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Analysis of Active-site Residues in Hyoscyamine 6β-Hydroxylase

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Abstract

Three amino acid residues (histidine-217, aspartic acid-219, and histidine-274) in *Hyoscyamus niger* hyoscyamine 6β-hydroxylase are strictly conserved among 2-oxoglutarate-dependent dioxygenases and other structurally related enzymes. These residues were investigated by chemical modification and site-directed mutagenesis. The hydroxylase was expressed in *E. coli* as a fusion protein to maltose-binding protein. Modification of histidine residues by diethyl pyrocarbonate inactivated the recombinant wild-type hydroxylase. Inactivation was prevented most effectively by the presence of 2-oxoglutarate. Mutation of histidine-217 to glutamine, histidine-274 to glutamine, or aspartic acid-219 to either histidine or asparagine inactivated the hydroxylase, whereas substitution of loosely conserved histidine-66 with glutamine did not decrease the catalytic activity of the enzyme. These results suggest that histidine-217, aspartic acid-219, and histidine-274 play important roles in the hydroxylase function, and may be the ligands to the active-site iron.

1. Introduction

Hyoscyamine 6β-hydroxylase (H6H; EC 1.14.11.11) from *Hyoscyamus niger* is a monomeric multifunctional enzyme that catalyzes hydroxylation of hyoscyamine, epoxidation of 6, 7-dehydrohyoscyamine by oxygen insertion into the double bond, and epoxidation of 6β-hydroxyhyoscyamine by 7-dehydrogenation [1-3]. In the medicinal solanaceous plants that produce anticholinergic tropane alkaloids, H6H catalyzes two consecutive oxidation reactions leading from hyoscyamine to its epoxide scopolamine by way of 6β-hydroxyhyoscyamine [3, 4]. For catalysis, H6H requires, in addition to the alkaloid substrate, Fe^{2+}, 2-oxoglutarate, O_2, and ascorbate, and is a member of the 2-oxoglutarate-dependent dioxygenase family.

Molecular cloning of the hydroxylase cDNA from *H. niger* has shown moderate but significant homology between primary structures of the hydroxylase and several other 2-oxoglutarate-dependent dioxygenases, such as flavanone 3β - hydroxylase, deacetoxycephalosporin C synthase, and deacetylcephalosporin C synthase [5]. Similar levels of significant homology in the amino acid sequences have also been noted between the hydroxylase and several oxygenases that require Fe^{2+} and ascorbate, but not 2-oxoglutarate, such as ethylene-forming enzyme and isopenicillin N synthase [5]. Alignment of these amino acid sequences has revealed that two histidine residues and one aspartic acid residue are strictly conserved among them (**Fig. 1**). Although vertebrate 2-oxoglutarate-dependent dioxygenases, such as prolyl 4-hydroxylase, lysyl hydroxylase, and aspartyl β-hydroxylase, are scarcely homologous in amino acid sequences to the enzymes listed in **Fig. 1**, two histidines and one aspartic acid can be aligned at similar spacings in their sequences [15]. Thus, these three amino acid residues may play important roles in the structure and function of this broad class of non-heme oxygenases.

We have previously screened H6H cDNA from *H. niger* root culture [6] and expressed hyoscyamine 6β-hydroxylase in *Escherichia coli* as a fusion protein to maltose-binding protein (MBP) [4]. The recombinant hydroxylase was recovered primarily in the soluble fraction, and was catalytically active. In this study, we first characterized the affinities of the recombinant hydroxylase toward substrates and co-factors, and then chemically modified histidine residues in the enzyme. Further, several conserved amino acid residues were mutated to examine their importance in the hydroxylase reaction.

2. Materials and Methods

2. 1 Bacterial strain, site-directed mutagenesis, and expression vectors

E. coli strain JM 109 was used as a host cell. *H. niger* H6H cDNA [6] was cloned into pMAL-c (New England Biolabs) to give pMH 1 that expressed a fusion protein between MBP and wild-type H6H, as described elsewhere [4].

