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# Mechanism-Based Inactivation of Rabbit Muscle Phosphoglucomutase by Nojirimycin 6-Phosphate†

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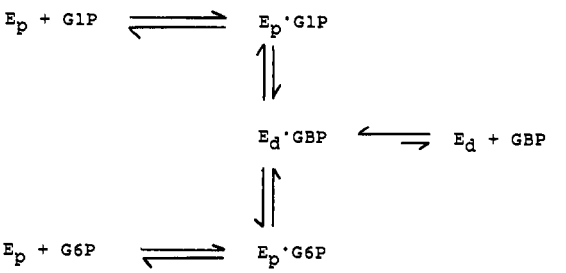
ABSTRACT: Nojirimycin 6-phosphate (N6P) was tested as a substrate and inhibitor for phosphoglucomutase (PGM). In the absence of glucose 1,6-bisphosphate (GBP), the incubation of PGM and N6P resulted in the complete inactivation of all enzyme activity. When equimolar amounts of N6P and GBP were incubated together with PGM, the GBP was quantitatively converted to glucose 6-phosphate (G6P) and phosphate. At higher ratios of GBP and N6P (>100) the final concentration of G6P produced was found to be 19 times the initial N6P concentration. These results have been interpreted to suggest that the phosphorylated form of PGM catalyzes the phosphorylation of N6P at C-1. This intermediate rapidly eliminates phosphate to form an imine and the dephosphorylated enzyme. The dephosphorylated enzyme is rapidly rephosphorylated by GBP and forms G6P. The imine is nonenzymatically hydrated back to N6P. Occasionally (5%) the imine isomerizes to a compound that is not processed by PGM.

Phosphoglucomutase (PGM)[1] catalyzes the interconversion of glucose 1-phosphate (G1P) and glucose 6-phosphate (G6P). The reaction catalyzed by phosphoglucomutase is activated by glucose 1,6-bisphosphate (GBP). Ray and Roscelli (1964) have proposed the mechanism shown in Scheme I. PGM has two forms; one is the phosphorylated enzyme ($E_p$), and the other is the dephosphorylated enzyme ($E_d$). Only the phosphorylated enzyme is active since it can phosphorylate either G1P or G6P to GBP in the enzyme active site. The newly formed GBP rephosphorylates the enzyme again, and the product, either G1P or G6P, is released from the phosphorylated enzyme. The thermodynamic equilibrium constant is 17.2 in favor of G6P in the pH range 6.2–7.5 at 30 °C (Najjar, 1962; Colowick & Sutherland, 1942). GBP can phosphorylate the dephosphorylated enzyme to the fully active phosphorylated form (Ray & Peck, 1972).

Nojirimycin is an antibiotic isolated from fermentation broths of several strains of *Streptomyces* such as *S. roseochromogenes* R-468, *S. lavendulae* SF-425, and *S. nojiriensis* n.sp. SF-426 (Inouye, 1968). Nojirimycin has been shown to be a potent inhibitor for $\alpha$- and $\beta$-glucosidase (Legler, 1984; Niwa et al., 1970). Nojirimycin 6-phosphate (N6P) is the product from the reaction of nojirimycin with hexokinase and ATP (Drueckhammer & Wong, 1985). In an attempt to use PGM to synthesize nojirimycin 1-phosphate we found that in the absence of GBP the incubation of PGM with N6P resulted in the inactivation of enzyme activity. When equimolar amounts of N6P and GBP were incubated together with PGM, the GBP was quantitatively converted to G6P. In this paper, the inhibitory mechanism of PGM by N6P and the role of GBP in this mechanism are elucidated.

Scheme I




## Materials and Methods

Hexokinase, glucose-6-phosphate dehydrogenase (G6PDH), GBP, G6P, PGM (300 units/mg), and G1P were purchased from Sigma Chemical Co. Nojirimycin was obtained from Dr. C.-H. Wong. Rabbit muscle PGM was the generous gift of Professor William J. Ray. [$\gamma$-$^{32}$P]ATP was purchased from ICN Radiochemicals. All other chemicals were purchased from either Sigma or Aldrich.

*Preparation of Nojirimycin 6-Phosphate.* The free form of nojirimycin was prepared from the nojirimycin bisulfite

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[1] Abbreviations: PGM, phosphoglucomutase; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; GBP, glucose 1,6-bisphosphate; N6P, nojirimycin 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase.

