# Site-Directed Mutagenesis of Residues in a Conserved Region of Bovine Aspartyl (Asparaginyl) β-Hydroxylase: Evidence That Histidine 675 Has a Role in Binding $Fe^{2+}$

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ABSTRACT: The roles in catalysis of several residues in bovine aspartyl (asparaginyl) β-hydroxylase that are located in a region of homology among α-ketoglutarate-dependent dioxygenases were investigated using site-directed mutagenesis. Previous studies have shown that when histidine 675, an invariant residue located in this highly conserved region, was mutated to an alanine residue, no enzymatic activity was detected. A more extensive site-directed mutagenesis study at position 675 has been undertaken to define the catalytic role of this essential residue. The partial hydroxylase activity observed with some amino acid replacements for histidine 675 correlates with the potential to coordinate metals and not with size, charge, or hydrophobic character. Furthermore, the increase in $K_m$ for $Fe^{2+}$ observed with the H675D and H675E mutant enzymes can account for their partial activities relative to wild type. No significant changes in the $K_m$ for α-ketoglutarate (at saturating $Fe^{2+}$) or $V_{max}$ were observed for these mutants. These results support the conclusion that histidine 675 is specifically involved in $Fe^{2+}$ coordination. Further site-directed mutagenesis of other highly conserved residues in the vicinity of position 675 demonstrates the importance of this region of homology in catalysis for Asp (Asn) β-hydroxylase and, by analogy, other α-ketoglutarate-dependent dioxygenases.

Aspartyl (asparaginyl) β-hydroxylase is a member of the α-ketoglutarate-dependent dioxygenase family of enzymes (Gronke *et al.*, 1989, 1990; Stenflo *et al.*, 1989), which includes prolyl-3, prolyl-4, and lysyl hydroxylases (Kivirikko *et al.*, 1989). β-Hydroxylation of aspartic acid (and asparagine) residues occurs posttranslationally in specific epidermal growth factor-like (EGF-like) domains of a number of proteins (Stenflo, 1991). A single conserved Asp (Asn) residue located within a putative consensus sequence of the EGF-like domains has been identified as the site of hydroxylation (Stenflo *et al.*, 1987; Przysiecki *et al.*, 1987). As a means to determine the physiologic role of hydroxylation, we have focused our efforts on first characterizing Asp (Asn) β-hydroxylase in order to develop either specific inhibitors of its enzymatic activity or methods to regulate its gene.

Gronke *et al.* (1989) initially identified *in vitro* Asp β-hydroxylase activity in mouse L-cells and in bovine liver using a 39 amino acid synthetic peptide substrate based on the structure of the first EGF-like domain of human coagulation factor IX. Enzymatic activity requires $Fe^{2+}$ and α-ketoglutarate (α-KG), and hydroxylation of the peptidyl substrate is stoichiometrically coupled to the decarboxylation of α-KG. A 52 kDa protein exhibiting Asp (Asn) β-hydroxylase activity was purified to homogeneity from detergent-solubilized bovine liver microsomes (Wang *et al.*, 1991). This fully active C-terminal-derived species resulted from proteolysis of the intact 85 kDa Asp (Asn) β-hydroxylase. The cDNA clone encoding the full-length 85 kDa Asp (Asn) β-hydroxylase has been isolated, and its deduced amino acid sequence has revealed that the enzyme has a compact C-terminal catalytic domain preceded by a potential transmembrane type II signal-anchor domain (Jia *et al.*, 1992). The catalytic domain of Asp (Asn) β-hydroxylase was recently expressed in *Escherichia coli* and purified to homogeneity (Jia *et al.*, 1994). This 52 kDa recombinant protein (P52)[1] retains full enzymatic activity with physical and kinetic properties indistinguishable from the 52 kDa fragment of the native enzyme.

