Phosphorylated Aminosugars: Synthesis, Properties, and Reactivity in Enzymatic Reactions†

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ABSTRACT: A number of phosphorylated aminosugars have been prepared and tested as substrates for metabolic reactions. 6-Aminoglucose is a slow substrate for yeast hexokinase with a V_{max} that is only 0.012% that for glucose. While V_{max} is pH independent, V/K decreases below the pK of 9.0 of the amino group. 6-Aminoglucose is a competitive inhibitor vs glucose with a K_i value increasing below the pK of 9 but leveling off at 33 mM below pH 7.16. Thus, protonation decreases binding affinity by 2.4 kcal/mol and only the neutral amine is catalytically competent. 6-Aminoglucose-6-P was synthesized enzymatically with hexokinase. Its pK's determined by ^{31}P NMR were 2.46 and 8.02 (α anomer) and 2.34 and 7.85 (β anomer), with a β:α ratio of 3.0. It is most stable at pH 12 (half-life 228 h at 22 °C), while as a monoanion its half-life is 3 h. The free energy of hydrolysis at 25 °C and pH 9.25 is −10.3 kcal/mol. The phosphorylated amino analogues of 6-P-gluconate, ribulose-5-P, fructose-6-P, fructose-1,6-bis-P (amino group at C-6 only), and glyceraldehyde-3-P were synthesized enzymatically. The ^{31}P NMR chemical shifts of these analogues are 8–8.5 ppm at pH 9.5. Their relative stability is 6-aminogluconate-6-P > 3-aminoglyceraldehyde-3-P > 6-aminoglucose-6-P > 6-aminofructose-1,6-bis-P ≃ 6-aminofructose-6-P > 5-aminoribulose-5-P. These analogues were tested as substrates for their respective enzymes. Phosphoglucomutase and alkaline phosphatase were tolerant of the O to N substitution, acting on 6-aminoglucose-6-P with V_{max} values 9 and 92% those for glucose-1-P and glucose-6-P. Of enzymes that do not transfer the phosphoryl group, phosphoglucoisomerase is least tolerant of the O to N change (V_{max} 17% that for glucose-6-P). Phosphorylated amino analogues were good substrates for phosphofructokinase, aldolase, and glucose-6-P and 6-P-gluconate dehydrogenases. Phosphorylated aminosugars are thus excellent isosteric analogues of normal metabolic intermediates, except for reactions catalyzed by kinases.

The phosphate monoester group is ubiquitous in nature and serves many functions, one of which is to provide a convenient handle by which enzymes can hold onto their substrates (Westheimer, 1987). These interactions can be probed by making slight alterations to the substrate and quantifying the effects of these changes with kinetic studies. This has been done with phosphorylated thiol analogues in the preceding paper in this issue, and results from studies with phosphorylated amino analogues will be presented in this paper. Knight and Cleland (1989) have reported a similar study of amino and thiol analogues as substrates for glycerokinase.

The reactions of phosphorylated amines are of interest for several reasons. First, since they are nearly isosteric with phosphate monoesters, they serve as a sensitive probe of enzyme interactions with the phosphate moiety and especially with the bridging heteroatom. The change in geometry in going from a bridging oxygen to a bridging NH group is modest, since the C–N and C–O bonds measure 1.47 and 1.43 Å (Huheey, 1983) while the P–N and P–O bond lengths are 1.77 and 1.57 Å (Corbridge, 1966). Although hydrogen-bonding capabilities to the phosphate nonbridge oxygen atoms are maintained, the bridging heteroatom has been altered so that rather than having two lone pairs that can accept hydrogen bonds, a nitrogen has only one lone pair, while the proton could possibly act as a hydrogen-bond donor. If the bridging nitrogen is protonated (Benkovic, 1971), however, it will have a positive charge and act only as a hydrogen-bond donor. Phosphorylated amines are closer analogues of phosphate monoesters than are phosphorylated thiols in terms of both bond lengths and hydrogen-bonding capabilities and should therefore produce more subtle effects on kinetic parameters.

A second reason for the study of phosphorylated amines is that the ability of phosphoryl-transferring enzymes to catalyze cleavage of the P–N bond may provide a better understanding of the transition states for these reactions. In the present work we will report the synthesis and chemical characterization of various phosphorylated amino analogues of phosphate-containing metabolites and the kinetics of their reactions with metabolic enzymes.

Materials and Methods

Nucleotides were from Boehringer. Glucose-6-P dehydrogenase from *Leuconostoc mesenteroides*, glutathione reductase from torula yeast, and pyruvate kinase and lactate and α-glycerophosphate dehydrogenases from rabbit muscle were from Sigma. Phosphoglucoisomerase from yeast, alkaline phosphatase from calf intestine, and phosphofructokinase, triose-P isomerase, phosphoglucomutase and glyceraldehyde-3-P dehydrogenase from rabbit muscle were from Boehinger. 1,2-*O*-Isopropylidene-D-glucofuranose, sodium azide, *p*-toluenesulfonyl chloride, ninhydrin, and solvents were from Aldrich. The following buffers were used in kinetic studies at or near the specified pH: trichloroacetic acid (pH < 1),

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$KHSO_4$ (pH 1.3), citrate (pH 3.2–5.1), Mes[1] (pH 6.0), Mops (pH 7.0), Hepes (pH 7.4), Taps (pH 8.0), Ches (pH 9.0), Caps (pH 9.8 and 10.7), and K_2HPO_4 (pH 11.8). No buffer was used above pH 13.

NMR. ^{31}P, ^{13}C and proton NMR spectra were obtained on Bruker AM500 or AM400 spectrometers. ^{13}C and proton chemical shifts were referenced to external TMS, while ^{31}P chemical shifts were referenced to 200 mM D_3PO_4 in D_2O.

Synthesis of 6-Aminoglucose. The synthesis was modified from that of Cramer (1962). 1,2-*O*-Isopropylidene-D-glucofuranose (0.9 M solution in dry pyridine) was tosylated at C-6 by adding dropwise at 5 °C an equal volume of 0.9 M *p*-toluenesulfonyl chloride in dry pyridine and letting the solution stir for 12 h. The solution was then quenched with water and extracted several times with chloroform. The chloroform was removed in vacuo to give solid 6-tosyl-1,2-*O*-isopropylidene-D-glucofuranose in 43% yield. Proton NMR ($CDCl_3$): δ 7.8 (d, J = 6.6 Hz), 7.4 (d, J = 6.6 Hz), 5.9 (s), 2.5 (s), 1.5 (s), 1.3 (s), 4–4.5 (m's).

