Galactose Mutarotase: Purification, Characterization, and Investigations of Two Important Histidine Residues†

Jane A. Beebe and Perry A. Frey*

Institute for Enzyme Research, University of Wisconsin−Madison, Madison, Wisconsin 53705

Received July 7, 1998; Revised Manuscript Received August 20, 1998

ABSTRACT: Galactose mutarotase catalyzes the interconversion of α- and β-anomers of aldoses and is a recently identified member of the *gal* operon of *Escherichia coli* and participant in the Leloir pathway [Bouffard et al. (1994) *J. Mol. Biol. 244,* 269−278]. We report the purification and characterization of this enzyme, as well as mechanistic studies involving chemical modification with diethylpyrocarbonate (DEPC) and site-directed mutagenesis demonstrating the significance of two conserved histidine residues. The enzyme lacks metal ions and oxidoreduction cofactors, and an extinction coefficient of $(6.2 \pm 0.4) \times 10^4$ M^{-1} cm^{-1} has been measured by quantitative amino acid analysis. The catalytic mechanism is likely concerted general acid/general base. Experiments involving modification with DEPC suggest that a histidine is essential and is protected by substrate. Furthermore, site-directed mutagenesis of two conserved histidines was performed, and characterization of these mutants (His104Gln and His175Asn) illustrates the significance of these residues. Kinetic analysis of H104Q demonstrates an increase in K_M of about 600-fold, a decrease in k_{cat} of approximately 7-fold, and a 4000-fold decrease in k_{cat}/K_M as compared to the wild-type enzyme. The activity of His175Asn mutant, on the other hand, was too low to be measured accurately, and His 175 remains a candidate for the general base. These mutants were also subjected to DEPC modification, and results are consistent with the presence of two important histidines positioned closely together in the active site.

Galactose mutarotase, a ubiquitous enzyme, catalyzes the equilibration of α- and β-anomers of certain sugars (D-galactose, D-glucose, D-xylose, D-arabinose, and D-fucose) (*1, 2*). It has recently been identified as a member of the *gal* operon of *Escherichia coli* and has been shown to be involved in the Leloir pathway as follows (*3*). After β-galactosidase cleaves lactose to produce glucose and β-D-galactose, galactose mutarotase catalyzes the equilibration of β-D-galactose with the α-anomer, the substrate for the next enzyme in this pathway, galactokinase, resulting in α-D-galactose-1-P. Galactose-1-P uridylyltransferase then catalyzes the transfer of a uridylyl group from UDP-glucose[1] to α-D-galactose-1-P to form UDP-galactose and α-D-glucose-1-P. UDP-galactose is then epimerized to UDP-glucose by UDP-galactose 4-epimerase, and the α-D-glucose-1-P is brought to equilibrium with α-D-glucose-6-P through the action of phosphoglucomutase prior to entering glycolysis. This sequence of reactions is also used in reverse for glycoprotein and glycolipid biosynthesis (*4, 5*). The active sites and chemical mechanisms of β-galactosidase, galactose-1-P uridylyltransferase, and UDP-galactose 4-epimerase have been described (*6*−*8* and references therein).

A role for mutarotase in sugar transport has also been proposed (*9, 10*). In addition, the transport of *n*-fluoro-*n*-deoxy-D-glucose derivatives across the human erythrocyte membrane has been studied by NMR, and it was found that α-anomers permeate up to 2.5-fold more rapidly than β-anomers (*11*).

Although the rate of uncatalyzed mutarotation is significant (0.032 min^{-1}) (*1*), nonenzymatic mutarotation is too slow to satisfy the needs of the cell for galactose utilization (*3*). Values of K_M are rather high for *E. coli* galactose mutarotase ($K_M^{galactose}$ = 6.5 mM, $K_M^{glucose}$ = 15 mM); however, k_{cat} is also quite high (k_{cat} = 9550 s^{-1}) (*2*), resulting in a k_{cat}/K_M of 6×10^5 $M^{-1}s^{-1}$ for the glucose substrate.

No mutarotase has yet been subjected to a thorough mechanistic analysis. Mutarotation could theoretically occur through any mechanism allowing cleavage of any one of the four bonds to C-1 of galactose. The most obvious chemical mechanism for the galactose mutarotase-catalyzed reaction is a concerted version of the nonenzymatic general acid/general base-catalyzed mechanism (Figure 1) (*12*−*15*). An oxidation/reduction mechanism similar to that of UDP-galactose 4-epimerase (*8*) would also be feasible if the enzyme contained an oxidoreduction cofactor. A third

† Supported by National Institutes of Health Grant GM 30480. J.A.B. was supported by Individual National Research Service Award number 5 F32 DK09306.

* To whom correspondence should be addressed. Phone: (608) 262-0055. Fax: (608) 265-2904.

[1] Abbreviations: DEPC, diethylpyrocarbonate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *p*-HMB, *p*-hydroxymercuribenzoate; ICP-OES, inductively coupled plasma optical emission spectrometry; IPTG, isopropyl thio-β-D-galactopyranoside; MES, 4-morpholineethanesulfonic acid; NAD, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; ppb, parts per billion; ppm, parts per million; TCA, trichloroacetic acid; UDP-galactose, uridine-5′diphosphate galactose; UDP-galactose 4-epimerase, uridine-5′-diphosphate galactose 4-epimerase; UDP-glucose, uridine-5′-diphosphate glucose.

10.1021/bi9816047 CCC: $15.00
Published on Web 09/26/1998

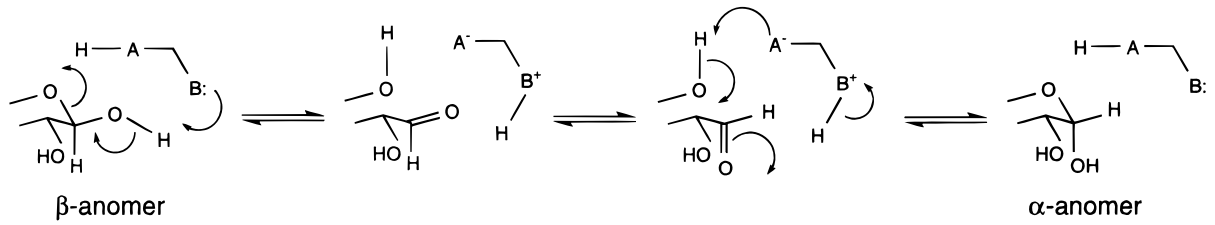

FIGURE 1: Proposed concerted general acid/general base-catalyzed mechanism for galactose mutarotase.

possibility is a mechanism similar to that of the glycosidases (*16*, *17*) involving cleavage between C-1 and the anomeric oxygen resulting in dehydration followed by rehydration. However, evidence supporting the general acid/general base-catalyzed mechanism was acquired for the *E. coli* enzyme using gas chromatography to observe the formation of products (*2*). When α-D-galactose was dissolved in water and allowed to come to equilibrium either in the presence or absence of galactose mutarotase, β-D-galactofuranose was observed as a minor product upon achieving equilibrium for both catalyzed and uncatalyzed mutarotation. These data suggest that, during both uncatalyzed and enzyme-catalyzed mutarotation, open-chain galactose is formed which can then be converted to either pyranoid or furanoid products (*12*, *13*). Additionally, a pH-rate profile for *E. coli* galactose mutarotase was obtained at saturating substrate concentrations that suggested a pK_a of 5.5 for a Brønsted base (*2*).

