The Enediolate Analogue 5-Phosphoarabinonate as a Mechanistic Probe for Phosphoglucose Isomerase*

(Received for publication, December 30, 1974)

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A stable analogue has been prepared of the enediolate anion believed to occur transiently in the reaction of phosphoglucose isomerase. This compound, 5-phosphoarabinonate, is the strongest known competitive inhibitor of the enzyme ($K_i = 3 \times 10^{-7}$ M below pH 7). A distinctive pH dependence of binding, also found for two other aldonic acid $\omega$-phosphates, 6-phosphogluconate and 4-phosphoerythronate, involves perturbation of a $pK_a$ from 7.0 in the free enzyme to 9.0 in the enzyme-inhibitor complex. This perturbation may reflect a catalytically advantageous increase in basicity which occurs around the transition state of the normal enzymatic reaction.

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EXPERIMENTAL PROCEDURES

Materials

Phosphoglucose isomerase was isolated from rabbit muscle and purified to homogeneity by column chromatography on CM-Sephadex as described previously (14). Enzyme preparations were stored as crystalline suspensions and had specific activities of approximately 800 units/mg of protein. Nicotinamide adenine dinucleotide phosphate was obtained from Boehringer Mannheim Corp., fructose 6-phosphate and yeast glucose-6-phosphate dehydrogenase from Boehringer and Sigma, and 6-phosphogluconate dehydrogenase from Sigma.

Inorganic reagents were obtained from Mallinckrodt. Biochemicals, buffers, carbohydrates, sugar phosphates, and reagents for various color tests were products of Sigma, Boehringer, or Calbiochem. Potassium arabinonate was purchased from K&K Laboratories, Irvine, Calif.

Methods

Preparation and Standardization of Sugar Phosphates—Milligram quantities of 4-phosphoerythronate (15) and 5-phosphoarabinonate were synthesized from erythrose 4-phosphate and arabinose 5-phosphate (16), respectively, by bromine oxidation according to the procedure for the synthesis of 6-phosphogluconate (17). The products were purified by ion exchange chromatography as described by Brun et al. for 6-phosphogluconate (9).

Bromine oxidation of arabinose 5-phosphate was not adaptable to easy or inexpensive preparation of 5-phosphoarabinonate in gram quantities. Therefore, a modification of the original synthesis of this compound published by Neuberg and Collatz (18) was developed.

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*This is Paper 22 in a series of studies on phosphohexose isomerases. The work was supported in part by Research Grant AM 07203 from the United States Public Health Service. The investigations described here are from a dissertation submitted in 1974 by John M. Chirgwin to the Graduate Division of the University of California, Riverside, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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1 In this paper the term transition state analogue is used to describe analogues of metastable intermediates (occurring at relative free energy minima during catalysis) was well as of true transition states.
For a typical synthesis Grade I Sigma disodium fructose 6-phosphate (10.0 g, about 27 mmol) was dissolved in 150 ml 0.5 N NaOH and vigorously stirred at 23° under 1 atm of oxygen for 48 hours, during which time oxygen consumption was 756 ml (33 mmol). The reaction mixture was adjusted with concentrated HCl to pH 1.5 and bubbled with N₂ to remove dissolved CO₂. This procedure was followed by adjustment to pH 5 with saturated aqueous barium hydroxide. The resulting solution (350 ml) was filtered and the acid barium salt precipitated by the addition of 2 volumes of absolute ethanol. The isolated barium salt was suspended in 50 ml of water, brought into solution with the minimum amount of concentrated HCl, and reprecipitated as before. A third precipitation was performed in the same manner but with only a single volume of ethanol. The material resulting solution (350 ml) was filtered and the acid barium 5-phosphoarabinonate (78% of theory, based upon the starting potassium arabinonate and of trisodium 5-phosphoarabinonate were used within a few tenths of a pH unit of their pK values to assure adequate buffering capacity. Measurements of pH were made with a Beckman model 1019 Research pH meter. Kinetic data were analyzed by the graphical method of Hofstee (25), and the reciprocal values of the slopes of the straight lines in the primary graphs were plotted against the inhibitor concentrations divided by the slope values. The slopes of these secondary plots correspond to the values of Kᵢ. (Representative primary and secondary plots are given in Fig. 2.) Each Kᵢ value is based upon about 25 assays performed at two or three inhibitor concentrations.

RESULTS

Characterization of 5-Phosphoarabinonate—Characterization of 5-phosphoarabinonate was performed on samples of the sugar phosphate prepared in gram quantities from fructose 6-phosphate by the modified procedure of Neuberg and Collatz (18).