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adduct according to the method of Inouye et al. (1968). N6P was synthesized enzymatically by the catalytic action of hexokinase. In a 30-mL solution of 100 mM Hepes buffer, pH 8.0, 5.0 mM nojirimycin was reacted with 10 mM ATP, 30 mM $MgCl_2$, and 100 units of hexokinase. The reaction was monitored with HPLC by following the decrease in the concentration of ATP and the increase in the concentration of ADP. The reaction was complete within 3 h. The solution was loaded on a Whatman DE52 anion-exchange column and eluted with triethylamine (TEA)/$HCO_3^-$ buffer, pH 8.0, using a 10–500 mM gradient. The fractions that were active to glucose-6-phosphate dehydrogenase (G6PDH) were collected and dried by rotary evaporation.[2] The isolated yield was 61.5 μmol (41%).

*Inhibition of Phosphoglucomutase by Nojirimycin 6-Phosphate.* The formation of G6P from G1P was followed by using a coupled assay system with G6P dehydrogenase. The 2.0-mL reaction mixtures contained 100 mM Tris/20 mM histidine buffer, pH 7.5, 0.4 mM $NAD^+$, 1.0 mM EDTA, 3.0 mM $MgCl_2$, 0.1 mM G1P, and 100 units of G6PDH. PGM (0.2 unit) was preincubated in a volume of 1.0 mL with either 100 mM Tris buffer, pH 7.5, and 30 μM N6P or 100 mM Tris buffer, pH 7.5, 30 μM N6P, and 30 μM GBP. The reactions were initiated by adding the preincubated solutions to the 2.0-mL assay solution. The increase in the concentration of NADH was followed at 340 nm. All experiments were conducted at 25 °C.

*Reaction of Nojirimycin 6-Phosphate with Phosphoglucomutase and Glucose 1,6-Bisphosphate.* The time course for the reaction of N6P and PGM was measured in a 3.0-mL reaction solution that contained 100 mM Tris/25 mM histidine buffer, pH 7.5, 1.0 mM EDTA, 3.0 mM $MgCl_2$, 0.4 mM $NAD^+$, 0.3 mM GBP, and 2.34 μM N6P. The reactions were started by adding 0.5 unit of PGM. At various times, the formation of G6P was quantitated by adding 5 units of G6PDH, and the increase in absorbance was followed at 340 nm. When the formation of G6P was almost complete, additional 2.34 μM N6P was added. The time course for the formation of glucose 6-phosphate was measured again by the same method. The effect of varying the concentration of GBP at fixed concentrations of N6P and enzyme was measured in a 3.0-mL reaction solution that contained 100 mM Tris/25 mM histidine buffer, pH 7.5, 1.0 mM EDTA, 3.0 mM $MgCl_2$, 0.4 mM $NAD^+$, 2.34 μM N6P, and various concentrations of GBP. The reactions were started by adding 10 units of PGM, and the formation of G6P was measured by the method described above. The time courses at different pH values were measured in 3.0-mL reaction solutions that contained 1.0 mM EDTA, 3.0 mM $MgCl_2$, 0.4 mM $NAD^+$, 2.34 μM N6P, and 0.25 mM GBP. The pH values of the solutions were controlled by 100 mM Tris/25 mM histidine buffer, pH 7.5, 8.5, and 9.5. The reactions were started by the addition of 10 units of PGM, and the total amount of G6P formed was measured after 60 min. The effect of varying the concentration of enzyme with fixed levels of N6P (1.17 or 2.34 μM) and GBP (0.25 mM) was determined in identical reaction mixtures at pH 7.5, with variable amounts of PGM. The time course for the formation of G6P was followed after the addition of 50 units of G6PDH.

*$^{31}P$ NMR Analysis.* The reaction products were identified by NMR spectroscopy. In an 10-mm NMR tube, the 3.0-mL reaction solution contained 100 mM Tris/12.5 mM histidine buffer, pH 7.5, 1.0 mM EDTA, 3.0 mM $MgCl_2$, 10 units of PGM, 0.5 mM GBP, and 25% $D_2O$. The reaction was initiated by adding 11.7 μM N6P. The $^{31}P$ NMR spectra were taken every 20 min. After 100 min, additional 117 μM N6P was added to complete the conversion, and the $^{31}P$ NMR spectrum was taken again. $^{31}P$ NMR spectra were obtained on a Varian XL-200 multinuclear spectrometer operating at a frequency of 81 MHz. Typical acquisition parameters were 10000-Hz sweep width, 1.6-s acquisition time, and 10-μs pulse width. The reported chemical shifts are referenced to an internal standard of phosphate.