E. coli galactose mutarotase, a monomer of molecular weight 38 000 (*18*), has been cloned, sequenced, and overexpressed (*3*). The objective of our research has been to develop a purification procedure for overexpressed galactose mutarotase, to characterize the enzyme, and to begin a detailed mechanistic analysis. The enzyme has been purified to homogeneity and characterized with respect to extinction coefficient, oxidoreduction cofactors, and metal ions. Additionally, the mechanism is being studied using a combination of chemical modification and site-directed mutagenesis techniques. The present findings demonstrate the importance of two conserved histidine residues, His 104 and His 175.

MATERIALS AND METHODS

Purification of Galactose Mutarotase. The plasmid pGB1001 which codes for *E. coli* galactose mutarotase (*3*) was a gift from Dr. Sankar Adhya, NIH. It was transformed into BL21(DE3)pLysS cells (Novagen). Seven liters of 2XYT media (*19*) containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol was inoculated with 5 mL of overnight culture and grown to an absorbance of 0.9−1 at 600 nm. Isopropyl thio-β-D-galactopyranoside (IPTG) was then added to 1 mM, and induction proceeded for 3.5 h. Cells were centrifuged at 5000*g* for 10 min, then pellets were frozen directly into liquid nitrogen. Typically 2.6 g cells were obtained from 1 L media.

For the entire procedure, 10 mM Hepes (pH 7.5) was used as the buffer with phenylmethanesulfonyl fluoride (PMSF) added daily to 1 mM to control proteases. Cells were resuspended at a concentration of 0.12 g/mL (18 g cells in each purification) and broken by sonication in eight 30 s bursts using a sonic dismembrator (Fisher Scientific). Cells were sonicated on ice and cooled between bursts to keep the temperature at <9 °C. Cell debris was removed by centrifugation at 25000*g* for 30 min. The following procedures were conducted in a cold room (9 °C). Streptomycin sulfate (20%) was added dropwise to the supernatant while stirring to a final concentration of 3%. The supernatant was stirred an additional 20 min, then the precipitate was removed by centrifugation at 8000*g* for 30 min. The supernatant was brought to pH 7.5 by the addition of 1 M NaOH and loaded onto a Q-Sepharose Fast Flow column (5 cm × 22 cm), which had been equilibrated with 10 mM Hepes buffer (pH 7.5). The enzyme was eluted at ∼0.3 M NaCl using a 4 L gradient containing 10 mM Hepes (pH 7.5) with an increasing NaCl concentration (0−0.4 M).

A coupled assay (27 °C, 10 mM Hepes, pH 7.5) involving the addition of freshly dissolved α-D-glucose to NAD (3 mM) and glucose dehydrogenase (USB or Sigma) (*3*, *20*) was used in purification of the enzyme and for several experiments. Only the β-form of glucose is a substrate for glucose dehydrogenase, which catalyzes the oxidation of β-D-glucose to D-glucono-δ lactone, and the concomitant reduction of NAD to NADH. The initial velocity (first 20% of the reaction) was determined by monitoring the increase in absorbance at 340 nm. For optimal activity, coupling enzyme was resuspended in 10 mM Hepes (pH 7.5), 3 mM NAD, and 125 mM KCl, and aliquots were frozen in liquid nitrogen and stored at −70 °C. All assays were carried out at 27 °C. For all time courses, the rate of the background reaction in the absence of galactose mutarotase was subtracted from that of the enzymatic reaction. Fractions from the Q-Sepharose column were assayed by this procedure, and those possessing the highest activity and purity as analyzed by SDS−PAGE were pooled and concentrated using Centriprep-10 concentrators (Amicon). Galactose mutarotase was dialyzed against 4 L of 10 mM Hepes (pH 7.5) (three changes, 6−14 h each session), then frozen directly in liquid nitrogen (20−30 μL drops) and stored at −70 °C.

Plasmids containing mutant genes of galactose mutarotase were transformed (*21*) into GB87 cells that possess a deletion in the gene for galactose mutarotase (*3*). These cells were a gift from Dr. Sankar Adhya, NIH. Mutants were purified in the same way as the wild-type enzyme but with some additions to the purification procedure due to the reduction in expression (from 30 mg of enzyme/g of cells for wild-type enzyme in BL21(DE3)pLysS cells to 0.4−0.7 mg of enzyme/g of cells for mutants in GB87 cells).

About 40 g of cells was obtained from a 7 L growth, and this amount was used in each purification. After the streptomycin sulfate precipitation, the supernatant was brought to 40% ammonium sulfate saturation while stirring in the cold room, and stirring was continued for an additional 20 min. The precipitate was removed by centrifugation at 8000*g* for 30 min, and the pellet was discarded. The

supernatant was brought to pH 7.5 using 1 M NaOH. Ammonium sulfate was then added to 50% saturation, and the supernatant was removed by centrifugation at 8000*g* for 30 min. The pellet was resuspended in 40 mL of 10 mM Hepes (pH 7.5) and dialyzed in 4 L of buffer for three sessions, 6−14 h each. The mutant enzyme was then diluted to 100 mL and purified over a Q-Sepharose column as described for the wild-type enzyme. After pooling the purest fractions as determined by SDS−PAGE, ammonium sulfate was added to 17.5%, and the enzyme was loaded onto a phenyl sepharose column that had been equilibrated with 10 mM Hepes (pH 7.5) and 17.5% ammonium sulfate. A 1.5 L gradient of decreasing ammonium sulfate concentration from 17.5% to 5% was applied, then a 600 mL gradient from 5% to 0%. The enzyme was eluted with 10 mM Hepes (pH 7.5). The pure fractions as determined by SDS−PAGE were pooled, and enzyme was concentrated, dialyzed, frozen in liquid nitrogen, and stored at −70 °C as described for the wild-type enzyme.

Polarimetric Assay. Polarimeters have been useful for assaying mutarotation (*1*, *2*) because of the difference in specific rotation of α- and β-anomers of aldoses. Due to the relatively high cost and impurity of commercially available coupling enzyme, a Perkin-Elmer model 141 polarimeter was used for assays in which [S] > 50 μM. Water-jacketed cells were used to maintain a reaction temperature of 27 °C. For all assays, α-D-glucose (Aldrich) was dissolved in 10 mM Hepes (pH 7.5) immediately before addition to enzyme, and the initial rate (first 20%) of the reaction was measured. The initial rate of the background reaction was subtracted from the initial rate of each enzyme-catalyzed reaction.