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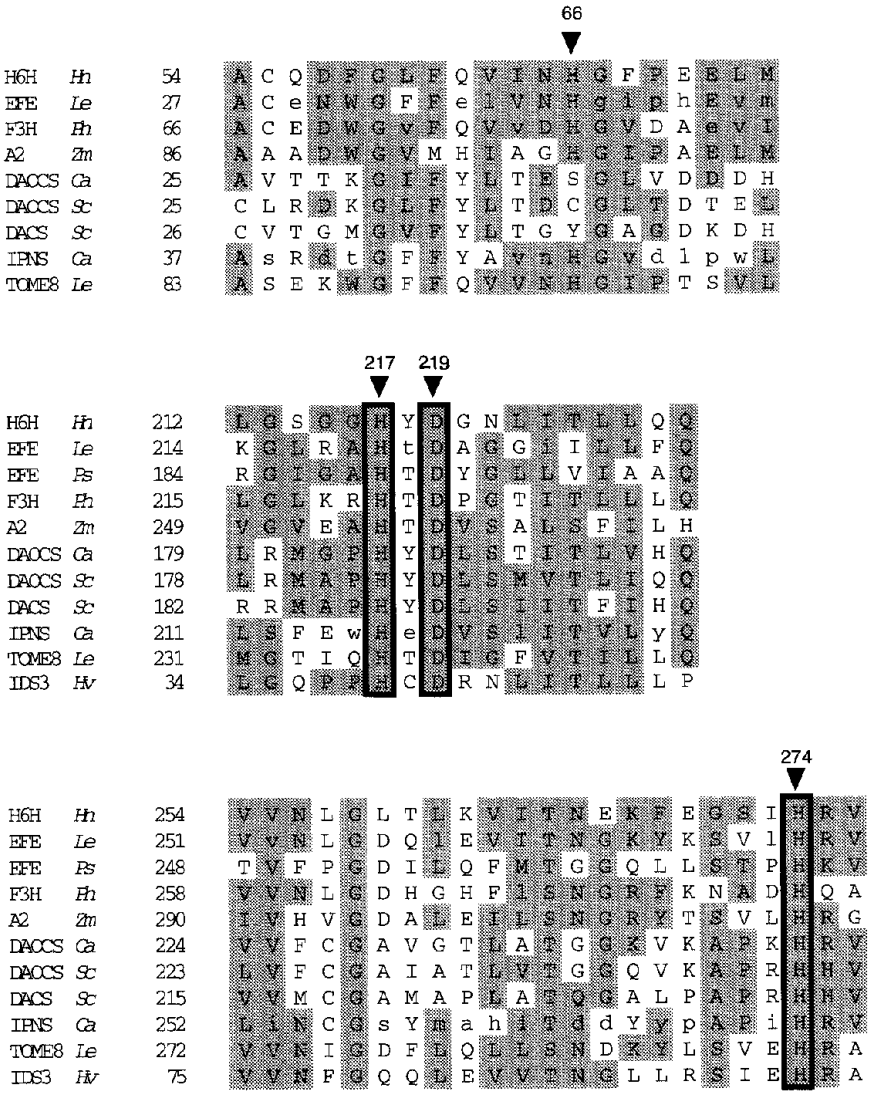

Fig. 1 Homology of H6H to other non-heme oxygenases.
Invariant histidine and aspartic acid residues are boxed and homologous residues are shaded. Mutated residues are indicated by triangles. H6H: hyoscyamine 6β-hydroxylase [6], EFE: ethylene-forming enzyme [7], F3H: flavanone 3β-hydroxylase [8], DAOCS: deacetoxycephalosporin C synthase [9, 10], DACS: deacetylcephalosporin C synthase [11], IPNS: isopenicillin N synthase [12], TOME8: tomato ripening-related protein [13], IDS3: iron deficiency-specific protein3 [14], Hn: *Hyoscyamus niger*, Le: *Lycopersicon esculentum*, Ps: *Pseudomonas syringe*, Ph: *Petunia hybrida*, Zm: *Zea mays*, Ca: *Cephalosporium acremonium*, Sc: *Streptomyces clavuligerus*, Hv: *Hordeum vulgare*

