Studies on the Mechanism of *Escherichia coli* Glucosamine-6-phosphate Isomerase†

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ABSTRACT: *Escherichia coli* glucosamine-6-phosphate isomerase is specific for removal of the 1-*pro-R* hydrogen of fructose 6-phosphate (fructose-6-P). The conversion of [2-^{3}H]glucosamine-6-P to fructose-6-P plus ammonia is accompanied by 99% exchange of tritium with water and 0.6% transfer to C-1 of fructose-6-P. The enzyme is active toward α-glucosamine-6-P and apparently inactive toward the β anomer. The combination of the above results supports a *cis*-enolamine intermediate for the reaction. The labeling of substrate and product pools in tritiated water shows that the two halves of the reaction are each freely reversible. No single step appears to be rate determining. 2-Amino-2-deoxyglucitol-6-P is an unusually strong competitive inhibitor ($K_I = 2 \times 10^{-7}$ M, compared with the $K_m = 4 \times 10^{-4}$ M for glucosamine-6-P), suggesting the enzyme has a strong affinity for the open-chain form of glucosamine-6-P.

The first step in amino sugar metabolism is the interconversion of fructose-6-P[1] and glucosamine-6-P catalyzed by the glucosamine-6-P isomerase (deaminase) and by fructose-6-P:glutamine amidotransferase (Roseman, 1959, 1970). The reaction represents a unique biochemical example of the Amadori rearrangement—a ketose/aldose isomerization coupled to an amination/deamination (Hodge, 1955). The glucosamine-6-P isomerase reaction is formally analogous to glucose-6-P isomerase, but some as yet undefined specificity or mechanistic properties ensure that the former does not isomerize glucose-6-P and that the latter does not isomerize glucosamine-6-P. The latter is also unique amongst the reversible enzymes of the isomerase class in being modulated by an allosteric activator, *N*-acetylglucosamine-6-P, which produces a pronounced lowering of the K_m's for all substrates (see Noltmann, 1972, for references).

Possible mechanisms for the reaction are considered in Scheme I. The α-β elimination mechanism (Davidson, 1967) would seem unlikely since the acidity of the α hydrogen would be far greater in the open-chain carbonyl form than in the valence-saturated carbinolamine form. A hydride shift mechanism has been proposed for aldose-ketose isomerases based on model studies of the acid-catalyzed reaction (Harris and Feather, 1975) and would proceed without hydrogen exchange with solvent. In addition to the above, the reaction could be assisted by covalent interaction of the C_2 amino group with an electrophilic center such as a dehydroalanine residue (Wickner, 1969; Givot et al., 1969; Hanson and Havir, 1970) or a terminal pyruvoyl residue (see Snell and DiMari, 1970, for references).

The present paper reports the stereochemistry of the glucosamine isomerase reaction and some properties of the hydrogen (proton) activation process. The results are interpreted in terms of a base-catalyzed carbonyl-enolization mechanism involving fructosimine-6-P as an intermediate. Attempts to inactivate the enzyme with $NaBH_4$ were negative but the experiments revealed a powerful competitive inhibition by 2-amino-2-deoxyglucitol-6-P ($K_i = 10^{-3} \times K_m$ for glucosamine-6-P).

Materials and Methods

E. coli Glucosamine-6-P Isomerase. E. coli were grown on glucosamine as sole carbon source in a minimal salts medium (Comb and Roseman, 1958, 1962). The enzyme was purified as follows: 400 g of packed cells were disintegrated by mild sonic oscillation with 400 mL of 20 mM K_2HPO_4 and centrifuged for 30 min at 25 000 rpm. The crude extract (specific activity, 0.4 unit/mg of protein) was diluted with 500 mL of cold H_2O and applied to a 2.5 × 40 cm DEAE-cellulose column (Whatman DE-52) equilibrated at pH 7.5 with 10 mM KH_2PO_4–HPO_4, pH 7.5. The column was eluted with 20 mM and then with 50 mM KH_2PO_4–K_2HPO_4 buffer. The isomerase appeared after the major protein peak in the latter buffer. The pooled fractions representing 70% of the activity applied to the column and a purification of 25- to 30-fold were concentrated to 10 mL by Amicon ultrafiltration. After adjustment to pH 5.4 with dilute H_3PO_4, the isomerase was absorbed to Amberlite-1RP64 (Rohm and Haas, Co.) and eluted batchwise with 200 mM KH_2PO_4–K_2HPO_4 buffers of steadily increasing pH (Comb and Roseman, 1962). The fractions of specific activity 80–100 units/mg of protein were combined and concentrated to 5 mL. The final concentrate represented about 30 mg of protein and a 30% overall recovery. It was stored either at 0–5 °C or in liquid N_2 with little loss of activity over extended periods (2–5 months). The protein displayed a single band on sodium dodecyl sulfate gels (Weber and Osborn, 1969), representing subunits of 28 000–30 000 M_r.