*Phosphoryl Transfer.* [$^{32}P$]N6P was prepared by the reaction of nojirimycin and [$\gamma$-$^{32}P$]ATP with hexokinase as described above. The 100-mL reaction solution contained 100 mM Hepes buffer, pH 7.5, 3.0 mM $MgCl_2$, 0.67 mM GBP, 30 units of PGM, and 8.43 μM N6P (202600 cpm). The reaction was monitored by measuring G6P formation and was complete in 50 min. Then 50 μM unlabeled N6P was added to convert all of the remaining GBP to G6P and inorganic phosphate. The solution was then loaded on a borax column, which was prepared according to the method of Lefebvre et al. (1963), and eluted by using a gradient from 0 to 4 M triethylammonium borate buffer. The fractions containing phosphate were assayed by the method of Bartlett et al. (1959), and those containing G6P were assayed by the reaction of G6PDH. The radioactivity in every fraction was measured by liquid scintillation counting.

*Trapping the Intermediate with β-Mercaptoethanol.* The putative intermediate in the inactivation of PGM was trapped with β-mercaptoethanol. The time course for the conversion of GBP to G6P in the presence of various amounts of β-mercaptoethanol was measured. Each 3.0-mL reaction solution contained 100 mM Tris buffer, pH 7.5, 1.0 mM EDTA, 3.0 mM $MgCl_2$, 0.4 mM $NAD^+$, 0.29 mM GBP, 10 μM N6P, and various amounts of β-mercaptoethanol. The reaction was started by the addition of 0.2 units of PGM. At various times the concentration of G6P was measured by adding 10 units of G6PDH.

*Michaelis Constant of Nojirimycin 6-Phosphate.* The initial velocities for the formation of G6P at various levels of N6P were measured. Each 3.0-mL reaction solution contained 100 mM Tris/20 mM histidine buffer, pH 7.5, 1.0 mM EDTA, 3.0 mM $MgCl_2$, 0.4 mM $NAD^+$, 0.25 mM GBP, and variable amounts of N6P. The reaction was started by the addition of 0.2 unit of PGM. Every minute, the amount of G6P formed was quantitated by adding 10 units of glucose-6-phosphate dehydrogenase.

*Relative Velocity.* The initial velocities for the N6P-induced formation of G6P from GBP were measured as above with 0.25 mM GBP, 0.1 unit PGM, and either 0.1 mM N6P or 0.1 mM G1P.

## Results

*Inhibition of Phosphoglucomutase by Nojirimycin 6-Phosphate.* The initial velocity of the reaction catalyzed by PGM was measured by using a coupled assay system with G6PDH. When PGM was preincubated with 30 μM N6P, the rate of formation of G6P from G1P was 0.31% of the observed rate when PGM was not preincubated with N6P. When PGM was preincubated with equivalent concentrations of N6P and GBP, the observed rate of formation of G6P from G1P was still only 0.31% of the control reaction rate.

*Reaction of Glucose 1,6-Bisphosphate with Nojirimycin 6-Phosphate and Phosphoglucomutase.* Figure 1 shows the time course for the formation of G6P when 0.3 mM GBP was

[2] Nojirimycin 6-phosphate is a substrate for glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*. The $K_M$ is 0.36 mM, and the $V_{max}$ is 0.60 times the maximal rate for the oxidation of glucose 6-phosphate when NAD is utilized as the other substrate.

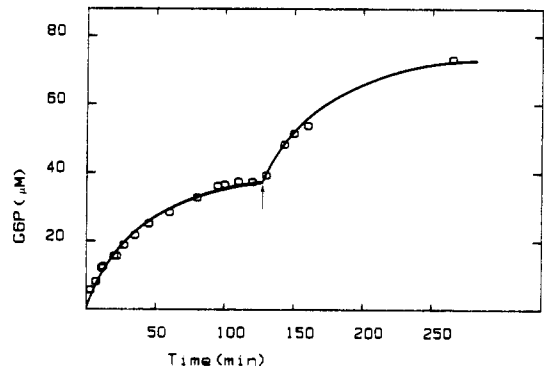



FIGURE 1: Time course for the formation of G6P when 2.34 μM N6P was incubated with 0.3 GBP in the presence of PGM. After 130 min, additional 2.34 μM N6P was added. Additional details are given in the text.

