Stereospecificity and Catalytic Function of Histidine Residues in 4a-Hydroxy-tetrahydropterin Dehydratase/DCoH†

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Received March 23, 1998; Revised Manuscript Received June 15, 1998

ABSTRACT: Three conserved histidines have been shown to be important for the enzymatic activity of 4a-hydroxy-tetrahydropterin dehydratase, a bifunctional enzyme which is involved in regeneration of tetrahydrobiopterin and is also a cofactor (DCoH) for the transcription factor HNF-1α. The 4a isomer dependent kinetics of the mutants of rat/human enzyme, H61A, H62A, and H79A, and the effect of diethylpyrocarbonate (DEPC) have been investigated to elucidate the dehydratase mechanism. At pH 6.5 wild-type enzyme is inactivated by DEPC after derivatization of one histidine, shown to be H61 by comparison to H61A. H79 is also derivatized by DEPC at pH 7.0 and above, whereas H62 does not react at any pH. Dehydratase activity of H61A with 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin was not detectable. In contrast, although K_m for the enantiomeric 4a(*S*)-hydroxy-6(*R*)-methyl-tetrahydropterin was 65-fold higher than with wild-type, k_{cat} was 86% of wild-type. H79A gave complementary results: activity with 4a(*S*)-hydroxy-6(*R*)-methyl-tetrahydropterin was undetectable, but 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin had almost normal K_m and 75% of wild-type k_{cat}. Replacing H62 with alanine decreased k_{cat}/K_m 80- and 60-fold, and k_{cat} to 24% and 89% of wild-type for the 4a(*R*),6(*S*)- and 4a(*S*),6(*R*)- isomers, respectively. Near neutral pH nonenzymatic dehydration catalyzed by solvated proton had a rate constant of 1.55×10^5 M^{-1} sec^{-1}. A break in the rate versus pH curve at 5.95 was tentatively assigned to protonation of the carbinolamine guanidinium system. The free acid of acetic acid and the imidazolium ion showed general acid catalysis of 18.5 and 1.5 M^{-1} sec^{-1}, respectively, in dehydrating the neutral carbinolamine. Compared to the later value, dehydratase effective molarity is 11 M. These results are consistent with a dehydratase mechanism in which H61 and H79 act as general acid catalysts for the stereospecific elimination of the 4a(*R*)- and 4a(*S*)-hydroxyl groups, respectively. The role of H62 is primarily binding substrate, with an additional component of base catalysis.

Dehydratase/DCoH is one of the few proteins currently known with two seemingly separate functions, one as an enzyme (*2*) and the other in regulation of transcription (*3*−*6*). In the cytoplasm, it catalyzes the dehydration of 4a-hydroxy-tetrahydrobiopterin to give quinoid dihydrobiopterin, the first step in the regeneration of tetrahydrobiopterin[1] cofactor for the aromatic amino acid hydroxylases (Figure 1). In the nucleus as DCoH it is a dimerization cofactor of the transcription factor HNF1α (*2*).

The cytoplasmic dehydratase is a tetramer of 4 identical subunits of 103 amino acids, the sequence of which is highly conserved. The amino acid sequences of human and rat proteins are identical, and the mouse varies by only one amino acid (*3, 4*). A similar protein from *Pseudomonas aeruginosa* is 33% identical and 57% homologous (*7*) and has catalytic activity which is very similar to the rat enzyme (*7*−*9*). Dehydration of 4a-hydroxy-tetrahydropterins occurs spontaneously, but at a rate which is insufficient in the liver to maintain tetrahydrobiopterin in the reduced state during a phenylalanine load (*10*). An inherited disorder of man which is characterized by transient hyperphenylalaninemia and excretion of 7-substituted pterins (*11, 12*) is associated with mutations in this enzyme (*13, 14*). 7-Pterins have been shown to accumulate in reactions of phenylalanine hydroxylase in vitro under conditions in which nonenzymatic dehydration is minimized (*15, 16*).

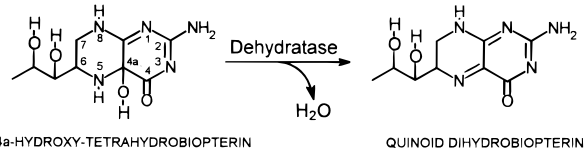

FIGURE 1: The reaction catalyzed by 4a-hydroxy-tetrahydropterin dehydratase.

One of the more intriguing aspects of its enzymatic activity is that the dehydratase is capable of catalyzing dehydration of both 4a-hydroxy stereoisomers of the carbinolamine

† This investigation was supported by Grant NS-26662 awarded by the National Institute of Neurological Disorders and Stroke and Grant 31-43380.95 awarded by the Swiss National Science Foundation. A preliminary report of part of this work has been presented (*1*).

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[1] Abbreviations: tetrahydrobiopterin, 6(*R*)-*l-erythro*-dihydroxypropyl-tetrahydropterin; tetrahydropterin, 2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine; DEPC, diethylpyrocarbonate; CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane; bis-Tris, bis-(2-hydroxyethyl)imino-tris(hydroxymethyl)methane; divicine, 2,6-diamino-5-hydroxy-4(3H)-pyrimidinone.

S0006-2960(98)00663-1 CCC: $15.00
Published on Web 07/21/1998

substrate with nearly equal efficiency (*17*). Since the dehydratase lacks metal (*17*) or other cofactors, the location of the catalytic site was proposed on the basis of sequence homology with the bacterial enzyme to be in a cleft surrounded by three conserved histidines (*18, 19*). The crystal structure of the enzyme complexed with a product analogue (*20*), together with site-directed mutagenesis (*21*−*23*), has indicated that these histidines are important for catalytic activity. However, their stereochemical specificity has not been investigated. Further, although several hypotheses have been made for the role which each histidine plays in catalysis (*20, 22*), evidence supporting any particular mechanism is lacking. From chemical precedence, acid catalysis would be expected as the most likely mechanism of carbinolamine dehydration (*24*), but we have shown that base catalysis also occurs in the nonenzymatic reaction (*25*).

In this paper the effect of site-directed mutagenesis on the reactivity of enzyme with the individual substrate isomers and chemical modification of enzyme with DEPC are investigated. These results have revealed specific functions for each of the three conserved histidine residues and have disclosed the mechanism by which native enzyme dehydrates both isomers of substrate.

EXPERIMENTAL PROCEDURES

Materials. Chromatofocusing reagents and Phenyl-Sepharose were from Pharmacia. Diethylpyrocarbonate (DEPC) and hydroxylamine HCl were from Aldrich, imidazole and Tris were from Sigma, bis-Tris was from Research Organics, and isopropyl-β-D-thiogalactopyranoside was from Boehringer. 4a-Hydroxy-tetrahydropterin dehydratase from rat liver and dihydropteridine reductase from bovine liver were purified as previously reported (*17*). Dehydratase substrates were stereospecifically synthesized as previously described (*10, 25*).