The glucosamine-6-P isomerase was assayed either at 25 or 37 °C in an incubation containing 100 mM Tris-HCl, pH 7.8, and 25 mM glucosamine-6-P (freshly adjusted to pH 7.8 with KOH). Fructose-6-P plus any glucose-6-P formed were measured after 10 or 15 min with the anthrone-H_2SO_4 reagent (Comb and Roseman, 1962). A unit of enzyme activity catalyzed the formation of 1 μmol of fructose-6-P min^{-1} at 25 °C. The reverse reaction was performed in an incubation containing 100 mM Tris-HCl, pH 7.8, 20 mM fructose-6-P, and 200 mM NH_4Cl, followed by the estimation of glucosamine-

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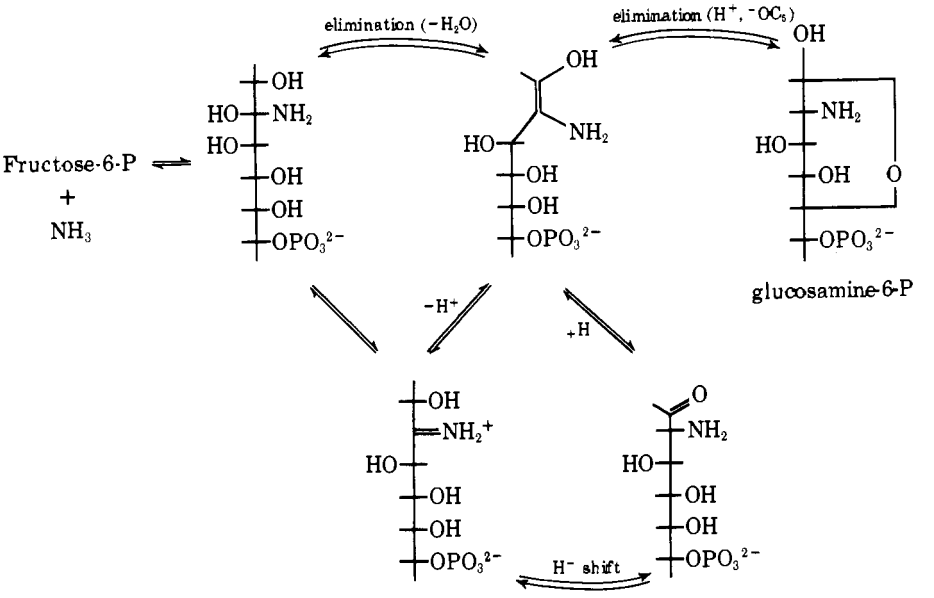
[1] Abbreviations used: P, phosphate (as in fructose-6-P for fructose 6-phosphate); DEAE, diethylaminoethyl; PEP, phosphoenolpyruvate.

Scheme I



6-P with a modified Morgan–Elson procedure (Ghosh and Roseman, 1962). Protein was estimated with the Lowry method.

Fructose-6-P and fructose-1,6-P_2 were isolated by chromatography on Dowex 1 (Cl^-) (Bartlett, 1969), glucosamine-6-P on Dowex 50 (H^+), eluting with the passage of 5–8 bed volumes of H_2O. [^{3}H]NADPH, gluconate-6-P, 2-amino-2-deoxygluconate-6-P, glucosamine-6-P, etc. were resolved on DEAE-cellulose (Cl^-) at pH 7.5 (Pastore and Friedkin, 1962).

When desired, fructose-1,6-P_2 was converted to fructose-6-P by a two-step procedure. First, fructose-1,6-P_2 (0.5 mM) was hydrolyzed with potato acid phosphatase overnight at pH 5 in a mixture containing 10 mM sodium acetate buffer and 3 mM magnesium acetate followed by passage through a small Dowex 1 (acetate$^-$) column to separate the free sugar. Second, fructose was rephosphorylated with a minimum of yeast hexokinase and ATP and the fructose-6-P isolated by Dowex 1 (Cl^-).

Crystalline α-glucosamine hydrochloride and β-glucosamine (free base) were prepared as described by Westphal and Holzman (1942). The α form had a specific rotation $[\alpha]^{20}_D = +103°$ and the β form $[\alpha]^{20}_D = +14°$. The equilibrium mixture, $[\alpha]^{20}_D = +47.5°$, was 63% β anomer and 37% α anomer, regardless of the state of protonation of the amino group. The free base approached anomeric equilibrium with $t_{1/2}$ = 7 min at 15 °C and pH 9, while the hydrochloride (pH 5) mutarotated more slowly ($t_{1/2}$ = 110 min at 15 °C). Rotation measurements were performed in a Schmidt and Haensch Model 10840 polarimeter. The apparent pK of the C_2-amino group was 7.5. In phosphorylating the purified anomers with yeast hexokinase, the α form was found to be a much better substrate (relative V_{max} = 0.5, K_m = 0.05 mM) than the β form (V_{max} = 0.2, K_m = 1 mM) and mixtures of the anomers gave biphasic kinetics. This is contrary to the reported behavior toward the best substrate, glucose (V_{max} = 1, K_m = 0.1 mM), where the two anomers are thought to be equally good substrates (Sols and Crane, 1954).