Measurement of the Extinction Coefficient. The extinction coefficient of galactose mutarotase was determined as described (*22*, *23*), with modifications. Ten microliters of 2.5 mM 4-transhydroxyproline (Sigma) was added to the amino acid standards and to the enzyme before hydrolysis for use as an internal standard. To determine whether complete hydrolysis had occurred, the enzyme was hydrolyzed for either 24 or 48 h. The phenylthiocarbamyl derivatives were separated using a Beckman 5 μ C18 Ultrasphere column with dimensions of 4.6 mm × 25 cm and a Beckman System Gold HPLC. The following gradient, which is effective at separating phenylthiocarbamyl derivatives (Dr. Frank Ruzicka, University of Wisconsin−Madison, personal communication) was used. Each step involves a linear gradient unless otherwise noted. The column was washed with solvent A (0.05 M ammonium acetate, pH 6.8) for 10 min between injections, then solvent B (44% acetonitrile, 10% methanol, and 46% H_2O in 0.1 M ammonium acetate, pH 6.8) was increased in a gradient from 0% to 10% in 35 min, 10% to 30% in the next 35 min using gradient curve 3 (a convex gradient), 30% to 55% in the following 35 min, and 55% to 100% in the next 10 min, then from 100% to 0% in 5 min.

Preparation and Quantification of Diethylpyrocarbonate (DEPC). DEPC (Aldrich) was diluted 1/10 into acetonitrile (Fisher, HPLC grade) (*24*, *25*), and its concentration was determined by reaction of an aliquot with 10 mM imidazole (pH 7.5) and measurement of the increase in absorption at 230 nm due to the formation of the N-carbethoxyimidazole derivative (*24*, *26*). Tenfold dilutions of DEPC in acetonitrile stored at 4 °C were used in subsequent experiments, with the concentration of DEPC measured before each experiment.

Table 1: Purification of Galactose Mutarotase Expressed in BL21(DE3)pLysS[a]

step	activity[b,c]	total activity[d]	% yield	yield (mg)	SDS−PAGE
crude	22.8[e]	7.1×10^7	100	3111	close to pure
3% streptomycin sulfate	26.8[e]	6.5×10^7	92	2419	close to pure
Q-Sepharose (0−400 mM NaCl)	79.2[f]	4.3×10^7	60	539	pure

[a] Conditions for purification: all steps were carried out in a coldroom (9 °C) and in 10 mM Hepes (pH 7.5) and 1 mM PMSF. [b] Conditions for assays: 10 mM Hepes (pH 7.5), 27 °C, [α-D-glucose] = 50 μM. [c] (μM product formed/min)/mg of protein. [d] μM product formed/min. [e] Protein concentration was determined as in ref *34*. [f] Enzyme concentration determined from the extinction coefficient (1.62 mL mg^{-1} cm^{-1} or 6.2×10^4 M^{-1} cm^{-1}).

Site-Directed Mutagenesis of Histidine Residues. Site-directed mutagenesis of three histidine residues was performed using the QuikChange site-directed mutagenesis kit (Stratagene). For the H104Q mutant, 29 base primers were used to substitute the Gln codon (CAG) codon for the His codon (CAC). This mutation generated a *Pst*I site that was used initially in screening for the H104Q mutation. The DNA was then transformed (*21*) into GB87 cells (described earlier). The entire sequence of this gene was then determined by the method of Sanger (*27*) to confirm that the desired mutation had indeed occurred, and that this had been the only mutation generated in the gene.

For the H175N and H190N mutants, 42 base and 36 base primers were used, respectively, to substitute the AAC codon for CAC. Sequencing (*27*) was used to screen for each desired mutation, then the sequence of the entire gene was verified by the University of Wisconsin Biotechnology Center using fluorescent dye-terminators (*28*).

Data Analysis. Data were fitted using the KaleidaGraph (Synergy Software) curve-fitting program using equations below describing simple exponentials (*29*).

$$A_t = A_o\exp(-kt) \quad (1a)$$

$$A_t = A_1\exp(-k_1t) + A_2\exp(-k_2t) \quad (1b)$$

$$A_t = A_o\exp(-kt) + C \quad (1c)$$

$$B_t = A[1 - \exp(-kt)] \quad (2a)$$

$$B_t = A_1[1 - \exp(-k_1t)] + A_2[1 - \exp(-k_2t)] \quad (2b)$$

RESULTS

Purification of Galactose Mutarotase. *E. coli* galactose mutarotase has been cloned, sequenced, and overexpressed in BL21(DE3) cells (*3*). A purification method for *E. coli* galactose mutarotase involving acid precipitation, ammonium sulfate precipitation, gel filtration chromatography, adsorption by calcium phosphate gel, and preparative polyacrylamide gel electrophoresis has been published (*2*). We have developed a simpler method for the purification of the overexpressed *E. coli* enzyme to homogeneity as determined by SDS−PAGE (Table 1).

Mutants of galactose mutarotase were expressed in GB87 cells which have a deletion in the gene coding for galactose

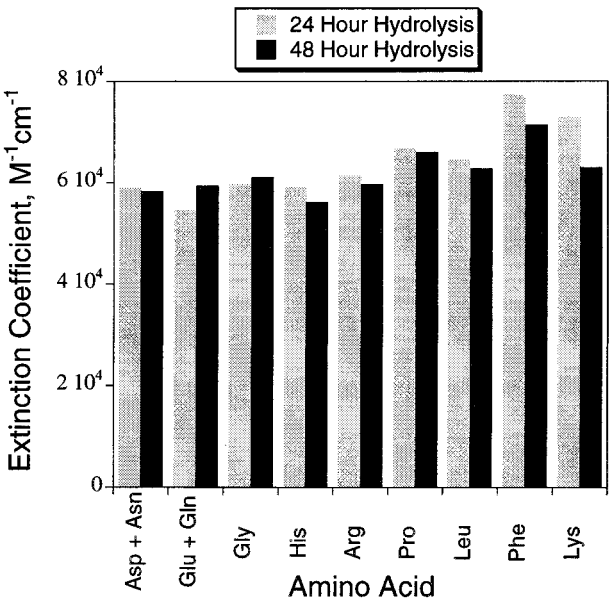

FIGURE 2: The extinction coefficients determined after a 24 or 48 h hydrolysis by quantitative amino acid analysis.

mutarotase (*3*). Expression of mutants in these cells was significantly less (0.4−0.7 mg of enzyme/g of cells) than the expression of the wild-type enzyme in BL21(DE3)pLysS (30 mg of enzyme/g of cells), and additional steps were added to purify the mutants to homogeneity.

Measurement of the Extinction Coefficient. The extinction coefficient was determined by quantitative amino acid analysis (*22, 23; Dr. Frank Ruzicka, University of Wisconsin—Madison, personal communication*). The extinction coefficients determined for nine amino acids [Asn and Gln are quantitatively hydrolyzed to Asp and Glu (*30*)] are shown in Figure 2, and the average value was $(6.2 \pm 0.4) \times 10^4$ M^{-1} cm^{-1} and 1.6 ± 0.1 mL mg^{-1} cm^{-1}. To determine whether hydrolysis was complete, one portion of the enzyme sample was hydrolyzed for 24 h and another for 48 h. The differences between these extinction coefficients were within the experimental error described above.