General Methods. Recombinant rat liver dehydratase was overexpressed in *Escherichia coli*, as described previously (*26*). For SDS−PAGE a method optimized for low molecular weight proteins was used (*27*), with 10% acrylamide in the running gel. The concentration of 4a-hydroxy-tetrahydropterin dehydratase was determined from its UV absorbance (*28, 29*). The absorption coefficients of model compounds of the component amino acids in 6 M guanidine−HCl (ϵ_{280} = 5450 M^{-1} cm^{-1} for tryptophan and ϵ_{280} = 1265 M^{-1} cm^{-1} for tyrosine) were used to calculate the concentration of denatured dehydratase from its absorbance in 6 M guanidine−HCl. The absorption coefficient of the native protein was then calculated from a comparison of its absorbance with the absorbance of the denatured protein. The subunit molar absorption coefficient of the native protein (12 kD) at 280 nm was found to be 13 470 M^{-1} cm^{-1}, and 13 875 M^{-1} cm^{-1} at λ_{max} = 283 nm.

Oligonucleotides. Oligonucleotide primers for PCR-directed mutagenesis and/or PCR amplifications were synthesized on a Gene Assembler Plus DNA synthesizer from Pharmacia Biotech Inc. To change histidine codons to alanine, the following primers were used (codons for alanine are underlined and nucleotide exchanges are in bold): for histidine 61, 5′-GAG AAA CTG GAC **GC**C CAT CCT GAA TG-3′; for histidine 62, 5′-CTG GAC CAC **GC**T CCT GAA TGG TT-3′; and for histidine 79, 5′-ACG CTG AGC ACC **GC**T GAG TGT GCC GG-3′. The primer encoding the N-terminal end of the dehydratase coding sequence was 5′-CG*GAATTCATATG* GCT GGC AAA GCA CAC AG-3′ which contains an *Eco*RI and an *Nde*I endonuclease recognition site (italics) plus the ATG start codon (underlined) that overlaps with the *Nde*I site. The primer for the C-terminus was 5′-CG*GGATCCTA* TGT CAT GGA CAC TGC TAC-3′ which has a *Bam*HI cloning site (italics) and the translational stop codon (underlined).

Construction of Expression Vectors. The coding sequences for expression of the individual mutant proteins H61A, H62A, and H79A were generated by two consecutive rounds of PCR amplifications. For the first round of amplification, which was a PCR-directed mutagenesis procedure, a plasmid DNA template harboring the human wild-type cDNA was used (26). Amplification was carried out by applying standard PCR conditions (*30*) using the C-terminal 3′-primer in combination with the 5′-primers for H61A, H62A, and H79A. The generated DNA fragments with lengths of 151, 145, and 97 bp, respectively, were gel purified and used as primers for the second round of PCR amplification in combination with the N-terminal primer. The products from the second PCR were gel purified, digested with *Eco*RI and *Bam*HI, and ligated into plasmid pUC18. Individual clones for the three mutant dehydratases were screened for correct sequences by DNA sequence analysis using the AutoRead sequencing kit and an Automated Laser Fluorescent (ALF) DNA sequencer from Pharmacia Biotech Inc. To generate *E. coli* plasmid vectors expressing the mutant proteins, the corresponding pUC18 plasmid derivatives were digested with *Nde*I and *Bam*HI. The fragments containing the coding sequences were ligated into a pGEMEX-2-Nde vector, linearized with *Nde*I/*Bam*HI, to fuse the ATG start codon of the phage T7 gene 10 with the reading frames for the mutant proteins. The pGEMEX-2-Nde expression vector is a derivative of the original pGEMEX-2 vector (Promega) in which the single *Nde*I site present in the plasmid backbone has been removed (unpublished). The generated plasmids pHDH56, pHDH57, and pHDH58 expressed the mutant dehydratases H61A, H62A, and H79A, respectively.

Growth and Harvesting of E. coli Cells. For overproduction of the recombinant mutant proteins, the pGEMEX-2-Nde plasmid derivatives were transformed into *E. coli* BL21(DE3)/pLysS (Promega). Bacteria were grown at 37 °C in Luria-Bertani medium (*30*) containing 25 mg/L chloramphenicol and 50 mg/L ampicillin to an OD at 600 nm of 0.5, and induced for recombinant expression for 3 h by the addition of isopropyl-β-D-thiogalactopyranoside, 0.2 mM final concentration. The cells were collected by centrifugation, and cells from 1 L of culture were resuspended in 40 mL of 5 mM Tris-HCl, pH 8.0, and frozen in liquid nitrogen.

Purification of Recombinant Dehydratases. The suspension of *E. coli* cells containing wild-type or mutant dehydratases was sonicated in 5 mL aliquots, 3 × 10 s on ice in a Branson model 250 sonifier set at 6, with cooling for 1 min on ice between each cycle. The lysed cells were centrifuged at 100000*g* at 4 °C for 1 h and dehydratase purified from the supernatant by column chromatofocusing, with a pH gradient of 7.4−5.4. After dialysis against 25 mM imidazole−HCl, pH 7.4 at 4 °C, the dialyzed extract

was applied to a 1 × 45 cm column packed with PBE 94 (Pharmacia) which had been equilibrated with 25 mM imidazole−HCl, pH 7.4, at 4 °C. The column was eluted with 500 mL of Polybuffer 74 (Pharmacia) which had been diluted 1:10 with water and adjusted to pH 5.4 with HCl at 4 °C. The fractions containing activity were concentrated in an Amicon stirred cell with a YM 30 membrane at 55 psi, and then reconcentrated twice diluting each time with 5 mM Tris-HCl, pH 8.0. The washed concentrate was fast frozen in aliquots in liquid nitrogen. Due to the high level of expression, this one-step purification produces ∼50 mg of dehydratase from 1 L of cells, which by SDS−PAGE appears to be >99% pure (see below).

Dehydratase Activity Assay. Enzyme activity was measured by an assay procedure developed in this laboratory (*10*, *17*), using chemically synthesized 4a-hydroxy-tetrahydropterins (*10*, *25*). Since the substrate is unstable, the last step in the synthesis, cyclization of a pH 9.1 solution, is done on the day of use, and the substrate is kept as a methanol solution in a dry ice/ethanol bath. Dehydratase is strongly inhibited by its product quinoid dihydropterins, which have K_i's that are typically lower than the substrate K_m (*17*). To eliminate product inhibition, NADH and an excess of dihydropteridine reductase are added to reactions so that quinoid dihydropterins are immediately reduced to tetrahydropterins which do not inhibit (*17*). Accurate measurement of low K_m's is made possible by running reactions to completion and calculating kinetic constants by progress curve analysis (see below). Reaction mixtures containing NADH (100−300 μM) and an excess of dihydropteridine reductase (2 units/mL) in 25 mM Tris-HCl, pH 7.4 were temperature equilibrated to 10 °C in a water-jacketed cuvette before addition of substrate in a volume of 5−10 μL (20−200 μM). Temperature in the cuvette was measured using a YSI type 729 thermistor probe. Dehydratase was added last, preferably in an amount that completed dehydration of substrate in 30−60 s. A short reaction time and low temperature (10 °C) minimize interference from the background rate due to nonenzymatic dehydration. Reactions were monitored at 340 nm for the consumption of NADH, and data were acquired by computer at a rate of 2−5 points/s from the time of initiation of reaction with dehydratase until about 1 min after the reaction is complete. The 340 nm absorbance values are divided by the net absorption coefficient (5700 M^{-1} cm^{-1}) to give the nanomoles of 4a-hydroxy-tetrahydropterin dehydrated as a function of time. The first-order rate constant for nonenzymatic dehydration was measured separately and entered as a constant (k_{nonenz}) in fitting data to the rate equation. Progress curves were analyzed with a PC compatible nonlinear regression program "Scientist" (Micromath, Salt Lake City, UT) by fitting data to the differential equation

