GDP-Mannose Mannosyl Hydrolase Catalyzes Nucleophilic Substitution at Carbon, Unlike All Other Nudix Hydrolases†

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ABSTRACT: GDP-mannose mannosyl hydrolase (GDPMH) from *Escherichia coli* is a 36.8 kDa homodimer which, in the presence of Mg^{2+}, catalyzes the hydrolysis of GDP-α-D-mannose or GDP-α-D-glucose to yield sugar and GDP. On the basis of its amino acid sequence, GDPMH is a member of the Nudix family of enzymes which catalyze the hydrolysis of nucleoside diphosphate derivatives by nucleophilic substitution at phosphorus. However, GDPMH has a sequence rearrangement (RE to ER) in the conserved Nudix motif and is missing a Glu residue characteristic of the Nudix signature sequence. By ^1H NMR, the initial hydrolysis product of GDP-α-D-glucose is β-D-glucose, indicating nucleophilic substitution with inversion at C1′ of glucose. Substitution at carbon was confirmed by two-dimensional $^1H-^{13}C$ HSQC spectra of the products of hydrolysis in 48.4% ^{18}O-labeled water which showed an additional C1′ resonance of β-D-glucose with a typical upfield ^{18}O isotope shift of 18 ppb and an intensity of 47.6% of the total signal. No ^{18}O isotope-shifted resonances (<4%) were found in the ^{31}P NMR spectrum of the GDP product. Thus, unlike all other Nudix enzymes studied so far, GDPMH catalyzes nucleophilic substitution at carbon rather than at phosphorus. A small solvent kinetic deuterium isotope effect on k_{cat} of 1.76 ± 0.25, independent of pH over the range of 6.0−9.3, suggests that the deprotonation of water may be part of the rate-limiting step.

GDP-mannose mannosyl hydrolase (GDPMH)[1] from *Escherichia coli* is a member of the Nudix family of enzymes which catalyze the hydrolysis of nucleoside diphosphate derivatives (NDP-X), and contain the consensus sequence **G**$(X)_5$E$(X)_7$**RE**UXE**E**XGU, where U is a hydrophobic residue (*1*). The NMR-determined solution structure of the prototypical Nudix enzyme, MutT nucleoside triphosphate pyrophosphohydrolase, shows that this consensus sequence forms a novel loop−helix−loop motif, which serves as the binding site for an essential divalent cation and the triphosphate moiety of the dNTP substrate (*2*). Site-directed mutagenesis studies of the MutT enzyme have established that this region contains four key catalytic residues (*3*−*5*) indicated in bold type above. Heretofore, on the basis of the reaction products and on the basis of direct ^{18}O incorporation studies, Nudix hydrolases were all shown to catalyze nucleophilic substitutions at phosphorus (*6*−*8*).

In the presence of Mg^{2+}, the Nudix enzyme GDPMH catalyzes the reactions

$$\text{GDP-α-D-mannose} + H_2O \rightarrow \text{GDP} + \text{mannose} + H^+ \quad (1)$$

$$\text{GDP-α-D-glucose} + H_2O \rightarrow \text{GDP} + \text{glucose} + H^+ \quad (2)$$

While GDP-α-D-mannose is the likely biological substrate, the k_{cat}/K_m of GDP-α-D-glucose is very similar to that of GDP-α-D-mannose. Both the k_{cat} and K_m values of GDP-α-D-glucose are approximately 5-fold greater than those of GDP-α-D-mannose (*9*). From the literature, only one other enzyme has been described which releases a free sugar from a nucleotide sugar, a GDP-glucose hydrolase from yeast with a specific activity (0.19 unit/mg) (*10*) an order of magnitude lower than that of GDPMH (1.7 units/mg). A recently characterized human lysosomal α-mannosidase has a specific activity of 0.29 unit/mg (*11*).

GDPMH is a 160-residue enzyme (subunit molecular mass of 18 405 Da) which contains a modified Nudix signature sequence differing from that of the more than 450 other members of the family by an amino acid rearrangement, and a substitution for a conserved glutamate as shown below (where U is a hydrophobic residue):

38	44	52	53	54	56	57	59	60	
G	**E**	**R**	**E**	**U**	.	**E**	**E**	. **G U**	(Nudix Box)
G	**E**	E	**R**	U	.	.	A **E**	. **G U**	(GDPMH)

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[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; EDTA, (ethylenedinitrilo)tetraacetic acid; GDPMH, guanosine diphosphate mannose mannosyl hydrolase; HSQC, heteronuclear single-quantum coherence; IPTG, isopropyl β-D-thiogalactoside; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TPPI, time-proportional phase incrementation.