Metal Ion and Cofactor Analysis. A sample of 15 μM purified galactose mutarotase and a control sample containing 10 mM Hepes (pH 7.5) were supplied to the Soil and Plant Analysis Lab at the University of Wisconsin where inductively coupled plasma optical emission spectrometry (ICP-OES) was performed. This technique has a sensitivity of 0.01−0.1 ppm. Amounts of the following metal ions in the galactose mutarotase sample were found to be less than or equal to that in the control buffer: P, K, Mg, Mn, Ca, Fe, Al, Cd, Co, Cr, Cu, Li, Mo, Ni, Pb, As, and Se. The amount of zinc was slightly higher than that in the control buffer (0.08 ions/molecule enzyme). A 0.9 μM sample of purified galactose mutarotase and a control sample of the Amicon Centriprep-10 filtrate from this preparation was supplied to the Soil and Plant Analysis Lab of the University of Wisconsin, where inductively coupled plasma mass spectrometry (ICP-MS) was performed. This technique has a sensitivity of 0.01−0.1 ppb. The zinc content of the enzyme preparation determined by this technique was 0.02 ions/molecule enzyme. The small amount of zinc present is likely adventitious. Additionally, enzyme activity decreased by a factor of 2.4 (from 2.4 μM/min to 1.0 μM/min) when 0.21 μM galactose mutarotase was incubated 30 min in the presence of 10 μM zinc sulfate. After the incubation, enzyme was diluted to 2.4 nM in 10 μM $ZnSO_4$, α-D-glucose was added to 50 μM, and enzyme was assayed as described. The rate of the uncatalyzed reaction was unchanged in the presence of 10 μM zinc sulfate (0.6 μM/min).

Since NADH and NADPH absorb light at 340 nm ($\epsilon_{340} = 6.22 \times 10^3$ $M^{-1}cm^{-1}$), and flavoproteins possess absorption spectra with maxima around 280, 350−380, and 450 (*31*), a spectrum from 320 to 800 nm using 92 μM galactose mutarotase was obtained to search for these cofactors. There were no peaks in this spectrum, which had an absorbance at 340 nm of 0.02. This absorbance appears to be from the peak at 280 nm. To quantify NAD, galactose mutarotase was precipitated with trichloroacetic acid (TCA), the acid was removed by extraction with ether, and the supernatant was enzymatically assayed for NAD using glucose dehydrogenase with negative results.

Steady-State Kinetic Parameters. The following steady-state kinetic parameters were measured for the conversion of α-D-glucose to β-D-glucose by galactose mutarotase using a polarimeter as described: $k_{cat} = 22\,000 \pm 1000$ s^{-1}, $K_M = 29 \pm 4$ mM, and $k_{cat}/K_M = (7.6 \pm 0.7) \times 10^5$ $M^{-1}s^{-1}$. These values are similar to those measured previously (*2*): $k_{cat} = 6700$ s^{-1}, $K_M = 15$ mM, and $k_{cat}/K_M = 4.5 \times 10^5$ $M^{-1}s^{-1}$.

Chemical Modification of Histidine Residues Using Diethylpyrocarbonate (DEPC). The pH-rate profile for galactose mutarotase under conditions of saturating substrate possesses a pK_a of 5.5, suggesting aspartic acid, glutamic acid, or histidine as a possible Brønsted base catalyst (*2*). A combination of chemical modification and site-directed mutagenesis techniques is being used to search for catalytic residues.

The involvement of histidine residues was examined by modification of the enzyme with DEPC, which produces a N-carbethoxy derivative absorbing at 240 nM with an extinction coefficient of 3200 $M^{-1}cm^{-1}$ (*24, 32, 33*) (Figure 3A). The concentration of the N-carbethoxy derivative was divided by the enzyme concentration to determine the number of histidine residues modified for each time point. The resulting biphasic data were described by a double exponential (eq 2b), with one histidine modified about 20-fold more rapidly than the others. A related experiment was also performed under identical conditions except that galactose mutarotase was incubated with 65 mM galactose [$K_M^{galactose} = 6.5$ mM (*2*)] for 40 min before the addition of DEPC. The increase in absorbance at 240 nm was monitored and lacked a fast phase, suggesting that the most rapidly reacting histidine was completely protected.

The effect of DEPC modification on the activity of galactose mutarotase was also examined using the coupled assay at 50 μM α-D-glucose (580-fold below K_M), and the polarimeter at 90 mM α-D-glucose (3-fold above K_M). The dependence of the disappearance of active galactose mutarotase on the amount of time since the addition of DEPC is shown (Figure 3B). Activity was completely lost, and the rate constant describing this loss ($k_{obs} = 0.078$ min^{-1}) is more similar to that for modification of the slower-reacting histidines as determined from the A_{240} measurement ($k_{obs} = 0.062$, Figure 3A) than that for the rapidly modified histidine ($k_{obs} = 1.2$ min^{-1}). When galactose mutarotase was incu-

