The Catalytic Mechanism of Glucose 6-Phosphate Dehydrogenases: Assignment and ^{1}H NMR Spectroscopy pH Titration of the Catalytic Histidine Residue in the 109 kDa *Leuconostoc mesenteroides* Enzyme†

Michael S. Cosgrove,*,‡,§ Stewart N. Loh,‖ Jeung-Hoi Ha,‖ and H. Richard Levy‡

Department of Biology, Syracuse University, Syracuse, New York 13244, and Department of Biochemistry & Molecular Biology, SUNY Upstate Medical University, Syracuse, New York 13210

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ABSTRACT: The chemical shifts of the $C^{\epsilon 1}$ and $C^{\delta 2}$ protons of His-240 from the 109 kDa *Leuconostoc mesenteroides* glucose 6-phosphate dehydrogenase (G6PD) were assigned by comparing ^{1}H and ^{13}C spectra of the wild-type and mutant G6PDs containing the His-240 to asparagine mutation (H240N). Unambiguous assignment of the His-240 $^1H^{\epsilon 1}$ resonance was obtained from comparing ^{13}C-^{1}H heteronuclear multiple quantum coherence NMR spectra of wild-type and H240N G6PDs that were selectively labeled with $^{13}C^{\epsilon 1}$ histidine. The results from NOESY experiments with wild-type and H240N variants were consistent with these assignments and the three-dimensional structure of G6PD. pH titrations show that His-240 has a pK_a of 6.4. This value is, within experimental error, identical to the value of 6.3 derived from the pH dependence of k_{cat} [Viola, R. E. (1984) *Arch. Biochem. Biophys. 228,* 415−424], suggesting that the pK_a of His-240 is unperturbed in the apoenzyme despite being part of a His-Asp catalytic dyad. The results obtained for this 109 kDa enzyme indicate that ^{1}H NMR spectroscopy in combination with heteronuclear methods can be a useful tool for functional analysis of large proteins.

Glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49)[1] from *Leuconostoc mesenteroides* is a 109 632 dalton enzyme that catalyzes the oxidation of glucose 6-phosphate (G6P) using NAD^+ or $NADP^+$. It has been cloned and expressed in *Escherichia coli* (*1*), and the X-ray crystal structure has been solved to 2.0 Å resolution (*2*). The protein is a dimer of identical 54 kDa subunits. Each subunit is composed of two domains: a coenzyme binding domain with a classic Rossman fold, and a large $\beta+\alpha$ domain that contains an antiparallel nine-stranded β-sheet that makes up part of the dimer interface (Figure 1). The pocket that is formed by the space between domains makes up the active site and is lined by conserved residues.

Site-directed mutagenesis has been used to identify residues involved in the catalytic mechanism of G6PD (*5*), and in binding ligands (*5*−*8*). His-240 is the general base that abstracts a proton from the C1-OH of G6P, allowing transfer of the C1 hydride to the C4 position of the nicotinamide ring of the coenzyme (*5*). From the three-dimensional structure of G6PD, His-240 $N^{\epsilon 2}$ is optimally positioned for proton abstraction, while its $N^{\delta 1}$ is hydrogen-bonded to the $O^{\delta 1}$ of Asp-177, forming a His-Asp catalytic dyad (*2*, *9*). Replacement of His-240 with asparagine by site-directed mutagenesis results in almost a 10^5-fold decline in k_{cat}, while replacement of Asp-177 with asparagine results

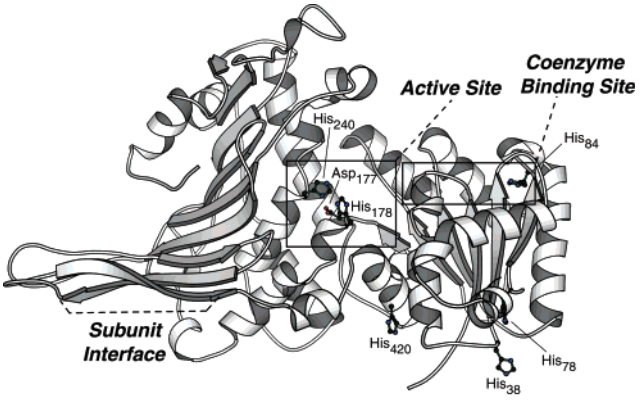

FIGURE 1: Ribbon diagram of the X-ray crystal structure of *L. mesenteroides* G6PD. One subunit of the dimer is shown with positions of the six histidine residues indicated. The general areas of the coenzyme binding domain, the dimer interface, and the active site are indicated. Nitrogen atoms are indicated in blue, and oxygen atoms are red. Aspartate 177 was added for reference. This ribbon diagram was generated using RASMOL (*3*) and MOLSCRIPT (*4*) using coordinates from Rowland et al. (*2*) (Protein DataBank code: 1DPG).

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* Correspondence should be addressed to this author at the Department of Biophysics and Biophysical Chemistry, Johns Hopkins School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205. Phone: (410) 955-3967; Fax: (410) 614-8648; E-mail: coz@cozzy.med.jhmi.edu.

‡ Syracuse University.

§ Current address: Department of Biophysics and Biophysical Chemistry, Johns Hopkins School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.

‖ SUNY Upstate Medical University.