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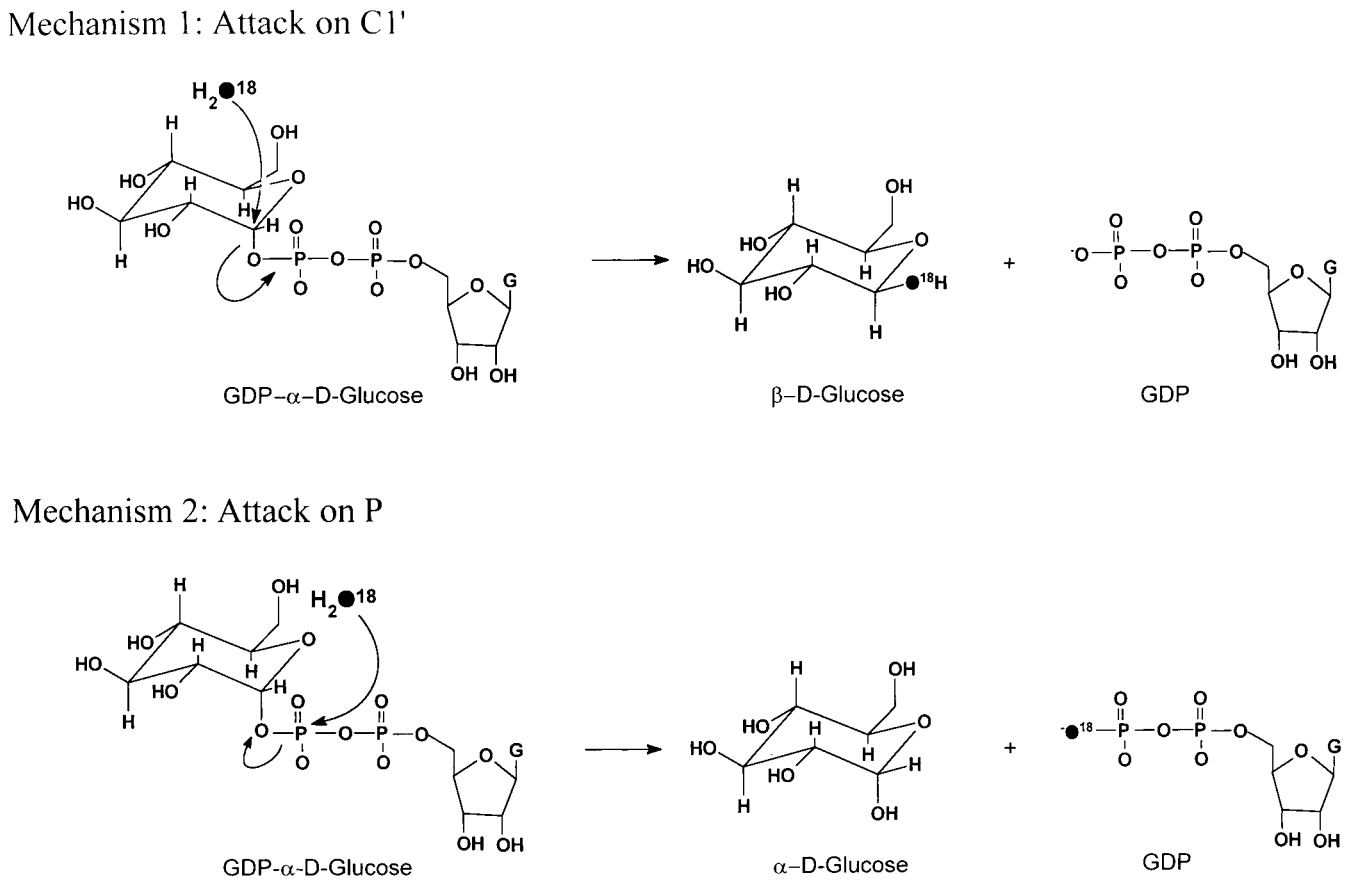

FIGURE 1: Alternative mechanisms and sites of bond cleavage for the GDPMH-catalyzed hydrolysis of GDP-α-glucose.

In the MutT-catalyzed pyrophosphohydrolysis of NTPs, Glu-53 (MutT sequence numbering shown above) positions and deprotonates the attacking metal-bound water molecule, and Arg-52 may orient Glu-53 (*2*, *3*, *5*).

The products of the GDPMH-catalyzed reactions could, in principle, result from nucleophilic substitution at either the β-phosphorus or C1′ of the GDP-sugar substrate (Figure 1). Substitution at C1′ is chemically more attractive than substitution at the β-phosphorus because GDP, in the presence of Mg^{2+}, is a better leaving group than mannose or glucose. However, in all known Nudix hydrolases, the attack is on phosphorus, resulting in cleavage of a pyrophosphate bond (*1*). For this reason, it was of interest to determine which mechanism was operative for GDPMH. A preliminary abstract of this work has been published (*12*).