$$d([NADH])/dt = -k_{cat}[S]/(K_m + [S]) - k_{nonenz}[S] - C$$

where the $t = 0$ initial conditions have [NADH] and [S] set equal to parameters, $[NADH]_0$ and $[S]_0$, the NADH and substrate concentrations in the reaction at the start of data collection. This approach improves on that used previously (*17*) by including in the equation, in addition to k_{cat} and K_m, a parameter for the final slow rate (C), due mostly to autooxidation and recycling of tetrahydropterin. Typically, about 20−25 s of data beyond the completion of the reaction is included in the fit to establish the value of C. This allows low K_m values to be more accurately determined, limited primarily by spectrophotometer noise.

Reaction of Dehydratase with DEPC. DEPC (0.1 M in absolute ethanol) was freshly prepared immediately before use. The concentration of the diluted DEPC was verified by making a further 250-fold dilution into 10 mM imidazole and determining the increase in absorbance at 230 nm due to *N*-carbethoxyimidazole (molar absorption coefficient = 3000 M^{-1} cm^{-1}) (*31*). DEPC (5 μL of 0.1 M) was added to dehydratase (35 μM, ≈0.21 mg) in 5 mM potassium phosphate in a total volume of 0.5 mL. Reactions were monitored spectrally at 25 °C for 1 h. Control reactions were treated identically except that 5 μL of absolute ethanol was added instead of DEPC. Histidine residues were quantitated from the increase in absorbance at 240 nm using a Δ absorption coefficient of 3200 M^{-1} cm^{-1} (*31*). Dehydratase concentration was determined from the 280 nm absorbance using the subunit molar absorption coefficient of 13 470 M^{-1} cm^{-1} (see above).

Circular Dichroism (CD) and Fluorescence Spectra. All spectra were run in 5 mM potassium phosphate, pH 6.8, at 25 °C. CD spectra from 190 to 300 nm were acquired on a Jasco J-700 spectropolarimeter. Fluorescence spectra were recorded on a Perkin-Elmer LS-50B luminescence spectrometer, with excitation and emission slit widths of 2.5 and 5 nm, respectively.

Chromatography. For determination of oligomeric structure, proteins were analyzed by high-pressure size exclusion chromatography on a Biosep-SEC-S3000 (Phenomenex) 30 cm × 0.78 cm column eluted at 0.6 mL/min with 50 mM Tris-acetate, pH 7.2, at 4 °C. Chromatographic data were recorded with a Waters Model 996 photodiode array detector.

Mass Spectrometry. Purified dehydratase was extensively dialyzed against distilled water and lyophilized to dryness. Electrospray ionization mass spectra were acquired on a Finnigan-MAT SSQ700 quadrupole mass spectrometer fitted with an Analytica of Branford electrospray source. The electrospray energy was −3.5 kV, and the nitrogen bath gas was heated to 160 °C. Samples were dissolved at a concentration of approximately 10 pmol/μL in 50% acetonitrile/0.5% acetic acid and infused into the electrospray interface at a flow rate of 1 μL/min. Spectral averaging for 1 min was employed prior to profile mode data acquisition. Deconvolution of the ESI mass spectra was accomplished by the BioMass program component of the SSQ data system software.

Nonenzymatic Dehydration of 4a-Hydroxy-6-methyl-tetrahydropterin. The rate of dehydration of 4a-hydroxy-6(*S*)-methyl-tetrahydropterin was determined at 17 ± 0.1 °C in imidazole-Cl, bis-Tris-Cl, and sodium acetate buffers with concentrations ranging between 0.01 and 0.1 M (0.2 M for imidazole) by observing the increase of absorbance at the isosbestic wavelength for the conversion of the initial quinoid 6-methyl-dihydropterin to 6-methyl-7,8-dihydropterin. Due to the varying proportion of the cationic forms of these products, this wavelength (which was determined by repeat scanning of separate reaction mixtures over 30 min) varied between 330 and 350 nm in the pH range studied (3.2−7.8). Since the effect of added KCl on dehydration is small (*25*), buffers were not made to constant ionic strength. The

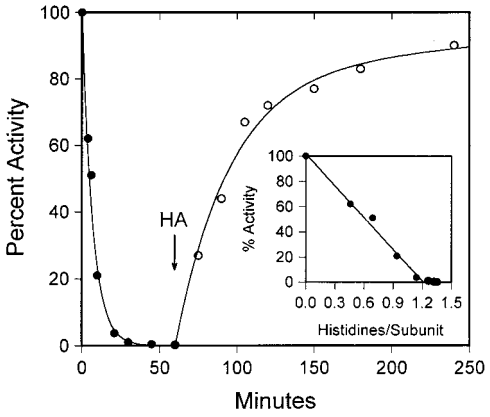

FIGURE 2: Inactivation of rat liver dehydratase by DEPC and reactivation by hydroxylamine (HA). The reaction was performed in 5 mM potassium phosphate, pH 6.5, at 25 °C and contained 35 μM (subunit) rat liver dehydratase and 1 mM DEPC. Aliquots were removed at timed intervals, the reaction was quenched by addition of an equal volume of 5 mM imidazole, and the activity was measured with 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin as substrate in 25 mM Tris-HCl, pH 7.4, at 10 °C. After 60 min, hydroxylamine (HA) was added to give a final concentration of 0.5 M, and incubation continued for an additional 180 min at 25 °C, with samples removed and assayed for activity at the indicated times. The inset shows the relationship between activity and the number of histidine residues derivatized per subunit.