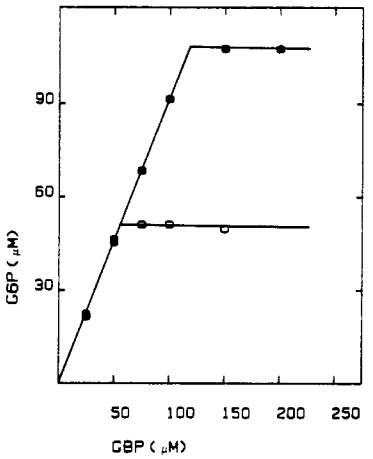



FIGURE 2: Maximum formation of G6P when various concentrations of GBP are incubated with fixed levels of N6P in the presence of PGM. N6P concentrations: 2.34 (○) and 4.68 μM (●).

incubated with 2.34 μM N6P and 0.1 unit of PGM. When the formation of G6P reached a maximum after about 130 min, additional 2.34 μM N6P was added to the reaction mixture. Another "burst" of G6P was formed after the second addition of N6P. The maximum amount of G6P formed was at least 16 times the concentration of the added N6P provided the initial concentration of GBP was sufficiently high. Shown in Figure 2 are the results when variable amounts of GBP are incubated with either 2.3 or 4.7 μM N6P in the presence of phosphoglucomutase at pH 7.5. This graph indicates that when the initial concentration of GBP is less than 19 times that of N6P, the GBP is quantitatively converted to G6P. When the initial concentration of GBP is higher than 19 times that of the initially added N6P, G6P is formed in an amount approximately 19 times the initial concentration of N6P regardless of the initial concentration of GBP. At pH 8.5 and 9.5 the maximal amount of G6P produced is 27 and 34 times, respectively, the initial N6P concentration. Since nojirimycin is unstable at pH values less than 6.5, this experiment was not performed in more acidic conditions. At a fixed concentration of N6P (2.34 μM) and GBP (0.25 mM), increasing the concentration of PGM affects only the velocity of conversion, not the total amount of formation of G6P (data not shown). A plot of the initial velocity versus enzyme concentration is linear with a slope of 0.017 μmol $min^{-1}$ (unit of phosphoglucomutase)$^{-1}$.

The identity of the reaction products was confirmed by $^{31}P$ NMR spectroscopy. Shown in Figure 3A is the spectrum of GBP shortly after the addition of PGM and N6P. The phosphoryl groups attached to C-6 and C-1 resonate at 1.85

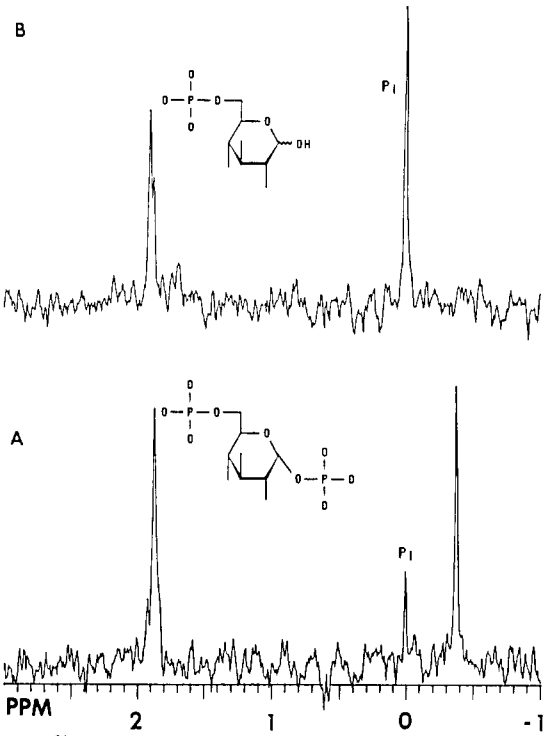



FIGURE 3: $^{31}P$ NMR spectra of reaction mixture after incubation of GBP (0.5 mM) and N6P (11.7 μM) in the presence of PGM. (A) Spectrum taken 20 min after mixing. (B) Spectrum taken after the N6P concentration was increased to 117 μM and the reaction had come to equilibrium.