EXPERIMENTAL PROCEDURES

Materials. GDP-α-D-glucose, GDP-α-D-mannose, GDP, and ultrapure $MgCl_2$ were purchased from Sigma (St. Louis, MO). The concentrations of GDP-α-D-mannose and GDP-α-D-glucose were measured by UV absorption at 252.5 nm using the extinction coefficient of guanosine ($\epsilon = 13.7\ mM^{-1}\ cm^{-1}$). The anomeric structure of GDP-α-D-glucose was confirmed by ^1H NMR at 600 MHz; the chemical shift of H1′ of the α-anomer was 5.58 ppm [d(d), $J = 3.5$, 7.2 Hz] and was similar to the H1′ chemical shift of GDP-α-xylose, 5.68 ppm [d(d), $J = 3.5$, 7.0 Hz] previously published (*13*): P_β −10.41 ppm [d(d), $J = 7$, 20 Hz], P_α −8.7 ppm [d(t), $J = 5$, 19.4 Hz]. The assignments and intactness of GDP were confirmed by ^{31}P NMR at 243 MHz: P_α −8.50 ppm [d(t), $J = 7$, 22 Hz], P_β −4.35 ppm [d, $J = 22$ Hz]. Sephadex G-50 fine was obtained from Pharmacia (Uppsala, Sweden). Calf intestinal alkaline phosphatase and AEBSF were from Boehringer Mannheim (1000 units/mL). ^{18}O-labeled water (98.5%) and deuterium oxide (99.9%) were from Isotec (Miamisburg, OH). Ultrafiltration concentrators were purchased from Vivaspin (Stonehouse, U.K.). The BCA Protein Assay Kit was purchased from Pierce (Rockford, IL). DTT was from GibcoBRL (Gaithersberg, MD). The construction of the pETorf1.9 vector was described previously (*9*).

GDPMH Preparation. The procedure of Frick et al. (*9*) was followed with slight modification to optimize yield. The pETorf1.9 vector was transformed into BL21(DE3) *E. coli*. Glycerol stocks of the transformed bacteria were made and stored at −80 °C. Half a liter of MOPS medium (*6*) containing 100 μg/mL ampicillin was innoculated with 10 mL of an overnight culture grown in LB medium (*14*), and grown at 37 °C with shaking at 250 rpm. When the cell density reached an A_{600} of 1.0, the culture was induced with 2 mM IPTG and grown for an additional 2 h. The cells were collected by centrifugation, weighed, and stored overnight at −80 °C. The cells were then resuspended in 10 volumes (w/v) of lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM sodium EDTA, 10% sucrose, 1 mM DTT, 0.1% Triton X-100, and 0.1 mg/mL AEBSF] and sonicated for 1 min. Cell debris was removed from the crude extract by centrifugation at 10000*g* for 30 min. Solid ammonium sulfate was added to attain 40% saturation, and the precipitate was collected and discarded. The ammonium sulfate concentration of the supernatant was raised to 60% saturation, and the precipitate which contained the GDPMH protein was dissolved in 2 mL of buffer A [50 mM Tris-HCl (pH 7.5) and

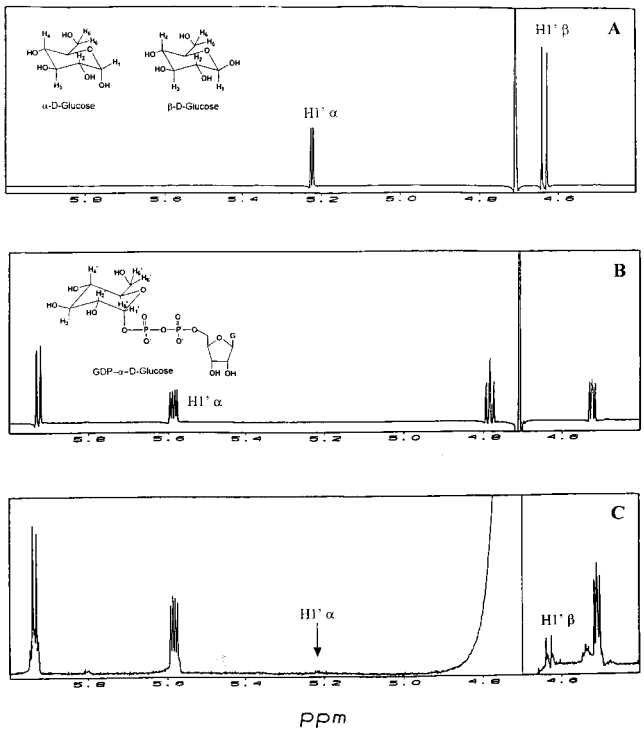

FIGURE 2: Downfield region of the one-dimensional ^{1}H spectra of substrate, reaction products, and standards. (A) Glucose at anomeric equilibrium showing H1′ of the α- and β-anomers. (B) GDP-glucose. (C) Reaction products at 15 min and 34 °C under the conditions described in text.