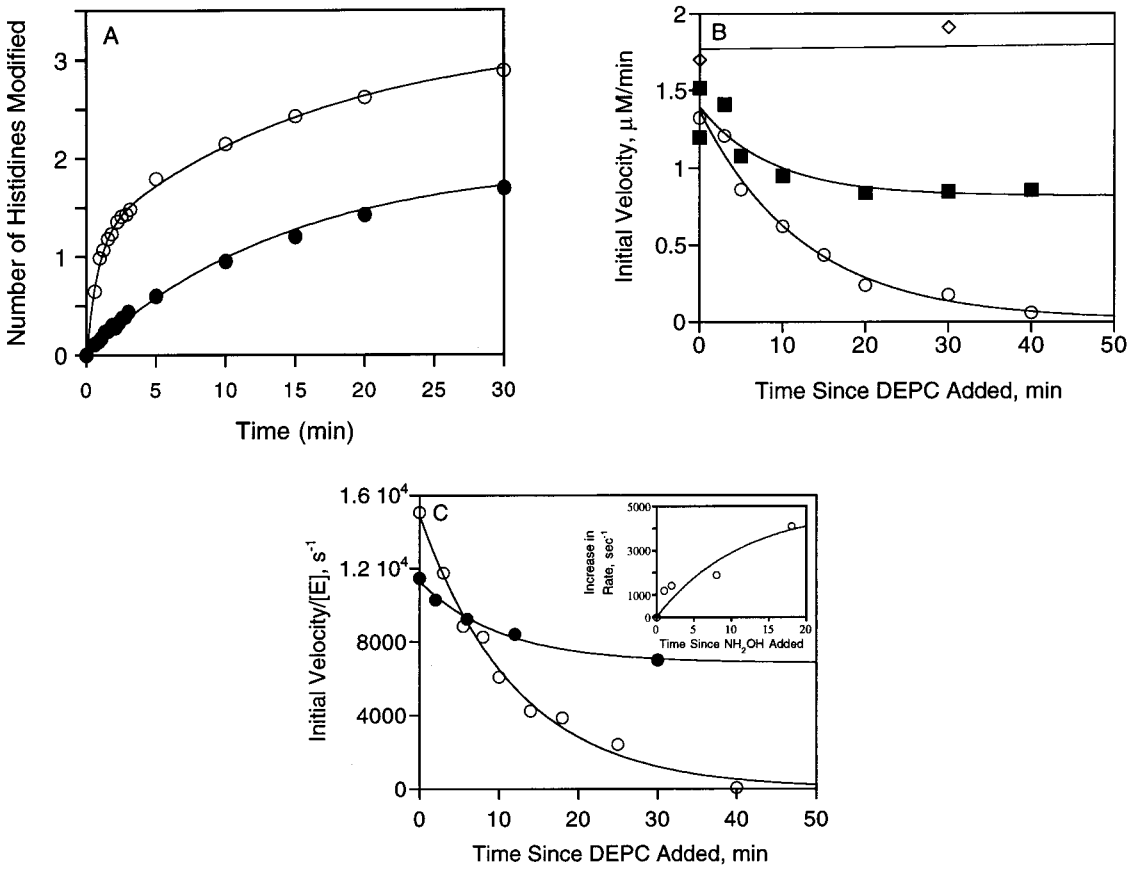

FIGURE 3: Changes in A_{240} and enzyme activity observed upon modification of galactose mutarotase with DEPC. The buffer for each experiment was 100 mM MES (pH 6), and each experiment was conducted at 27 °C. (A) The absorbance at 240 nm was monitored (27 °C) after DEPC (2.2 mM) was mixed with galactose mutarotase (9.8 μM). Data (○) were biphasic and were described by a double exponential (eq 2b) with $A_1 = 1.18 \pm 0.04$ His modified and $k_{obs1} = 1.22 \pm 0.09$ min^{-1}, $A_2 = 2.06 \pm 0.03$ His modified, and $k_{obs2} = 0.062 \pm 0.003$ min^{-1}. An experiment was also performed under identical conditions except that the enzyme had been incubated with 65 mM galactose for 40 min prior to the addition of DEPC, and the increase in A_{240} possessed one phase. Data (●) were fitted to a single first-order exponential (eq 2a): $A = 1.96 \pm 0.02$ His modified, $k_{obs} = 0.070 \pm 0.003$ min^{-1}. (B) The influence of DEPC modification on enzyme activity using the coupled assay at 50 μM α-D-glucose is shown. Galactose mutarotase (24.4 μM) was incubated with 4.4 mM DEPC, and 5 μL aliquots were removed and diluted 200-fold into 10 mM Hepes (pH 7.5) that had been incubated on ice. This dilution was then diluted into the assay mixture for a final galactose mutarotase concentration of 1.2 nM, and the rate of the reaction was measured after the addition of α-D-glucose to 50 μM. Data (○) were described by a single-exponential decay (eq 1a) with $A_0 = 1.37 \pm 0.05$ μM/min and $k_{obs} = 0.078 \pm 0.006$ min^{-1}. Additionally, enzyme (24.4 μM) was incubated with 65 mM galactose for 40 min before the addition of DEPC to 4.4 mM, and assays were performed as described for experiments conducted in the absence of galactose. Data (■) were described by the equation for a single-exponential decay plus a constant (eq 1c) with $A_0 = 0.6 \pm 0.1$ μM/min, $k_{obs} = 0.12 \pm 0.08$ min^{-1}, and a constant $= 0.8 \pm 0.1$ μM/min. A control experiment was also performed in which galactose mutarotase (2.44 μM) was incubated (27 °C) in the absence of chemical modification reagents, and aliquots were withdrawn and assayed. Data (◇) were fit to the equation for a straight line with slope $= (6 \pm 9) \times 10^{-4}$ μM/min^2 and intercept $= 1.77 \pm 0.09$ μM/min. (C) The effect of DEPC modification on the ability of galactose mutarotase to catalyze mutarotation of 90 mM α-D-glucose was determined using a polarimeter. Galactose mutarotase (9.8 μM) was incubated with DEPC (2.2 mM), then 4 μL aliquots were removed and diluted 250-fold into ice-cold 10 mM Hepes (pH 7.5). A portion of this dilution was added to freshly dissolved α-D-glucose (90 mM) for a final enzyme concentration of 2.2 nM, and the initial rate was measured. The dependence of the initial rate on the time since the addition of DEPC (○) was described by a single-exponential decay (eq 1a), with $A_0 = 14\,900 \pm 700$ s^{-1} and $k_{obs} = 0.08 \pm 0.01$ min^{-1}. When 65 mM galactose was incubated with 9.8 μM galactose mutarotase for 40 min before the addition of DEPC to 2.2 mM, 61% of the activity was retained, and data (●) were described by the equation for a single-exponential decay plus a constant (eq 1c): $A_0 = 4400 \pm 400$ s^{-1}, $k_{obs} = 0.10 \pm 0.02$ min^{-1}, and constant $= 6900 \pm 400$ s^{-1}. The inset shows the reactivation of galactose mutarotase by hydroxylamine (100 mM) which was added 10 min after the addition of DEPC. Data were fitted using a single first-order exponential (eq 2a), demonstrating an increase in rate of 5000 ± 3000 s^{-1}.

bated with 65 mM galactose for 40 min prior to the addition of DEPC to 2.2 mM, 56% of the activity was retained (Figure 3B).

A control experiment was performed to determine whether galactose mutarotase loses activity when incubated in the absence of chemical modification reagents. Galactose mutarotase (2.44 μM) in 10 mM Hepes (pH 7.5) was incubated at 27 °C, and aliquots were periodically removed for assay for up to 4.8 h without a significant decrease in activity (Figure 3B). Additionally, 2.44 μM enzyme incubated at 27 °C in 100 mM MES (pH 6) retained full activity for at least 4.8 h (data not shown).

To measure the influence of DEPC modification on enzyme activity at a higher substrate concentration, a similar experiment was performed using a polarimeter to measure the rate of the reaction (Figure 3C). As with the experiment involving assays conducted at a lower substrate concentration (Figure 3B), enzyme activity was completely lost. However, when 65 mM galactose was added to galactose mutarotase 40 min before the addition of DEPC to 2.2 mM, 61% of the

activity was retained. The effect of DEPC on galactose mutarotase activity (Figure 3, panels B and C) together with the quantification of the N-carbethoxy derivative (Figure 3A) suggest that the histidine residue most rapidly modified is protected by galactose but is not essential, and that one or more slowly modified residues is essential.

To determine whether hydroxylamine was capable of reactivating enzyme that had been modified with DEPC (*24*, *32*), 9.8 μM galactose mutarotase was incubated with 2.2 mM DEPC and aliquots were withdrawn, diluted, and assayed as described using 90 mM α-D-glucose. After 10 min, hydroxylamine was added, and aliquots were diluted and assayed (Figure 3C, inset). In addition, the coupled assay was used to investigate reactivation by hydroxylamine. Galactose mutarotase (9.8 μM) was incubated with DEPC (2.2 mM) in 100 mM MES buffer (pH 6) and aliquots were withdrawn and assayed using 50 μM α-D-glucose. As described above, hydroxylamine was added to a concentration of 100 mM after 10 min. Two trials were performed in which 16 ± 2% and 20 ± 1% of the activity was recovered (data not shown). These experiments demonstrate that modification of one or more histidine residues is at least partially responsible for the complete loss of activity upon modification with DEPC.