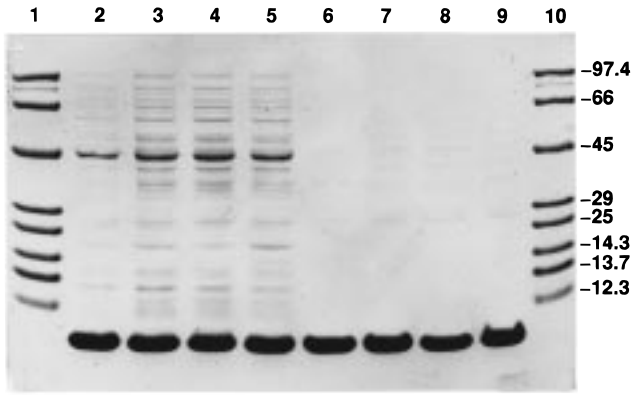

FIGURE 3: SDS−PAGE of 100000*g* supernatants of lysed cells (20 μg of each), wild-type, H61A, H62A, and H79A (lanes 2, 3, 4, and 5); and purified dehydratase (10 μg of each), wild-type, H61A, H62A, and H79A (lanes 6, 7, 8, and 9). Molecular weight standards (kDa) (lanes 1 and 10).

pH values were measured in the final reaction mixtures while at 17 °C. Data were collected and fitted to a first-order equation as described previously (*25*).

The ultraviolet absorption spectrum of 4a-hydroxy-6(*S*)-methyl-tetrahydropterin at pH 4.5 was obtained by continuous flow analysis by injecting 1 μL of a 0.5 mM solution in methanol into a stream of cooled 10 mM sodium acetate pH 4.5, and pumped at 2.0 mL/min into a Water's 996 photodiode array detector set to acquire 5 spectra/sec. The delay between injection and appearance in the flow cell was about 1.2 s. After some of the injections, the flow was stopped and the reaction followed with time. Dehydration of the carbinolamine was complete by 5 s. The spectrum of the reaction at 0 time was approximated by subtracting from the initial spectrum of the carbinolamine the spectrum of the quinoid 6-methyl-dihydropterin found at 5 s multiplied by a factor such that no absorbance at 325 nm appeared in the difference spectrum.

RESULTS

Reaction of Rat Liver Dehydratase with Diethylpyrocarbonate (DEPC). In preliminary experiments the role of histidine residues in catalyzing dehydration of 4a-hydroxy-tetrahydropterins was ascertained by incubating dehydratase with DEPC, which is relatively selective for histidyl residues. The activity of the enzyme and the increase in absorbance at 240 nm due to formation of *N*-carbethoxy-histidine were measured as a function of time after addition of DEPC. There was no change in absorbance at 280 nm, ruling out reaction at tyrosine residues (*31*). A direct relationship was observed between the extent of reaction with DEPC and the loss of activity. At 25 °C and pH 6.5 half of the activity was lost in 6 min (Figure 2). Inactivation was complete when about 1.2 histidines/subunit had been derivatized (Figure 2, inset). The DEPC inactivated dehydratase was then incubated with hydroxylamine. After 3 h 90% of the activity was restored indicating that reaction of DEPC was specifically with a histidine residue (*31*) (Figure 2). The competitive inhibitor, quinoid 6(*S*)-propyl-dihydropterin, when added before DEPC, protected dehydratase from inactivation in a concentration-dependent manner. Thus, the inactivation by DEPC was due to reaction with a histidine at the catalytic site.

Structure of Histidine Mutants and Wild-Type Recombinant Dehydratases. The three histidine residues in dehydratase which are conserved in mammals and in bacteria, H61, H62, and H79, (*7*), were individually mutated to alanine. The mutant proteins were produced at levels comparable to that of recombinant wild-type enzyme, and on SDS−PAGE all showed a major band at ~12 kDa (Figure 3, lanes 2−5). The high level of expression allowed cell extracts to be purified to >99% homogeneity by a one-step column chromatofocusing procedure (Figure 3, lanes 6−9).

The molecular weights of each of the recombinant dehydratases were determined by electrospray mass spectrometry and found to be 11 867 Da for wild-type and 11 800 Da for all 3 mutants, consistent with replacement of histidine by alanine and also cleavage of the N-terminal methionine. In contrast to rat liver dehydratase, which is acetylated on the N-terminal amino group to give a molecular weight of 11 909 Da (*4*), the dehydratases expressed in *E. coli* were not post-translationally modified beyond removal of the N-terminal methionine, as previously reported (*32*). Since there is the possibility of an effect of the N-terminal acetyl group on function, both rat liver dehydratase and recombinant wild-type dehydratase have been used as controls in characterization of the mutants.

To examine the effect of the mutations on secondary or tertiary structure, fluorescence emission spectra were measured from 310 to 450 nm with excitation wavelength at 295 nm with 2.5 μM enzyme in 5 mM potassium phosphate, pH 6.8. The intensity and maxima (330 nm) of the emission spectra of the 3 mutants, wild-type recombinant, and rat liver dehydratase were identical to each other, indicating that the local environment of the tryptophan residues is unchanged. To further verify that the histidine to alanine mutations had not significantly altered the secondary structure, the CD spectrum of each mutant dehydratase was acquired and compared to the spectrum of rat liver and wild-type dehydratase. The CD spectra of the mutants were superimposable

Table 1: Catalytic Efficiency of Histidine Mutant and Wild-Type Recombinant Dehydratases in the Dehydration of 4a(*R*)-Hydroxy-6(*S*)-methyl-tetrahydropterin as Substrate[a]

			catalytic efficiency[b]	
dehydratase	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} sec^{-1})	(%)
rat liver	10	1.5	6.7	100
wild-type	10	1.5	6.7	100
H61A			$<3 \times 10^{-3}$	<0.06
H62A	2.4	30	0.08	1.2
H79A	7.5	2.5	3	45

[a] Reactions were at 10 °C in 25 mM Tris-HCl, pH 7.4, with 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin as substrate. Dehydratase concentrations ranged from 0.1 to 1 nmol/mL of reaction for rat liver, wild-type, and H79A, and from 1 to 7 nmol/mL of reaction for H62A. The kinetic constants for each dehydratase were determined in at least 5 separate experiments with 3−5 progress curves in each experiment. With H61A at concentrations of up to 60 nmol/mL, no enzyme-dependent rate consistent with direct dehydration of 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin at concentrations up to 200 μM could be detected. [b] Percent catalytic efficiency = 100(k_{cat}/K_m for mutant)/(k_{cat}/K_m for wild-type).

Table 2: Catalytic Efficiency of Histidine Mutant and Wild-Type Recombinant Dehydratases in the Dehydration of 4a(*S*)-Hydroxy-6(*R*)-methyl-tetrahydropterin as Substrate[a]

			catalytic efficiency	
dehydratase	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} sec^{-1})	(%)
rat liver	10	6	1.67	100
wild-type	10	6	1.67	100
H61A	8.6 ± 3.5	390 ± 160	0.022	1.3
H62A	8.9 ± 1.9	330 ± 70	0.027	1.6
H79A			$<3 \times 10^{-3}$	<0.18

[a] Reaction conditions were the same as for Table 1, except that 4a(*S*)-hydroxy-6(*R*)-methyl-tetrahydropterin was substrate, and dehydratase concentrations were 0.1−1 nmol/mL for rat liver and wild-type, and 2−7 nmol/mL for H61A and H62A. With up to 50 nmol H79A/mL, no enzyme-dependent rate could be detected with 4a(*S*)-hydroxy-6(*R*)-methyl-tetrahydropterin at concentrations of up to 200 μM.

on the spectra of wild-type and rat liver. Therefore, since the fluorescence and CD spectra of each of the three mutants were found to be similar to the spectra of wild-type and rat liver dehydratases, the mutations do not appear to have resulted in any major distortion of structure.