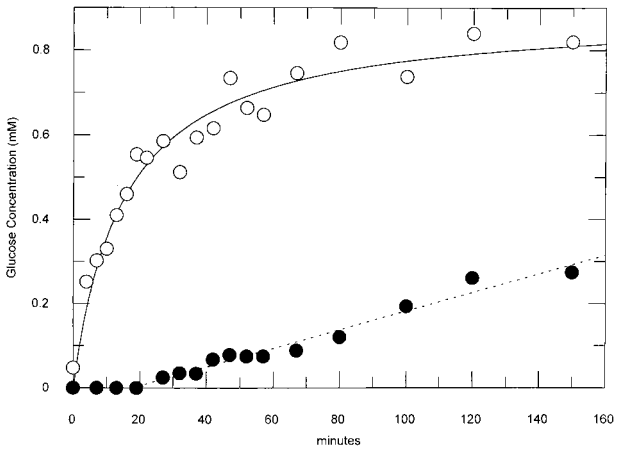

FIGURE 3: Glucose formation vs time in the presence of 99% D_2O and GDPMH. The specific activity measured from the initial slope of the curve was 1.03 units/mg at 34 °C. The open symbols refer to β-D-glucose and the filled symbols to α-D-glucose.

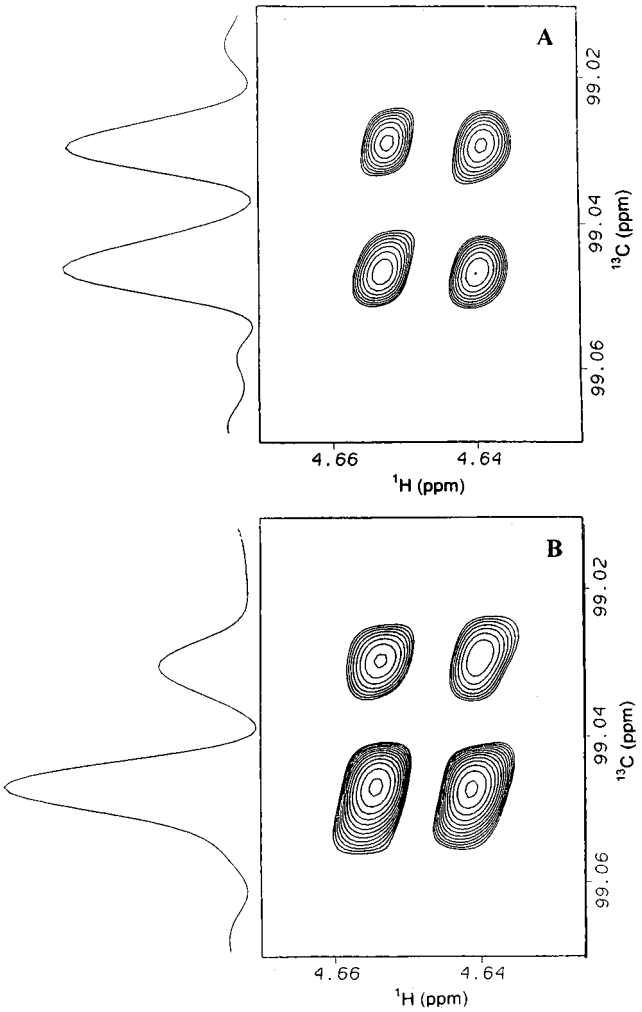

FIGURE 4: Expanded region of the two-dimensional ^{1}H−^{13}C HSQC spectrum of the reaction products formed in the presence of GDPMH and 48.4% $H_2{}^{18}O$. (A) ^{13}C1′ and ^{1}H1′ cross-peaks of β-D-glucose. (B) The same region after the addition of 1.8 mM unlabeled glucose.

Table 1: Solvent Deuterium Kinetic Isotope Effects on the GDPMH-Catalyzed Hydrolysis of GDP-α-D-Glucose at 34 °C

pH	$^Hk_{cat}$ (units/mg)	$^Dk_{cat}$ (units/mg)	$^Hk_{cat}/^Dk_{cat}$
6.0	0.45	0.29	1.55
6.7	1.20	0.80	1.50
7.5	1.72	0.95	1.81
8.0	2.10	1.26	1.67
8.5	1.94	1.05	1.85
9.3	1.86	0.85	2.19
			average = 1.76 ± 0.25

1 mM EDTA], loaded onto a gel-filtration column (Sephadex G-50 fine; 2.5 cm × 110 cm), and eluted with 200 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 1 mM sodium EDTA. Two major peaks were separated, the former containing large molecular weight impurities (42−46 kDa), and the latter, corresponding to a molecular mass of 39 kDa, contained the GDPMH activity. Active fractions containing the highly purified GDPMH (>98% pure as judged by SDS−PAGE) were pooled (36 mL) and concentrated by ultrafiltration (Vivaspin, 10 000 molecular weight cutoff, polyethersulfone membrane).