Site-Directed Mutagenesis of Conserved Histidine Residues. *E. coli* galactose mutarotase contains seven histidines, two of which are conserved (His 104 and His 175), and one residue (His 190) that is histidine in all cases tested except for *Saccharomyces carlsbergensis*, which has a threonine in this position (*3*). To investigate the roles of these histidines, the following mutants were generated: H104Q, H175N, and H190N. Each gene was completely sequenced to ensure that it contained only the desired mutation. Plasmids were then transformed into the GB87 cell line, which lacks mutarotase, and the activities of crude lysates were compared to those in which no plasmid or the plasmid for the wild-type gene was present. The assays involved the use of the coupling enzyme glucose dehydrogenase and 50 μM α-D-glucose. In the absence of any plasmid, the crude lysate of GB87 cells containing 2.3 μg/mL protein (*34*) catalyzed mutarotation at a rate of 0.04 μM product formed/min after subtraction of the background rate. Preparations of crude lysate (protein concentration = 2.3 μg/mL) from GB87 cells containing the wild-type plasmid catalyzed the reaction at a rate of 0.5 to 1 μM product formed/min. When 4.3 μg of protein/mL of the H190N crude extract was assayed, a rate of 2.5 μM product formed/min resulted, demonstrating that the histidine residue at 190 is not likely to be important in substrate binding or catalysis. However, both H104Q and H175N crude extracts were devoid of measurable activity even when protein concentrations were increased to 43 μg/mL.

To ascertain whether these decreases in activity were due to decreased expression or impaired enzyme function, both H104Q and H175N mutants were purified to homogeneity. The following kinetic parameters were determined for H104Q: $k_{cat} = 3300 \pm 1900$ s^{-1}, $K_M = 17 \pm 10$ M, and $k_{cat}/K_M = 195 \pm 10$ M^{-1}s^{-1}. The error in the measurements of k_{cat} and K_M for this mutant was high because the substrate α-D-glucose was insoluble at concentrations greater than 2 M. These data suggest that His 104 plays an important role in substrate binding. The activity of His 175, however, was so low that no rate above background could be detected, and kinetic parameters could not be determined. To set limits on the reduction in activity, assays were performed at 0.6 and 2 M α-D-glucose with 12.5 μM mutant enzyme, producing initial velocities of −0.0027 and 0.000 17 min^{-1}, respectively, after subtraction of the background rate. The rate of the background reaction is 0.0286 ± 0.0014 min^{-1}, and assuming that an initial velocity 10-fold larger than the standard deviation could have been measured, the rate at 0.6 M α-D-glucose is ≤10 s^{-1} (2000-fold less than the value for wild-type enzyme) and the rate at 2 M α-D-glucose is ≤37 s^{-1} (600-fold less than that for wild-type enzyme).

These mutants were also characterized with respect to the increase in absorbance at 240 nm upon reaction with DEPC, reflecting an increase in the concentration of the N-carbethoxy derivative (Figure 4, panels A and B). In these experiments involving 9.8 μM wild-type or mutant galactose mutarotase, DEPC was initially added to 2.2 mM, then brought to 6.6 mM at 9 min to compensate for the side reaction involving the hydrolysis of DEPC (*24*). At early times (0−9 min, Figure 4B), data were best described by a double exponential (eq 2b) and indicate that the amplitude of the first phase is reduced by a factor of 3.2 for H175N and 3.6 for H104Q as compared to that of the wild-type enzyme. At longer times (>9 min), DEPC modifies histidines of the mutants somewhat more quickly than those of the wild-type enzyme.

The increase in absorbance at 240 nm upon reaction with DEPC in the presence of galactose was investigated for the H104Q and H175N mutants (Figure 4C). For the H104Q mutant, no protection by galactose was observed. However, some protection was seen for the H175N mutant, and the amplitude determined in the presence of galactose is about 2-fold smaller than the sum of the amplitudes measured in the absence of galactose.

To determine whether the H104Q mutant could be inactivated by DEPC, H104Q (19 μM) was incubated in 100 mM MES (pH 6, 27 °C) for 10 min, then DEPC was added to 2.2 mM. Aliquots were withdrawn and diluted 16-fold into 10 mM Hepes (pH 7.5) that had been incubated on ice. Assays were performed using the polarimeter, with 2 M α-D-glucose and 1.2 μM H104Q. Complete inactivation occurred, and the dependence of the disappearance of active enzyme on the amount of time since the addition of DEPC was determined. Data were fitted using a single-exponential decay (eq 1a) with $A_0 = 346.7 \pm 0.4$ s^{-1} and $k_{obs} = 0.787 \pm 0.004$ min^{-1} (data not shown).

The involvement of aspartic and glutamic acid residues was investigated by the modification of galactose mutarotase with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and aminomethanesulfonic acid in 70 mM MES (pH 6) as described (*32*, *33*, *35*). Assays were performed (50 μM α-D-glucose) with the modified enzyme, and a time course demonstrating the relationship between the initial rate of the reaction catalyzed by 1.2 nM galactose mutarotase and the time of incubation of enzyme with the chemical modification reagents is shown in Figure 5.

Similar experiments were performed in which the enzyme was incubated with 65 mM galactose 15 min before the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and aminomethanesulfonic acid in order to determine whether substrate protects against modification of essential carboxylic acid residues. In both experiments,