The mutant and wild-type recombinant dehydratases and the enzyme isolated from rat liver were analyzed by high-pressure size exclusion chromatography. The retention times and peak shapes of each were found to be identical and, when compared to molecular weight standards, showed that all were tetramers with molecular weights in the region of 46 000 kDa. Additionally, all of the proteins were retained by a 30 kDa ultrafiltration membrane (Amicon YM 30). Thus, there appears to be no effect of the histidine → alanine mutations nor of the N-terminal acetyl group on the oligomeric state of the protein.

Dehydratase Activity with 4a(R)-Hydroxy-6(S)-methyl-tetrahydropterin. The kinetic parameters of rat liver, wild-type, and the 3 histidine mutant dehydratases were determined under standard assay conditions at pH 7.4, with chemically synthesized 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin as substrate over a range of enzyme concentrations. This substrate has the same configuration at the 6-position as 6(*R*)-tetrahydrobiopterin.[2] The results summarized in Table 1 show that the change of histidine 79 to alanine has only a small effect on K_m and k_{cat}. The effect of the mutation of histidine 62 to alanine is more pronounced with an increase in K_m of 20-fold and a decrease in k_{cat} of 4-fold. With H61A, although some NADH consumption was detected in the presence of 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin, experiments employing a wide range of dehydratase (up to 60 μM) and initial substrate concentrations indicated that these rates were due to the presence of a small amount of the 4a(*S*) epimer.

To ascertain whether the reactions of recombinant wild-type and mutant dehydratases are affected by pH in the same manner as the rat liver enzyme, K_m and k_{cat} with 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin were also determined at pH 8.4. With an increase in pH from 7.4 to 8.4, k_{cat} decreased 3-fold for wild-type similar to the effect of pH on rat liver dehydratase (*17*), but decreased 5−6-fold for H62A and H79A. However, as with rat liver enzyme (*17*), there was no significant difference in affinity for substrate between pH 7.4 and 8.4 for H62A, H79A, or wild-type dehydratase.

Dehydratase Activity with 4a(S)-Hydroxy-6(R)-methyl-tetrahydropterin . We have previously reported that rat liver dehydratase catalyzes dehydration of both isomers generated in the chemical synthesis of substrate (*17*). To shed further light on this unusual lack of stereospecificity, the substrate properties of 4a(*S*)-hydroxy-6(*R*)-methyl-tetrahydropterin were evaluated with each of the histidine → alanine mutant dehydratases, over a range of enzyme concentrations. As in all other analyses, wild-type recombinant dehydratase was indistinguishable from the rat liver enzyme. However, the relative abilities of the histidine mutants to dehydrate 4a-(*S*)-hydroxy-6(*R*)-methyl-tetrahydropterin (Table 2) differed considerably from their catalytic properties demonstrated with 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin (Table 1). As summarized in Table 1, with 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin as substrate, k_{cat}/K_m values for H79A and H62A are, respectively, 45% and 1.2% of that of wild-type. Comparison of the kinetic parameters determined with the 6(*R*) isomer in Table 2 with the value in Table 1 using the 6(*S*) isomer shows that the H62A mutant has nearly the same percent catalytic efficiency regardless of which isomer is the substrate. In contrast, H61A, which shows no activity with 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin, is able to catalyze dehydration of 4a(*S*)-hydroxy-6(*R*)-methyl-tetrahydropterin with a catalytic efficiency of 1.3% of that of wild-type. Furthermore, with H79A there was no enzymatic activity attributable to dehydration of 4a(*S*)-hydroxy-6(*R*)-methyl-tetrahydropterin when examined at enzyme concentrations up to 50 μM.

Effect of DEPC on Histidine → Alanine Mutant Dehydratases. As noted above, reaction of rat liver dehydratase with DEPC at pH 6.5 resulted in a total loss of activity after the covalent reaction of DEPC with ~1.2 histidines (Figure 2). To identify which histidine(s) had been derivatized, H79A, H62A, and H61A were incubated with DEPC for 60 min and the extent of reaction was compared with wild-type and rat liver dehydratases. The results show that at pH 6.5, H62A and H79A react with DEPC to the same extent as wild-type and rat liver dehydratase. However, there appears to be almost no reaction of H61A with DEPC (Figure 4).

[2] A simple 6(*S*)-alkyl-tetrahydropterin has the same configuration as 6(*R*)-tetrahydro-biopterin due to alteration of side-chain priority by the 1′-hydroxyl group.

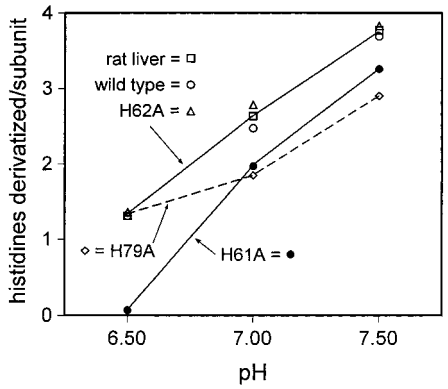

FIGURE 4: The number of histidine residues derivatized by DEPC (1 mM) in rat liver, wild-type, and in three histidine → alanine mutants of dehydratase (each at 35 μM) after incubation for 1 h at 25 °C in 5 mM potassium phosphate at the indicated pH.

This implies that at pH 6.5 DEPC reacts primarily with histidine 61.

When DEPC was incubated with rat liver, wild-type, and the three histidine mutant dehydratases at higher pH's, further difference between the reactivities of each of the dehydratases was observed. The number of histidine residues derivatized per subunit of enzyme at each pH after 60 min is summarized in Figure 4. Although H79A is modified to the same extent at pH 6.5 as wild-type, at pH 7.0 both H61A and H79A have about 0.7 fewer histidines carbethoxylated. The H62A mutant behaves identically to wild-type suggesting that H62 does not react with DEPC within the pH range studied. At pH 7.5, approximately three histidines are modified in H61A and H79A, but close to four are modified in H62A, wild-type, and rat liver dehydratases. The latter observation indicates that at least two histidines outside of the catalytic domain also react with DEPC. There was no change in 280 nm absorbance at any pH indicating the absence of reaction of DEPC with tyrosine residues.