A region of the primary sequence of P52 that may comprise the active site was identified through sequence alignment of the C-terminal region of the enzyme with the C-terminal regions of other vertebrate α-KG-dependent dioxygenases (Jia *et al.*, 1994). This proposed active site region includes the His-2 motif, a region of high homology previously identified in the prolyl and lysyl hydroxylase enzymes (Jia *et al.*, 1994; Myllylä *et al.*, 1992). The motif is characterized most notably by an invariant histidine, a proline, and a conserved basic residue. Initially, the highly conserved His residue of Asp (Asn) β-hydroxylase (H675) was mutated to an alanine residue (Jia *et al.*, 1994). No detectable enzymatic activity was found in the resulting H675A mutant protein, and it appears that this residue may be important in the binding of α-KG and/or $Fe^{2+}$, substrates common to all α-KG-dependent dioxygenases. In the present study, many substitutions were made at position 675 to determine the precise catalytic role of this essential residue.

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[1] Abbreviations: WT P52, wild-type 52 000 Da recombinant Asp (Asn) β-hydroxylase; p52, pFLAG-1 plasmid carrying cDNA coding for P52 enzyme; WT p52, 1.4 kb insert from p52 encoding wild-type P52 enzyme; mutant p52, 1.4 kb cDNA from p52 encoding mutant P52 enzymes.

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In addition, a site-directed mutational analysis of several other residues within or adjacent to the His-2 motif of Asp (Asn) $\beta$-hydroxylase was conducted in order to evaluate the role of the His-2 motif as a possible active site region.

## EXPERIMENTAL PROCEDURES

*Materials.* Standard protocols for DNA manipulations and cell transformations were either those published by Sambrook *et al.* (1989) or those described in Promega Technical Manual 1 (1992). The *E. coli* bacterial strains used in the mutagenesis procedures were BMH 71-18 *mut*S electrocompetent cells, obtained from Clontech, and chemically competent JM109 from Promega Corp. All restriction endonucleases, T4 ligase, T4 polynucleotide kinase, and calf intestinal alkaline phosphatase were purchased from New England Biolabs and used according to manufacturer's instructions. The Luria Broth (LB) used in cell culturing was obtained from Gibco BRL, and LB agar plates as well as LB agar plates containing 100 $\mu$g/mL ampicillin (amp) were purchased from Remel. Difco was the source for the tryptone and yeast extract.

*Oligonucleotide-Directed Mutagenesis.* The cDNA cloning and construction of the expression plasmid containing the 1.4 kb insert (p52) coding for the 52 000 Da catalytic domain of Asp (Asn) $\beta$-hydroxylase (P52) have been described previously (Jia *et al.*, 1994). The 1.4 kb fragment (WT p52) was excised by *Hind*III/*Xho*I digestion of the p52 expression plasmid, purified from a 1% low-melting agarose (Sigma) gel using Magic PCR preps (Promega Corp.), and ligated into the *Hind*III/*Sal*I-digested pALTER-1 phagemid vector (Promega Corp.). Site-directed mutagenesis was carried out using the Promega Altered Sites *in vitro* mutagenesis system. This method employs two mutagenic primers: one to produce the desired mutation in the cloned DNA and the other to restore ampicillin resistance by correcting a deletion mutation in the $\beta$-lactamase gene on the pALTER-1 plasmid. Phagemid single-stranded DNA of pALTER-1 containing WT p52, prepared using the Magic M13 DNA purification system (Promega Corp.), was used as the template for mutagenesis. All mutants in this study were derived from the recombinant wild type and were mutated independently using synthetic oligonucleotides. The mutations were identified through DNA sequencing of ampicillin-resistant clones by the dideoxy chain termination method (Sanger *et al.*, 1977; Tabor & Richardson, 1987) using the T7 promoter primer and the Sequenase version 2.0 sequencing kit (United States Biochemical). [After ligation into the pFLAG expression vector, the entire P52 gene of each mutant was sequenced using pFLAG-1 C24 primer (IBI) and synthetic sequencing primers.]