Assay of GDPMH. The enzyme assay is based on the formation of inorganic orthophosphate in a coupled system containing both GDPMH and calf intestinal alkaline phosphatase, as previously described (*9*). Phosphate was quantified by the method of Ames and Dubin (*15*), and protein was quantified using a bicinchoninic acid assay kit (Pierce) with bovine serum albumin as a standard. A unit of activity is defined as 1 μmol of GDP-α-D-mannose hydrolyzed per minute.

Molecular Properties of GDPMH. The native molecular weight of GDPMH was determined by its elution volume from a Sephadex G-50 fine column calibrated with ovalbumin and lysozyme as molecular weight standards. The subunit molecular weight was determined by SDS−PAGE in reference to molecular weight standards, and independently by matrix-assisted laser desorption ionization time-of-flight

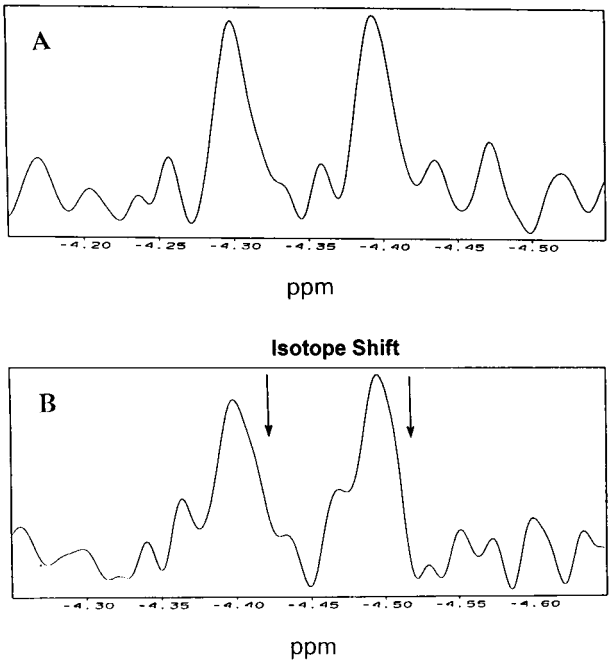

FIGURE 5: Expanded region of the ^{31}P NMR spectrum of GDP showing the P_β doublet. (A) Unlabeled standard solution (10 mM). (B) Reaction product formed in the presence of 47.4% $H_2{}^{18}O$ and GDPMH. The downfield shift of the P_β doublet is due to the presence of the $MgCl_2$ (5 mM) in the reaction mixture. The arrows indicate where an upfield isotope shift of 22 ppb would have been seen.

mass spectrometry using saturated sinapinic acid and 10.6% formic acid in 50% methanol.

Preparation of NMR Samples. For the NMR studies, the enzyme was exchanged with the Vivaspin ultraconcentrators into NMR buffer [5.4 mM deuterated Tris-d_{11}-DCl (pH 7.5), 18 mM NaCl, 0.3 mM NaN_3, 10 mM DTT, and 0.1 mg/mL AEBSF]. For field/frequency locking and to avoid a strong HDO signal, 99.9% D_2O was present in the reaction mixtures. Stock solutions of ultrapure $MgCl_2$ and GDP-α-D-glucose used in the one-dimensional ^{1}H NMR experiment were lyophilized once and redissolved in 99.9% D_2O. The pH values of buffer solutions were measured in H_2O prior to lyophilization and redissolved in 99.9% D_2O.

NMR Spectroscopy. All NMR experiments were carried out at 34 °C on a Varian Unity Plus 600 MHz NMR spectrometer equipped with a pulse field gradient unit and four independent RF channels. The proton and natural abundance carbon spectra were collected with a Varian 5 mm triple-resonance probe with an actively shielded *z*-gradient. The phosphorus spectra were collected with a Varian 5 mm broadband probe. The States−TPPI method was employed in the indirect dimension for the natural abundance two-dimensional ^{1}H−^{13}C HSQC spectra (*16*).

