to the procedure of O’Connell and Rose (5).

Theory—Assume that phosphorylase isomerase prefer one of the anomers of fructose-6-P occurring in solution and that the other anomer converts to the substrate at a relatively slow rate on an experimental time scale. Let fructose-6-P be mixed with sufficient isomerase to rapidly establish an equilibrium between the active forms of fructose-6-P and glucose-6-P, and let the reaction be terminated at varying times. Thus the glucose 6-P produced will be the result of the initial rapid reaction plus the slower spontaneous anomerization, and the extent of the initial reaction can be obtained by extrapolation back to zero time. This value may then be compared with predications based on known...
equilibrium values with the various anomeric forms, as follows.
(a) The α-pyranose form of glucose-6-P is the major or only product (2) which then can anomerize relatively slowly to the equilibrium mixture of 38% α-glucose-6-P and 62% β-glucose-6-P. (b) Fructose-6-P in aqueous solution is predominantly in the furanose form with about 20% α- and 80% β-fructose-6-P (6). (c) The equilibrium for the interconversion of glucose-6-P and fructose-6-P, including the major cyclic forms, is fructose-6-P/glucose-6-P = 0.27, 25°C (7). From α, β, and ε the equilibrium proportions are α-glucose-6-P:β-glucose-6-P:α-fructose-6-P:β-fructose-6-P = 1:1.63:0.147:0.59, respectively. Thus, the amount of α-glucose-6-P to be expected from an initial equilibrium mixture of fructose-6-P anomers can be shown, if α-fructose-6-P is the substrate, to be 20% × 1/1.147 or 17% of the total fructose-6-P and 80% × 1/1.59 = 50% if β-fructose-6-P is used. On the other hand, starting with an equilibrium mixture of glucose-6-P forms, the amount that will be converted to fructose-6-P during the rapid phase is expected to be: 38% × 0.147/1.147 = 5% of the total glucose-6-P if α-fructose-6-P is the exclusive product and 38% × 0.59/1.59 = 14% if it is β-fructose-6-P. These calculations make the reasonable assumption (see below) that there is no contribution by epimerase activity or unfavored anomer utilization in the fast initial equilibrium formation.

RESULTS

In the first experiment with yeast phosphoglucone isomerase the time course of formation of glucose-6-P from fructose-6-P was measured at three concentrations of enzyme (Fig. 1). The results with the lowest amount of enzyme appear to represent a combination of rates, an initial fast reaction and subsequent slower utilization of substrate. Replotting the data as a first order approach to equilibrium (Fig. 2), using for the infinite time value the over-all glucose-6-P/fructose-6-P equilibrium determined under these conditions, the points fall on a straight line that does not pass through zero. This suggests the occurrence of a rapid initial phase. With greater amounts of enzyme this first phase is completed before the earliest sampling time and the second phase is increased in rate as a linear function of the amount of enzyme (Fig. 3). By extrapolation of the second phase (Fig. 2) to zero time one can estimate the fraction of substrate converted to glucose-6-P in approaching the earlier equilibrium. The values obtained for the three cases are 17.3%, 16%, and 5%, which are to be compared with 17% and 50% as α-glucose-6-P to be expected from α- and β-fructose-6-P as substrate, respectively (see “Theory”). Thus the rapid reaction must represent the utilization of α-fructose-6-P. It is clear from the enzyme dependence of the second phase slope (Fig. 3) that yeast phosphoglucone isomerase must also catalyze the conversion of β-fructose-6-P to glucose-6-P, but at a significantly lower rate than for α-fructose-6-P. Under the conditions of this experiment with β fructose-6-P present initially at 0.4 mM and with 0.08 mM glucose-6-P resulting from fructose-6-P turnover, this rate is about 30 to 45% of the maximum steady state velocity of fructose-6-P conversion to glucose-6-P. On the other hand, the rate of α-fructose-6-P isomerization in this experiment at its initial concentration, 0.1 mM, seems to be about 10 times that of the β-fructose-6-P initially at 0.4 mM. Further experiments will be necessary to determine the $K_m$ of reaction with β-fructose-6-P and the true $K_m$ of α-fructose-6-P. The $K_m$ measured with all forms of fructose-6-P present is 0.11 to 0.23 mM (8), so that the concentration of total fructose-6-P (0.5 mM) in the experiment was initially close to saturation.