*Expression of Recombinant Mutant Proteins.* Once each mutant was verified by DNA sequence analysis, the mutant p52 fragments were excised from the mutagenesis plasmids by double digestion with *Hind*III and *Xba*I, gel-purified as described above, and then ligated into the *Hind*III/*Xba*I site of the pFLAG-1 (IBI) expression vector used for expression of the recombinant wild-type enzyme. Ligation mixtures were used to transform *E. coli* DH5 F'IQ competent cells (Bethesda Research Labs), and positive clones were obtained by selecting single-colony isolates with slow growth rates on isopropylthio-$\beta$-galactoside (IPTG) from replica plates of transformants on LB amp (100 $\mu$g/mL) plates with 1 mM IPTG (Bethesda Research Labs) and LB amp (100 $\mu$g/mL) only plates. Transformants containing the mutant p52 inserts were further verified by *Hind*III/*Xho*I digestion of miniprep DNA prepared from IPTG-sensitive clones using Promega's Magic minipreps. These mutant p52 expression vectors were then used to transform chemically competent *E. coli* BL21 protease-deficient cells (Novagen). Single-colony ampicillin-resistant transformants were grown at 37 °C in 5 mL of LB containing 100 $\mu$g/mL amp to an $OD_{600}$ of 0.7−1.0 and induced with 1 mM IPTG (final concentration). Expression of the mutant aspartyl (asparaginyl) $\beta$-hydroxylases was confirmed by Western blot analysis of supernatants prepared by lysis of cell pellets obtained from 1 mL of liquid culture. The mutants were expressed as fusion proteins with the FLAG peptide N-terminal to the mutant P52 genes as described previously (Jia *et al.*, 1994). Mutant expression levels, monitored by immunoblot analysis, were comparable to wild type, which was cultured, induced, and expressed in parallel.

*Partial Purification of Mutant Hydroxylases.* For large-scale expression and purification of the mutant P52 enzymes, *E. coli* BL21 p52 mutant transformants were grown in 3× LB (30 g of tryptone, 15 g of yeast extract, and 5 g of NaCl/L, pH 7.5) containing 100 $\mu$g/mL ampicillin. After overnight incubation, the cultures were diluted 1:50 with fresh 3× LB media and grown to an $OD_{600}$ of 1.0−1.2. Protein expression was induced by incubation at 28−30 °C for 2.5 h with 1 mM IPTG. The cells were harvested and washed with ice-cold phosphate-buffered saline (PBS) and then frozen at −70 °C. The frozen pellets from 500 mL of culture (2−3 g of cell paste) were resuspended in 15 mL of 50 mM Tris, pH 7.0, lysed by addition of 1 mg/mL lysozyme and 10 mM $MgCl_2$ (final concentration), and then frozen at −70 °C for 20 min. DNase (15 $\mu$g/mL) was added to the thawed lysates which were then centrifuged for 10 min at high speed in a microfuge. The mutant enzymes were partially purified from the supernatant using Anti-FLAG M2 affinity gel (IBI) according to manufacturer's instructions except that 50 mM Tris, pH 7.0, was substituted for PBS and the protein was eluted from the column with 150 $\mu$g/mL FLAG peptide in 50 mM Tris, pH 8.0. Column fractions were assessed for purity by SDS−PAGE, and those containing mutant P52 were identified by Western blot analysis using antibodies monospecific to native bovine Asp (Asn) $\beta$-hydroxylase (Wang *et al.*, 1991; Jia *et al.*, 1994). Mutant P52-containing fractions were approximately 10−25% pure, and these were assayed for hydroxylase activity. Wild-type P52 enzyme was also expressed and partially purified from cell lysates as described above and assayed in parallel.

*Hydroxylase Assays.* Unless otherwise indicated, all assays for hydroxylase activity utilized the peptide EGF-$S_{2B}$, whose structure is based on the second EGF-like domain of bovine protein S (Monkovic *et al.*, 1992). Both $^{14}CO_2$ release from the labeled cofactor α-keto[1-$^{14}$C]glutaric acid and $\beta$-hydroxyaspartic acid (Hya) determination of the isolated hydroxylated product were carried out as described previously (Gronke *et al.*, 1990; Przysiecki *et al.*, 1987; Wang *et al.*, 1991). Assays for $^{14}CO_2$ release were performed in duplicate at 37 °C in a final volume of 20 $\mu$L in the presence and absence of 50 $\mu$M peptide substrate. Reaction mixtures contained 50 mM Pipes, pH 7.0, 0.1 mg/mL BSA, 2 mM L-ascorbic acid, 0.1 mg/mL catalase, 137 $\mu$M α-KG (25 cpm/pmol), and 100 $\mu$M ferrous ammonium sulfate. All