Enzymic assays of the synthesized compound gave the following contaminations: fructose 6-phosphate, 0.01%; glucose 6-phosphate, 0.1%; 6-phosphogluconate, 0.3%. Bial's orcinol assay (26) yielded 0.4% pentose, while Roe's resorcinol assay (27) gave 0.1% ketose. Contamination with inorganic phosphate was about 5% (20). The synthetic material chromato-
To determine the pKₐ values of the aldonic acid phosphates, 10 mg of 5-phosphoarabinonate or 6-phosphogluconate in a volume of 2 ml were acidified and titrated with 1 N NaOH in a Radiometer TTTlc/ABUlc titrator system equipped with microelectrodes. At 30°c values of 3.7 and 6.3 for pK₂ and pK₃, respectively, were found for both compounds.

**Inhibition Studies on Phosphoglucone Isomerase—**Results from competitive inhibition experiments were plotted according to the procedure of Dixon and Webb (31). In plots of this type upward breaks in the curve are caused by ionizations in the enzyme-inhibitor complex and downward breaks by ionizations in free enzyme or free inhibitor. Fig. 4 shows a Dixon plot for the inhibition of rabbit muscle phosphoglucone isomerase by 5-phosphoarabinonate. The theoretical straight-line intersections indicate pKₐ values of 7.0 and 9.0. Below pH 7, Kᵢ is 3 x 10⁻⁴ M. Fig. 5 shows a similar Dixon plot for 4-phosphoerythronate, fitted to the pKₐ values of Fig. 4. The curve is displaced downward, the Kᵢ being 2 x 10⁻¹ M below pH 7. The fact that the Kᵢ of 5-phosphoarabinonate below pH 7 is 10⁻² times the best value for substrate Kᵦ, rising to 1/10 Kᵦ at pH 9, supports the reasoning that this inhibitor is a good analogue of the cis-1,2-enediolate anion in the enzymatic reaction.

As can be seen from Fig. 6 (which summarizes all the inhibitor binding results from this laboratory) the four-, five-, and six-carbon enediolate analogues (for structures see Fig. 7) bind to phosphoglucone isomerase in a manner very different from that of inhibitors without carboxyl groups at C-1 (32).

Both 6-phosphogluconate and 4-phosphoerythronate bind to the enzyme less well than 5-phosphoarabinonate. A reasonable explanation is that the former must experience some steric crowding to position the C-1 carboxylate correctly, while the
latter may need to be reoriented to achieve proper binding of C-1 and the phosphate group. It is notable that all three aldonic acid phosphates show the same pH dependence, involving pKₐ values of 7.0 and 9.0. The very tight binding of 5-phosphoarabinonate and the pH dependence of binding peculiar to the aldinate inhibitors provide strong confirmation of our original supposition (Fig. 1) that 5-phosphoarabinonate should be a potent transition state analogue of phosphoglucoisomerase.

The two pKₐ values involved in aldinate binding are most readily explained by assigning, as did Wolfenden for triosephosphate isomerase (10), the one at 7.0 to the catalytically active base which governs Vₘₐₓ (8). The second pKₐ at 9.0, which was not seen with triosephosphate isomerase (5), we interpret to reflect an upward perturbation of the pKₐ at 7.0, which is caused by the binding of the enediolate form or its analogues.

According to a previously postulated mechanism (8), maximum catalytic activity requires the protonation of a residue with a pKₐ equal to 9.3 and the deprotonation of another with a pKₐ equal to 6.9. On first sight these two pKₐ values could be the same as those observed to govern the binding of 5-phosphoarabinonate. For the lower pKₐ such identity is reasonable since in both cases the pKₐ values at around pH 7 are assigned to the free enzyme. However the higher pKₐ at about pH 9 is associated with the enzyme-inhibitor complex in the case of 5-phosphoarabinonate binding but with the free enzyme in the case of productive catalysis. It is not possible to assign a lysine ε-amino group the pKₐ value of 9.0 which governs binding of the enediolate analogue, because this fails to explain two observations: (a) the involvement of the lysine in ring-opening and (b) the uptake of a proton upon formation of the enzyme-5-phosphoarabinonate complex above pH 7.

**DISCUSSION**

Studies with transition state analogues for triosephosphate isomerase (5, 33) suggest that planar double-bond geometry and a negative charge at C 2 are the essential features of the enediolate intermediate which must be incorporated into a good analogue inhibitor, such as first shown by Wolfenden for phosphoglycolate (5). In the case of phosphoglucoisomerase, 5-phosphoarabinonate, which possesses these features, binds to the enzyme and perturbs from 7 to 9 the pK of what may be the base responsible for proton transfer. This pK shift places constraints on the possible chemical identity of the base catalyst. Regarding the mechanism of interaction between enzyme and ligand, Pauling's original theory (34) has been extended to suggest (5, 6, 35) that transition state analogues may owe their tight binding to direct interaction with catalytic groups at the active site.