Acid Catalysis of Nonenzymatic Dehydration. The change in the rate of nonenzymatic dehydration of 4a-hydroxy-6(*S*)-methyl-tetrahydropterin at 17 °C in imidazole-Cl, bis-Tris-HCl, and sodium acetate as a function of buffer concentration was extrapolated to 0 buffer ($k_{\text{dehyd}[0]}$) and fitted to eq 1:

$$k_{\text{dehyd}[0]} = k_0 + k_{\text{H1}}[\text{H}^+]f_0 + k_{\text{H2}}[\text{H}^+]f_+ \qquad (1)$$

where k_0 is the first-order solvent-dependent rate of dehydration of the neutral carbinolamine in the absence of acid or base catalysis (set to 0.002 s^{-1} as previously published, *25*), and k_{H1} and k_{H2} are the rate constants for proton-catalyzed dehydration of the neutral and cationic species of the carbinolamine which are present in fractions f_0 and f_+, respectively. Since the p*K* for formation of the anion is 9.8 (*25*), $f_0 = 1 - f_+$ in the pH region studied in the present investigation. The value of k_{H1} obtained by nonlinear least-squares fit to the buffer-independent rates, shown in Figure 5, was found to be $1.55 \pm 0.3 \times 10^5$ M^{-1} sec^{-1}, only slightly higher than previously estimated using a more limited pH range (*25*). Proton catalysis of the dehydration of the cation, k_{H2}, was found to be 600 M^{-1} sec^{-1}. A p*K* value of 5.95 ± 0.05 was calculated for the transition between the neutral and cationic species. A break in the rate of dehydration of 4a-hydroxy-6-methyl-tetrahydropterin versus pH in this region has been previously reported to have a somewhat

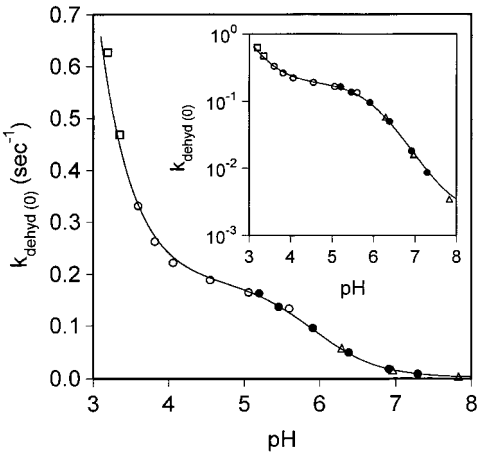

FIGURE 5: The buffer-independent rate of nonenzymatic dehydration of 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin as a function of pH at 17 °C. The rates in imidazole-Cl (triangles), bis-Tris-HCl (closed circles), and sodium acetate (open circles) were extrapolated to 0 buffer concentration. The open squares are with dilute HCl. Inset: the same buffer-independent rates are plotted on a log scale. The lines are from the fit to eq 1.

higher p*K* value (*22*), possibly due to differences in reaction temperature and the model used to fit the data.

Imidazole catalysis of dehydration at pH 6.29, 6.96, and 7.83 was found to have catalytic constants of 0.93, 0.84, and 0.28 M^{-1} sec^{-1}, respectively. Fitting the individual data points to eq 2

$$k_{\text{Imid}[\text{total}]} = k_{\text{dehyd}[0]} + k_{\text{Imid}}\, f_0 + k_{\text{ImidH+}}\, f_0 \qquad (2)$$

showed that the constant for general acid catalysis by the imidazolium ion of dehydration of neutral 4a-hydroxy-6(*S*)-methyl-tetrahydropterin ($k_{\text{ImidH+}}$) was 1.5 ± 0.1 M^{-1} sec^{-1}, whereas the catalytic constant for the action of imidazole free base on the neutral carbinolamine (k_{Imid}) was 0.05 ± 0.02 M^{-1} sec^{-1}. The ability to detect dehydration of the carbinolamine cation by the imidazolium ion (catalytic constant is probably low) was limited by the lack of buffer capacity by imidazole in the pH region where the cation is in abundance.

Catalysis of dehydration by sodium acetate buffer showed a maximum value at pH 5.1 due to the overlap between the p*K* for acetate and that of the carbinolamine. The rate observed at pH 5.6 was 0.76 M^{-1} sec^{-1}, rose to 0.86 M^{-1} sec^{-1} at pH 5.1, and then gradually fell to 0.58 M^{-1} sec^{-1} by pH 3.6. Fitting the individual data points to eq 3

$$k_{\text{acetate}} = k_{\text{dehyd}[0]} + k1_{\text{HOAc}}\, f_0 + k2_{\text{HOAc}}\, f_+ \qquad (3)$$

indicated that the action of the acid species of acetic acid on the neutral carbinolamine ($k1_{\text{HOAc}} = 18.5 \pm 2$ M^{-1} sec^{-1}) is about 30-fold higher than that of the carbinolamine cation ($k2_{\text{HOAc}} = 0.55 \pm 0.15$ M^{-1} sec^{-1}). Inclusion in eq 3 of a term for catalysis due to the acetate ion did not significantly improve the fit to the data. Catalysis of dehydration by bis-Tris was very low and essentially undetectable near neutral pH as might be expected for such a weak base.

The UV spectrum of 4a-hydroxy-6(*S*)-methyl-tetrahydropterin in 10 mM sodium acetate, pH 4.5, was found to have absorbance maxima at 224 and 279 nm ($\epsilon_{279}/\epsilon_{224} \approx 1.05$) and lacked the strong 246 nm peak of the neutral species (*25*). Repeat spectra of the decay of the carbinolamine

between pH 3 and 8 indicated that the large majority of the products can be accounted for by appearance first of quinoid 6-methyl-dihydropterin and subsequently of 6-methyl-7,8-dihydropterin.

DISCUSSION

We have previously shown that rat liver dehydratase acts almost equally well on both the 6(*R*) and 6(*S*) isomers of 4a-hydroxy-6-methyl-tetrahydropterin, both having identical k_{cat} and nearly the same K_m values (*17*). An especially striking result of the current work is that mutation of H79 or H61 confers stereospecificity, but for opposite substrate isomers. This observation is most readily understood in terms of acid catalysis in which these histidines selectively protonate the 4a(*S*)- and 4a(*R*)-hydroxyl groups, respectively.

Since no metal or cofactors are required for activity (*17*), general acid and/or base catalysis, which we have observed in the nonenzymatic reaction (*25*), by protein residues appeared to be a likely mechanism. Many classical chemical studies of carbinolamines illustrate both specific and general acid catalysis of water elimination (*24*, *33*−*36*). This has been demonstrated in the particular case of the 4a-hydroxy-tetrahydropterins for a number of buffers at pH 7 and above (*25*). In the current work, the extension to lower pH values using several buffer types clearly shows that nonenzymatic dehydration of 4a-hydroxy-6(*S*)-methyl-tetrahydropterin is subject to both proton and general acid catalysis. A break in the curve of the buffer-independent rate versus pH (Figure 5) and also the changes in buffer constants with pH are consistent with decreased catalysis upon protonation of 4a-hydroxy-6-methyl-tetrahydropterin with a p*K* value of 5.9 at 17 °C. This dissociation constant is tentatively assigned to protonation of the extended guanidinium system on the basis of spectral similarity to N^6-(2′(*S*)-aminopropyl)-quinoid divicine in 0.1 M HCl (λ_{max} = 224 and 270 nm), and the similarity of the p*K* to that of quinoid divicine (5.2) (*25*). Even below pH 5, however, acid catalysis of dehydration is not completely abolished; the effectiveness of proton and the acid form of acetate buffer being decreased about 260- and 34-fold, respectively, in comparison to their actions on the more neutral species. Nonetheless, it is clearly advantageous for the dehydratase to bind substrate in a manner that avoids putting a positive charge on the guanidinium system. The X-ray crystal structure of dehydratase with 7,8-dihydrobiopterin bound (*20*) shows that all of the hydrogen-bonding interactions of the guanidinium system involve donation of hydrogen from ligand to protein.