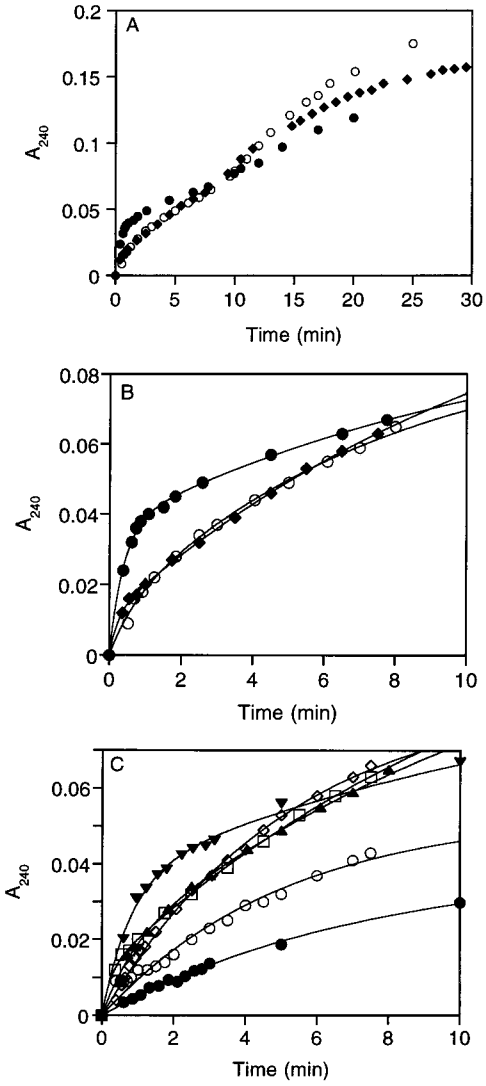

FIGURE 4: Characterization of the mutants H104Q and H175N with respect to the effects on formation of the N-carbethoxy derivative as determined by the change in A_{240}. (A) The absorbance at 240 nm was monitored for wild-type enzyme (●), H104Q (○), and H175N (◆) (9.8 μM each) after DEPC was brought to 2.2 mM, then to 6.6 mM after 9 min to compensate for DEPC hydrolysis. (B) Data from early times (0−9 min) were described by a double exponential (eq 2b): for wild-type enzyme (●), $A_1 = 0.036 \pm 0.002$, $k_{obs1} = 2.6 \pm 0.2$ min^{-1}, $A_2 = 0.06 \pm 0.02$, $k_{obs2} = 0.09 \pm 0.05$ min^{-1}; for the H175N mutant (◆), $A_1 = 0.0103 \pm 0.0009$, $k_{obs1} = 4 \pm 1$ min^{-1}, $A_2 = 0.11 \pm 0.01$, $k_{obs2} = 0.08 \pm 0.02$ min^{-1}; and for H104Q (○): $A_1 = 0.010 \pm 0.006$, $k_{obs1} = 2 \pm 1$ min^{-1}; $A_2 = 0.08 \pm 0.01$, $k_{obs2} = 0.14 \pm 0.06$ min^{-1}. (C) As described for the wild-type enzyme (Figure 3A), the effects of the presence of galactose (65 mM) on the formation of the N-carbethoxy derivative as measured by the increase in A_{240} were determined for the H104Q and H175N mutants. Data for the reaction of the wild-type enzyme in the absence of galactose (▼) were fitted using a double exponential (eq 2b) and have been described (Figure 3A). Here, the amplitude is expressed as A_{240}: $A_1 = 0.037 \pm 0.001$, $A_2 = 0.064 \pm 0.001$. In the presence of galactose (●), $A = 0.0612 \pm 0.0007$. For the H104Q mutant, data obtained in the absence of galactose (▲) are described in Figure 4B. In the presence of galactose (◇), data were fitted using a single first-order exponential (eq 2a): $A = 0.086 \pm 0.002$, $k_{obs} = 0.0190 \pm 0.009$ min^{-1}. For the H175N mutant, data obtained in the absence of galactose (□) are described in Figure 4B. Data (○) measured when the H175N mutant was incubated with 65 mM galactose for 40 min before the addition of DEPC were described by a single first-order exponential (eq 2a): $A = 0.054 \pm 0.005$ and $k_{obs} = 0.19 \pm 0.03$ min^{-1}.

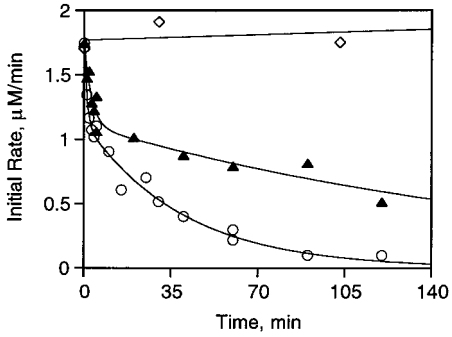

FIGURE 5: The investigation of the possible involvement of aspartic acid or glutamic acid residues in catalysis by chemical modification. Galactose mutarotase (4.9 μM) was incubated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (100 mM) and aminomethanesulfonic acid (250 mM) (27 °C). Aliquots were removed and diluted 200-fold into ice-cold 10 mM Hepes (pH 7.5) and assayed using 50 μM α-D-glucose. Complete biphasic inactivation occurred, and data (○) were described by the equation for a double exponential decay (eq 1b) with $A_1 = 0.61 \pm 0.09$ μM/min, $k_{obs1} = 0.9 \pm 0.3$ min^{-1}, $A_2 = 1.14 \pm 0.06$ μM/min, and $k_{obs2} = 0.025 \pm 0.003$ min^{-1}. When galactose mutarotase (4.9 μM) was incubated with 65 mM galactose for 15 min before the addition of the chemical modification reagents, dilution, and assay, some protection occurred. These data (▲) were also described by a double-exponential decay (eq 1b): $A_1 = 0.6 \pm 0.1$ μM/min, $k_{obs1} = 0.3 \pm 0.1$ min^{-1}, $A_2 = 1.1 \pm 0.1$ μM/min, and $k_{obs2} = 0.005 \pm 0.002$ min^{-1}. Additionally, galactose mutarotase (2.4 μM) was incubated in 10 mM Hepes (pH 7.5, 27 °C) in the absence of chemical modification reagents. Aliquots were removed and assayed, and data (◇) (also shown in Figure 3B) were described by a straight line with slope $= (6 \pm 9) \times 10^{-4}$ μM/min^2 and intercept $= 1.77 \pm 0.09$ μM/min.

activity was completely abolished in a biphasic fashion. In the presence of galactose, the amplitudes remained unchanged; however, modification was slowed.

To investigate the involvement of cysteine residues, 2.44 μM galactose mutarotase was incubated with 50 μM *p*-hydroxymercuribenzoate (*p*-HMB) (*32*, *33*, *36*) and aliquots were diluted 100-fold into ice-cold 10 mM Hepes (pH 7.5), then to 1.2 nM galactose mutarotase and assayed using 50 μM α-D-glucose and the coupling enzyme glucose dehydrogenase (data not shown). Depletion of activity leveled off within the first 30 min and decreased little (0.12 μM/min) for the next 3 h 15 min. Data were described by the equation for a single-exponential decay plus a constant (eq 1c): $A_0 = 1.7 \pm 0.1$ μM/min, $k_{obs} = 0.5 \pm 0.1$ min^{-1}, and constant $= 0.27 \pm 0.06$ μM/min, demonstrating that 16% of the activity remains. These data suggest that cysteine residues are not required for catalysis. *E. coli* galactose mutarotase contains no conserved cysteine residues (*3*). Additionally, the low concentrations of chemical modification reagents in all of the chemical modification experiments described have no effect on the rate of the background reaction (~0.7 μM/min at 50 μM α-D-glucose) (data not shown).

DISCUSSION

Characterization of Galactose Mutarotase. Galactose mutarotase, a 38 kDa monomer (*3*, *18*), has been further characterized with respect to the extinction coefficient and has been shown to lack metal ions and oxidoreduction cofactors. The lack of oxidoreduction cofactors rules out a mechanism involving reversible oxidation and reduction at C-1. Steady-state kinetic parameters have also been evaluated and are similar to those measured previously (*2*).

Galactose mutarotase possesses a few interesting features that are currently being investigated in greater detail. The enzyme is rather stable to heat, retaining 50% activity (50 μM substrate) after a 13 μL aliquot was placed in boiling water 5 min and allowed to cool 15 min before assay. In addition, reaction of 5.3 μM galactose mutarotase with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of 3 M guanidine thiocyanate, 3 mM EDTA, and 38.5 mM potassium phosphate buffer (pH 8) (*37*) suggested the presence of four free sulfhydryls. Interestingly, the enzyme activity is completely depleted in the presence of DTT (data not shown). Galactose mutarotase contains a total of four cysteine residues (*3*).