The binding of 5-phosphoarabinonate to phosphoglucoisomerase shows two aspects which call for explanation: its very low Kᵢ and its unusual pH dependency. Below pH 7 the Kᵢ is 10⁴ times smaller than Kᵢ for the substrates, suggesting that the specific interactions between enzyme and this inhibitor must be quite different from those in the Michaelis complexes. During catalysis a cationic residue at the active site could stabilize the negative charge of the enediolate intermediate and enhance its binding.

Rose has suggested the presence at the active site of an acidic residue which polarizes the substrate carbonyl and thus enhances enediolate formation (36). One would expect such a group also to interact electrostatically with the enediolate species and its analogues, thereby increasing their binding. This amino acid residue has recently been implicated in epoxide ring opening of the active site reagent 1,2-anhydromannitol 6-phosphate (37), and it was suggested that this critical side chain might be the ε-amino portion of a lysine residue, possibly that involved in hemiacetal ring opening (4, 8). Alternatively, we propose that it may be an arginine side chain, which has the additional function of liganding the oxygens of C-1 and C-2 of the substrates and of the enediolate intermediate in the cis configuration.

In addition to the tight binding of 5-phosphoarabinonate to phosphoglucoisomerase (the highest value of Kᵢ), is still 10 times smaller than Kᵢ for the substrates), there exists a second type of interaction between enzyme and inhibitor at the active site. This interaction is manifested in the shift from 7.0 to 9.0 of a pKₐ which may be the same pKₐ involved in catalysis (8). If one assigns the shifted pKₐ to the catalytically active base, it can be interpreted as the result of a direct electrostatic effect or of a conformational change which alters the environment of the base. Both interpretations are compatible with the base being a histidine imidazole group as advocated by our laboratory (4).

Shifts in pKₐ of this type have been postulated to occur in the hemoglobin alkaline Bohr effect (38) and in the activation of chymotrypsinogen. In both cases a pK shift occurs on breaking a noncovalent imidazole-carboxyl bond: in the Bohr effect the magnitude of the pKₐ change is 1.6 units (39); with chymotrypsinogen it is 1.2 units (40). Such a pKₐ shift is difficult to envisage for phosphoglucoisomerase if the base is a glutamyl γ-carboxylate group as suggested by O'Connell and Rose (37). Since this carboxylate group would already have its pKₐ perturbed from 4 to 7, it seems unlikely that it could then be perturbed upward by another 2 units as a consequence of interaction with a second carboxyl. Nevertheless, the data of these authors indicate the presence at the active site of a glutamate residue possessing an unusually nucleophilic γ-carboxyl group. However, the heat of ionization associated with the base catalyst in rabbit muscle phosphoglucoisomerase has been estimated at 7700 cal/mol (8). This value argues against the identification of the base with a simple carboxyl, for which ΔH° would be expected to be between −1500 and +1500 cal/mol, while the value found is typical.

![Fig. 7. Structures of various sugar phosphates as they are thought to bind in phosphoglucoisomerase.](image-url)
for the N—H bond in the imidazolyl portion of a histidine residue.

An attractive explanation for the apparent high reactivity and the abnormal pKₐ of a glutamate γ-carboxyl group would be the formation of an imidazole-carboxyl pair similar to that first seen by Blow et al. (41) in the x-ray structure of the active site of chymotrypsin, which shows a ΔH° of 9600 cal/mol (42), a value of the same magnitude as that seen for phosphoglucose isomerase. It should be noted, however, that the side chain reactivities and the catalytic functions of phosphoglucose isomerase and of the serine proteases differ considerably. Furthermore, the charge localization in the protease imidazole-carboxyl pairs is unresolved (43, 44); therefore, such a pair, if it occurs in phosphoglucose isomerase, may be quite different from that seen in chymotrypsin.

One possible interpretation is that substrate interaction with the active site may induce transient conformational changes which alter the geometry of an imidazole-carboxyl pair and thence its pKₐ. A transient increase in basicity could in this manner enhance catalysis (45). Thus the pKₐ shift from 7.0 to 9.0, seen upon inducing phosphoglucose isomerase with 5-phosphoarabinonate into its enediolate binding conformation, may represent part of a larger increase in basicity at the transition state. Such an increase in basicity would explain the enzyme's ability to catalyze at physiological pH the abstraction from substrate of a carbon-bound proton, a reaction which otherwise occurs only in strong alkalies (4).

This explanation for the mode of binding of aldonic acid ω-phosphates to phosphoglucose isomerase is supported by the observation of a conformational change with 6-phosphogluconate. Upon treatment of crystals of the pig muscle enzyme with the inhibitor, a small region of intensity difference is observed at the University of Bristol.

Acknowledgments—We would like to thank Drs. J. L. Sudmeier and P. D. Curb for obtaining the 13C NMR spectra, Ms. Adele Register for preparing the enzyme and for technical assistance, and Dr. Michael Dunn for critical reading of the manuscript.

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The enediolate analogue 5-phosphoarabinonate as a mechanistic probe for phosphoglucone isomerase.
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