The tosylate was then displaced with azide by refluxing a solution of 0.2 M 6-tosyl-1,2-*O*-isopropylidene-D-glucofuranose and 1.4 M sodium azide in 60% acetone for 72 h. Reaction products were then extracted into chloroform several times. Thin-layer chromatography on silica gel, using 3:1 chloroform ethyl acetate, indicated one major species containing sugar (sulfuric acid charring) with an R_f of 0.22 and a minor byproduct with an R_f of 0.60. The major product was purified on a 4 × 25 cm silica gel column with use of this solvent system. Fractions were assayed with TLC, pooled, and concentrated in vacuo to remove solvent to give 6-azido-1,2-*O*-isopropylidene-D-glucofuranose in 54% yield. Proton NMR ($CDCl_3$): δ 6.1 (s), 4.7 (s), 1.5 (s), 1.3 (s), 3.0–4.0 (m's).

The azido group was reduced by stirring 0.2 M 6-azido-1,2-*O*-isopropylidene-D-glucofuranose and 0.6 M dithiothreitol for 24 h at pH 8.5 under N_2. The amine product was purified on a 2.5 × 30 cm column of Dowex-50-X2-H^+, by washing first with water and then eluting the product with 0.5 M ammonia. Fractions were assayed by spotting on silica gel TLC plates, removing ammonia with a heat gun and spraying with a fluorescamine solution (Dawson et al., 1986). Fractions containing amine were pooled and concentrated, and a 40 mg/mL solution in 0.1 N sulfuric acid was heated at 60 °C for 17 h and then neutralized with $Ba(OH)_2$. The solution was filtered free of $BaSO_4$ with a Millipore filter, and the product was purified on a Dowex-50 column as above. Fractions containing the aminosugar were pooled and lyophilized after removal of ammonia in vacuo to give 6-aminoglucose in 76% yield. Proton NMR ($CDCl_3$): δ 5.2 (d, J = 4.0), 4.6 (d, J = 8.0), 3–3.6 (m's). ^{13}C NMR (D_2O): δ 95.9, 92.1, 75.6, 74.0, 72.4, 71.4, 67.7, 40.4.

Enzymatic Synthesis of Phosphorylated Aminosugars. 6-Aminoglucose-6-P was synthesized in a reaction mixture containing 100 mM PEP, 10 mM ATP, 12 mM $MgCl_2$, 20 mM 6-aminoglucose, 50 mM Ches, pH 9, 25 units/mL pyruvate kinase, and 1500 units/mL yeast hexokinase. After 7 h at 22 °C, the solution was filtered through an Amicon PM30 membrane. The filter was washed to recover enzyme for reuse. Three or four such reaction sequences were carried out, increasing the reaction time by 2 h after each filtration step, until the hexokinase activity became too low. 6-Aminoglucose-6-P was purified on a 5 × 50 cm column of QAE Sephadex in the formate form. After the column was charged with the sample, it was washed with 10 mM ammonia and eluted with a linear gradient from 0 to 200 mM KCl in 10 mM ammonia. Fractions containing 6-aminoglucose-6-P were pooled, and 3 volumes of 10% $BaCl_2$ and 10 volumes of 95% ethanol were added to precipitate the product. The solution was centrifuged and the pellet washed twice with 80% ethanol. The precipitate was then stirred with potassium Chelex beads until it dissolved. This slurry was added to a potassium Chelex column and the eluent was concentrated to 20 mM. Proton NMR (D_2O): δ 5.42 (d, J = 3.7, 1 H), 4.82 (d, J = 7.2, 3 H), 3–4 (m's). ^{13}C NMR (H_2O): δ 97.4, 92.9, 75.7, 73.0, 71.4, 43.2. ^{31}P NMR (H_2O, pH 12.8): δ 9.12 (t, J = 7.8, integration = 1.0), 9.03 (t, J = 7.4, integration = 3.0).

Other phosphorylated amine analogues and phosphate monoesters were synthesized enzymatically from 6-aminoglucose-6-P and glucose-6-P, respectively, at pH 9 (50 or 100 mM Ches) and 22 °C. Unless otherwise specified, reactions were run for 15 min, and the mixtures were ultrafiltered through Centricon 10 membranes at 8000 rpm for 30 min at 5 °C to remove enzymes.

6-Aminogluconate-6-P was synthesized from 5 mM 6-aminoglucose-6-P with 10 units/mL glucose-6-P dehydrogenase/1 mM NADP/10 mM oxidized glutathione/4 units/mL glutathione reductase. 6-P-Gluconate was synthesized similarly from glucose-6-P. 5-Aminoribulose-5-P was synthesized from 5 mM 6-aminoglucose-6-P with 20 units/mL glucose-6-P dehydrogenase/15 units/mL 6-P-gluconate dehydrogenase/10 mM NADP/5 mM $MgCl_2$/0.5 mM EDTA. The enzymes were not removed by ultrafiltration because the product was unstable.

6-Aminofructose-6-P was synthesized from 6 mM 6-aminoglucose-6-P with 7 units/mL phosphoglucoisomerase, and fructose-6-P was prepared similarly from glucose-6-P. 6-Aminofructose-1,6-bis-P was synthesized from 1 mM 6-aminoglucose-6-P with 20 units/mL phosphoglucoisomerase/20 units/mL phosphofructokinase/2 mM ATP/3 mM $MgCl_2$. Enzyme ultrafiltration was done after 5 min of incubation and at pH 10 to minimize product decomposition. Fructose-1,6-bis-P was prepared similarly from glucose-6-P. 3-Aminoglyceraldehyde-3-P was synthesized from 2 mM 6-aminoglucose-6-P with 20 units/mL each phosphoglucoisomerase, phosphofructokinase, aldolase, and α-glycerophosphate dehydrogenase/2 mM ATP/3 mM $MgCl_2$/1.8 mM NADH.