Desired mutations were introduced into a plasmid pNH6HE, in which the entire coding region of H6H cDNA was inserted into the polylinker site of pTV118N (Takara Shuzo, Kyoto). Mutants were generated by oligonucleotide-directed mutagenesis using a Transformer™ Site-Directed Mutagenesis Kit (CLONTECH), except that aspartic acid-219 was changed to asparagine by PCR [16]. Five mutation primers were synthesized using a DNA synthesizer (model 381A, Applied Biosystems):
5′-GGTCTCTTTCAGGTGATTAATCAAGGATTTCCAGAAG, 5′-CATTGGGATCCGGAGGACAATATGATGGTAACC, 5′-CATTGGGATCCGGAGGACACTATCATGGTAACC, 5′-CATTGGGATCCGGAGGACACTATAATGGTAACC, and 5′-GAAAAGTTTGAAGGATCCATCCAAAGGGTAGTGACAGATCC (underlined letters indicate altered nucleotides). These oligonucleotides were designed to change a target amino acid to the desired amino acid, and simultaneously to introduce a new restriction site (*Ase*I or *Bam*HI) near the target site without changing the original amino acid. As a selection primer for Transformer™, an oligonucleotide (5′-GGCGTATCACGAGGCCTTTTCGTCTCGCGCG) was designed to eliminate a unique restriction site, *Eco*O109 I in pTV118N, and to create a new unique restriction site, *Stu*I. Mutants were selected by cutting the wild-type plasmid with *Eco*O109 I, according to

the manufacturer's instructions. Selected plasmids were checked by the newly introduced restriction sites in the mutation primers, and the selection primer. Introduced mutations were also verified by DNA sequencing. When PCR was used for mutagenesis, the entire length of the mutated H6H coding region was sequenced to verify that no unintentional mutations were introduced.

2. 2 Isolation and partial purification of recombinant H6H protein

An overnight culture of 2 m*l* was diluted 100-fold with LB broth containing 60 μg/m*l* ampicillin, and grown at 37°C until OD_{660} became 0.4. The culture was cooled to 10°C, and isopropylthio-β-D-galactoside (IPTG) was added to 0.4 mM. After incubation at 10°C for three days, the culture was centrifuged at 3,000 g for 10 min., and the pellet was suspended in TD buffer (20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol). The bacterial cells were lysed by a single freeze-thaw cycle and sonication. The lysed cells were centrifuged at 10,000 g for 20 min., and the protein in the supernatant was precipitated at ammonium sulfate concentrations of between 40% and 60% (saturated). The precipitate was dissolved in TDG buffer (TD buffer with 30% glycerol), and desalted by passage through PD-10 columns (Pharmacia). The crude enzyme solution (5 μg of protein) was loaded on a hydroxylapatite column, Econo-Pac® HTP Cartridge (5 m*l*; Bio-Rad), that had been equilibrated with TDG buffer. This column was washed with 10 m*l* of the same buffer, after which the protein was eluted with a linear gradient of 0 to 100 mM potassium phosphate in TDG buffer at a flow rate of 0.2 m*l*/min. All fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the fractions that contained recombinant H6H protein were pooled and concentrated with an Amicon YM-30 ultrafiltration membrane (Amicon Corporation). The enzyme solutions were stored in small aliquots at −20°C until assayed.

2. 3 SDS-PAGE and immunoblotting

SDS-PAGE on 10% separation gel and immunoblotting using a mouse anti-H6H monoclonal antibody (mAb5) were performed according to Hashimoto *et al.* [17]. A rabbit antiserum against MBP was purchased from New England Biolabs.