1 Sample calculation assuming α-fructose-6-P as substrate and α-glucose-6-P as product. The ratio of α-fructose-6-P:α-glucose-6-P in rapid enzymatic equilibrium is equal to 0.147:1.0 (taken from the equilibrium distribution), and will be given by the ratio of α-fructose-6-P present initially − α-glucose-6-P formed:α-glucose-6-P formed, or 20% fructose-6-P initial − $x$: $x$, where $x$ is the glucose-6-P (in percentage of total fructose-6-P initial) that is formed rapidly by enzymatic isomerization.

\[ 0.147 = \frac{(20\% \text{ fructose-6-P}_{\text{initial}} - x)}{x} \]

thus $x = 20\% \text{ fructose-6-P}_{\text{initial}}/1.147$
FIG. 4. Time course of fructose-6-P formation from (α + β)-glucose-6-P. Procedure was as in Fig. 1 and \( f_t = \text{fructose-6-P/glucose-6-P} + \text{fructose-6-P} \). Phosphoglucose isomerase was 120 units per ml (△) and 420 units per ml (●). A and B represent results from corresponding experiments done on different occasions.

FIG. 5. Log plot of the approach to equilibrium in the direction glucose-6-P + fructose-6-P from Fig. 4, where \( f_t \) is defined as in Fig. 4 and 0.215 is the value of \( f_t \) at equilibrium. Symbols are as in Fig. 4.

FIG. 6. Effect of phosphoglucose isomerase concentration on the rate of β-glucose-6-P utilization. (Slopes from Fig. 5) × 2.3 give the first order rate constant for the corresponding enzyme concentration. Symbols are as in Fig. 4.

In the second series of experiments with yeast phosphoglucose isomerase, the time course of formation of fructose-6-P from the anomeric equilibrium of glucose-6-P forms was measured at 120 and 420 units of isomerase per ml and plotted as above in Figs. 4, 5, and 6. The occurrence of fast and slow reactions is evident in the log plot (Fig. 5) in which the amount of fructose-6-P formed from glucose-6-P initially represents 7.2% of the substrate. The predictions (see "Theory") from equilibrium considerations are 5.2 and 14.5% for enzyme formation of α- and β-fructose-6-P, respectively, from α-glucose-6-P. Thus, α-fructose-6-P is released from phosphoglucose isomerase. It is clear from Fig. 6 that the slow isomerization of β-glucose-6-P at an initial concentration of 0.31 mM and in the presence of the mixture formed rapidly by isomerization of 0.19 mM α-glucose-6-P is at most only about 20% of that of the α form. Thus, it is understandable that, using much lower amounts of enzyme, Salas et al. (1) were unable to observe the isomerization of β-glucose-6-P.

The finding that the sample of yeast phosphoglucose isomerase is able to catalyze the aldose-ketose isomerization of both α and β forms can be interpreted in several ways: (a) a composite of two distinct isomerases; (b) an α + α specific isomerase plus contaminating epimerases for glucose-6-P and fructose-6-P (1- and 2-epimerases, respectively); or (c) a single enzyme with specificities for both anomeric forms of glucose-6-P and fructose-6-P.