Calibration of Stock Solutions. The concentration of 6-aminoglucose was determined with ninhydrin (Moore & Stein, 1948). The standard curve for this assay was generated with 2-aminoglucose whose concentration had been calibrated by phosphorylation by MgATP in the presence of hexokinase/pyruvate kinase/lactate dehydrogenase/NADH/phosphoenolpyruvate. Enzymatic endpoint assays at pH 9 and 22 °C were used for phosphate esters and their amino analogues. These assays were also used to determine half-lives at 22 °C for nonenzymatic decomposition of the phosphorylated amines. Assays for 6-aminoglucose-6-P and 6-aminogluconate-6-P employed either glucose-6-P or 6-P-gluconate dehydrogenase (10–17 units/mL) plus 5 mM nucleotide. The concentration of 5-aminoribulose-5-P was determined by ^{31}P NMR using the sum of the pyrophosphate resonances of NADP and NADPH as an internal reference. The concentration of 6-aminofructose-6-P was measured with a phosphoglucoisomerase, glucose-6-P dehydrogenase couple, while

[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonate; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonate; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Mops, 3-(*N*-morpholino)propanesulfonate; Caps, 3-(cyclohexylamino)-1-propanesulfonate; TMS, tetramethylsilane.

Table I: Kinetic Parameters for 6-Aminoglucose-6-P in the Hexokinase Reaction

pH	relative[a] V_{max}	relative[a] V/K	K_m (mM)	K_i[b] (mM)	K_m (glucose) (mM)
8.2	0.0094 ± 0.0007	0.00051 ± 0.00004	5.2 ± 0.7	4.7 ± 0.1	0.283 ± 0.009
9.0	0.012 ± 0.0004	0.0018 ± 0.0001	1.7 ± 0.2	1.65 ± 0.08	0.25 ± 0.01
10.0	0.012 ± 0.0004	0.0029 ± 0.0002	1.3 ± 0.1	2.4 ± 0.3	0.31 ± 0.04

[a] Value relative to 100 for glucose. [b] Value as competitive inhibitor vs glucose.

the assay for the bisphosphate employed 4 units/mL aldolase/8 units/mL glyceraldehyde-3-P dehydrogenase/2.4 mM NAD/10 mM arsenate. The concentration of 3-aminoglyceraldehyde-3-P was determined with glyceraldehyde-3-P dehydrogenase.

Kinetic Assays. Continuous assays were run in a 1-mL volume in 1-cm cuvettes by measuring absorbance changes at 340 nm from reduced nucleotides (ϵ_{340} = 6.22 mM^{-1} cm^{-1}). Assays were run at 25 °C unless otherwise specified. Fixed time assays were conducted by removing aliquots at various times from the reaction mixture and quenching by dilution into an endpoint assay solution.

Initial velocity studies with glucose or 6-aminoglucose as substrates for yeast hexokinase were carried out at pH values from 7.5 to 10. Reaction mixtures contained 100 mM buffer, enough KCl to give I = 0.05 M, 4 mM ATP, 6 mM $MgCl_2$, 2 mM NAD, 6 units/mL glucose-6-P dehydrogenase, and an appropriate level of hexokinase.

Data Processing. Data were fitted to appropriate equations by use of the least-squares method (Cleland, 1977). The titration curve for 6-aminoglucose was fitted to eq 1 where

$$Y = a + b/(1 + H/K) \quad (1)$$

Y is milliliters of base added, a and b are plateaus in the titration curve, and K is the acid dissociation constant of the amine. The ^{31}P NMR chemical shifts for 6-aminoglucose-6-P as a function of pH were fitted to eq 2, where Y is chemical

$$Y = \frac{[YL(H^2/(K_1K_2)) + YM(H/K_2) + YH]}{[H^2/(K_1K_2) + H/K_2 + 1]} \quad (2)$$

shift, YL, YM, and YH are chemical shifts of the neutral, monoanion, and dianion forms, and K_1 and K_2 are acid dissociation constants. Values for V_{max} and K_m were obtained by fitting initial velocities to eq 3. The V/K profile for the

$$v = V_{max}A/(K_m + A) \quad (3)$$

phosphorylation of 6-aminoglucose was fitted to eq 4 where

$$\log (V/K) = \log [V/K_o/(1 + H/K)] \quad (4)$$

V/K_o is the value for the unprotonated amine and K the apparent acid dissociation constant of the amine. K_i values for competitive inhibition of 6-aminoglucose vs glucose were obtained from fits of the kinetic data to eq 5. The pK_i profile

$$v = V_{max}A/[K_m(1 + I/K_i) + A] \quad (5)$$

for competitive inhibition by 6-aminoglucose was fitted to eq 6, where K_1 and K_2 are acid dissociation constants of the

$$pK = \log [K_{io}(1 + K_2/H)/(1 + H/K_1)] \quad (6)$$

enzyme-bound 6-aminoglucose and an enzymic group, respectively, after the known pK of free 6-aminoglucose was corrected for by adding log $(1 + H/10^{-9.0})$ to each pK_i value. The pH stability profile for 6-aminoglucose-6-P was fitted to eq 7 or 8. Eq 7 assumes specific base catalysis of decom-

$$\log k = \log [k_o(1 + H/K_1)/(1 + K_2/H) + c/H] \quad (7)$$

$$\log k = \log [k_o(1 + H/K_1)/(1 + K_2/H) + c/(1 + K_3/H)] \quad (8)$$

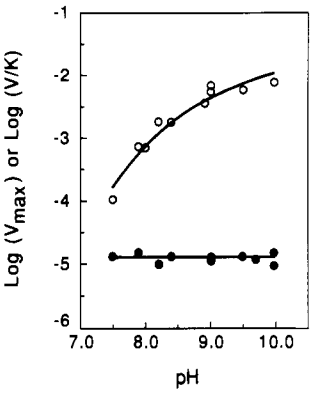


FIGURE 1: pH variation of log V_{max} (●) and log (V/K) (○) for 6-aminoglucose as a substrate for hexokinase with 4 mM MgATP. The V/K data are fitted to eq 4 with a pK value of 9.0 ± 0.2. The units of V_{max} and V/K are arbitrary.

position, while eq 8 assumes general base catalysis. In eqs 7 and 8, k is the rate constant for decomposition of 6-aminoglucose-6-P, k_o is the rate constant for the monoanion, K_1 and K_2 are acid dissociation constants of the compound, K_3 is the acid dissociation constant for the general base catalyst, and c is a constant.