2. 4 Enzyme assay

Partially purified preparations of recombinant H6H were assayed for hydroxylase activity by measuring the formation of 6β-hydroxyhyoscyamine from hyoscyamine by gas-liquid chromatography [3]. The standard reaction mixture contained 50 mM Tris/HCl buffer (pH 7.8), 0.4 mM ferrous sulfate, 4 mM sodium ascorbate, 1 mM 2-oxoglutaric acid, 0.2 mM *l*-hyoscyamine hydrobromide, 1 mg/m*l* catalase (Calbiochem) and the enzyme. In the experiments shown in **Figs. 2C** and **4**, enzyme solutions and buffers were treated with chelate ion-exchange resin (Chelex 100; Bio-Rad) to remove residual iron in the solutions. This treatment somewhat decreased specific activity of the enzyme solutions.

2. 5 Modification by diethyl pyrocarbonate (DEPC)

The enzyme buffer was changed to potassium phosphate buffer (100 mM, pH 6.0) with 30% glycerol using PD-10 columns (Pharmacia). DEPC was diluted with ice-cold anhydrous ethanol before use, and kept on ice. The concentration of ethanol in the reaction mixture was 4% (v/v). The enzyme solution in 50 mM potassium phosphate buffer, pH 6.0, was incubated on ice with 8 mM DEPC. An equal amount of ethanol was added to the reference sample. At various time intervals, samples (10 μl) were withdrawn from the incubation mixture, and a histidine solution was added to the samples to a final concentration of 10 mM to stop the DEPC reaction. The samples were then assayed for enzyme activity. In protection studies, hyoscyamine (20 mM), 2-oxoglutarate (40 mM), Fe^{2+} (1.25 mM), and ascorbate (25 mM), either alone or in combination, were first preincubated with the enzyme for 15 min. on ice, and then treated with DEPC for an additional 15 min. on ice as described above.

3. Results

3. 1 Characterization of H6H protein fused with MBP

The recombinant fusion protein between MBP at the N-terminus and wild-type H 6 H at the C-terminus was purified to greater than 90% purity (see **Fig. 5A** below). The partially purified fusion protein had a specific activity of 1,584 pKat/mg protein, which corresponded with the catalytic constant of at least 0.13 s^{-1}. This figure compares very well with the catalytic constant of 0.13 s^{-1} for the native H6H purified from *H. niger* root culture [18]. Thus, the addition of MBP at the N-terminus of H6H does not significantly affect its catalytic constant. The fusion protein was then characterized for its affinity toward substrates and cofactors. The enzyme showed typical Michaelis-Menten saturation curves in response to increasing concentrations of hyoscyamine, 2-oxoglutarate, and Fe^{2+} (**Fig. 2 A, B,** and **C**). Residual iron in the enzyme preparation was removed by an ion-exchange resin and used for the experiment shown in **Fig. 2C.** K_m values (±S. D.) were 71±10 μM for hyoscyamine, 159±19μM for 2-oxoglutarate, and 4.8±1.1 μM for