Anomeric specificity experiments were performed at ice temperature in 0.5 mL total volume in 15-mL centrifuge tubes with rapid magnetic stirring. The reactions were initiated by rapid addition of 20 μL of freshly dissolved glucosamine (α or β) (5 mM) with an Eppendorf pipettor followed 2 s later by the addition of 0.1 mL of 2 N $HClO_4$ with a second Eppendorf pipet. Protein was removed after the addition of 0.05 mL of bovine serum albumin (10 mg/mL) and centrifugation followed by a second addition of albumin and centrifugation. The samples were neutralized to pH 4.5 with 2 N KOH and centrifuged to remove precipitated $KClO_4$. ADP and fructose-6-P assays were performed on 0.05-mL aliquots. Controls lacking hexokinase or isomerase were treated in the same way. ADP was assayed in 0.1 mL total volume with 0.1 unit of pyruvate kinase, 0.1 unit of lactate dehydrogenase, 0.01 μmol of NADH, and 0.1 μmol of PEP. After exactly 15 min the samples were acidified with 1 N HCl to destroy NADH. A solution (1.5 mL) containing 6 N NaOH and 0.02% H_2O_2 was added and the alkaline fluorescent form of NAD was developed at 60 °C in the dark as described by Lowry and Passonneau (1972, Chapter 1). ADP standards were included each time, as were blanks which lacked enzyme. Fructose-6-P was assayed in a similar manner in 0.1 mL containing 1 mM ATP, 3 mM $MgCl_2$, 0.01 mM NADH, 0.1 unit of fructose-6-P kinase, aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase.

(1R)-[1-^{3}H]Fructose-6-P was prepared by incubating glucose-6-P with phosphoglucose isomerase in tritiated water and pulling the reaction to fructose-1,6-P_2 with phosphofructokinase and ATP. The fructose-1,6-P_2 was isolated from Dowex 1 (Cl^-) by elution with 0.1 N HCl and treated as above for conversion to (1R)-[1-^{3}H]fructose-6-P. [2-^{3}H]Glucosamine-6-P was prepared by incubation in tritiated water of fructose-6-P, NH_4Cl, and glucosamine-P isomerase. The acidified solution was placed on a column of Dowex 50 (H^+), eluted with water, and maintained as it was eluted at pH 4.

N-Acetylglucosamine-6-P was prepared as described by Leloir and Cardini (1962).

Materials obtained from commercial sources were: yeast hexokinase, yeast phosphoglucose isomerase, glucose-6-P dehydrogenase, creatine kinase potato acid phosphatase (Grade I) (Boehringer-Mannheim); NADH, NADP, and ATP (P-L Biochemicals); fructose-6-P, glucosamine-6-P, glucosamine hydrochloride, phosphocreatine, muscle aldolase, phosphofructokinase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase (Sigma); and ^3H_2O (1 Ci/mL), [1-^{3}H]glucose, and [U-^{14}C]fructose (New England Nuclear).

TABLE I: Requirements for Tritium Exchange into Water from (1*R*)-[1-^{3}H]Fructose-6-P by Glucosamine-6-P Isomerase.

Additions[a]	Exchange into H_2O (cpm) (80 000 cpm total) 2 h	15 h
None	2 200	3 440
Glucosamine-6-P isomerase (0.05 unit)	2 600	3 200
Glucosamine-6-P isomerase (0.05 unit) + NH_4Cl (0.15 M)	28 500	47 600
Glucosamine-6-P isomerase (0.05 unit) + CH_3NH_3Cl (0.15 M)	1 600	2 100
Glucose-6-P isomerase (1.0 unit)	60 000	70 000

[a] Reaction mixtures contained 100 mM Tris-HCl buffer, pH 7.8, 2 mM (1*R*)-[1-^{3}H]fructose-6-P (80 000 cpm/μmol labeled with ^{3}HOH and glucose-6-P isomerase). Aliquots (0.5 mL) were removed at the indicated times after incubation at 25 °C and the H_2O was separated by lyophilization in a closed system.

Results and Discussion

Hydrogen Exchange: Requirements and Stereospecificity. The *E. coli* glucosamine-6-P isomerase catalyzes an efficient tritium exchange between (1*R*)-[1-^{3}H]fructose-6-P and water. Table I shows some of the properties of this exchange which could be used as an alternate assay method for the enzyme. It requires the addition of NH_4Cl, confirming that the preparation is free of contaminating glucose-6-P isomerase and more importantly, demonstrating that the enzyme does not enolize fructose-6-P before the addition of ammonia. It would appear that the C-1 tritium is removed from the 2-imino or 2-carbinolamine derivative, as suggested in Scheme I, though the effect of ammonia could be merely to activate the enzyme. Methylamine does not substitute for ammonia in the tritium exchange implying that, if ammonia only functions as an activator, its simplest analogue is without effect.

When fructose-6-P and NH_4Cl were incubated in tritiated water with an excess of the glucosamine-6-P isomerase and the fructose-6-P was reisolated, it was found to contain 0.8–0.9 equiv of tritium in a stable position. The almost stoichiometric incorporation of a solvent proton suggests that glucosamine-6-P isomerase is specific for removal and exchange of only one of the two C-1 hydrogens of fructose-6-P. As indicated in Table II, approximately 99% of this label was reexchanged into the water by incubation with the stereospecific glucose-6-P isomerase. In a complementary experiment, [1-^{3}H]glucose was phosphorylated with hexokinase and isomerized to glucosamine-6-P by the combined action of glucose-6-P isomerase and excess glucosamine-6-P isomerase in unlabeled medium. The isolated, tritiated glucosamine-6-P had almost the same specific activity as the starting [1-^{3}H]glucose (7800 cpm/μmol vs. 8100 cpm/μmol); i.e., the conversion occurred without measurable exchange of tritium into the water. The location of the tritium in glucosamine-6-P at C-1 was shown by oxidizing it with $NADP^+$ and glucose-6-P dehydrogenase to 2-amino-2-deoxygluconate-6-P. When the latter was isolated (Table IIB) it contained no detectable tritium counts.[2] The location of the tritium at C-1 of the glucosamine-6-P was substantiated by conversion to [^{3}H]NADPH by combined action of glucosamine-P isomerase, glucose-6-P isomerase, and glucose-6-P dehydrogenase. The above experiments taken