Results

Synthesis of 6-Aminoglucose. 6-Aminoglucose was synthesized from 1,2-*O*-isopropylidene-D-glucofuranose in four steps with an overall yield of 18%. The tosylation, azide displacement, and acid-deblocking steps were slight modifications of those used by Cramer (1962, 1963), but the reduction of the azide was done under mild conditions with dithiothreitol (Staros et al., 1978) rather than by catalytic hydrogenation. In the proton NMR spectrum, the protons at C-1 resonate at 5.2 and 4.6 ppm for the α and β anomers, respectively. The ^{13}C resonances at 95.9 and 92.1 ppm are from C-1 of the β and α anomers (the corresponding resonances for glucose are 96.8 and 93.0 ppm). The O to N change has the greatest effect on the C-6 resonance, moving it 22 ppm upfield (61.9 ppm for glucose; 40.4 ppm for 6-aminoglucose).

Kinetic Studies with Yeast Hexokinase. 6-Aminoglucose was a slow substrate for hexokinase (Table I). The V_{max} was pH independent at 0.012 ±0.0004% that for glucose, while V/K for the aminosugar decreased below a pK of 9 (Figure 1). At pH 10, V/K for 6-aminoglucose was 0.0029 ± 0.0002% that for glucose and the K_m was 1.3 mM, compared to 0.3 mM for glucose.

6-Aminoglucose was a competitive inhibitor vs glucose, with the pK_i decreasing above a pK of 9.4 and decreasing below the pK of 9 (Figure 2). The decrease on the acid side is partial, leveling off to a pH-independent value for K_i of 33 ± 4 mM below a pK of 7.16. The K_m for MgATP in the presence of saturating (50 mM) 6-aminoglucose at pH 8.7 was 0.083 ± 0.019 mM. This is similar to the K_m of 0.063 ± 0.004 mM reported for MgATP with saturating glucose at pH 8.0 by Viola and Cleland (1978).

Enzymatic Synthesis of 6-Aminoglucose-6-P. Yeast hexokinase was used to catalyze phosphorylation of 6-amino-

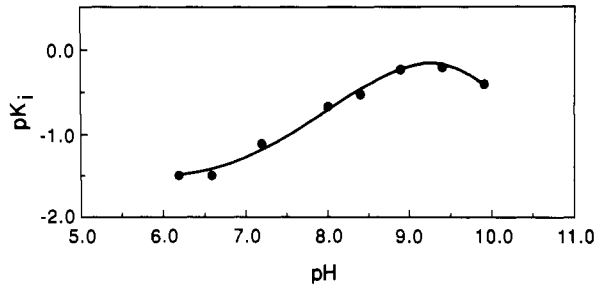


FIGURE 2: pH variation of K_i for 6-aminoglucose as a competitive inhibitor vs glucose in the hexokinase reaction with 4 mM MgATP. The data were fitted to eq 6 with pK values of 7.16 ± 0.09 and 9.4 ± 0.1 after adding $\log(1 + H/10^{-9.0})$ to each pK_i value to correct for the pK of the amine. The latter factor was then subtracted from the fitted curve to give the one plotted.

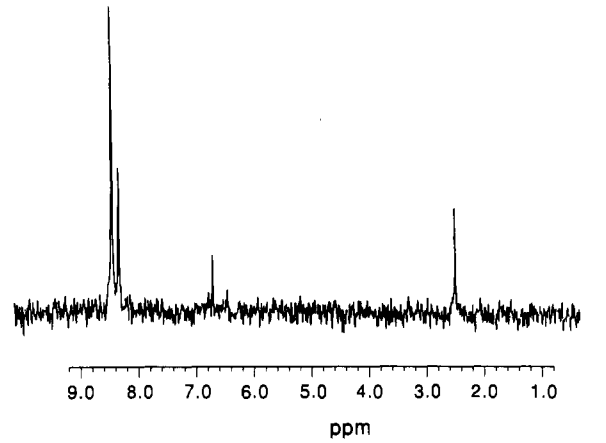


FIGURE 3: Proton-decoupled ^{31}P NMR spectrum of purified 6-aminoglucose-6-P at pH 9.5 and 22 °C after some hydrolysis. The resonance at 2.51 is from phosphate, while the peaks at 8.46, 8.34, and 6.72 correspond to the β and α anomers and an unknown form of 6-aminoglucose-6-P.

glucose by MgATP. The ^{31}P NMR spectrum after 2.5 h indicated production of a phosphorylated amine (8.3 ppm) and phosphate (2.5 ppm) at the expense of PEP (all consumed) and ATP (−5.5, −10.6, −19.1 ppm). After purification, 6-aminoglucose-6-P was obtained in 18% yield. Lyophilization resulted in some hydrolysis, so 6-aminoglucose-6-P was stored as a 20 mM solution at 5 °C at pH 11–12 where it is most stable. The ^{31}P, ^{13}C, and proton NMR spectra were consistent with the structure. Integration of the proton resonances at 5.42 and 4.82 ppm from C-1 of the α and β anomers indicated that the β/α ratio was 3.0. The ^{13}C resonances at 97.4 and 92.9 ppm correspond to the β and α anomers, respectively (those for glucose-6-P resonate at 100.0 and 95.2 ppm). Again, the O to N substitution has the largest effect on the C-6 resonance, which is 43.2 in the amino analogue vs 63.2 ppm in glucose-6-P. In a proton-coupled ^{31}P NMR spectrum (not shown), each resonance (β anomer at 8.46 and α anomer at 8.34 ppm) was split into a triplet from coupling to the protons on C-6. A small resonance at 6.72 ppm in the proton-decoupled spectrum in Figure 3 probably represents another equilibrium form of 6-aminoglucose-6-P, since complete enzymatic oxidation to 6-aminogluconate-6-P with glucose-6-P dehydrogenase resulted in the disappearance of this resonance along with those at 8.46 and 8.34 ppm.