[1] Abbreviations: G6PD, glucose 6-phosphate dehydrogenase; G6P, glucose 6-phosphate; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid; DEP, diethyl pyrocarbonate; TSP, 3-(trimethylsilyl)propionic acid-*2,2,3,3-d*; HMQC, heteronuclear multiple quantum coherence; RMSD, root-mean-square deviation.

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in a 10^2-fold decline (*5*). Additional structure−function studies showed that the loss of activity in D177N G6PD was associated with the absence of the negatively charged functional group provided by the Asp at position 177 in the wild-type enzyme (*9*).

Studies on the His-Asp catalytic dyad from various enzymes suggest that the function of the aspartate is not general. For example, investigations of the His-Asp catalytic dyads of rat trypsin (*10*) and ribonuclease (*11*) suggest that the aspartate functions to stabilize the productive tautomer of the catalytic histidine of the dyad. However, similar studies with mandelate racemase (*12*), and glucose 6-phosphate dehydrogenase (*9*), suggest that the aspartate functions to affect the pK_a of the histidine, making it a better general base. One would expect the negative electrostatic potential of the aspartate to stabilize the protonated form of histidine, thus increasing its pK_a. Consistent with this, kinetic studies with mandelate racemase suggest that removal of Asp-270 results in the depression of the pK_a of the (*R*)-specific acid/base catalyst, His-297, from the positive electrostatic potential of the active site (*12*). Similar experiments with G6PD were less clear; removal of Asp-177 in *L. mesenteroides* G6PD resulted in the disappearance of a pK_a at pH 4.5 in the k_{cat}/K_m profile, and the appearance of a group ionizing at pH 6.8 (*9*), suggesting either that the Asp functions to lower the pK_a of the catalytic base His-240 or that the pK_a at 4.5 reflects the ionization of Asp-177. It would be of interest, therefore, to determine the microscopic pK_a of His-240 in wild-type and D177N G6PDs to distinguish these possibilities and to better assess the impact of the aspartate on the pK_a of the histidine in the His-Asp catalytic dyad.

In this investigation, we report the determination of the pK_a of His-240 in wild-type *L. mesenteroides* G6PD, using the H240N mutant to assign the His-240 $^1H^{\epsilon 1}$ and $^{13}C^{\epsilon 1}$ chemical shifts in ^{13}C-^1H HMQC NMR spectroscopy experiments. Despite the large size of the protein, the proton histidine resonances in the wild-type enzyme are sufficiently resolved over the pH range 5.3−8.6, allowing the determination of the pK_a for His-240 from ^1H NMR spectroscopy experiments. Comparison of wild-type and H240N ^1H NMR spectra suggests the assignment for His-240 $^1H^{\delta 2}$ which produces NOE connectivities consistent with the three-dimensional structure of G6PD. Replacement of Asp-177 with asparagine results in line broadening or shifting such that the pK_a of His-240 in the D177N enzyme could not be determined.

MATERIALS AND METHODS

Materials. Coenzymes were obtained from Boehringer Mannheim; Matrex gel Purple A, Matrex gel Orange B, and CF-50 Centriflo membrane cones from Amicon Corp.; G6P from Sigma; Coomassie protein assay reagent and bovine serum albumin standard from Pierce; Sequenase Version 2.0 DNA sequencing system and Sculptor in vitro mutagenesis system from Amersham International; Prep-A-Gene DNA purification system from Bio-Rad; pUC-19, M13 bacteriophage, T4 polynucleotide kinase, and DNA ligase from Gibco-BRL; and restriction endonucleases from New England Biolabs. NaOD, DCl, and D_2O were obtained from Aldrich. The oligonucleotide for site-directed mutagenesis was synthesized by Ransom Hill Bioscience Inc. The oligonucleotide sequence was as follows (change from wild-type sequence underlined):

H240N: GATTCAAAAC<u>A</u>ACACCATGC

Site-Directed Mutagenesis. All standard DNA techniques were performed as described by Sambrook et al. (*14*) and as previously described (*7*). Site-directed mutagenesis was performed with the Amersham in vitro mutagenesis system, using the oligonucleotide listed above. The H240N mutation was constructed in M13 bacteriophage, subcloned into pUC19, and transformed into *E. coli* strain SU294, which lacks the G6PD gene (*6*). The plasmid was isolated and the entire G6PD gene was sequenced to ensure that no other mutations were introduced.

Purification and Assay of G6PD Activity. Recombinantly expressed wild-type and H240N G6PDs were purified to homogeneity as described previously (*5*). Routine assays for G6PD activity were performed at 25 °C in a Gilford 240 spectrophotometer at 340 nm by following the rate of appearance of NADPH. Assays were initiated by the addition of enzyme to 1.0 mL of 33 mM Tris-HCl, pH 7.8, containing 2.28 mM G6P and 0.160 mM $NADP^+$. Protein concentrations were determined from the extinction coefficient of wild-type G6PD at 280.5 nm (*13*).