TABLE II: Stereochemistry of Glucosamine-6-P Isomerases for Hydrogen Removal at C_1 of Fructose-6-P.

	cpm/μmol	Total cpm
A. Position of Exchange Incorp. of ^{3}H from ^{3}HOH into Fructose-6-P (NH_4^+ present)[a]		
Fructose-6-P $\xrightarrow[\text{Glucosamine-6-P Isomerase}]{^3\text{HOH } (NH_4Cl)}$ [1-^{3}H]Fructose-6-P (I)		
Solvent protons	82 000	4.5×10^{10}
Isolated [1-^{3}H]fructose-6-P (I)	70 300	360 000
Anal. of I:[b] $H^+ + I \xrightarrow[\text{Glucose-6-P Isomerase}]{} \text{Glucose-6-P} + H^+$		
Starting: fructose-6-P (I)	70 300	85 000
Isomerase products		
Fructose-6-P + glucose-6-P	800	1 060
Solvent protons	0.8	84 000
B. Reaction with (1*R*)-[1-^{3}H]Fructose-6-P[c]		
[1-^{3}H]Glucose-6-P $\xrightarrow[\text{Glucose-6-P Isomerase}]{H_2O}$ (1*R*)-[1-^{3}H]Fructose-6-P $\xrightarrow[\text{Glucosamine-P Isomerase}]{(NH_4Cl)}$ glucosamine-6-P (II)		
[1-^{3}H]Glucose[c]	8 100	1 510 000
Isolated glucosamine-6-P (II)	7 800	460 000
Solvent protons		<10 000
Anal. of II:[d] II + $NADP^+$ $\xrightarrow[\text{Glucose-6-P Dehydrogenase}]{}$ 2-Amino-2-deoxygluconate-6-P + NADPH		
Starting: [1-^{3}H]glucosamine-6-P (II)[e]	7 560	81 000
Dehydrogenase products		
NADPH	610[f]	3 100
2-Amino-gluconate-6-P	20	100
Glucosamine-6-P (unreacted)	13 500	70 100

[a] The reaction contained in a total volume of 4 mL: fructose-6-P (10 mM), NH_4Cl (200 mM), Tris-HCl, pH 7.8 (50 mM), glucosamine-6-P isomerase (1.25 units/mL), and tritiated water. After incubation for 2 h at 25 °C, fructose-6-P (I) was isolated. [b] The reaction mixture contained in 1 mL: fructose-6-P (I) (1.1 μmol), Tris-HCl, pH 7 (20 μmol), and glucose-6-P isomerase (Boehringer, 6 units). Incubation was for 10 h at 25 °C. The sample was lyophilized in a closed system to separate the water from the nonvolatile counts. [c] Reaction mixture contained in a total volume of 10 mL: [1-^{3}H]glucose (20 mM), ATP (2 mM), $MgCl_2$ (5 mM), phosphocreatine (25 mM), NH_4Cl (100 mM), Tris-HCl, pH 7.5 (50 mM), hexokinase (2 units/mL), glucose-6-P isomerase (2 units/mL), creatine kinase (5 units/mL), and glucosamine-6-P isomerase (4 units/mL). Incubation was for 5 h at 25 °C followed by isolation of glucosamine-6-P (II) on a Dowex 50 (H^+) column. [d] The reaction mixture contained in 10 mL: [1-^{3}H]glucosamine-6-P (II) (1 mM), $NADP^+$ (2 mM), Tris-HCl, pH 7.5 (20 mM), and glucose-6-P dehydrogenase (5 units/mL). Incubation was continued until 50% reaction (3 h). The mixture was then diluted to 50 mL and applied to DEAE-cellulose column at pH 7.5 for isolation of NADPH, 2 amino-gluconate-6-P, and unreacted glucosamine-6-P. [e] The tritium acts as if at C-1 in the coupled sequence: glucosamine-P isomerase, glucose-6-P isomerase, and glucose-6-P dehydrogenase, which resulted in transfer of all the tritium into NADPH. [f] The observed specific activity is $^1/_{12}$ that of the starting glucosamine-6-P because of a large tritium isotope effect on the V_{max}/K_m ratio. The calculated $(V^H/K^H)/(V^T/K^T)$ is 17-fold after correction for the increase in tritium labeling in the unreacted glucosamine-6-P pool (Collins and Lietzke, 1959).

[2] The difference in labeling of [^{3}H]NADPH in Table IIB by glucosamine-6-P (610 cpm/μmol) and by glucose-6-P (7320 cpm/μmol) is due to the fact that the former reduction went only part way to completion while the latter went 100% to completion. The calculated value for k_H/k_T = 17 (after correction for 50% reaction) for the very poor substrate glucosamine-6-P is close to the theoretical maximum V_{max}/K_m isotope effect calculated from zero-point energies and is rarely found for dehydrogenase reactions (Klinman, personal communication).