The pK values for the phosphate group of 6-aminoglucose-6-P were determined at I = 0.1 M by titrating with base and monitoring the effect on the ^{31}P NMR chemical shifts for the two anomeric forms. The α anomer had pK's of 2.46 ± 0.13 and 8.02 ± 0.06, while the β anomer had pK's of 2.34 ± 0.16 and 7.85 ± 0.06. A fit of the data to eq 2 gave the

Table II: ^{31}P NMR Chemical Shifts for 6-Aminoglucose-6-P

protonation state	δ^a (ppm) α anomer	β anomer
neutral species	0.51 ± 0.14	0.82 ± 0.16
monoanion	4.44 ± 0.06	4.79 ± 0.05
dianion	12.1 ± 0.06	11.9 ± 0.05

[a] I = 0.1 M, 25 °C. Values from fits of chemical shifts vs pH to eq 2.

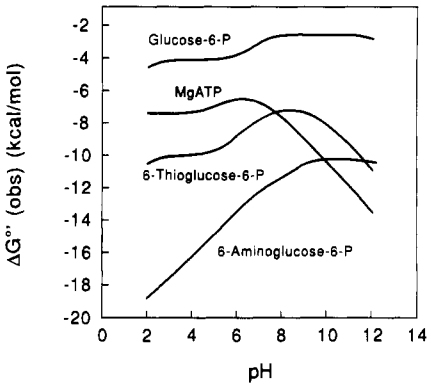


FIGURE 4: Calculated free energies of hydrolysis for MgATP, glucose-6-P, 6-thioglucose-6-P, and 6-aminoglucose-6-P at 25 °C. The pK values for the various molecules were from Jencks and Regenstein (1968), Pecoraro et al. (1984), Knight et al. (1991), Izatt & Christensen (1968), and the present work.

chemical shifts for the neutral, monoanionic, and dianionic forms of both anomers (Table II).

Free Energy of Hydrolysis of 6-Aminoglucose-6-P. A reaction mixture containing 38.6 mM 6-aminoglucose, 15 mM ATP, 17 mM $MgCl_2$, 100 mM Ches, pH 9.25, and 4000 units/mL yeast hexokinase at 25 °C was followed by ^{31}P NMR and allowed to come to equilibrium. The initial concentration of 6-aminoglucose was determined by a ninhydrin assay while that of ATP was determined spectrophotometrically (ϵ_{259} = 15.4 mM^{-1} cm^{-1}). After 21 h, equilibrium was reached and the ^{31}P NMR intensities were used to calculate an equilibrium constant of 0.182, corresponding to a free energy of reaction of 1.01 kcal/mol. Assuming a free energy of hydrolysis of MgATP at pH 7.0 of −6.8 kcal/mol (Atkinson et al., 1959; Benzinger et al., 1959; Rosing & Slater, 1972), the free energy of hydrolysis of MgATP at pH 9.25 will be −9.3 kcal/mol. The free energy of hydrolysis of 6-aminoglucose-6-P at pH 9.25 and 25 °C is thus −10.3 kcal/mol. At pH values above 10, however, the free energy of hydrolysis of MgATP is more negative than that for 6-aminoglucose-6-P, while at lower pH's the value for the amino analogue begins to decrease strongly with decreasing pH. Calculated pH profiles for the free energies of hydrolysis of MgATP, glucose-6-P, and its amino and thiol analogues are shown in Figure 4.

Equilibrium Constant for the Phosphoglucoisomerase Reaction. This value could not be determined accurately at pH 9 and 22 °C because of the instability of 6-aminofructose-6-P. Glucose-6-P dehydrogenase endpoint assays (±phosphoglucoisomerase) were used at 5.7 °C, after filtering off enzymes, to determine concentrations of the aldose and the ketose, allowing calculation of the equilibrium constant at this temperature. The equilibrium constant for ketonization of glucose-6-P at 5.7 °C and pH 8.7 was 0.214 ± 0.006, while at 22.0 °C it was 0.264 ± 0.004. From eq 9, ΔH was calculated as 2.10 ± 0.13 kcal/mol. Assuming the same ΔH for

$$\ln (K_2/K_1) = (-\Delta H/R)(1/T_2 - 1/T_1) \quad (9)$$

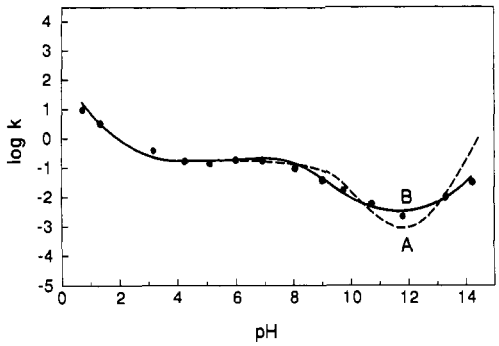


FIGURE 5: Stability of 6-aminoglucose-6-P as a function of pH at 22 °C. The lines represent fits to either eq 7 (A) or eq 8 (B). The fit to eq 7 gave pK's of 2.5 ± 0.2 and 9.3 ± 0.2, and σ = 0.645, while the fit to eq 8 gave pK's of 2.4 ± 0.1, 8.6 ± 0.2, and 13.2 ± 0.2 (the latter for the general base) and σ = 0.358. The units of k were hours^{-1}.

reaction of the amino analogue, the equilibrium constant for ketonization of 6-aminoglucose-6-P at pH 8.7 and 5.7 °C of 0.202 ± 0.002 corresponds to a value of 0.250 ± 0.002 at 22.0 °C.

pH Stability Profile for 6-Aminoglucose-6-P. The pH dependence of the rate constant for the hydrolysis/decomposition of 6-aminoglucose-6-P was determined by assaying for residual 6-aminoglucose-6-P with a glucose-6-P dehydrogenase endpoint assay at fixed time intervals. The resulting profile in Figure 5 shows that there is maximum stability at pH 11.8 with a half-life of 228 h. This profile is similar to that of phosphoramidate (Chanley & Feageson, 1962), with protonation of the monoanion causing an increase in rate and deprotonation of the monoanion causing a decrease in rate. The lability at high pH, however, is unique to 6-aminoglucose-6-P. These data were fitted to eq 7, which assumes specific base catalysis at high pH, and to eq 8, which assumes general base catalysis. Equation 8 gave a better fit (σ of 0.36 vs 0.65) and a pK of 13.2 ± 0.2 for the general base catalyzing hydrolysis at high pH. The rate constant for monoanion hydrolysis was 0.234 ± 0.038 h^{-1}, with this rate increasing below a pK of 2.4 ± 0.1 and decreasing above a pK of 8.6 ± 0.2. ^{31}P NMR analysis of the decomposition at pH 13 revealed residual 6-aminoglucose-6-P (9.0 ppm), phosphate (3.2 ppm), and several other unidentified phosphorylated amino species (9.3, 8.8, 8.4 ppm).