Preparation of NMR Spectroscopy Samples. Purified wild-type and H240N G6PDs were concentrated with an Amicon stir pressure concentrator using a YM10 membrane and then dialyzed once against 50 mM Tris-HCl (pH 7.8), once against 10 mM Tris-HCl (pH 7.8), and 3 times against doubly distilled water. The proteins were lyophilized and taken up in 100% D_2O (Aldrich), and the pH was adjusted to ∼9.0 with NaOD. The samples were then incubated for 6 h at 37 °C to facilitate amide proton exchange and lyophized 2 more times. The sample was then dissolved in 10 mM unbuffered 2-(*N*-morpholino)ethanesulfonic acid (MES) in 100% D_2O to a final concentration of ∼0.5 mM (subunit concentration). The pH of the samples treated in this way was ∼6.0 (uncorrected for the deuterium isotope effect).

Temperature Dependence of Stability and Activity. The ^1H NMR spectrum of wild-type G6PD at 25 and 37 °C showed that the higher temperature resulted in sharper peaks with no adverse effect on solubility or catalytic activity (data not shown). At 42 °C, however, the protein solution became significantly cloudy due to protein precipitation. An apparent T_m of ∼52 °C was determined by incubating wild-type G6PD (25 mg/mL) at different temperatures for 30 min and assaying for remaining activity at 25 °C. The experiments reported here were carried out at 35 °C to minimize the possibility of aggregation due to thermal denaturation.

^1H NMR Spectroscopy. NMR spectra were recorded at 35 °C using a Bruker DRX500 spectrometer operating at 500 MHz for ^1H. 2D NOESY spectra were acquired with a 150 ms mixing time. A presaturation pulse was applied during the 1.7 s relaxation delay to suppress residual ^1H_2O. Each spectrum consisted of 400 t_1 increments (128 scans) of 4096 t_2 points. Prior to Fourier transformation, each dimension was apodized by a squared sine-bell function and zero-filled to a final matrix size of 4096 × 2048 ($t_2 \times t_1$). Data processing was performed using the XWINNMR software package (Bruker Instruments). Chemical shifts are

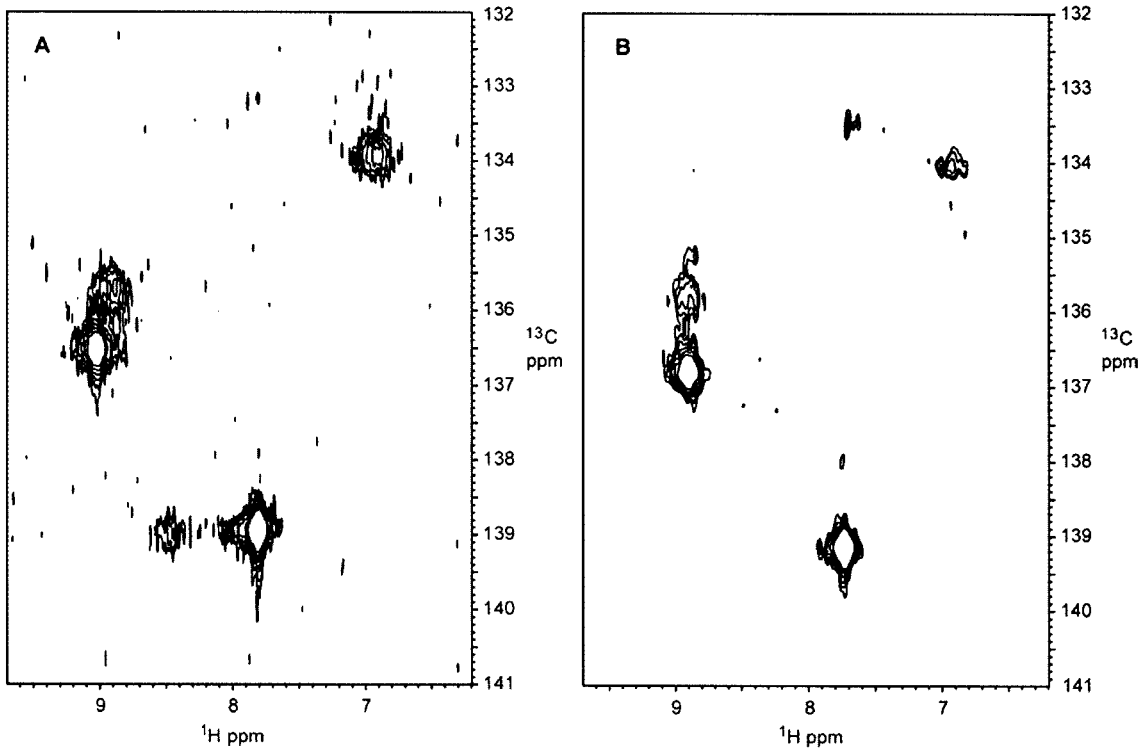

FIGURE 2: 600 MHz ^{13}C-^{1}H HMQC spectra of G6PD WT (A) and H240N (B), recorded in 99% ^2H_2O, pH* 5.64 (glass electrode reading uncorrected for the deuterium isotope effect).

referenced to an internal standard of 3-(trimethylsilyl)-propionic acid-*2,2,3,3-d* (TSP).