TABLE III: Intramolecular Tritium Transfer

$$[2\text{-}^3H]\text{glucosamine-6-P} \rightarrow {}^3H_2O + NH_4^+ + \text{fructose-6-P} \xrightarrow{ATP} \text{fructose-1,6-P}_2{}^a$$

	Start		33% Reaction		90% Reaction		100% Reaction	
	cpm/μmol	cpm total	cpm/μmol	cpm total	cpm/μmol	cpm total	cpm/μmol	cpm total
[2-^{3}H]Glucosamine-6-P	119 000	1 750 000	117 000	1 130 000	54 200	80 000		1 000
^3H_2O	0.1	10 100	0.4	620 000	1.1	1 680 000	1.1	1 750 000
[1-^{3}H]fructose-1,6-P_2		200	950	4 500	850	14 200	790	15 000

[a] Reaction mixture contained, in 1.4 mL, Tris-HCl, pH 8.2 (100 μmol), [2-^{3}H]glucosamine-6-P (15 μmol, 120 000 cpm/μmol), *N*-acetylglucosamine-6-P (0.5 μmol), ATP (20 μmol), $MgCl_2$ (25 μmol), glucosamine-6-P isomerase (1.5 units), and fructose-6-P kinase (6 units). Reaction at 25 °C was initiated by the addition of the isomerase. Aliquots (0.25 mL) were removed at 0, 25, 70, and 100 min and the glucosamine-6-P, fructose-1,6-P_2, and H_2O isolated.

together show that the glucosamine-6-P isomerase and glucose-6-P isomerase are specific for removal of the same prochiral C-1 hydrogen of fructose-6-P. Given the specificity of the latter for the 1-*pro-R* hydrogen (Rose and O'Connell, 1960), it is concluded that the glucosamine-6-P isomerase is also specific for removal of this hydrogen in proton exchange with water and possibly in transfer to C-2 of the product glucosamine-6-P.

The extent of nonstereospecificity of labeling of glucosamine-6-P by the isomerase in tritiated water is judged from the above experiments to be less than 1%. The data to be discussed below (cf. Table III) allow an even lower limit to be set (0.1%).

Hydrogen Exchange vs. Hydrogen Transfer. The stereochemistry of the reaction at C-1 of fructose-6-P relative to the *R* stereochemistry at C-2 of the glucosamine-6-P and the property of proton exchange with water follow the pattern established with a number of enzymes in the isomerase class (Rose, 1975); that is, proton removal and readdition to the vicinal carbons must occur either from the same face of a *cis*-enolamine intermediate or from opposite faces of a *trans*-enolamine. Evidence for *cis*-enediol intermediates in other isomerases has been obtained by the demonstration of intramolecular proton transfer between vicinal carbons which makes the antarafacial approach seem less acceptable. In some cases, notably glucose-6-P isomerase (Rose and O'Connell, 1961), ribose-5-P isomerase (McDonough and Wood, 1961), and three pentose isomerases (Rose et al., 1967), the level of transfer is very high, suggesting mediation by a single nonprotonic base or an efficient shuttle between nonprotonic bases at the active site.

To examine the possibility of intramolecular proton transfer in the present case, [2-^{3}H]glucosamine-6-P was prepared by incubation of glucosamine-6-P and ammonia in tritiated water with excess isomerase. The isolated [2-^{3}H]glucosamine-6-P (120 000 cpm/μmol) was then treated with glucosamine-6-p isomerase, in the absence of NH_4^+ (<1 mM), with an excess of fructose-6-P kinase and ATP present to phosphorylate fructose-6-P as soon as it was formed. As shown in Table III under these irreversible conditions, the isomerase liberated most of the counts (99.3%) into water. However, the isolated fructose-1,6-P_2 contained tritium, at a specific activity much greater than that of the water, although only 0.7% of that of the [2-^{3}H]glucosamine-6-P. To establish that the tritium was truly in the fructose-1,6-P_2 and to specify its location, the compound was hydrolyzed to fructose, then rephosphorylated to fructose-6-P with hexokinase and ATP. The fructose-6-P, after limited treatment with glucose-6-P isomerase (6 units for 2 h) gave up most of its tritium to water. Since only 1–2% of the counts would have been liberated nonenzymatically under these conditions (see Table I), it is concluded that at least 4/5 of the tritium was present in the 1-*pro-R* position of fructose-6-P and that approximately (4/5) × 0.7 = 0.6% intramolecular transfer of tritium occurred. Although the level of tritium transfer is quite low, the result nevertheless suggests that proton addition to the vicinal carbon is suprafacial across a *cis*-enolamine.