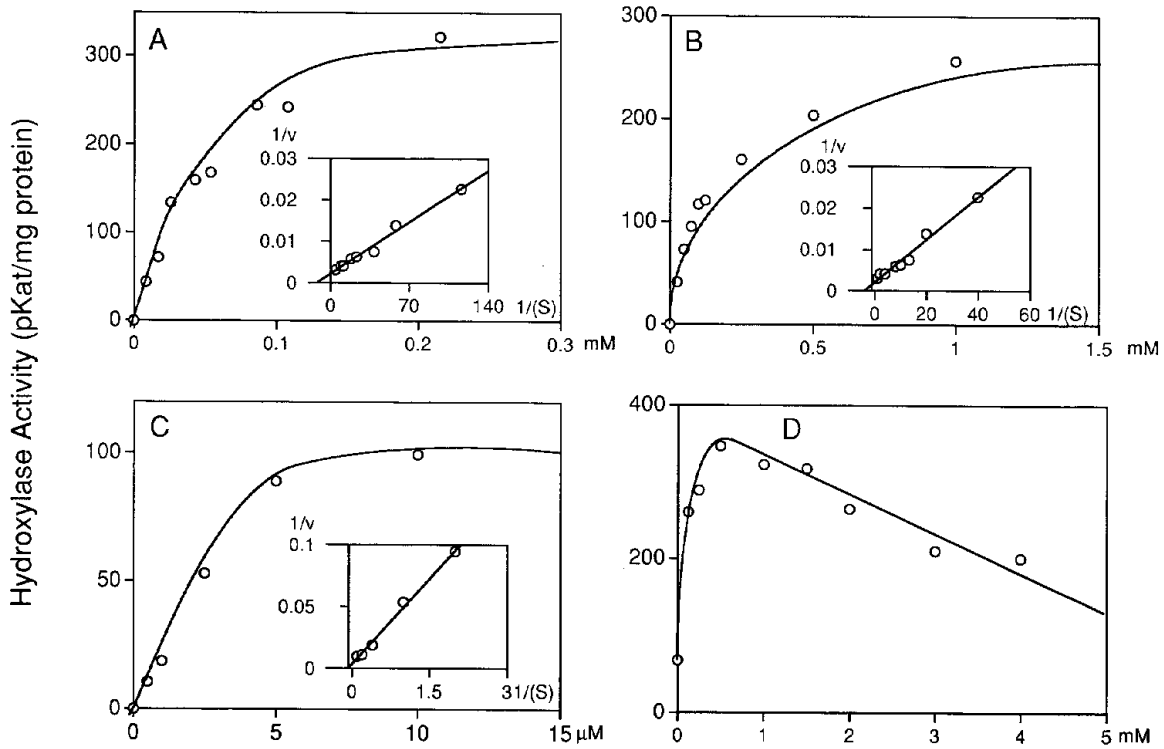

Fig. 2 Effects of various concentrations of reaction components on the activity of recombinant wild-type H6H.

Standard assay conditions were used except that the concentration of hyoscyamine (A), 2-oxoglutarate (B), Fe^{2+} (C), or ascorbic acid (D) was varied with the fixed concentrations of other reaction components. For the experiment (C), the enzyme solution and the assay mixture were treated with chelate ion-exchange resin to remove residual iron in the solutions.

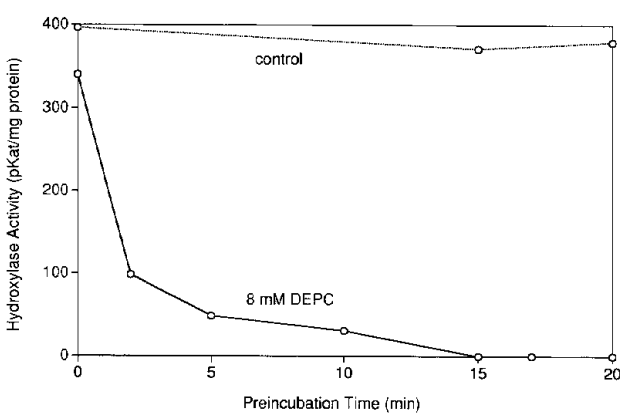

Fig. 3 Inactivation of H6H by DEPC.

Recombinant wild-type H6H was incubated with 8 mM DEPC (solid line), or with an equal amount of ethanol (control: dotted line), and then assayed for enzyme activity.

Fe^{2+}. Although ascorbate was not absolutely required, it markedly increased H6H activity (**Fig. 2D**). The highest activity was observed at 0.5 mM, and higher concentrations of ascorbate were inhibitory.

The K_m values for hyoscyamine and 2-oxoglutarate were about two to three times higher than those of the H6H enzyme that had been partially purified from *H. niger* root culture [2]. The reason for the lower affinity of the recombinant H6H fusion protein is not clear, but the N-terminal maltose-binding domain may cause some steric hindrance at the active site.

3. 2 Diethyl pyrocarbonate treatment

The partially purified recombinant H6H was rapidly inactivated by incubation with 8 mM DEPC at pH 6.8 on ice (**Fig. 3**). The enzyme activity dropped to less than 30% of the original activity after two min. of the DEPC treatment, and to an undetectable level after 15 min. The enzyme treated with ethanol alone was scarcely inactivated during the course of the experiment. When the spectra of the inactivated and original enzymes were monitored at 210-310 nm, only the inactivated enzyme showed one peak around 240 nm (data not shown). This indicates that histidine residues were chemically modified by DEPC.