$^{13}C^{\epsilon 1}$-Histidine Labeling and ^{13}C NMR Spectroscopy. To increase protein yields for ^{13}C labeling, the G6PD gene was inserted into the T7 expression vector pET23a (Novagen). $^{13}C^{\epsilon 1}$-His-labeled G6PD was expressed in BL21(DE3) *E. coli* cultures, grown in minimal M9 medium (*14*) containing 100 mg/L $^{13}C^{\epsilon 1}$-L-histidine (99% enriched; Cambridge Isotopes Laboratories, Inc.). Cultures were induced by the addition of 100 mg/L isopropyl-β-D-thiogalactopyranoside at A_{600} = 1.0 and allowed to express for 12 h at 37 °C. Protein was purified as described (*5*). NMR spectroscopy samples were prepared by desalting G6PD samples into 10 mM MES, 99% ^2H_2O using a Sephadex G10 spin column. ^{13}C-^{1}H HMQC spectra were acquired at 35 °C on a Bruker DRX600 spectrometer. Protein concentration was 0.3−0.5 mM.

pH Titrations. The pH was adjusted by the addition of 0.1 M NaOD or 0.1 M MES (free acid) in 100% D_2O. All pH measurements are glass electrode readings, made at room temperature and not corrected for the deuterium isotope effect (indicated throughout as pH*). pH* values were measured immediately before and immediately after obtaining spectra. The reported pH* is the average of the two measurements, which never deviated more than 0.1 pH* unit. Chemical shifts are referenced to internal TSP. Curve-fitting was carried out using the PRISM program (GraphPad Software). pK_a* is used to denote the acid dissociation constant uncorrected for the deuterium isotope effect.

RESULTS

Unambiguous Assignment of the His-240 $^1H^{\epsilon 1}$ Resonance. *L. mesenteroides* G6PD contains a total of six histidine residues per subunit (*15*), two of which are conserved and located in the active site. The others are all located in or near the coenzyme binding domain of the protein (Figure 1). Figure 2 compares ^{13}C-^{1}H HMQC spectra for wild-type and H240N G6PDs that have been selectively labeled with $^{13}C^{\epsilon 1}$-L-histidine. In the wild-type spectrum, peaks are observed for five of the six histidines. When His-240 is replaced with asparagine, the cross-peak at 8.5, 138.9 ppm disappears from the H240N spectrum. The three-dimensional structures of wild-type and H240N G6PDs were determined previously (*5*), and show that replacing His-240 with asparagine does not affect the overall three-dimensional structure of G6PD (main chain RMSD = 0.3 Å). A similar conclusion is reached when comparing the wild-type and H240N G6PDs in solution as their ^{1}H NMR spectra are highly similar (shown for the aromatic region in Figure 3). These results indicate that since the structures of the wild-type and H240N enzymes are highly similar, the differences in the HMQC spectra can only be accounted for by the mutation. Therefore, the ^{13}C-^{1}H chemical shift for His-240 is unambiguously assigned.

Assignment of His-240 $^1H^{\delta 2}$ Suggested by Comparison of Wild-Type and H240N Spectra. Figure 3 compares the ^{1}H NMR spectra of the aromatic region of wild-type G6PD with that of the H240N enzyme at pH* 7.8. The largest difference occurs at 7.74 ppm, consistent with the chemical shift of a histidine δ2 proton. Comparison of NOESY spectra of fully deuterated wild-type and H240N G6PDs shows a weak NOE cross-peak between peaks at 7.75 and 8.49 ppm in the wild-type spectrum that is missing in the H240N spectrum, suggesting intramolecular $^1H^{\delta 2}$-$^1H^{\epsilon 1}$ dipolar coupling (Figure 4). Additional NOE connectivities consistent with the three-dimensional structure of G6PD suggest that the His-240 $^1H^{\delta 2}$ assignment is correct. An NOE cross-peak can be observed between the putative His-240 $^1H^{\delta 2}$ resonance at 7.75 ppm and two protons in the aromatic region (6.6 and 6.1 ppm).

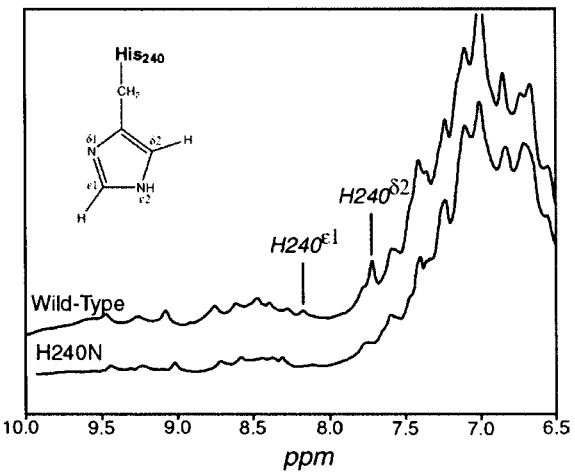

FIGURE 3: ^{1}H NMR spectra (500 MHz) of aromatic regions of wild-type *L. mesenteroides* G6PD and the H240N mutant at 35 °C. The protein concentration is 0.5 mM (subunit concentration) in 50 mM Tris (pH* 7.8) and 100% ^2H_2O. Each spectrum is the sum of 1024 accumulations. The assignment for His-240 $^1H^{\delta 2}$ is indicated at 7.74 ppm. The assignment for His-240 $^1H^{\epsilon 1}$ is indicated at 8.25 ppm.