One-Dimensional ^{1}H NMR Spectroscopy. Because the k_{cat} value for GDP-α-D-glucose was 5-fold greater than that for GDP-α-D-mannose (*9*), and the competitive spontaneous anomerization reaction, GDP-α-D-glucose was used as the substrate for the NMR studies. The GDPMH reaction was initiated by adding 11 milliunits of GDPMH to an NMR tube containing 0.6 mL of 99.9% D_2O, 5 mM GDP-α-D-glucose, 25 mM ultrapure $MgCl_2$, and 5 mM deuterated Tris-d_{11}-DCl (pH 7.5). Each spectrum consisted of 16 transients with a spectral width of 8000 Hz using 32 000 complex points and a recycle time of 8 s. The first spectrum was obtained in 6.5 min, and subsequent spectra were recorded every 3 min for 36 min and then at larger intervals (5, 20, and 30 min) for a total of 154 min. A one-dimensional ^{1}H spectrum was also collected after 24 h to verify that the glucose α- and β-anomers had come to equilibrium (33% α and 67% β). The NMR data were processed and analyzed on an Indigo Silicon Graphics workstation using the Felix 2.3 software package (Molecular Simulations, Inc.). For the one-dimensional spectra, a 35° sine-bell window function was applied to 16 000 points before Fourier transformation and baseline correction. Determination of the peak intensities of the α- and β-proton resonances was achieved after peak simulation using the pick peak/peak optimization protocol in Felix 2.3.

^{31}P NMR Spectroscopy. The GDPMH reaction (1.0 mL) was carried out in 47.4% ^{18}O-labeled water, 10% D_2O, 20 mM GDP-α-D-glucose, 5 mM ultrapure $MgCl_2$, 5 mM Tris-d_{11}-DCl (pH 7.5), and 0.75 unit of GDPMH for 19 h at room temperature (23.5 °C). To the reaction mixture was added 12 μL of 0.5 M sodium EDTA to stop the reaction. The sample was then passed through three 200 μL Chelex [5 mM Tris-d_{11}-DCl (pH 7.5) and 99.9% D_2O] columns to remove trace amounts of paramagnetic metal ions. To avoid sample dilution, 100 μL of the reaction mixture was initially used to replace the buffer in the void volume of the Chelex column, and discarded. The sample was then lyophilized and redissolved in 99.9% D_2O. The GDP product of the reaction was analyzed by one-dimensional ^{31}P NMR spectroscopy as follows: sweep width of 12 000 Hz, 66 000 complex points, 790 transients, and a recycle time of 5.8 s. The spectrum was processed by applying a 35° window function on 1024 real data points and zero-filled to 32 000 points. The spectra were referenced to 77.4% H_3PO_4 containing 10% D_2O.

Two-Dimensional Natural Abundance ^{1}H−^{13}C Heteronuclear NMR Spectroscopy. The two-dimensional ^{1}H−^{13}C HSQC spectra were recorded for a 0.6 mL reaction mixture containing 48.4% ^{18}O-labeled water, 10% D_2O, 5 mM GDP-α-D-glucose, 10 mM ultrapure $MgCl_2$, 5 mM deuterated Tris-d_{11}-DCl (pH 7.5), and 110 milliunits of GDPMH. The reaction was stopped, and the sample was treated as described above for ^{31}P NMR studies. The parameters of the spectrum were as follows: spectral widths of 2000 Hz (^{1}H, t_2, 1024 complex points) and 500 Hz (^{13}C, t_1, 1024 complex points), using 24 transients with the ^{1}H carrier frequency set to water (4.687 ppm at 34 °C) and the ^{13}C carrier frequency set to the ^{13}C resonance of C1′ of glucose (99.88 ppm).

Solvent Kinetic Isotope Effects. Initially, kinetic parameters (K_m and k_{cat}) for GDP-α-D-glucose hydrolysis were determined in H_2O and in 90% D_2O at 34 °C in the presence of 80 mM Na HEPES buffer (pH 7.5), 20 mM $MgCl_2$, 18 mM NaCl, 0.5 unit of alkaline phosphatase, and 1.12 milliunits of GDPMH at six substrate concentrations ranging from 0.6 to 6.0 mM. Solvent kinetic deuterium isotope effects on k_{cat} were determined at 34 °C, using saturating levels of ultrapure $MgCl_2$ (20 mM) and GDP-α-D-glucose (10 mM) over the pH range of 6.0−9.3 using the following buffers: 80 mM MES (pH 6.0−6.7), Na HEPES (pH 7.5−8.0), glycinamide-HCl (pH 8.5), and glycine (pH 9.3) in the presence of 100% H_2O or 90% D_2O. After the pH of each buffer solution was adjusted in H_2O, buffers were lyophilized and redissolved in 99.9% D_2O. $MgCl_2$ and GDP-α-D-glucose were also

lyophilized once and redissolved in 99.9% D_2O. Other components present in the final reaction mixture were 18 mM NaCl, 0.09 mM EDTA, and 0.5 unit of calf intestinal alkaline phosphatase. The reactions were initiated by the addition of 1.54 or 2.16 milliunits of GDPMH in aqueous buffer. Rates were measured at 34 °C and were linear versus time and enzyme concentration during the 15 min assay. At all pH values, corrections for the spontaneous hydrolysis of GDP-α-D-glucose were made.