Enzymatic Synthesis of Phosphorylated Amines. Both glucose-6-P and 6-aminoglucose-6-P were metabolized by the reactions of glycolysis in vitro. Reaction mixtures contained 100 mM Ches, pH 9.0, 10 mM $MgCl_2$, 0.9 mM ATP, 0.5 mM NAD, 2 mM arsenate, 52 μM sugar phosphate, 25 units/mL glyceraldehyde-3-P dehydrogenase, and 50 units/mL each phosphofructokinase and aldolase. The reaction was started with 35 units/mL phosphoglucoisomerase and allowed to reach an endpoint. Triose-P isomerase (100 units/mL) was added, and a second endpoint was reached. The first endpoint (from carbons 4–6, which become glyceraldehyde-3-P or its amino analogue during the aldolase reaction) was reached ~8 times faster with glucose-6-P than with 6-aminoglucose-6-P, while the second endpoints (from carbons 1–3, which become dihydroxyacetone-P in both cases) were reached at the same rate.

The amino analogues of glycolytic intermediates from the above system were synthesized enzymatically at pH 9, starting with 6-aminoglucose-6-P. Enzymes were removed by ultrafiltration, and the analogues were used for stability and kinetic studies without further purification. Similar syntheses were carried out with glucose-6-P and 6-P-gluconate dehydrogenases. The ^{31}P NMR chemical shifts for these compounds are in Table III. The chemical shifts are pH dependent, shifting upfield as the phosphate groups are protonated, but fall in the 8–8.5-ppm range for the dianionic phosphates at high pH.

Table III: ^{31}P NMR Chemical Shifts of Phosphorylated Amines and the Corresponding Phosphate Esters

compound	δ (ppm) at pH 9.5	δ (ppm) at pH 9.2 of phosphate ester
6-aminoglucose-6-P	8.4	4.32
6-aminoglucose-1-P	2.04	2.32
6-aminofructose-6-P	8.29	3.81
6-aminofructose-1,6-bis-P	4.24, 8.18	4.08, 3.92
3-aminoglyceraldehyde-3-P	8.18	4.45
6-aminogluconate-6-P	8.16	4.75
5-aminoribulose-5-P	8.24	3.86

Table IV: Stability of Phosphorylated Amines at 22 °C

compound	pH	half-life	relative stability[a]
6-aminogluconate-6-P	5.0	12.5 h	120
	9.0	45.0 h	210
3-aminoglyceraldehyde-3-P	5.0	6.93 h	67
	9.1	2.15 h[b]	10
6-aminoglucose-6-P	5.0	2.96 h	28
	9.0	13.2 h	61
6-aminofructose-6-P	5.0	6.24 min	1.0
	8.7	1.64 h	7.6
6-aminofructose-1,6-bis-P	5.1	8.17 min	1.3
	9.1	13.2 min	1.0
5-aminoribulose-5-P	5.0	<5 min	<0.8
	9.0	12.9 min	1.0

[a] Stability relative to 6-aminofructose-6-P at pH 5 or 5-aminoribulose-5-P at pH 9. [b] The half-life for glyceraldehyde-3-P at pH 9 is 8.67 h. The decomposition of these molecules at high pH is by elimination rather than hydrolysis.

The reactions of 6-aminoglucose-6-P catalyzed by glucose-6-P dehydrogenase, phosphoglucomutase, and phosphoglucoisomerase were monitored with ^{31}P NMR. A new resonance was observed in each case in addition to those from 6-aminoglucose-6-P and phosphate. In the glucose-6-P dehydrogenase reaction, which went to completion, all of the 6-aminoglucose-6-P resonances disappeared.

Stability of Phosphorylated Amines. Half-lives for each of the amino analogues were determined at pH 5 where they are monoanions and at pH 9 where they are dianions (Table IV). In all cases except for 3-aminoglyceraldehyde-3-P the monoanion is less stable.

Kinetic Parameters for Amino Analogues. Values for V_{max} and K_m were determined both for the normal phosphate esters and for the amino analogues as substrates for their respective enzymes (Table V).

Discussion

6-Aminoglucose was reported by Machado de Domenech and Sols (1980) not to be a substrate for hexokinase, but these studies were done at pH 7.4 where the amino group is so strongly protonated that activity is difficult to determine. We have found that 6-aminoglucose is a slow substrate for yeast hexokinase with a V_{max} 4 orders of magnitude less than that for glucose and a K_m 6.8-fold larger at pH 9 (3.4-fold if one corrects for the fraction present as free amine). The amino group is thus clearly a poor substitute for the hydroxyl group in the hexokinase reaction, although not as poor as a thiol group (Knight et al., 1991). The fact that the V/K profile shows a decrease below the pK of 6-aminoglucose while the V_{max} profile is pH independent indicates that only the neutral form of 6-aminoglucose is catalytically competent. However,