Anomer Specificity of the Glucosamine-6-P Isomerase. Although aldose–ketose isomerases are generally thought to catalyze enolization of their substrates while they are in their open-chain carbonyl forms—analogous to the triosephosphate isomerase (Reynolds et al., 1971; Trentham et al., 1969)—activity toward a closed ring form of the aldose substrate has been demonstrated in four cases (Salas et al., 1965; Schray et al., 1973). For these four, ring-opening steps have been added to the overall mechanism with substrate and product. It is not surprising that, with the notable exception of glucose-6-P isomerase which has a mutarotase activity toward its substrates (Salas et al., 1965; Wurster and Hess, 1973; Schray et al., 1973), the ring-opening step shows specificity for only one of the anomers of the aldose substrate. In each of the cases examined, the active anomeric form of the aldose is the one which, in the predominant chair ($^4C_1$) conformation, has a cis orientation of the glycosyl and C_2 hydroxyls. This is the α-anomer which would lead directly to a *cis*-enediol intermediate without C_1–C_2 bond rotation. Thus, the evidence has suggested either that a "minimal motion," rigid complex exists between the enzyme and the open-chain carbonyl form or that the same catalytic residue functions as a base in removing the C_1–OH proton for ring opening and as an electrophile in polarizing the C_1–carbonyl. If this pattern applies to glucosamine-6-P isomerase, activity toward α-glucosamine-6-P would be consistent with a *cis*-enolamine intermediate, while activity toward β-glucosamine-6-P would be consistent with a *trans*-enolamine intermediate.

An investigation of the active substrate form of glucosamine-6-P was undertaken using an approach similar to that of Salas et al. (1965) in their study of the anomeric specificity of glucose-6-P dehydrogenase. The purified anomeric forms of glucosamine were added to a coupled reaction system containing yeast hexokinase, ATP, glucosamine-6-P isomerase, and a saturating concentration of *N*-acetylglucosamine (0.2 mM) to lower the K_m for glucosamine-6-P (~0.05 mM at 0 °C, pH 7.8). Large amounts of hexokinase, a low temperature, and a short incubation time (2 s) were used because of the expected rapid mutarotation rate of glucosamine-6-P at pH

TABLE IV: Anomer Specificity of Glucosamine-6-P Isomerase.[a]

(α or β)-glucosamine $\xrightarrow[\text{hexokinase}]{\text{ATP}}$ (α or β)-glucosamine-6-P $\xrightarrow[\text{isomerase}]{}$ fructose-6-P + NH_4

Glucosamine Anomer	Isomerase Act. (μmol min^{-1} mL^{-1} at 0 °C)	Concn Found at 2 s	
		Glucosamine-6-P[b] (μM)	Fructose-6-P (μM)
α	1.9	36	17
	3.8	23	36
	7.6	10	51
β	1.9	70	6
	3.8	49	7
	7.6	53	8

[a] THE]/5-ML incubation mixture at 0 °C contained: ATP (1 mM), $MgCl_2$ (2.5 mM), Tris-HCl, pH 8.0 (50 mM), *N*-acetylglucosamine-6-P (0.2 mM), α- or β-glucosamine (0.2 mM, added last), yeast hexokinase, and the noted amount of glucosamine-6-P isomerase (assayed at 0 °C with an equilibrated solution of glucosamine-6-P). Hexokinase was 56 units/mL for experiments with α-glucosamine and 730 units/mL for experiments with β-glucosamine (see Materials and Methods). ADP and fructose-6-P were determined (by the method of Lowry and Passonneau (1973) as described in the Materials and Methods section) in all the incubations, which were quenched at 2 s. [b] Calculated from ADP minus the fructose-6-P of the terminated incubation.

7.8 (see Materials and Methods section). As shown in Table IV, increasing concentrations of the isomerase converted the α-glucosamine-6-P formed in the hexokinase reaction to fructose-6-P in approximate proportion to the isomerase added, although the glucosamine-6-P present at termination time decreased progressively. On the other hand, β-glucosamine-6-P was isomerized only to a limited extent that was independent of isomerase concentration. The results suggest that the conversion of β-glucosamine-6-P to fructose-6-P is not catalyzed by the isomerase but that the formation of fructose-6-P in this case may reflect the amount of α-glucosamine-6-P formed by nonenzymatic anomerization. Thus, the anomeric specificity for the α anomer is consistent with a *cis*-enolamine intermediate.

Effect of Borohydride. Evidence for participation of modified amino acid residues in certain deaminase reactions has been described. Reduction of a dehydroalanine residue by borohydride in the phenylalanine (Hanson and Havir, 1970) and histidine ammonia lyases (Wickner, 1969; Givot et al., 1969) is stoichiometric with loss in activity, as in reduction of an $-NH_2$ terminal pyruvyl residue in certain transaminases (Snell and DeMari, 1970). Glucosamine-6-P isomerase was incubated either with sodium borohydride (10 mM, pH 7) alone or in combination with one or more substrates. Apparent inactivation of the isomerase was observed when NH_4Cl and fructose-6-P were both present or when glucosamine-6-P alone was present but not with enzyme alone, reflecting the reduction of a Schiff's base compound of enzyme with substrate. However, in these cases complete enzyme activity could be recovered after passage of the enzyme through a Sephadex G-25 column. Further investigation showed that a potent inhibitor was formed when borohydride was incubated with enzyme, fructose-6-P, and NH_4Cl or, in the absence of enzyme, with glucosamine-6-P alone. This inhibitor, purified on Dowex 50 (H^+) is tentatively identified as 2-amino-2-deoxyglucitol-6-P. When tested in the presence of 0.2 mM *N*-acetylglucosamine-6-P, the inhibition was linear competitive, $K_i = 2 \times 10^{-7}$ M, using glucosamine-6-P as the variable substrate, $K_m = 4 \times 10^{-4}$ M. The failure of borohydride reduction to irreversibly inactivate the isomerase would appear to rule out both the existence of modified amino acids at the active site and the formation of a Schiff's base between the enzyme and fructose-6-P.