Additionally, an NOE cross-peak is observed between the His-240 $^1H^{\epsilon 1}$ resonance at 8.5 ppm and another proton in the histidine epsilon region of the spectrum at 9.15 ppm (Figure 4). The crystal structure (*2*) reveals that the only aromatic residues within 8 Å of His-240 are His-178 and Tyr-179 (Figure 5): Tyr-179 $C^{\epsilon 1}$ and $C^{\delta 1}$ are, respectively, 3.73 and 3.94 Å distant from His-240 $C^{\delta 2}$, and His-178 $C^{\epsilon 1}$ is 3.5 Å distant from His-240 $C^{\epsilon 1}$. Each of the NOE interactions described above is missing in the NOESY spectrum of H240N (Figure 4).

pK$_a$* *Determination.* pH* titrations were conducted by following the His-240 $^1H^{\epsilon 1}$ and $^1H^{\delta 2}$ chemical shifts over the pH* range 5.3−8.6 in one-dimensional ^{1}H NMR spectroscopy experiments (Figure 6). The assignments for His-240 $^1H^{\epsilon 1}$ at various pH* values were confirmed by a similar pH* titration of the ^{13}C-labeled sample in HMQC experiments over the pH* range 5.6−7.2 (signal-to-noise limitations prevented extending the range further, not shown). Figure 7 shows plots of the wild-type G6PD His-240 $^1H^{\delta 2}$ and $^1H^{\epsilon 1}$ chemical shifts as a function of pH*. p*K*$_a$* values were determined by three-parameter fits of the following equation, which is derived from a simple acid dissociation equilibrium:

$$\delta_{obs} = \frac{\delta_{HA} + \delta_{A^-} \times 10^{pH-pK_a}}{1 + 10^{pH-pK_a}}$$

where δ_{HA} is the chemical shift in the acidic pH limit and δ_{A^-} represents the chemical shift in the basic pH limit.

The fitted p*K*$_a$* values for His 240 $^1H^{\delta 2}$ and $^1H^{\epsilon 1}$ are (±SD) 6.3 ± 0.1 and 6.4 ± 0.1, respectively. These values are within error identical as required for protons from the same amino acid, and are typical for that of histidine in solution (*16*).

DISCUSSION

Assignment of His-240 $^1H^{\epsilon 1}$ and $^1H^{\delta 2}$ Resonances. In this investigation, we have assigned the $^1H^{\delta 2}$ and $^1H^{\epsilon 1}$ chemical shifts for His-240 in wild-type G6PD. The His-240 $^1H^{\delta 2}$ chemical shift was assigned by comparing ^{1}H NMR spectra of wild-type and H240N enzymes. The overall ^{1}H NMR spectra for wild-type and H240N G6PDs were similar, indicating that changing His-240 to asparagine does not alter the overall conformation of the protein. This conclusion is supported by the fact that the X-ray structures of both enzymes are nearly identical [main chain RMSD = 0.3 Å (*5*)]. In the aromatic region of the NMR spectrum (Figure 3), the largest difference occurs at 7.74 ppm at pH* 7.8 where a peak is missing in the H240N spectrum. We have assigned this peak to His-240 $^1H^{\delta 2}$ based on its disappearance from the H240N spectrum, and two additional pieces of evidence. First, it titrates with an apparent p*K*$_a$* very similar to that of His-240 $^1H^{\epsilon 1}$, and it exhibits a narrow 0.05 ppm range between ionized and un-ionized forms (consistent with a $\delta 2$ proton). Second, it displays NOE connectivities consistent with the known three-dimensional structure of G6PD (Figure 4).

Comparison of the ^{1}H NMR spectra of wild-type and H240N G6PDs in the histidine epsilon region reveals other changes that prevent unambiguous assignment of the His-240 $^1H^{\epsilon 1}$ (Figure 3). To obtain this assignment, selectively labeled ^{13}C-histidine samples were produced and used to compare two-dimensional ^{13}C-^{1}H HMQC spectra of wild-type and H240N G6PDs. These experiments with the wild-type enzyme at pH* 5.6 showed a cross-peak at 8.5, 138.9 ppm that is missing in the H240N spectrum (Figure 2). This information unambiguously assigns the His-240 $^1H^{\epsilon 1}$ in ^{1}H NMR spectra of wild-type G6PD.

^{1}H NMR spectroscopy pH* titration of His-240 $^1H^{\epsilon 1}$ fits with a p*K*$_a$* of 6.4 ± 0.1 (Figure 7). The ^{1}H NMR spectroscopy assignments of the His-240 $^1H^{\epsilon 1}$ at different pH* values were confirmed with a similar titration with ^{13}C-labeled enzyme and HMQC experiments in the pH* range 5.6−7.2. The titration data for the His-240 $^1H^{\delta 1}$ proton fit with a p*K*$_a$* of 6.3 ± 0.1, within experimental error of that determined for the His-240 $^1H^{\epsilon 1}$ proton as expected. This indicates that the p*K*$_a$* of His-240 in the wild-type G6PD apoenzyme is typical for histidine in solution. While the data were fit well by a simple acid dissociation equation with only one apparent p*K*$_a$, the His-240 $^1H^{\epsilon 1}$ displays an uncharacteristically narrow range (∼0.25 ppm) between ionized and un-ionized forms for a histidine (typically ∼1 ppm) (Figure 7), raising the possibility that a nearby residue protonates with a p*K*$_a$ of 6.4 affecting the chemical shift and activity of His-240. Due to its proximity to His-240, the most likely candidate for such a residue is His-178. However, we note that based on the putative assignment of the His-178 $^1H^{\epsilon 1}$ from the NOE experiment (Figure 4), its chemical shift did not change over the pH range used in the titration experiment (compare peak at ^{1}H 9.1 ppm in Figures 2 and 3), indicating that its p*K*$_a$ is not typical. Therefore, it is not likely that the p*K*$_a$ of His-178 is affecting the titration behavior of His-240.