The imidazolium ion was found to be an effective general acid catalyst of nonenzymatic dehydration, having a rate constant at 17 °C of 1.5 M^{-1} sec^{-1} in its action on the neutral carbinolamine. This is about 30 times greater than that which was found for imidazole free base. A Brønsted plot of k_{H1}, k_{ImidH+}, and $k1_{HOAc}$ gave an α value of 0.58, which is only slightly lower than that found for dehydration of the carbinolamines formed from benzaldehyde with dimethylamine (α = 0.73) (*33*) or from *p*-chlorobenzaldehyde with hydrazine (α = 0.62) (*34*). Since the turnover number of rat liver dehydratase is about 16 s^{-1} at 17 °C (*17*), the effective molarity of the enzyme is about 11 M relative to nonenzymatic catalysis by the imidazolium ion. This level of enhancement of an intramolecular reaction over the intermolecular reaction is typical of general acid/base catalysis (*37*, *38*).

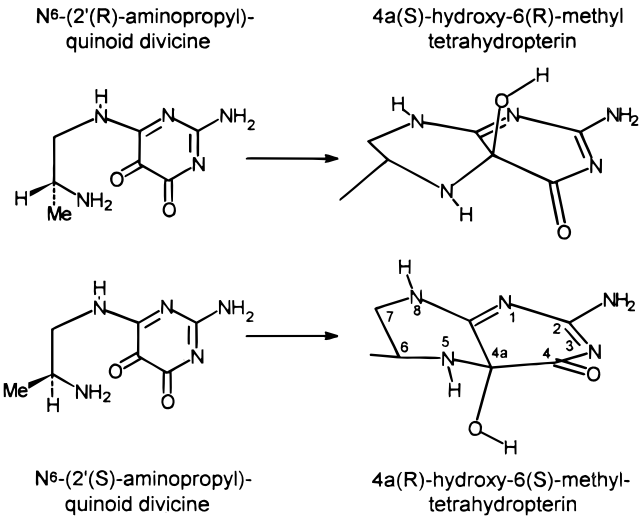

FIGURE 6: Cyclization of quinoid 6*N*(2′*S*-aminopropyl)-divicine and quinoid 6N(2′*R*-aminopropyl)-divicine to their predominant 4a epimers.

Acid catalysis as a mechanism for 4a-hydroxy-tetrahydropterin dehydratase requires protonation of a hydroxyl group that occupies markedly different positions in the two 4a isomers of substrate. Modeling of these isomers by molecular mechanics[3] indicates that due to the tetrahedral 4a carbon, their hydroxyls project perpendicularly to opposite sides of the pterin ring. The chemical synthesis of, for example, 4a-hydroxy-6(*S*)-methyl-tetrahydropterins involves the intramolecular cyclization of quinoid 6*N*(2′*S*-aminopropyl)-divicine (*10*, *25*). We have observed by CD spectroscopy a high degree of chiral induction in this reaction at the incipient 4a center (Bailey, S. W., and Ayling, J. E., manuscript in preparation) and that the resulting 4a hydroxyl group is trans to the 6-substituent.[4] Thus, when the 6-alkyl substituent of the product is (*S*), the 4a-hydroxyl is primarily in the (*R*) configuration, and vice versa (Figure 6). The H79A mutant dehydratase possesses 75% of the k_{cat} of wild-type in its reaction with 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin, but no activity attributable to the 4a(*S*)-hydroxy-6(*R*)-methyl-enantiomer could be detected. Conversely, H61A was found to have approximately 86% of wild-type activity with 4a(*S*)-hydroxy-6(*R*)-methyl-tetrahydropterin, but none due to the 4a(*R*)-hydroxy-6(*S*)-methyl-isomer. These data are consistent with acid catalysis by protonation of the 4a(*R*)-hydroxyl group specifically by H61 and, conversely, protonation of the 4a(*S*)-hydroxyl group specifically by H79. The physiological relevance of the ability of the enzyme to dehydrate both 4a-hydroxy-isomers is currently under investigation and will be the topic of a future communication.

[3] Calculated for the endocyclic tautomer using PCMODEL, Serena Software, Bloomington, IN.

[4] It appears that the previous assignment of a cis configuration to the 4a-hydroxyl group in the product of the phenylalanine hydroxylase reaction (*39*) was a result of the use of the racemic 6(*R*,*S*)-methyl-tetrahydropterin.

[5] Since the N-terminal methionine is cleaved from the recombinant dehydratase as well as from the enzyme isolated from liver, methionine is not included in the numbering of the amino acid residues (*1*, *4*, *32*). The numbers in the X-ray data deposited in the Brookhaven Protein Data Bank (1DCO and 1DCP) include methionine and therefore are greater by 1 than the final protein numbering.

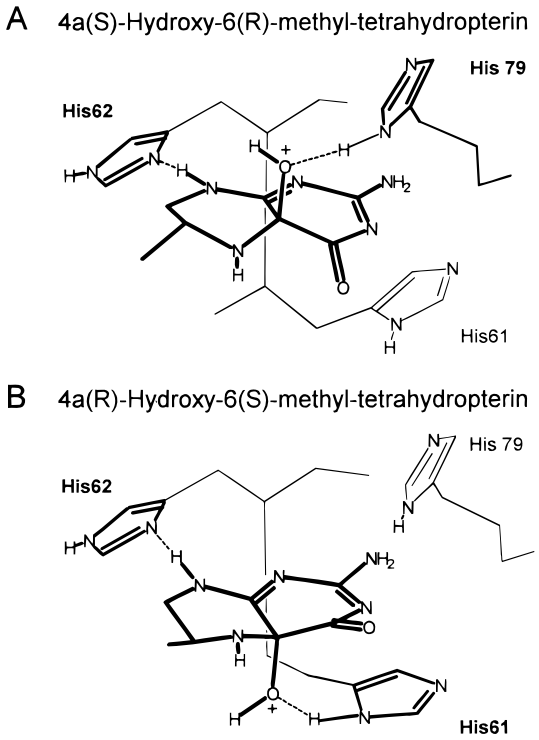

FIGURE 7: The differential interaction of 4a(*S*)-hydroxy-6(*R*)-methyl-tetrahydropterin (A) and 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin (B) with the dehydratase active site histidines. The main component of catalysis by general acid attack on the 4a-hydroxyl group is provided (A) by H79 in the case of the 4a(*S*) isomer or (B) by H61 when the 4a(*R*) isomer is used as substrate. With both isomers, H62 also promotes the reaction by abstraction of the proton on N8.