Concurrent studies by O'Connell in this laboratory (5) tend to rule out the first possibility. It was shown that two active-site reagents, differing by 50-fold in their maximal reactivity, both fully inactivate the isomerase activity of the yeast preparation (as well as a muscle phosphoglucose isomerase preparation) with simple first order kinetics. This result seems highly unlikely if two independent isomerases of different specificities were involved. Likewise the simple kinetic behavior shown by the yeast and muscle preparations in many studies with highly specific competitive inhibitors such as n-erythrose-4-P and 6-P-glucosonate is evidence of the same nature. In addition, three different phosphoglucose isomerase preparations (yeast, rabbit muscle, and pig heart muscle) all have the ability to utilize both substrates. It is unlikely that all purifications would lead to a similar ratio of an enzyme mixture.

The second possibility, that of distinct epimerases, was tested as follows. In the rapid quench studies already presented it is evident that the isomerization of α-fructose-6-P is much more rapid than that of the β anomer. Thus, if a distinct fructose-6-P 2-epimerase is present in the enzyme preparation it must be rate determining for the conversion of β-fructose-6-P to glucose-6-P. Using enzyme that had been inactivated by 1,2-anhydro-d-mannitol-6-P, which is known to inactivate the isomerase activity of the yeast preparation, a test of the survival of the epimerase activity was made (Table I). A low amount (100 units) of phosphoglucose isomerase converts about 50% of substrate to product in 150 ms. As expected from Fig. 1, four times this level of phosphoglucose isomerase converts approximately 75%. However, 100 units of phosphoglucose isomerase plus 300 "units" of inactivated phosphoglucose isomerase are equivalent to 100 units of active phosphoglucose isomerase...
Hess have discovered an enzyme in yeast that anomerizes substrates were predicted and confirmed (1). Salas et al. (1) suggested that the anomeric substrate specificities of these enzymes may be understood and predicted by reference to a cis-enediol intermediate and the known direction of protonation of the C-2 carbonyl group of the aldose. These considerations for the ketoses lead to the predicted formation of α-fructose-6-P in the case of phosphoglucose isomerase. In agreement with these considerations, α-fructose-6-P has been found to be the preferred substrate and preferred product for the isomerase reaction catalyzed by yeast phosphoglucose isomerase.

It remains to ask how the utilization of the β anomers of both glucose-6-P and fructose-6-P relates to the earlier mechanistic considerations. Three mechanisms will be considered. In the first (Scheme I) it is supposed that the enzyme uses only the β anomer as the preferred substrate and preferred product for the isomerase reaction. The isomerization of the acyclic form which thereby is prevented from returning to the β-glucose-6-P form. In this view the rate of isomerization of β-glucose-6-P should be limited by the rate of uncatalyzed ring opening, \( k_{\beta,0} \), which may be calculated from the rate of uncatalyzed anomerization as follows.

\[
\beta\text{-glucose-6-P} \xrightarrow{k_{\beta,0}} \text{OPEN} \xrightarrow{k_{\alpha}} \alpha\text{-glucose-6-P}
\]

\[
K_{eq} = k_{\beta,0}/k_{\alpha} = 1.66 (1)
\]

Therefore, \( k_{\beta,\alpha} = (\beta\text{-glucose-6-P} \rightarrow \alpha\text{-glucose-6-P}) = 0.06 \text{ s}^{-1} \) (2, 11)

\[
v_{\alpha,\beta} = \frac{k_{\beta,\alpha}}{k_{\beta,\alpha} + k_{\alpha}} \cdot (\beta) = 0.036 \text{ s}^{-1} (\beta)
\]

Therefore, \( k_{\beta,0} = 0.036 \text{ s}^{-1} \left( 1 + \frac{k_{\alpha}}{k_{\beta,0}} \right) \)

As seen in Fig. 6, enzyme-catalyzed rates of utilization of β-glucose-6-P in excess of 10 s\(^{-1}\) have been reached. In order to achieve such rates the ratio \( k_{\beta,0}/k_{\alpha} \) must exceed 275 which is very unlikely and is contradicted by polarographic studies on glucose (12).