RESULTS AND DISCUSSION

Molecular Properties of GDPMH. The native molecular mass of GDPMH was 39 kDa on the basis of its elution from a calibrated Sephadex G-50 fine column. The subunit molecular mass, estimated by SDS−PAGE, and determined by mass spectrometry, was 18 472 ± 67 Da, consistent with its calculated molecular mass of 18 405 Da. Hence, the native enzyme is a dimer.

One-Dimensional ^1H NMR Studies. In the GDPMH-catalyzed reaction, two potential sites of bond cleavage were considered: mechanism 1, substitution at C1′ with inversion at the anomeric carbon; and mechanism 2, substitution at phosphorus with retention of configuration at C1′ of glucose (Figure 1). To distinguish between these two mechanisms, the reaction was followed by one-dimensional ^1H NMR using GDP-α-D-glucose as the substrate which has a k_{cat} that is 5-fold greater than that of GDP-α-D-mannose. The H1′ proton resonance of GDP-α-D-glucose was assigned on the basis of its chemical shift and on the basis of its coupling to H2′ and to the β-phosphorus of the GDP moiety (see Figure 2 and Experimental Procedures).

The appearance of the C1′ proton resonance of the resulting glucose product was monitored during the reaction and was assigned on the basis of its chemical shift and on the basis of its coupling to H2′ (Figure 2). Initially, the ^1H NMR spectrum of the GDP-glucose substrate with selective decoupling established that it was GDP-α-D-glucose on the basis of the ^3J(H1′−H2′) coupling of 3.5 Hz. In addition, the ^3J(H1′−P_β) coupling of 7.2 Hz was found, resulting in a doublet of doublets for the H1′ resonance. The reaction was initiated by the addition of GDPMH. A burst of the β-anomer of glucose [δ = 4.64 ppm, ^3J(H1′−H2′) = 7.9 Hz] was observed between 0 and 22 min with no appearance of the α-anomer (Figures 2 and 3). The rate of appearance of β-glucose, measured from the initial slope of the curve shown in Figure 3, was 1.03 units/mg (34 °C) after 4 min. This value was slightly lower than the expected activity based on the standard assay performed in H_2O due to a solvent kinetic deuterium isotope effect, which was confirmed by the standard assay (see below, Table 1).

After a lag of 20 min, the resonance of the α-anomer of glucose appeared [δ = 5.22 ppm, ^3J(H1′−H2′) = 4.0 Hz], and its magnitude slowly increased with time (Figure 3) with a half-time of 140 min. This value agrees with the half-time for spontaneous anomerization of glucose measured under similar conditions [$t_{1/2,reported}$ = 130 min (*17*)].

The initial formation of β-glucose from GDP-α-D-glucose establishes inversion at C1′ of the substrate. Hence, GDPMH catalyzes the direct attack by water at the C1′ of GDP-α-D-glucose, with no evidence for a covalent glucosyl−enzyme intermediate, as shown in mechanism 1 (Figure 1).

Two-Dimensional $^1H-^{13}C$ HSQC Studies. As an independent test of the site of bond cleavage in the GDPMH-catalyzed reaction, the products formed in 24 h at 23 °C in the presence of 48.4% $H_2{}^{18}O$ were studied by natural abundance ^{13}C NMR spectroscopy. If substitution occurred at the C1′ position of glucose, an ^{18}O isotope upfield shift in the carbon resonance at position 1′ would be observed (*18*). Indeed, such an isotope shift was observed (Figure 4A) with two resonances in the carbon dimension appearing at 99.0295 and 99.0472 ppm, with the attached proton centered at 4.648 ppm in the proton dimension with the appropriate ^3J(H1′−H2′) coupling of 7.8 Hz. To determine which carbon resonance was due to the ^{18}O isotope shift, [^{16}O]glucose was added to the reaction mixture to yield a final concentration of 1.8 mM. The $^1H-^{13}C$ HSQC spectrum collected after the addition then revealed an approximate doubling in intensity of the downfield doublet, indicating that the 18 ppb upfield shift in the C1′ resonance was due to the ^{18}O isotope shift (Figure 4B). An otherwise identical reaction carried out in the absence of $H_2{}^{18}O$ showed only the downfield doublet (data not shown). Volume integration of the cross-peaks in Figure 4A showed the upfield carbon resonances to consititute 47.6 ± 1.0% of the total signals, indicating 47.6 ± 1.0% ^{18}O incorporation, consistent with the solvent concentration of 48.4% ($H_2{}^{18}O$). This further supports nucleophilic substitution at C1′ of GDP-α-D-glucose in accord with mechanism 1 (Figure 1).