Inspection of the X-ray crystal structure of dehydratase/DCoH with 7,8-dihydrobiopterin bound (*20*) reveals that this compound indeed resides directly between histidines 61 and 79[5] (Figure 7). Placing the pyrimidine rings of either 4a-(*S*)- or 4a(*R*)-hydroxy-tetrahydropterin in the average position of the pyrimidine ring of the analogue in the 3D crystal data indicates that H61 would be close to the trans-configured hydroxyl group in 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin[2] with Nδ of H61 located about 3.5 Å from the 4a(*R*)-hydroxyl group (Figure 7). This is consistent with the lack of activity of H61A with this substrate. H79 sits at the mouth of the catalytic domain and, perhaps due to the poor affinity of 7,8-dihydrobiopterin,[6] half of the residues in the crystal structure are in a conformation that places them mostly in solvent. Of those H79 residues oriented in the direction of the analogue, their imidazole Nδ is close to the calculated position of the oxygen of the isomeric 4a(*S*)-hydroxyl group. A much greater decrease in affinity was found with H61A for its preferred 4a(*S*),6(*R*) substrate isomer than with H79A and the 4a(*R*),6(*S*) isomer. Although this may be a result of a minor disruption in the conformation of the catalytic domain in H61A, H61 may also serve to directly anchor both substrate isomers through van der Waals contact with some of the more planar atoms in the pyrimidine ring, for example, N1, C2, 2-NH_2, and C8a.

[6] The inhibitory potency of 7,8-dihydrobiopterin (*17*) has been re-examined at even higher concentrations and the K_i found to be about 400 μM at pH 7.4 and 10 °C against 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin as substrate.

The functioning of H61 or H79 as an acid catalyst is supported by their behavior with diethylpyrocarbonate. Of the four histidine residues that can be derivatized by DEPC, the histidine mutants show that H79 and especially H61 are particularly susceptible at low pH. Thus, for example, at pH 6.5 although approximately one histidine is derivatized in the wild-type and in the H62A and H79A mutant dehydratases, there is very little derivatization of the H61A mutant. At pH 7, both H61 and H79 are derivatized, whereas H62 is not derivatized even when the pH is increased to 7.5. The extent of derivatization shown in Figure 4 reveals only the relative reactivities of these residues in the absence of substrate rather than specific p*K* values. Nonetheless, since only the free base of a given histidine residue should react at an appreciable rate with DEPC, these data suggest that H61 and H79 have the lowest p*K* values among those to which the reagent is accessible, consistent with a role as general acid catalysts (*24*). Although the reactivity with DEPC does not address the protonation state of each histidine in the presence of substrate, the simplest conclusion that can be drawn from the complementary enzymatic activity of H61A and H79A, their orientation in the crystal structure, and their reaction with DEPC is that these histidines selectively protonate 4a-hydroxyls having opposite configurations.

Base catalysis of nonenzymatic dehydration is usually observed only with carbinolamines having a relatively acidic nitrogen formed by condensation of an aldehyde or ketone with a weakly basic amine such as an oxime (*24*, *34*). However, even though 4a-hydroxy-tetrahydropterins are the intramolecular cyclization products of a strongly basic alkylamine (Figure 6), we have previously observed both hydroxide and general base promotion of nonenzymatic dehydration. Since the proton on N5 is not especially amenable to removal by base, it was proposed that nonenzymatic base catalysis would most likely occur by interaction with N8, or possibly on the 2-NH_2 or N3 (*25*). The kinetics of the mutant with alanine replacing H62 suggest that this residue has a major role in binding of substrate, and that it also is involved in some base catalysis. With 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin and the 4a(*S*),6(*R*)- enantiomer, H62A was found to have 20- and 55-fold increased K_m values, respectively, compared to wild-type dehydratase. Since the atoms of the extended guanidinium system of the two isomers are nearly coplanar, the similarity of the effects of this mutation on affinity is consistent with a binding mode for both 4a(*S*) and 4a(*R*) which corresponds to that of 7,8-dihydrobiopterin in the X-ray crystal. Nitrogen-8 of this latter ligand is located about 2.6 Å from H62−Nδ, and both hydrogens of the 2-amino group are coordinated to backbone carbonyl oxygens (*20*).

Although the effect of the H62A mutation on k_{cat}/K_m is nearly identical for both 4a isomers, the decrease in k_{cat} with 4a(*R*)-hydroxy-6(*S*)-methyl-tetrahydropterin was greater than that found with the 4a(*S*)-hydroxy-6(*R*)-enantiomer. However, with the latter, the extent of the decrease is somewhat uncertain due to the high K_m of this substrate (Table 2). In any event, the slower turnover suggests that H62 may promote dehydration via base catalysis. According to the mechanism proposed for the nonenzymatic reaction (*25*), this could occur either by H62 promoted formation of the substrate anion in the E·S complex or by a concerted action

with acid catalysis by H61 or H79 in the transition state, a question that remains to be addressed. The lack of reaction of H62 with DEPC (i.e., H62A was found to be derivatized to the same extent as wild-type dehydratase) at any pH may be due to either lack of reagent accessibility or the fact that in the absence of substrate it remains largely cationic under the derivatization conditions investigated. The latter possibility would indicate that H62 is fairly basic compared to free imidazole.

These observations resolve the confusion as to which histidine residues might be acting as general acid or general base catalysts (*20*, *22*). The H61A and H79A mutants clearly demonstrate that the enzyme does not need both H61 and H79 for a given substrate isomer. Instead, each acts as general acid catalyst for the stereospecific elimination of a 4a(*R*)- or 4a(*S*)-hydroxyl group, respectively. The base catalysis by abstraction of a proton from N5 that has been proposed (*20*, *22*) would not be expected considering the precedence of nonenzymatic carbinolamine dehydration (*24*, *34*). A molecule of solvent water would be an adequate acceptor for the final removal of proton from N5 of the quinoid dihydropterin. Potentially, this deprotonation could also occur after release of product from the dehydratase. The data presented indicate that the major mechanism of dehydration is acid catalysis by H61 or H79 (but with opposite 4a isomers), with H62 promoting binding and some base catalysis via N8. Although 7,8-dihydrobiopterin is a very weak inhibitor, the published crystal structure is consistent with these conclusions.

Several important aspects of the interaction of substrate with the dehydratase await further clarification. Since 7,8-dihydrobiopterin lacks a 4a-hydroxyl group and has markedly different conformational and electronic properties than the carbinolamine substrate, this analogue was not well localized within the catalytic domain, and only small changes in protein conformation were observed upon its binding. A high-resolution crystal structure with a tight binding stable 4a-hydroxy-tetrahydropterin would help to resolve the remaining mechanistic and structural questions.

ACKNOWLEDGMENT

We wish to thank Dr. C. Heizmann for his interest and support, Dr. Susan Weintraub for the ES-MS analysis, and F. Neuheiser for technical assistance.

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BI980663H