A second mechanism to be considered is shown in Scheme II. This scheme proposes that the active site utilizes both substrate forms but via different pathways involving no common intermediates and no crossover from the intermediates of one pathway to the other. These involve a cis-enediol with the \( \alpha \) substrates and a trans-enediol with the \( \beta \) forms. This scheme is based on the observed labeling pattern during the enrichment of the C-5 hydroxyl to the C-1 carbonyl group should be from the \( \beta \) face and the approach of the C-5 hydroxyl to the C-1 carbonyl group should be from the \( \alpha \) face. Therefore, the approach of the C-5 hydroxyl to the C-1 carbonyl group should be from the \( \alpha \) face to produce \( \alpha \text{-glucose-6-P} \) specifically. However, the present study shows that this enzyme catalyzes the isomerization to fructose-6-P of the β anomer of glucose-6-P also, although more slowly. This conclusion has been reached independently by Wurster and Hess (9)

It has previously been impossible to extend these concepts to the ketose substrates due to the unavailability of the pure ketose anomers and the lack of information concerning the proportion of the appropriate anomers in solution. This latter deficiency has recently been overcome for fructose-6-P (6) which has made the present study of phosphoglucose isomerase possible. The prediction of the anomeric specificity for the ketoses can be understood and predicted by reference to a cis-enediol intermediate for the phosphoglucose isomerase. The prediction of the anomeric specificity for the ketoses assumes that the C-5 hydroxyl approaches the C-2 carbonyl of the acyclic ketose from the same face that it departed/approached the carbonyl group of the aldose. The latter was defined by the direction of approach of the conjugate acid group to C-2 which, assuming restricted rotation around C-5-C-6 and that a cisoid relation between the two oxygens was conserved, would force an antarafacial approach at C-1. Similar considerations for the ketoses lead to the predicted formation of α-fructose-6-P in the case of phosphoglucose isomerase. In agreement with these considerations, α-fructose-6-P has been found to be the preferred substrate and preferred product for the isomerase reaction catalyzed by yeast phosphoglucose isomerase.

Previous considerations of the mechanism of the aldose-ketose isomerases by Schray and Rose (10) have led to the suggestion that the anomeric substrate specificities of these enzymes can be understood and predicted by reference to a cis-enediol intermediate and the known direction of protonation of the two enediol carbons. With this background, the specificities of \( \alpha \)-xylose isomerase and \( L \)-arabinose isomerase for their aldose substrates were predicted and confirmed (10). Salas et al. (1) showed previously that yeast phosphoglucose isomerase uses \( \alpha \text{-glucose-6-P} \) preferentially. Since in the mechanism of isomerization, proton addition at C-2 of the cis-enediol comes from

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**Table I**

Effect of epoxide-inactivated phosphoglucose isomerase on the rate of β-fructose-6-P utilization by active phosphoglucose isomerase

<table>
<thead>
<tr>
<th>Addition</th>
<th>Percentage of glucose-6-P formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>52.9 54.0 53.4</td>
</tr>
<tr>
<td>0.6 mg phosphoglucose isomerase (300 units)</td>
<td>75.0 75.3 75.2</td>
</tr>
<tr>
<td>0.6 mg phosphoglucose isomerase (epoxide treated)</td>
<td>53.2 52.8 53.0</td>
</tr>
</tbody>
</table>

**Table II**

Effect of epoxide-inactivated phosphoglucose isomerase on the rate of β-glucose-6-P utilization by active phosphoglucose isomerase

<table>
<thead>
<tr>
<th>Addition</th>
<th>Percentage of fructose-6-P formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.8 7.6 7.7</td>
</tr>
<tr>
<td>0.5 mg phosphoglucose isomerase (220 units)</td>
<td>20.0 20.2 20.1</td>
</tr>
<tr>
<td>0.5 mg phosphoglucose isomerase (epoxide treated)</td>
<td>9.3 7.6 8.4</td>
</tr>
</tbody>
</table>

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Scheme I. In this mechanism the enzyme uses both the α and acyclic forms directly. The isomerization of the β anomers proceeds through the free acyclic compound by a noncatalyzed ring opening. This mechanism is essentially that of Salas et al. (1). Only those compounds shown in a box are enzyme bound. F6P, fructose-6-P; G6P, glucose-6-P.