Significance of the pK$_a$ *of His-240.* The pH dependence of kinetic parameters for *L. mesenteroides* G6PD was reported by Viola (*17*) for the $NADP^+$-linked reaction, and similar results were found by Olive et al. (*18*) for the NAD^+-linked reaction. With G6P as the variable substrate in the $NADP^+$-linked reaction, the dependence of log k_{cat} on pH is described by two apparent p*K*$_a$ values of 6.3 and 8.7. Protonation of these residues reduces enzymatic activity. It was suggested that the p*K*$_a$ of 6.3 may be associated with a change in the overall charge of the protein, resulting in a

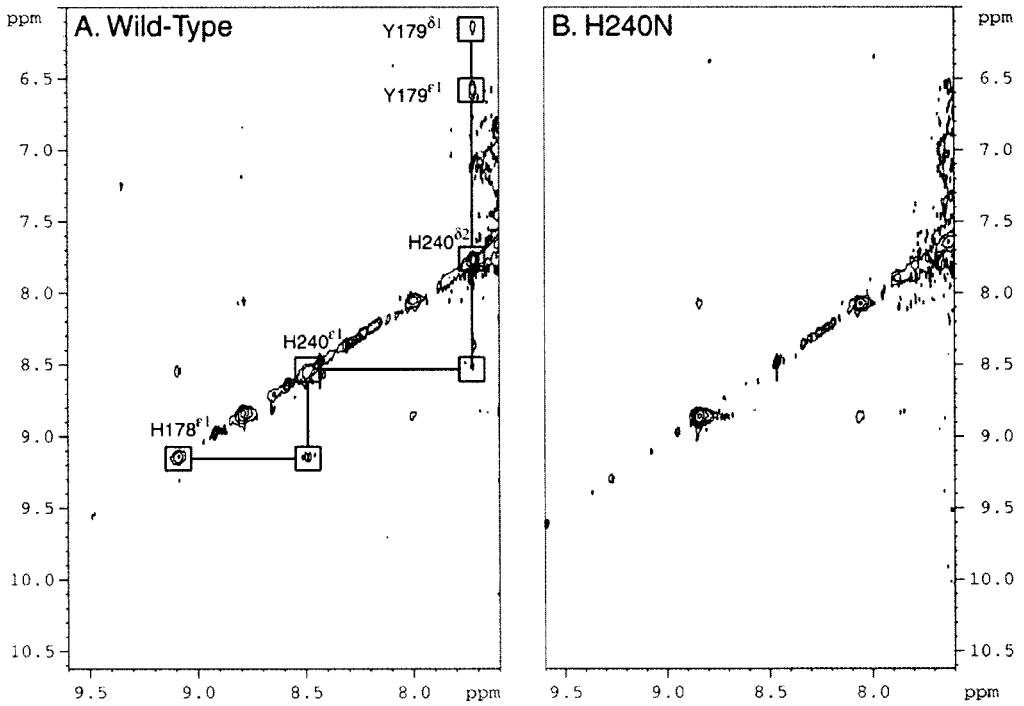

FIGURE 4: NOESY spectra of 0.5 mM samples of wild-type and H240N *L. mesenteroides* G6PDs recorded at 500 MHz in 100% ^2H_2O at 35 °C, pH* 5.6. The mixing time was 150 ms. (A) Wild-type G6PD. (B) H240N G6PD.

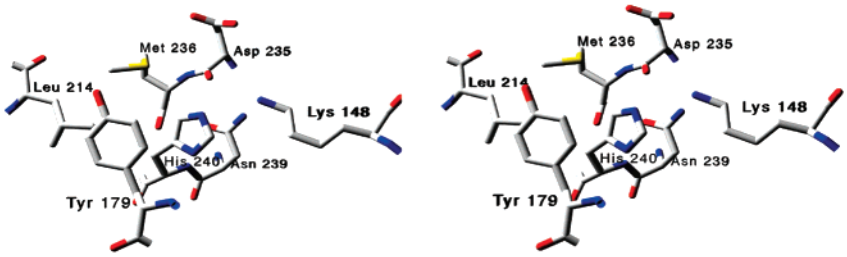

FIGURE 5: Stereo diagram of the active site X-ray crystal structure of *L. mesenteroides* G6PD (*2*) showing all amino acid residues within 5 Å of His-240 $^1H^{\delta 2}$.