Possible Functions of the Conserved Histidine Residues His 104 and His 175 in the Mechanism of Galactose Mutarotase. Results of DEPC modification and protection experiments with wild-type enzyme clearly indicate the presence of at least one protected histidine as demonstrated by the measurement of the formation of the N-carbethoxy derivative in the presence compared to the absence of galactose (Figure 3A). Results also suggest the presence of an essential histidine residue (Figure 3, panels B and C) because of the complete loss of activity occurring in the presence of DEPC and the reversal of this loss by hydroxylamine. In addition, 60% of this activity is protected by galactose, implying that this necessary residue resides in the active site. The histidine reacting most rapidly with DEPC (1.2 min^{-1}) as demonstrated by the appearance of the N-carbethoxy derivative is obviously protected (Figure 3A); however, the essential histidine loses activity at a significantly slower rate (Figure 3, parts B and C, 0.08 min^{-1}). This slower rate is similar to the rate of appearance of the N-carbethoxy derivative during the slower phase (0.06 min^{-1}), however, although 60% of enzyme activity was protected by galactose, the slower-reacting histidines do not appear to be protected by galactose (Figure 3A). One possible explanation for these data is that because there are a total of seven histidine residues in galactose mutarotase, and DEPC is hydrolyzed over time as a side reaction (*24*), the data may not be sensitive enough to detect the protection of one slowly reacting histidine. Additionally, if the faster-reacting histidine is not *completely* protected, the protection of one slower-reacting residue would not be easy to observe. An alternative explanation for these results is that because the fast-reacting histidine is clearly not essential (Figure 3, panels B and C) but 60% of the activity is protected by galactose in the presence of DEPC, *either* the fast-reacting or a slow-reacting histidine is necessary for activity, but activity is lost when both are modified.

We also present two models for explaining the differences in rates of DEPC modification. One possibility is that the rapidly modified histidine is on the surface of the enzyme and is thus more accessible to DEPC modification. The remaining histidine residues are located on the interior of the protein and are shielded from modification. A second possibility is that two histidines are located near each other, and since the experiments are conducted at pH 6, one residue is protonated and the other is not. The unprotonated residue will react rapidly with DEPC, while the protonated residue will react more slowly. If galactose interacts with the protonated histidine and diminishes its interaction with the unprotonated one, the pK_a of the unprotonated His will rise, thus decreasing the rate with which it reacts with DEPC.

Characterization of mutants of the two conserved histidine residues has also been informative. Steady-state kinetic parameters of the H104Q mutant demonstrate that this histidine is likely important for binding, while no measurable activity for the H175N mutant could be demonstrated. His 175 remains a candidate for the putative Brønsted base. These mutants were also characterized with respect to their reactions with DEPC. Interestingly, the quantification of modification by DEPC as demonstrated by the appearance of the N-carbethoxy derivative (Figure 4, panels A and B) is very similar for these mutants. *Both* mutants display a diminishing of the fast phase, but an increase in the rate of the slow phase. It is possible that either His 104 or His 175 is the histidine residue that reacts with DEPC rapidly, thus explaining the abatement of the fast phase. The decrease in the amplitude of the fast phase in the other mutant could be explained by the model described above involving two histidine residues positioned near one another. Both mutation of the fast-reacting histidine and mutation of the protonated, slower-reacting histidine would result in the disappearance of the fast phase. Additionally, the H104Q mutant is inactivated by DEPC, suggesting that the complete loss of activity that results upon modification of the wild-type enzyme with DEPC is not simply due to modification of His 104.

The increase in the rate of DEPC modification for the mutants during the slow phase might be caused by a conformational change increasing the accessibility of the histidine residues. Mutations of histidine to asparagine or glutamine are considered to be conservative and unlikely to cause significant structural perturbations (*38*), however, the structure of these mutants compared to that of the wild-type enzyme has not yet been investigated. Structural studies of galactose mutarotase are currently underway and will also be conducted for these mutants once the analysis of the wild-type enzyme is complete.

The mutant enzymes were also characterized with respect to the ability of galactose to protect them from DEPC modification (Figure 4C). Unlike the wild-type enzyme, the H104Q mutant exhibits no protection by galactose, while the H175N mutant displays some protection. The protection by galactose of the H175N mutant may suggest that this mutant retains the ability to bind substrate and that the mutant's structure is stabilized when substrate is bound.

Besides histidine, aspartic and glutamic acid residues are considered candidates for the general base and could possess a pK_a consistent with that measured at saturating substrate (*2*). As described (Figure 5), chemical modification with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and aminomethanesulfonic acid results in complete biphasic loss of activity, and galactose slows this loss. However, there are no conserved aspartic or glutamic acid residues in *E. coli* galactose mutarotase (*3*).

As mentioned earlier, the analysis of reaction products by gas chromatography of both the uncatalyzed and the mutarotase-catalyzed mutarotation of α-D-galactose indicate the presence of a small amount of the furanose form once equilibrium is reached (*2*). In the absence of enzyme, no furanose form was seen after 5 min, and most of the galactose was still in the α-form, but the furanose form was observed at 3 h. For the enzyme-catalyzed reaction, about the same

amount of furanose form was present in 5 min as that seen for the uncatalyzed reaction after 3 h. Since galactose mutarotase apparently catalyzes the pyranose-furanose equilibration, it was concluded (*2*) that enzyme-catalyzed mutarotation proceeds through the open-chain form which can be converted to either the pyranoid or furaniod form.

We wondered whether galactose mutarotase might simply catalyze the opening of the ring, then release the open-chain intermediate into solution, allowing it to close on its own. Rate constants for opening and closing of the ring have been measured for α-D-glucose by a polarographic method in which the reducible, free aldehyde form was quantified (*39*). Data were obtained at a variety of phosphate buffer concentrations, and since mutarotation is catalyzed by phosphate buffer, rate constants increased as phosphate buffer concentration increased. To compare the rate constant for ring closure to the rate of the enzyme-catalyzed reaction, the rate constant for ring closure was extrapolated to zero phosphate concentration. We fitted the data of Los et al. (*39*), which consisted of measurements of the rate constant at 0.655 M α-D-glucose for ring closure acquired from 70 to 153 mM phosphate buffer, to the equation for a straight line using the computer program KaleidaGraph (Synergy Software), resulting in a slope $= 0.5 \pm 0.1$ mM^{-1} s^{-1} and intercept $= 3 \pm 15$ s^{-1}. Although the error in the intercept is fairly high, it is clearly less than the rate of the enzyme-catalyzed reaction at 0.655 M glucose (21 067 s^{-1}), suggesting that galactose mutarotase catalyzes closure as well as opening of the ring.

Since the enzyme catalyzes ring closure, a Brønsted acid is likely involved, and we plan to search for one. Galactose mutarotase, while devoid of conserved lysines, contains two conserved argenines and two conserved tyrosines (*3*), which may be candidates for chemical modification and site-directed mutagenesis experiments. Currently, X-ray crystallographic studies of the wild-type enzyme are underway, which may help identify residues in the active site.

ACKNOWLEDGMENT

We are very grateful to Professor Laurens Anderson for the use of his polarimeter.

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BI9816047