Scheme II. In this mechanism the α-glucose-6-P → α-fructose-6-P conversion represents that part of the process in which proton is transferred, i.e., single base and no proton exchange. The β glucose 6 P → β fructose 6 P conversion proceeds through a trans-enediol formed by antarafacial proton abstractions by either of two bases and hence explains the proton exchange (13, 14) and the stereochemistry of labeling (15). F6P, fructose-6-P; G6P, glucose-6-P.

of fructose-6-P in tritiated water (14) and the observation of intramolecular proton transfer (13) which is most easily explained by a suprafacial proton transfer by way of a single base at the re face. However, it was observed that about half of the isomerization reactions occurred with incorporation of hydrogen from the medium. Although this was interpreted as partial exchange of the itinerant proton with water at the conjugate base stage, an alternative explanation would be that a portion of the isomerization represented by the β glucose 6 P to β fructose-6-P conversion follows a two base mechanism, allowing antarafacial proton additions to a trans-enediol. Thus, the two pathways lead to the same stereochemistry of proton activation at C-1 of fructose-6-P. If this were not the case, the “slow” interconversion of the anomeric forms would lead to an apparent nonstereospecificity of labeling at C-1 of fructose-6-P from tritiated water. A similar case of co-existence of two parallel paths has previously been established for dioldehydrase which acts on both components of a propanediol solution by independent and stereochemically distinct pathways (16, 17).

Two arguments can be raised in opposition to this mechanism. It seems clear from the work of Salas et al. (1) and also of Wurster and Hess (2) that phosphoglucone isomerase catalyzes the conversion of α-glucose-6-P to β-glucose-6-P. In addition, Carlson et al. presented evidence that phosphoglucone isomerase catalyzed the anomerization of β-glucose 6-sulfate (18). The findings cannot be explained in terms of this scheme in which the α and β forms are processed by nonintersecting pathways. A second objection relates to the direction of ring closure. The direction of approach of the C-5 hydroxyl must be opposite in the formation of the two forms of fructose-6-P. This requires that an entirely different mechanism of orienting the C-5 hydroxyl and hence of binding the phosphate bearing portion of the chain is necessary.

The final proposal (Scheme III) requires that the enzyme combine with and open both the α- and β-cyclic forms and that torsion around C–C bonds leads to a cisoid conformation of the C-1 and C-2 oxygens prior to enolization. This proposal is not at variance with any of the kinetic or stereochemical data. It is required, however, that a similar torsion about C2-C3 cannot occur at the enediol stage presumably due to an interaction of the enzyme site with the oxygen functions of the enediol. If this restriction were not present, mannose-6-P would be a component of the equilibrium, which is not the case. Previous isotope effect studies (13) have provided evidence that the proton transfer step determines the initial rate of fructose-6-P to glucose-6-P conversion when it is studied under the usual steady state conditions. In such studies, which less than 0.1 unit of enzyme is used, the spontaneous anomerization rate is sufficient to maintain the anomeric equilibrium during the rate determination. Hence the isotope effect applies to the isomerization of
the better substrate, α-fructose-6-P. Since the β anomer passes through the same intermediate form prior to proton abstraction, according to this mechanism, an early step must be rate determining in the 10-fold slower isomerization of the β anomer. This may be either the ring opening step or a subsequent conformational rearrangement.

Apparently nature has adapted the active site of this isomerase to carry out the special function of ring opening. The ability of phosphoglucose isomerase to utilize both anomers of glucose-6-P and fructose-6-P may satisfy a very real metabolic need since both anomers of glucose are known to be phosphorylated by hexokinase (I), and this may be true for the furanose forms of fructose as well. It will be of interest to determine whether the same catalytic groups are involved in both the isomerization and ring opening functions.

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