Table V: Kinetic Parameters for Oxygen- and Nitrogen-Bridged Substrates for Selected Enzymes

		oxygen-bridged substrate		nitrogen-bridged substrate		
enzyme	substrate	pH	K_m (μM)	V_{max}[a]	K_m (μM)	V/K[a]
G6PDH[b]	glucose-6-P	8.9	99 ± 5[c]	52 ± 7	360 ± 10[c]	14.4 ± 0.3[c]
6PGDH[b]	6-P-gluconate	8.9	460 ± 20	14.8 ± 0.4	500 ± 30	13.5 ± 0.5
PGI[b]	glucose-6-P	9.1	270 ± 20[d]	17 ± 1	340 ± 60[d]	13.9 ± 0.7[d]
PFK[b]	fructose-6-P	8.8	44 ± 6	102 ± 7	38 ± 6	114 ± 12
aldolase	fructose-bis-P	8.9	8.0 ± 0.9	47 ± 1	4.7 ± 0.3	80 ± 4
alkPase[b]	glucose-6-P	9.1	130 ± 20	92 ± 4	160 ± 20	74 ± 7
PGM[b]	glucose-1-P	9.0	55 ± 7			
	6-aminoglucose-6-P			9 ± 3	51 ± 4[d]	9.9 ± 0.6

[a] Values relative to 100 for the oxygen-bridged substrate. [b] Enzymes: G6PDH, glucose-6-P dehydrogenase; 6PGDH, 6-P-gluconate dehydrogenase; PGI, phosphoglucoisomerase; PFK, phosphofructokinase; AlkPase, alkaline phosphatase; PGM, phosphoglucomutase. Cosubstrates were saturating. [c] Corrected for fraction present as β anomer. [d] Corrected for fraction present as α anomer.

since the K_i for 6-aminoglucose as a competitive inhibitor vs glucose increases below the pK of 9 but levels off to a pH-independent value below pH 7.16, we conclude that protonated 6-aminoglucose binds weakly to hexokinase and has a pK on the enzyme of 7.16. This perturbation in pK shows that the protonated amine binds 2.4 kcal/mol less favorably than the neutral amine, so the enzyme does tolerate a positive charge in the active site at some energetic expense. Similarly, 6-thioglucose does not bind appreciably to hexokinase when it is ionized, although it might bind weakly (Knight et al., 1991). It is reasonable that a negative charge is not tolerated, since an aspartate acts as a general base to remove a proton from the 6-hydroxyl of glucose (Anderson et al., 1978; Viola & Cleland, 1978). It is somewhat surprising that a positive charge is not well tolerated, but the global charge in the active site, rather than the local charge near C-6 of the substrate is clearly what is important.

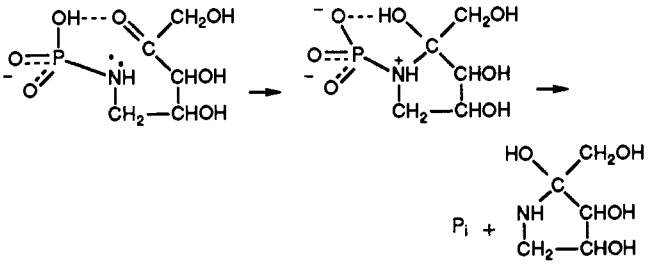
The K_m for MgATP in the presence of saturating 6-aminoglucose is essentially equal to that for glucose, indicating that the O to N substitution has not affected the synergistic binding between sugar and MgATP. With glycerokinase, Knight and Cleland (1989) also found that synergism between substrate and nucleotide was retained with an amino analogue, while the rate was drastically reduced. Since the synergistic binding of sugar and nucleotide is thought to reflect the conformation change that sets the stage for catalysis, it is not clear why the amino group is so poorly phosphorylated. Possibly there is extensive nonproductive binding, such as incorrect hydrogen bonding, in the active site.

6-Aminoglucose-6-P was synthesized enzymatically with hexokinase, since attempts to deblock chemically synthesized material always resulted in hydrolysis. The pK values for the β anomer (2.34 and 7.85) are lower than those for the α anomer (2.46 and 8.02), which is also the case for glucose-6-P (Knight et al., 1986). These authors proposed that the decrease in pK for the β anomer was the result of hydrogen-bonding interactions between the anomeric hydroxyl and the phosphate group. In the α anomer, the anomeric hydroxyl is anti to the phosphate and no interaction is possible.

The stability profile for 6-aminoglucose-6-P in Figure 5 shows that the monoanion has a half-life of 3 h and that protonation to the neutral species leads to an increase in hydrolysis rate, while deprotonation to the dianion gives a decrease in rate. Phosphoramidate shows a similar profile (Chanley & Feageson, 1962), but unlike phosphoramidate 6-aminoglucose-6-P is labile at high pH. The data best fit a model that assumes general base catalysis with a pK of 13.2 for the general base. Base lability is also seen to a lesser extent with glucose-6-P, where the dianion is less stable than the monoanion (Degani & Halmann, 1966; Bunton & Chaimovich, 1966). These authors proposed that the anomeric hydroxyl group of the β anomer was acting as a general acid or base catalyst at high pH, and a pK of 13.2 is consistent with this proposal. Further, at high pH there may be reactions occurring with glucose-6-P other than simple hydrolysis (Degani & Halmann, 1966). With 6-aminoglucose-6-P we observed several new and unidentified phosphorylated amines in the ^{31}P NMR spectrum during decomposition at high pH, but only phosphate at low pH.

6-Aminoglucose-6-P is thermodynamically much less stable than glucose-6-P. The free energy of hydrolysis is −10.3 kcal/mol at pH 9.25 and 25 °C, which is 7.5 kcal/mol more negative than for glucose-6-P. The pH profiles in Figure 4 show that above pH 10 6-aminoglucose-6-P is more stable than MgATP while at low pH the opposite is true. The enzymatic synthesis with hexokinase should thus be carried out at as high a pH as the enzyme will tolerate. By contrast 6-thioglucose-6-P is more stable than MgATP above pH 8, and less so below this pH. 6-Aminoglucose-6-P has the same thermodynamic stability as 6-thioglucose-6-P at pH 12 but is less stable than the phosphorylated thiol at lower pH values.