Table 1: Summary of pK_a Values from NMR Spectroscopy and Kinetic Experiments

	NMR titration	pH dependence of kinetic parameters	
enzyme	pK_a* His-240	pK_a log k_{cat}	pK_a log (k_{cat}/K_m)
wild-type	6.4	6.3[a]	4.5[a]
D177N	ND[c]	ND	6.8[b]

[a] From Viola (*17*). [b] From Cosgrove et al. (*9*). [c] ND: not determined.

loss of activity at low pH (*17*). The log (k_{cat}/K_m) profile was bell-shaped with limiting slopes of 2 and −2, suggesting the presence of four ionizable groups (*17*). At low pH, an amino acid residue with a pK_a of 4.5 was assigned to the general base of the reaction, likely a carboxylate containing amino acid such as aspartate or glutamate (summarized in Table 1).

The results of several other experiments, however, suggest that the imidazolium of a histidine is the general base. For example, site-directed mutagenesis (*5*), covalent modifications with diethyl pyrocarbonate (DEP) (*19*−*21*), and X-ray crystallographic analyses (*2, 9*) indicate that the general base that abstracts a proton from the C1-OH of G6P is His-240. Replacement of His-240 with asparagine results in a 10^5-fold decease in k_{cat} for both the NAD^+- and $NADP^+$-linked reactions of *L. mesenteroides* G6PD (*5*). Additionally, the crystal structure of the *L. mesenteroides* D177N−G6P−NADPH ternary complex shows that $N^{\epsilon 2}$ of His-240 is 2.75 Å distant from the C1-OH of G6P (*9*). It is interesting, therefore, that the microscopic pK_a* of 6.4 for His-240 in the wild-type enzyme reported in this investigation is similar to the pK_a of 6.3 observed in the log k_{cat} versus pH profile reported previously (*17*) (Table 1), suggesting that this kinetically derived pK_a may represent the ionization of His-240. This hypothesis deserves further investigation.

An important question is why the pK_a of a carboxylate (4.5) and not the pK_a of a typical histidine is observed in the log (k_{cat}/K_m) vs pH profile of wild-type G6PD (*17*). The k_{cat}/K_m for a substrate is the apparent first-order rate constant for a variable substrate when at near-zero levels (*22*), i.e., an apoenzyme [in the case of G6PD, k_{cat}/K_m reflects ionizations occurring in an enzyme−$NADP^+$ binary complex since all kinetic experiments were conducted with saturating $NADP^+$ (*17*)]. As in mandelate racemase (*12*), perhaps the pK_a of His-240 in G6PD is lowered under k_{cat}/K_m conditions by the positive electrostatic potential of surrounding residues in the active site, or by the positively charged nicotinamide moiety of $NADP^+$. In the absence of the negatively charged phosphate group of the substrate, there is an excess of positively charged residues that may influence the pK_a of His-240 [such as Lys-182 and Lys-343 (*8*)]. Binding of

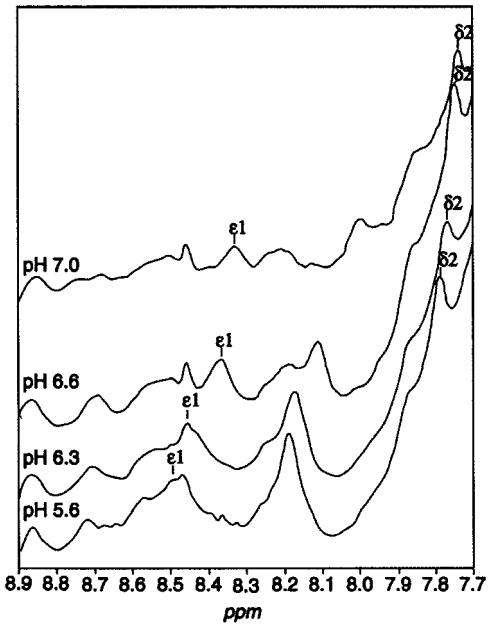

FIGURE 6: Selected spectra from the pH* titration of the wild-type G6PD apoenzyme. Data were recorded at 35 °C with a protein concentration of approximately 0.5 mM (subunit concentration) in 10 mM MES, 100% ^2H_2O. Each spectrum resulted from the sum of 1024 accumulations. The symbols $\epsilon 1$ and $\delta 2$ indicate His-240 $^1H^{\epsilon 1}$ and $^1H^{\delta 2}$, respectively.

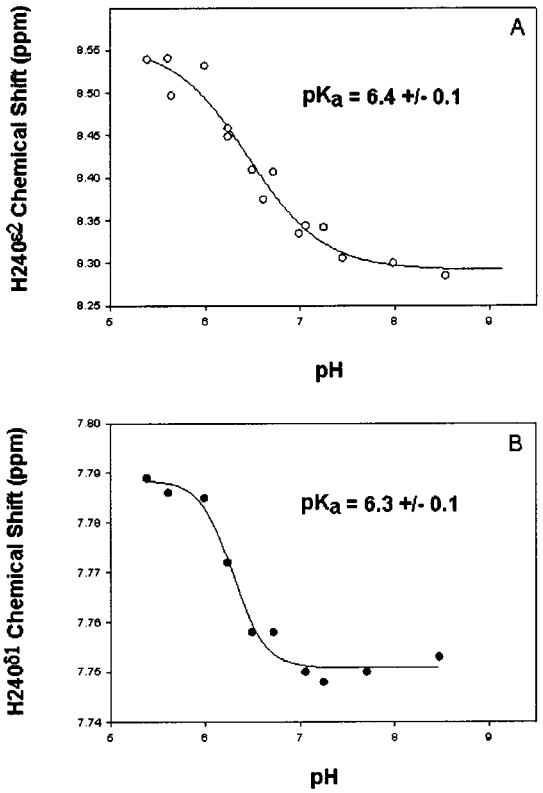

FIGURE 7: pH* titration curves for (A) His-240 $^1H^{\epsilon 1}$ and (B) His-240 $^1H^{\delta 2}$ for wild-type *L. mesenteroides* G6PD apoenzyme. The pK_a* values were determined by nonlinear least-squares fit of the equation in the text.