2-Amino-2-deoxyglucitol-6-P is an analogue of the straight-chain form, *aldehydo*-glucosamine-6-P. Strong binding of the isomerase with the straight chain form is to be expected for an enolization mechanism since the reducing form of aldohexoses is a very minor component in solution (Los et al., 1956) and arguments of catalytic efficiency predict that this equilibrium would be shifted toward the reactive component at the active site. The failure to observe a strong inhibition by straight-chain polyol analogues in other aldose-ketose isomerases (see Rose, 1975, for references) is puzzling in light of the present results.

^3H_2O Partitioning into Glucosamine-6-P and Fructose-6-P. The finding of only 0.6% intramolecular tritium transfer from the C_2–H of glucosamine-6-P to the C_1–H of fructose-6-P indicates that almost all the 1-*pro-R* hydrogen is derived from solvent. At a maximum isotope effect, $k_H/k_T = 20$, the greatest amount of ^1H transfer could have been only 10%. Thus, when solvent is labeled with tritium the presumed enzyme-enolamine intermediate will incorporate almost 1 equiv of tritium into the exchanging group as it partitions toward product or back toward substrate. The tritium partition ratio will yield information about the reversibility and relative rates of various steps in the reaction mechanism (Rose, 1962; Knowles et al., 1971). Of course, 1 equiv of tritium will be incorporated only if there is no kinetic isotope discrimination between $^1H^+$ and $^3H^+$ in the transfers to C-1 or C-2. Table V shows the results of such ^3H_2O partitioning experiments carried out in both the glucosamine-6-P → fructose-6-P + NH_3 and fructose-6-P + NH_3 → glucosamine-6-P directions. In each case, the substrate and product were examined after early times (before 5% of equilibrium had been reached) to minimize their recycling by the enzyme. In the first case, an apparent 3.3-fold discrimination against tritium from water was found in the initially formed glucosamine-6-P. This would be diminished only slightly to 3-fold if as much as a 10% direct intramolecular transfer of hydrogen occurs in the formation of fructose-6-P. Since isotope effects in the range $k_H/k_T = 8$ (Knowles et al., 1971), 20 (Rose et al., 1965), and 15 (Robinson and Rose, 1972) have been found for other enzyme-catalyzed enolization reactions, the low isotope effect of 3.3 in the present case can best be explained if the enzyme-product complex that undergoes enolization is slow to dissociate, allowing multiple enolizations to occur in each cycle of product formation. Where equilibrium between the conjugate acid proton in the enzyme-enolamine and the medium is complete, the fraction of E·product that dissociates is given by $(m - 1)/(n - 1)$, where m and n are the measured and intrinsic isotope effects.[3] For values of n between 8 and 15 and $m = 3.3$, product release is three- to sixfold slower than enolization. The

[3] For

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow{k_2} C$$

where $n = k_1^H/k_1^T = k_{-1}^H/k_{-1}^T$ and $m = [k_1^H k_2/(k_{-1}^H + k_2)][(k_{-1}^T + k_2)/k_1^T k_2]$, and $P = k_2/(k_{-1} + k_2)$ defines the portion of B that goes to C. Substitution of P into the equation for m gives the relation $P = (m - 1)/(n - 1)$.

TABLE V: [^{3}H]H_2O Isotope Partition and Discrimination.

[NH_4Cl] (mM)	Substrate cpm	Substrate cpm/μmol	Product cpm	Product cpm/μmol	Isotope Effect (^{1}H)/(^{3}H)	^{3}H Partition [^{3}H]Glucosamine-6-P / [^{3}H]Fructose-6-P
			Glucosamine-6-P → Fructose-6-P + NH_4^+ [a]			
			^3H_2O = 10 000 cpm/μatom of H^+			
	(Glucosamine-6-P)		(Fructose-6-P)			
0	6 500	490	3 460	3 260	3.3	1.9
	12 900	1010	6 650	3 390	3.4	1.9
	18 400	1460	9 050	3 230	3.2	2.0
	36 200	3810	30 100	4 790		
			Glucosamine-6-P → NH_4^+ + Fructose-6-P $\xrightarrow{ATP}$ Fructose-1,6-P_2 [b]			
			^3H_2O = 53 000 cpm/μatom of H^+			
	(Glucosamine-6-P)		(Fructose-6-P)			
0	70 700	1910	38 500	15 800	3.3	1.8
50	88 100	2380	37 000	21 000	2.5	2.4
200	130 600	3530	44 200	25 700	2.1	3.0
			Fructose-6-P + NH_4^+ → Glucosamine-6-P [c]			
			^3H_2O = 32 000 cpm/μatom of H^+			
	(Fructose-6-P		(Glucosamine-6-P)			
40	1 860	95	6 060	11 300	3.0	3.3
200	2 310	119	7 340	9 050	3.6	3.2
400	1 420	73	6 150	9 040	3.3	4.3