A number of phosphorylated amino analogues of metabolic intermediates have been synthesized enzymatically and their relative stabilities determined. The values in Table IV show that all of these molecules are acid labile, with the relative stabilities of the monoanions being 6-aminogluconate-6-P > 3-aminoglyceraldehyde-3-P > 6-aminoglucose-6-P > 6-aminofructose-1,6-bis-P ≃ 6-aminofructose-6-P > 5-aminoribulose-5-P. This is the same relative stability that was observed for phosphorylated thiol analogues by Knight et al. (1991). By analogy with the reaction observed with 5-thioribulose-5-P, which yields the cyclic thiosugar, the reaction of 5-aminoribulose-5-P presumably goes via



This chemistry is similar to that found by Jencks and Gilchrist (1964) for the formaldehyde-catalyzed hydrolysis of phosphoramidate. In that case, the nitrogen of phosphoramidate formed an adduct with one or two formaldehyde molecules with the structures

$$HO\text{-}CH_2\text{-}NH_2^+\text{-}PO_3^{2-} \qquad (HO\text{-}CH_2)_2\text{-}NH^+\text{-}PO_3^{2-}$$

The 6-aminofructose analogues could react similarly with the formation of a 6-membered ring, although the only ~2% of

open-chain form expected for these compounds (Benkovic & Schray, 1976) would decrease the rate. 3-Aminoglyceraldehyde-3-P is unusual in being more stable as a monoanion than at pH 9. This presumably reflects the tendency toward elimination rather than hydrolysis at high pH; glyceraldehyde-3-P decomposes by this route at high pH.

Phosphorylated amines undergo several enzymatic phosphoryl-transfer reactions where the P–N bond is cleaved or formed at rates close to those seen for the corresponding phosphate esters. In contrast to the hexokinase reaction where the V_{max} with 6-aminoglucose is 0.012% that with glucose, alkaline phosphatase accepts phosphorylated amines at a rate only 2–3-fold lower than phosphate esters (Snyder & Wilson, 1972), and 6-aminoglucose-6-P shows a V_{max} 92% that of glucose-6-P and nearly the same K_m. With phosphoglucomutase, 6-aminoglucose-6-P reacts with a V_{max} 9% that for glucose-1-P and a similar K_m. Glycerokinase phosphorylates (*S*)-1-aminopropanediol on nitrogen with a V_{max} 0.4% that of glycerol (Knight & Cleland, 1989), so it appears that kinases are unique in not readily accepting amino groups for phosphoryl transfer.

Of the enzymes that catalyze reactions at positions other than the phosphoryl group, phosphoglucoisomerase is the most sensitive to the N for O substitution, with a V_{max} 17% that of glucose-6-P. Aldolase and phosphofructokinase show almost no effect on kinetic parameters from the O to N change. Of the two dehydrogenases of the hexose monophosphate shunt, glucose-6-P dehydrogenase shows a V_{max} 52% that for glucose-6-P and a K_m 3.6-fold higher, while 6-phosphogluconate dehydrogenase shows a V_{max} 15% that of 6-phosphogluconate with no change in K_m. Although there are subtle differences in individual cases, the O to N change is well tolerated by all of the enzymes tested that do not transfer the phosphoryl group to or from nitrogen, and thus phosphorylated amines are good isosteric analogues of metabolic intermediates. Their inherent instability is a problem, but by working at low temperature and elevated pH, one can readily use them to evaluate the effects of the O to N change on metabolic reactions.

References

Anderson, C. M., Stenkamp, R. E., McDonald, R. C., & Steitz, T. A. (1978) *J. Mol. Biol. 123*, 207.

Atkinson, M., Johnson, E., & Morton, R. (1959) *Nature 184*, 1925.

Benkovic, S. J., & Sampson, E. J. (1971) *J. Am. Chem. Soc. 93*, 4009.

Benkovic, S. J., & Schray, K. J. (1976) *Adv. Enzymol. Relat. Areas Mol. Biol. 44*, 139.

Benzinger, T., Kitzinger, C., Hems, R., & Burton, K. (1959) *Biochem. J. 71*, 400.

Bunton, C. A., & Chaimovich, H. (1966) *J. Am. Chem. Soc. 88*, 4082.

Chanley, J. D., & Feageson, E. (1962) *J. Am. Chem. Soc. 85*, 1181.

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1.

Corbridge, D. E. C. (1966) in *Topics in Phosphorus Chemistry* (Grayson, N., & Griffith, E. J., Eds.) Vol. 3, p 208, Interscience, New York.

Cramer, F. D. (1962) *Methods Carbohydr. Chem. 1*, 242.

Cramer, F. D. (1963) *Methods Carbohydr. Chem. 2*, 244.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1986) *Data for Biochemical Research*, 3rd ed., p 457, Clarendon Press, New York.

Degani, Ch., & Halmann, M. (1966) *J. Am. Chem. Soc. 88*, 4075.

Huheey, J. E. (1983) *Inorganic Chemistry*, 3rd ed., p A37, Harper & Row, New York.

Izatt, R. M., & Christensen, J. J. (1968) in *CRC Handbook of Biochemistry*, p J90, Chemical Rubber Co., Cleveland, OH.

Jencks, W. P., & Gilchrist, M. (1964) *J. Am. Chem. Soc. 86*, 1410.

Jencks, W. P., & Regenstein, J. (1968) in *CRC Handbook of Biochemistry*, p J150, Chemical Rubber Co., Cleveland, OH.

Knight, W. B., & Cleland, W. W. (1989) *Biochemistry 28*, 5728.

Knight, W. B., Weiss, P. M., & Cleland, W. W. (1986) *J. Am. Chem. Soc. 108*, 2759.

Knight, W. B., Sem, D. S., Smith, K., Miziorko, H. M., Rendina, A. R., & Cleland, W. W. (1991) *Biochemistry* (preceding paper in this issue).

Machado de Domenech, E. E., & Sols, A. (1980) *FEBS Lett. 119*, 174.

Moore, S., & Stein, W. H. (1948) *J. Biol. Chem. 176*, 367.

Pecoraro, V. L., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry 23*, 5262.

Rosing, J., & Slater, E. C. (1972) *Biochim. Biophys. Acta 267*, 275.

Snyder, S. L., & Wilson, I. B. (1972) *Biochemistry 11*, 1616.

Staros, J. V., Bayley, H., Standring, D. N., & Knowles, J. R. (1978) *Biochem. Biophys. Res. Commun. 80*, 568.

Viola, R. E., & Cleland, W. W. (1978) *Biochemistry 17*, 4111.

Westheimer, F. H. (1987) *Science 235*, 1173.