Components of the reaction mixture were tested, both alone and combination, for their ability to protect the recombinant H6H from inactivation by DEPC (**Fig. 4**). Residual iron in the enzyme solution and reagents had been removed by chelate resin. Preincubation with 2-oxoglutarate alone provided very effective protection (68% activity of the non-DEPC-treated control) as compared to the sample without any protection (9% activity with DEPC). However, neither hyoscyamine, Fe^{2+}, nor ascorbate alone protected the enzyme from inactivation. Combinations of 2-oxoglutarate with other components also protected the enzyme to some extent, but were less effective than 2-oxoglutarate alone. Preincubation with combinations of reaction components that did

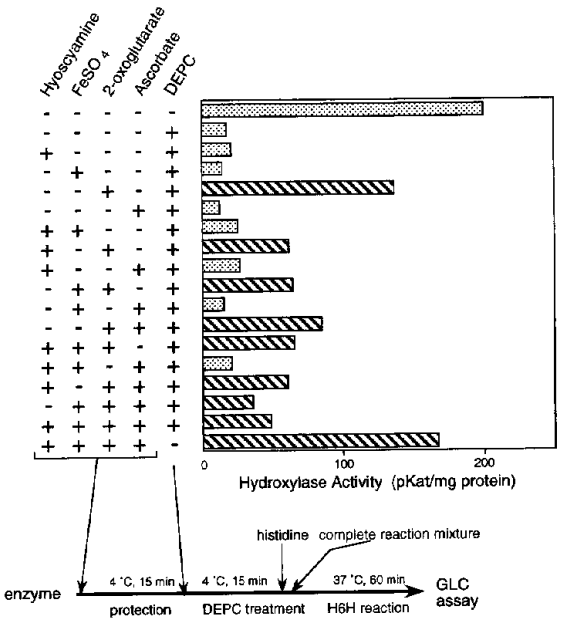

Fig. 4 The ability of reaction component to protect H6H from DEPC-mediated inactivation.
Recombinant wild-type H6H was preincubated with hyoscyamine, $FeSO_4$, 2-oxoglutarate and ascorbate, either alone or in combination, before DEPC treatment. Fifteen minutes after DEPC treatment, H6H activity was measured. The dotted bars indicate preincubation without 2-oxoglutarate while the hatched bars indicate preincubation with the dicarboxylic acid.

not include 2-oxoglutarate scarcely protected the enzyme.

3. 3 Mutation of the conserved amino acid residues

Two histidine residues are strictly conserved among several structurally related non-heme oxygenases (**Fig. 1**), and may be modified by DEPC to cause inactivation. These two residues (histidine-217 and histidine-274), as well as one loosely conserved histidine residue (histidine-66), were independently changed to glutamine residues by site-directed mutagenesis to respectively yield mutant enzymes,

Table 1.
Enzyme activities in mutated H6H proteins.

Protein	Hydroxylase activity	
	pKat/mg H6H	% of wild type
Wild type	1584±65	100
H66Q	1540±496	97
H217Q	ND*	0
D219H	ND*	0
D219N	ND*	0
D274Q	ND*	0

The amount of H6H protein in the assay mixture was estimated from the immunoblot using an anti-MBP antiserum (**Fig. 5C**).
* ND, not detected. Detection limit was 3 pKat/mg H6H.

H217Q, H274Q, and H66Q. In addition, the other strictly conserved residue (aspartic acid-219) was similarly replaced by either histidine or asparagine to give D219H or D219N. These mutant H6H proteins with MBP at their N-terminal regions were expressed in *E. coli*, partially purified from the bacterial crude extracts, and examined after SDS-PAGE separation by staining with Coomassie brilliant blue (**Fig. 5A**), or by immunoblotting with antibodies specific to H6H (**Fig. 5B**). Four mutants (H217Q, D219H, D219N, and H274Q) as well as wild-type recombinant H6H were highly purified (>90% purity), while the purity of H66Q was low for unknown reasons. Since introduced mutations might modify the epitope of the H6H-specific monoclonal antibody mAb5, the immunoblot with the antibody against MBP (**Fig. 5C**) was used to quantify the amounts of the fusion proteins. When equivalent amounts of the fusion proteins were analyzed for hydroxylase activity, wild-type enzyme and the mutant H66Q showed similar catalytic activities, whereas the other mutants (H217Q, D219H, D219N, and H274Q) were inactive (**Table 1**).