[a] The reaction mixture at 25 °C contained in 3.5 mL: glucosamine-6-P (15 mM), Tris-HCl, pH 8.5 (50 mM), and glucosamine-6-P isomerase (0.1 unit/mL). Aliquots (0.8 mL) were removed at 10, 20, 30, and 100 min, quenched with 0.1 mL of 2 N $HClO_4$, and assayed for fructose-6-P and glucosamine-6-P. Fructose-6-P was isolated on Dowex 1 (Cl^-), glucosamine-6-P on Dowex 50 (H^+). [b] The reaction mixtures contained in 2 mL: glucosamine-6-P (20 mM), Tris-HCl, pH 8.5 (50 mM), ATP (2 mM), $MgCl_2$ (5 mM), fructose-6-P kinase (2 units/mL), glucosamine-6-P isomerase (0.15 unit/mL), and the indicated concentration of NH_4Cl. The reactions were stopped after 10 min (no NH_4Cl), 13 min (50 mM NH_4Cl), and 22 min (200 mM NH_4Cl) by addition of 0.2 mL of 2 N $HClO_4$. [c] The reaction mixtures contained in 1 mL: Tris-HCl, pH 8.5 (50 mM), fructose-6-P (20 mM), glucosamine-6-P isomerase (0.06 unit/mL), and the indicated concentrations of NH_4Cl. The reactions were stopped with 0.1 mL of 2 N $HClO_4$ after 20 min at 25 °C.

limiting case of $m \sim 1$ has been reported for the labeling of glyceraldehyde-3-P by triosephosphate isomerase in tritiated water (Knowles et al., 1971).

The labeling of substrate and product pools by glucosamine-6-P isomerase favors glucosamine-6-P over fructose-6-P by a factor of 1.8–2.0. As shown in Table V, this factor was increased when NH_3 was present. The parallel decrease in tritium partition and isotope discrimination is consistent with the interpretation that NH_3 acts by delaying the irreversible dissociation of fructose-6-P.

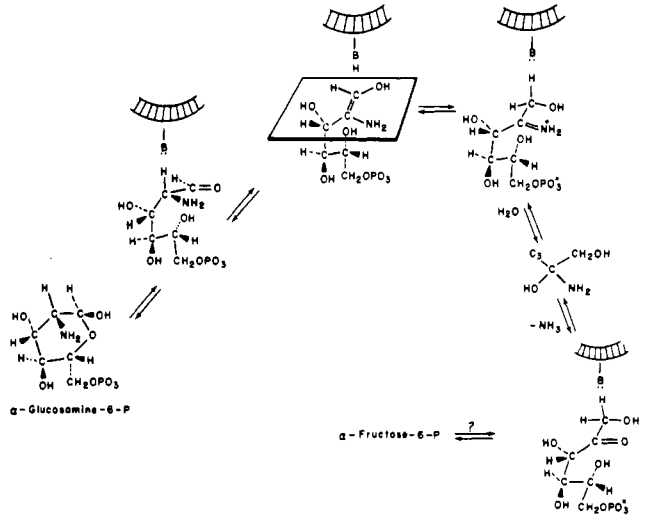
When the formation of glucosamine-6-P was measured in tritiated water, a similar apparent isotope effect of 3.3 was observed. Although precise measurements of intramolecular tritium transfer have not been made in this direction due to difficulties in maintaining [1-^{3}H]fructose-6-P in chiral purity, it can be stated that the extent of direct tritium and therefore hydrogen transfer will be small. Therefore the measured isotope discrimination will reflect the incorporation of medium protons into ≥90% of the product, and again product release must be slower than enolization.

The partition of tritium between glucosamine-6-P, as product, and fructose-6-P, as substrate, is at least as great as when the same partition is examined in the reverse direction at similar concentrations of NH_3. This result indicates that only one kinetically significant intermediate in the reaction pathway is capable of exchange with the medium. This is suggestive of the simple symmetrical mechanism previously proposed for glucose-6-P isomerase (Rose, 1962).

Concluding Remarks

Although the requirement of NH_4^+ for enolization of fructose-6-P could have indirect causes, it seems likely that the real species that enolizes is the imine as shown in Scheme II.

Scheme II



With the exception of the extra steps following ring opening that are required for formation and hydrolysis of the imine, the mechanism of glucosamine-P isomerase is shown to have the stereochemical and anomeric specificity properties of the other

(2*R*)-aldose isomerases including intramolecular proton migration from the 1-re face of an enol. These results add another example, the seventh, to the generalization that these reactions are based on a *cis*-endiol plan for the intermediate and a *single* base, *single* electrophile participation by the enzyme.

As already noted, the anomeric specificity of the isomerase agrees with the generalization found with all the isomerases studied in that the C_5 hydroxyl group closes the ring from the face of the enol plane opposite to that from which the proton is added. Glucosamine-P isomerase is unique in that, during the course of the overall reaction three, and possibly four, different groups must be able to attack the C_2 carbon at different stages: H^+, H_2N:, H_2O:, and possibly C_5–O– if a cyclic form of fructose-6-P proves to be a substrate form. This factor alone suggests a remarkable versatility in the functional groups surrounding the C-2 region in the active site. Concerning the proton-abstracting base shown in Scheme II, it retains the proton poorly, 0.006 relative to the rate of product formation. Thus, proton exchange with the medium (k_x) is at least 160 times faster than the turnover rate of the enzyme, which, from a subunit weight of 30 000 and from the highest specific activity obtained of 100, is at least 50 s^{-1}. Proton exchange must be at least 8×10^3 s^{-1}. This implies a pK_a for the conjugate acid of 6.8, based on the assumption that protonation of a carboxylate base of the enzyme from the medium is diffusion limited at $\sim 5 \times 10^{10}$ M^{-1} s^{-1} (Eigen et al., 1964).

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