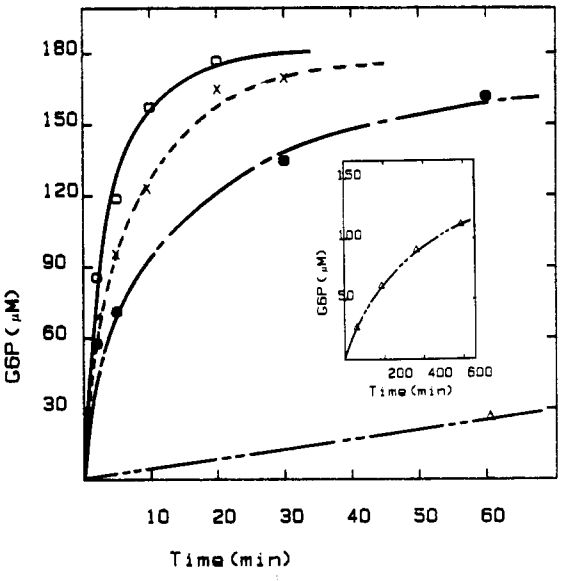


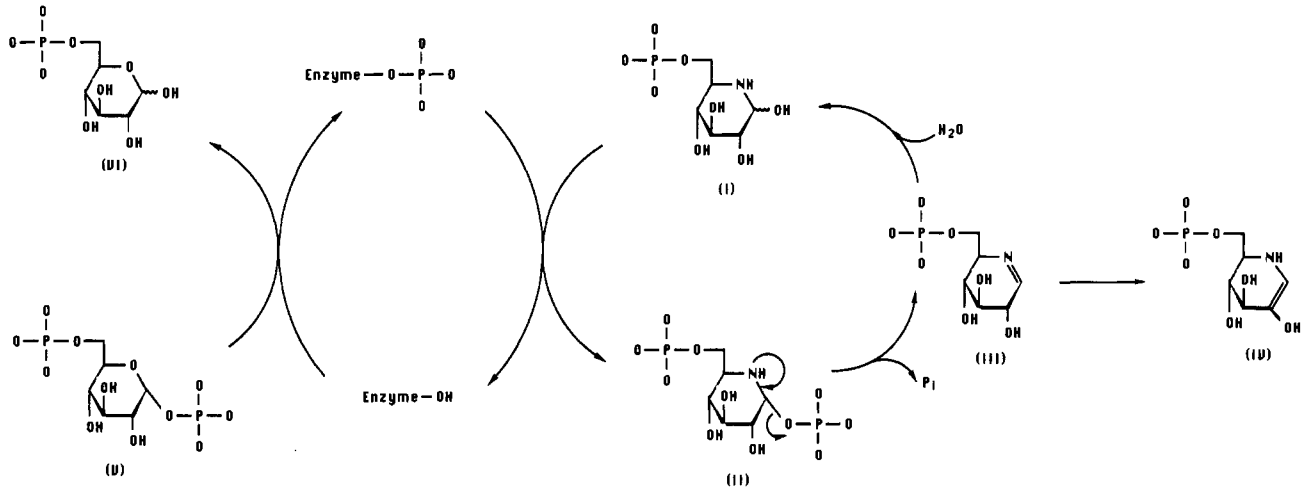

FIGURE 4: Time course for the formation of G6P after mixing 10 mM N6P, 0.29 mM GBP, and PGM in the presence of various concentrations of β-mercaptoethanol: 0 (○), 1.0 (×), 5.0 (●), and 50 mM (△).

and −0.38 ppm, respectively. After the reaction is complete, the signals for GBP disappear completely and are replaced by the resonances corresponding to the α- and β-anomers of G6P (1.92 and 1.85 ppm) and inorganic phosphate at 0 ppm (Figure 3B).

The time courses for the formation of G6P in the presence of PGM, N6P, and various amounts of β-mercaptoethanol are illustrated in Figure 4. The rate is dramatically altered, but the final concentration of G6P formed is approximately the same.

The rate of G6P formation as a function of the initial concentration of N6P at saturating levels of GBP was mea-

Scheme II



sured (data not shown). The $K_M$ value for N6P is 12.4 $\mu$M. The maximal rate for G6P formation in these experiments is 41% of the rate for the net enzymatic conversion of G1P to G6P under identical reaction conditions.