One-Dimensional ^{31}P NMR Studies. ^{18}O isotope shifts in ^{31}P NMR spectra provide a method for detecting sites of cleavage of bonds to phosphorus (*19*−*21*). After 19 h of GDPMH-catalyzed hydrolysis of GDP-α-D-glucose at 23 °C in 47.4% $H_2{}^{18}O$, the ^{31}P NMR spectrum revealed GDP as the only phosphorus-containing product. The β-phosphorus doublet of the GDP product at −4.35 ppm [$^2J(P_\alpha-P_\beta)$ = 22.7 Hz] was well-resolved and showed no evidence of an isotope shift (Figure 5). The arrows in Figure 5B show where a 22 ppb (5.4 Hz) upfield shift would have occurred, as was found for ADP β ^{18}O (*20*). Integrals of the resonances in Figure 5B, which improved the signal/noise ratio, indicated that no detectable ^{18}O (<4%) was bonded to the β-phosphorus. Similarly, no isotope shift of the α-phosphorus of the GDP product was found, in agreement with the ^1H and ^{13}C NMR studies (data not shown).

Solvent Kinetic Deuterium Isotope Effect in the GDPMH Reaction. Because the GDPMH-catalyzed reaction could only be monitored in D_2O by ^1H NMR, the effect of 90% D_2O on the velocity of the GDPMH-catalyzed hydrolysis of GDP-α-D-glucose (10 mM) was studied by the standard assay. Increasing the substrate concentration by 50% to 15 mM GDP-α-D-glucose resulted in a ≤10% increase in the rate over the pH range of 6.0−9.3, indicating approximate k_{cat} conditions. At pH 7.5, in the presence of 90% D_2O no kinetic isotope effect on the K_m of GDP-α-D-glucose was observed (0.83 ± 0.19 mM), but a solvent deuterium isotope effect on k_{cat} of 1.85 ± 0.22 was detected. Over the range of pH values from 6.0 to 9.3, a solvent kinetic isotope effect $^Hk_{cat}/{}^Dk_{cat}$ of 1.76 ± 0.25 was observed, showing no significant dependence on pH (Table 1). The rates were linear over time,

[2] T. K. Harris and A. S. Mildvan, unpublished oberservations, 2000.
[3] S. Desai, C. A. Dunn, and M. J. Bessman, unpublished observations, 2000.

indicating that the observed solvent kinetic isotope effect was not due to enzyme instability in the D_2O solvents. Therefore, the solvent kinetic isotope effect, while small, suggests the deprotonation of water in the rate-limiting step (*22*). On the basis of NMR and pH−rate studies, the homologous MutT enzyme uses a metal-bound hydroxide ion as the attacking nucleophile with deprotonation of a metal-bound water ligand as part of the rate-limiting step (*3*). A very similar solvent kinetic isotope effect of 1.8 ± 0.2 has been found with the MutT pyrophosphohydrolase.[2] Also, *N*-acetylornithine deacetylase, a metal-activated hydrolase with a similar mechanism, shows a solvent kinetic isotope effect of 2.1 (*23*). Solvent kinetic isotope effects are usually small, typically ranging from 2 to 3 (*24*). Like MutT, GDPMH requires Mg^{2+} for activity, suggesting that a metal-bound water may also be used in hydrolysis, and that deprotonation of this metal-bound water may be rate-limiting. The same metal or a second metal may assist the departure of the GDP leaving group.

CONCLUSIONS

Unlike all other Nudix hydrolases which catalyze nucleophilic attack on phosphorus and cleave a pyrophosphate bond, GDPMH catalyzes nucleophilic substitution at carbon rather than at phosphorus (mechanism 1, Figure 1), leaving the pyrophosphate moiety in GDP intact. The observed pH-independent solvent deuterium kinetic isotope effect on k_{cat} in the GDPMH-catalyzed reaction suggests that deprotonation of water is at least partially rate-limiting.

It is of interest to note that the unique attack on C1′ by GDPMH is not attributable to GDP-α-D-mannose being a sugar nucleotide and hence different from all other Nudix hydrolase substrates. We have recently discovered a new member of the Nudix family, having the canonical Nudix signature sequence, that hydrolyzes UDP-glucose to UMP and glucose 1-phosphate, cleaving the pyrophosphate bond.[3] It is reasonable to ask whether the different mechanism of GDPMH is related to the uncharacteristic arrangement of amino acids in its Nudix box. Site-directed mutants of the enzyme are being constructed to address this question.

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