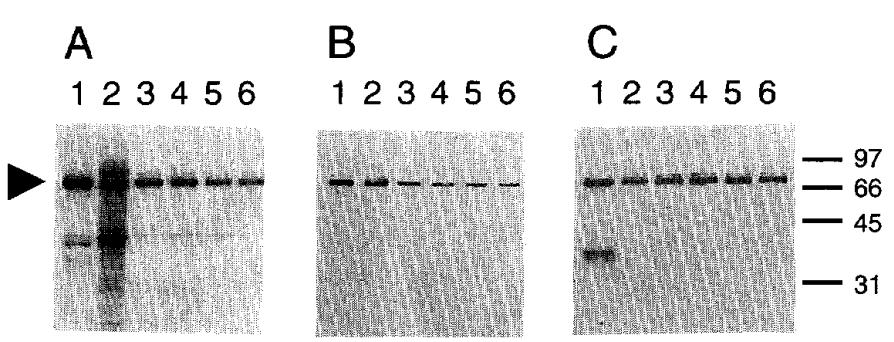

Fig. 5 SDS-PAGE and immunoblot analysis of mutagenized H6H proteins.
A: stained with Coomassie brilliant blue, B: immunoblot with an anti-H6H monoclonal antibody mAb5, C: immunoblot with an anti-MBP antiserum. Proteins were partially purified from *E. coli* cell extracts; lane 1: wild-type, lane 2: H66Q, lane 3: H217Q, lane 4: H274Q, lane 5: D219H, lane 6: D219N

4. Discussion

Kinetic studies with prolyl 4-hydroxylase [19, 20], lysyl hydroxylase [21], thymine 7-hydroxylase [22], and desacetoxyvindoline 4-hydroxylase [23] have suggested that 2-oxoglutarate-dependent dioxygenases function by an Ordered Ter Ter mechanism in which 2-oxoglutarate is the first substrate to bind, followed by O_2 and the substrate to be oxidized. Fe^{2-} and ascorbate are not consumed stoichiometrically with the reactions [20], and are thus not considered to be true substrates. Studies on prolyl 4-hydroxylase have proposed that Fe^{2+} binds first to the hydroxylase, and is not released between most catalytic cycles [19, 20]. The absolute requirement for 2-oxoglutarate, either alone or in combination with other substrates, to protect H6H from inactivation by DEPC (**Fig. 4**) is consistent with a reaction mechanism in which 2-oxoglutarate is the first real substrate to bind to the enzyme. The fact that Fe^{2+} is not required for efficient protection is not unexpected, since Fe^{2+} does not remain bound to the enzymes under non-turnover conditions [19, 20]. Inactivation by DEPC has also been reported for two other 2-oxoglutarate-dependent dioxygenases, 1-aminocyclopropane-1-carboxylate oxidase [24] and flavone 3β-hydroxylase [25].

Histidine-217, histidine-274, and aspartic acid-219 are all required for the function of H6H. These three residues are strictly conserved among the non-heme oxygenases that have moderate homology in their primary structures (**Fig. 1**). A theoretical model of the prolyl 4-hydroxylase reaction suggests that the active-site iron is coordinated with the enzyme by three side-chains [26]. The involvement of two or three histidine ligands and one aspartic acid ligand in iron binding has also been suggested from various spectroscopic analyses of isopenicillin N synthase [27-30]. Recent determination of cristal structure of isopenicillin N synthase [31] revealed that protein ligands for the active-site iron include two histidines and one aspartic acid at the positions equivalent to histidine-217, histidine-274, and aspartic acid-219 in H6H. Thus, these three conserved residues may well coordinate the active-site iron in 2-oxoglutarate-dependent dioxygenases.

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