$^{32}$P-Labeled N6P (8.4 $\mu$M) was incubated with 0.67 mM GBP and 30 units of PGM in order to monitor the fate of the phosphoryl group at C-6 of nojirimycin during the reaction. After 50 min, the concentration of G6P was 145 $\mu$M. Then 50 $\mu$M unlabeled N6P was added to convert all of the remaining GBP to G6P and inorganic phosphate. The G6P was then chromatographically separated from G1P, inorganic phosphate, N6P, and other products. No radioactivity (<1%) was observed in those fractions that contained the G6P. All of the radioactivity coeluted with the unresolved N6P and $P_i$ peaks.

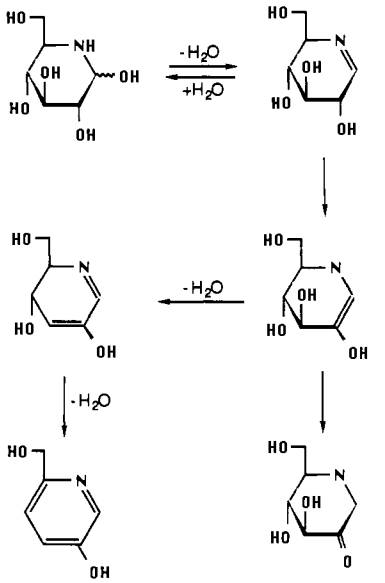
## Discussion

A model for the interaction of N6P with PGM in the presence of GBP is illustrated in Scheme II. In this proposed mechanism N6P (I) binds to the phosphorylated form of PGM and subsequently becomes phosphorylated at C-1. The resulting complex thus consists of the dephosphorylated enzyme and nojirimycin 1,6-bisphosphate (II). This very unstable intermediate rapidly eliminates phosphate and is converted to an imine (III). Since monophosphorylated sugars are unable to phosphorylate dephosphorylated enzyme at a significant rate (<0.01%),[3] the imine (III) is released from the active site into the bulk solution where it is rapidly rehydrated back to N6P. In the presence of GBP (V) the dephosphorylated form of phosphoglucomutase is rephosphorylated and the GBP is converted to G6P (VI) (actually an equilibrium mixture of G6P and G1P). The scheme is cyclical, and thus everytime N6P is processed by the enzyme, a molecule of GBP is converted to G6P and $P_i$. The evidence in support of this model is presented below.

(1) In the absence of GBP the incubation of N6P and PGM results in the inactivation of almost all enzymatic activity. The inactivation process is due directly to the dephosphorylation of PGM that is caused by the rapid loss of phosphate from the intermediate bisphosphate (II). Since the phosphorylated enzyme is significantly more active than the dephosphorylated enzyme in the interconversion of G6P and G1P,[3] the catalytic activity is dramatically reduced. Therefore, N6P can be considered to be a suicide or mechanism-based inhibitor of phosphoglucomutase. However, it should be noted that high concentrations of GBP can restore the original activity.

[3] William J. Ray, Jr., personal communication.

Scheme III



(2) In the presence of GBP and PGM with an excess of N6P, all of the GBP is converted to G6P and $P_i$. The formation of G6P and $P_i$ in this transformation is clearly established by enzymatic analysis with glucose-6-phosphate dehydrogenase and confirmed by $^{31}$P NMR spectroscopy.

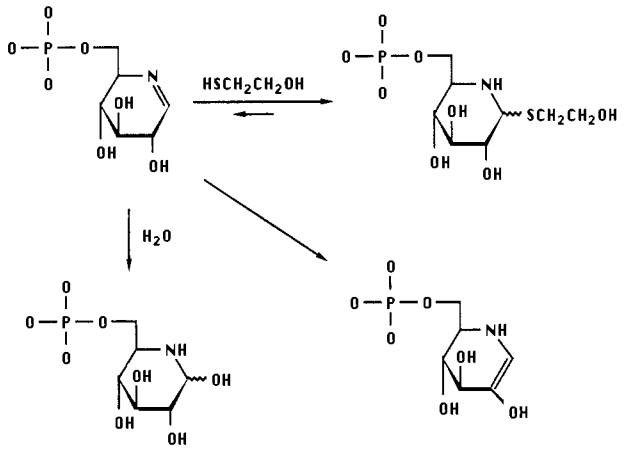
(3) The cyclical conversion of N6P and thus the catalytic effect on the dephosphorylation of PGM and GBP is supported by the observation that the observed GBP turnover is up to 19 times greater than the initial N6P concentration.