substrate (k_{cat} conditions) would be expected to neutralize this potential, resulting in the elevation of the pK_a of the base, as seen in the k_{cat} profile of G6PD (*17*).

However, the results from previous experiments, and the results reported in this investigation, suggest otherwise. First, from comparison of log (k_{cat}/K_m) profiles of wild-type and D177N enzymes, the pK_a that is observed in the wild-type enzyme at pH 4.5 appears to shift to 6.8 when Asp-177 is replaced with asparagine (*9*). This suggests that replacement of Asp-177 with asparagine does not result in the lowering of the pK_a for His-240 as expected. Second, the NMR spectroscopy experiments reported here were conducted in the absence of substrate, and an unperturbed pK_a* for histidine is observed for His-240 (Figure 7). Therefore, the electrostatic potential of active site residues does not influence the pK_a* of His-240. Third, ^1H NMR spectroscopy titrations carried out in the presence of 0.4 mM $NADP^+$ did not produce a detectable shift in the pK_a* of His-240 (data not shown), suggesting that the positive charge from the oxidized form of nicotinamide does not influence the pK_a of His-240.

Alternatively, when a substrate dissociates from the collision complex faster than it reacts to give products, the log (k_{cat}/K_m) versus pH profile can yield the pK_a values of groups participating either in binding or in catalysis (*22*). Since the pK_a of 4.5 is not present in any pK_i profile for a competitive inhibitor (*17*), it is likely that the group giving rise to this ionization behavior is not involved in substrate binding. This pK_a therefore represents the acid dissociation constant either of the general base in the reaction or of a group whose protonation state is important for the chemical reaction indirectly but not for binding (*22*). Since the preponderance of evidence suggests that His-240 is the general base with an unperturbed pK_a in the apoenzyme, it is likely that this group with a pK_a of 4.5 in the log (k_{cat}/K_m) vs pH profile is an amino acid whose protonation state is indirectly important for catalysis. A likely candidate for this amino acid is Asp-177, which interacts with His-240 in a His-Asp catalytic dyad. In support of this, replacement of Asp-177 with asparagine causes the pK_a at pH 4.5 in the k_{cat}/K_m profile to disappear, and is replaced with the appearance of a pK_a at 6.8, most likely that of His-240 (*9*). Additionally, this group behaves like a neutral acid in solvent perturbation studies [Viola, (*17*)]. Unfortunately, we were unable to determine the microscopic pK_a* of His-240 in the D177N enzyme due to either excessive line broadening or shifting of the His-240 peak (not shown).

Taken together, these data suggest that the general base of the G6PD enzyme-catalyzed reaction, His-240, has an unperturbed pK_a in the G6PD apoenzyme and enzyme−$NADP^+$ binary complex, despite being involved in a His-Asp catalytic dyad. Additionally, the group responsible for the pK_a of 4.5 in the log (k_{cat}/K_m) profile is likely to belong to Asp-177, and not the catalytic base as previously suggested. It has been estimated that the negative charge supplied by Asp-177 increases the rate of the G6PD reaction ~450-fold (*9*). Since the replacement of Asp-177 with asparagine does not affect the orientation or tautomeric form of His-240 (*9*), and since the pK_a* of His-240 is unperturbed in the apoenzyme, this 450-fold increase in the rate of the G6PD reaction is likely due to ionization effects on His-240 in the transition state, perhaps with the formation of a low-barrier hydrogen bond (*23*). Consistent with this, a pK_a of 6.8 was identified from the k_{cat}/K_m dependence on pH for the D177N enzyme. Assuming this reflects the ionization of His-240 in the D177N enzyme, then removal of Asp-177 in the wild-type enzyme causes the pK_a of His-240 to shift ~0.4 pH

unit. Perhaps this is enough to account for the 450-fold increase in the rate of the G6PD reaction in the presence of Asp-177.

CONCLUSION

In this investigation, we report the assignment of the ^{1}H NMR spectroscopy chemical shift for His-240, and determination of its pK_a*, in *L. mesenteroides* G6PD. The pK_a* of His-240 in the G6PD apoenzyme is found to be 6.4, which corresponds to an unidentified pK_a value of 6.3 that was previously derived from the dependence of k_{cat} on pH (*17*). These results suggest that the pK_a of His-240 is unperturbed in the apoenzyme despite its role in a His-Asp catalytic dyad. The results for this 109 kDa enzyme indicate that ^{1}H NMR spectroscopy in combination with heteronuclear methods can be a useful tool for the functional analysis of some large proteins.

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