(4) However, the catalytic turnover with a large excess of GBP is limited to about 19 times the initial N6P concentration. The lack of additional processing of G6P is not due to some irreversible inactivation of the phosphoglucomutase or the transformation of the GBP to something other than G6P. Figure 1 clearly demonstrates that after G6P production has ceased, it can be resumed by a second addition of N6P. Therefore, it can be concluded that the N6P is periodically, but irreversibly, converted to something that cannot be processed by this enzyme at a reasonable rate. This loss of catalytic activity is probably related to rearrangements that are known to occur with nojirimycin in acidic or basic conditions. All of these rearranged products are initiated from the imine intermediate and are illustrated in Scheme III (Inouye et al., 1968; Paulsen & Todt, 1968). Similar schemes

are now proposed for the imine of nojirimycin 6-phosphate.

(5) Alternatively, the limiting turnover ratio of 19 could be due to the actual formation of nojirimycin 1-phosphate in the active site. The release of nojirimycin 1-phosphate into solution followed by the irreversible loss of phosphate would break the catalytic cycle after nojirimycin is formed. However, this would require that the nojirimycin 1,6-bisphosphate intermediate (II) transfer the phosphoryl group at C-6 to the enzyme faster than the loss of phosphate at C-1. This is unlikely because it has been demonstrated that when the $^{32}P$-labeled N6P is incubated with PGM and GBP, none of the label is ultimately found in the G6P. If the $^{32}P$ label is ever transferred to the enzyme, then a signficant fraction of this label would eventually be found in the G6P/G1P pool.

(6) Additional support for the formation of the imine intermediate in the bulk solution comes from the trapping experiments with β-mercaptoethanol. Figure 4 shows that as the concentration of β-mercaptoethanol increases, the rate for the formation of the G6P from GBP becomes slower. This result is due to the nucleophilicity of the β-mercaptoethanol. When the imine intermediate is formed, the anomeric carbon is attacked by the thiol group of the mercaptoethanol.




The thioether product is in equilbrium with the imine of N6P and β-mercaptoethanol. Even though the overall rate for G6P production becomes very slow in the presence of β-mercaptoethanol, the total conversion of GBP is independent of the β-mercaptoethanol concentration. When the imine is slowly reformed by the loss of β-mercaptoethanol, the imine is again partitioned back to N6P via hydration or irreversibly lost through rearrangement. The regenerated N6P then recycles through the reaction pathway until it eventually becomes the product of the irreversible rearrangement.

The following model can be used to quantitatively evaluate the partitioning of the nojirimycin 6-phosphate intermediates

$$\mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{B} \xrightarrow{k_3} \mathrm{C} \qquad (1)$$
$$\mathrm{B} \rightarrow \mathrm{P}$$

where A is N6P, B is the imine intermediate, P is G6P, and C is the product of the irreversible Amadori rearrangement.

When GBP is saturating and the initial concentration of N6P is lower than its $K_M$ value, the rate of formation for G6P is proportional to the concentration of N6P and enzyme. It can be shown that the partitioning ratio between $k_2$ and $k_3$ can be calculated from the $P/A_0$ ratio when $t$ is very large.

$$P/A_0 = (k_2 + k_3)/k_3 \qquad \text{when } t = \infty \qquad (2)$$

The expression for the initial velocity ($dP/dt$) can be obtained from

$$\frac{1}{dP/dt} = \frac{1}{A_0(k_2 + k_3)} + \frac{1}{A_0 k_1} \qquad (3)$$

where $k_1$ is proportional to the enzyme concentration ($k_1 = k_1'E$). When 2.34 and 4.7 μM N6P are incubated with 0.3 mM GBP and PGM, the partitioning ratios calculated by using eq 2 are 19 and 20, respectively. This result indicates that for every 19 cycles one N6P becomes nonreactive by the irreversible Ammadori rearrangement. Therefore, the hydration rate is 19 times faster than the Amadori rearrangement. The double-reciprocal plot for the initial velocity of G6P formation versus the PGM concentration intersects the vertical axis at the origin. This indicates that the nonenzymatic rehydration ($k_2$) and the Amadori rearrangement ($k_3$) are much faster than the enzymatic conversion since saturation was not achieved at concentrations of enzyme that are convenient to use as predicted by eq 3.

*Summary.* Nojirimycin 6-phosphate has been shown to be a potent inhibitor of phosphoglucomutase. The inhibitory mechanism is consistent with the substrate-induced dephosphorylation of the essential phosphoenzyme intermediate.

## ACKNOWLEDGMENTS

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