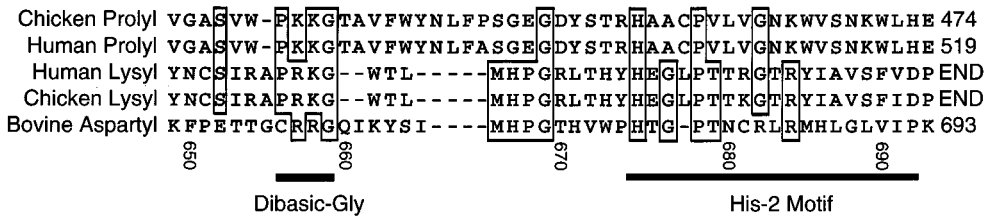


FIGURE 1: Multiple sequence alignment of the C-terminal regions of vertebrate α-KG-dependent dioxygenases showing the His-2 motif region. Sequence identities observed in three or more sequences are boxed. The dibasic-Gly region and the His-2 motif are underlined. The His-2 motif is characterized by an invariant histidine, a proline, and a conserved basic residue (lysine or arginine). These correspond to His-675, Pro-678, and R684 in bovine aspartyl hydroxylase. Other regions of interest include the MHPG sequence containing an invariant glycine (H667 and G669 in the aspartyl enzyme) and the highly conserved dibasic-glycine region (including G659).

enzyme dilutions were in 50 mM Tris-HCl pH 7.2, 200 mM NaCl, and 1.0 mg/ml BSA. Enzymatic activities, picomoles of $CO_2$ released per microgram of protein, of the wild-type and mutant P52 enzymes were adjusted for the actual amount of hydroxylase in each sample by scanning densitometry of the hydroxylase band on a Coomassie-stained SDS−polyacrylamide gel. Determinations of enzymatic activity using $^{14}CO_2$ release assays were repeated 2 or more times at the same and different enzyme concentrations. Activity assays on those mutants with no apparent activity were repeated at 10 times the initial enzyme concentration. In control experiments, a known amount of hydroxylase activity was added to several inactive mutant enzyme assay mixtures. There was no effect on the activity of the added active enzyme, eliminating the possibility of inhibition effects at these high enzyme concentrations. In addition, pure enzyme (both P52 and native) added to partially purified preparations gave the expected amount of hydroxylase activity, indicating that enzymatic activity assessments on the partitially purified wild-type and mutant P52 enzymes were not affected by the impurities present in these samples. Limits of detection for mutant enzyme activity were 1% of wild-type activity. Kinetic data were obtained using the $^{14}CO_2$ release assay described above at varying concentrations (6 or more to saturation) of α-KG and $Fe^{2+}$ using the peptide substrate EGF-$S_{2B}$ and were analyzed using the Hewlett-Packard kinetic package. The data were fit according to the Marquardt method (Marquardt, 1963).

*Protection of P52 Mutants from Iodo[$^{14}$C]acetamide Labeling by α-KG/$Fe^{2+}$.* Partially purified recombinant wild-type and mutant P52 proteins were reacted with 1 mM iodo-[$^{14}$C]acetamide ([$^{14}$C]IA) in the presence and absence of substrates as described previously (Jia *et al.*, 1994). Final concentrations of substrates were 200 μM α-KG and 50 μM $Fe^{2+}$. Following the reaction, the samples were run on SDS−polyacrylamide gels, the labeled P52 bands were excised from the gel and solubilized, and the amount of incorporated radioactivity was measured by scintillation counting. The dpm values were adjusted to correct for the actual amount of β-hydroxylase as judged by scanning densitometry of the Coomassie blue-stained SDS−polyacrylamide gel prior to excision of the hydroxylase bands. Calculation of percent protection from [$^{14}$C]IA by α-KG and $Fe^{2+}$: 100% minus [(dpm from [$^{14}$C]IA + α-KG + $Fe^{2+}$)/(dpm from [$^{14}$C]IA only)](100). Percent protection reflects the extent that the substrates prevent the incorporation of [$^{14}$C]IA.

*Other Procedures.* Protein concentrations were determined using the BioRad protein assay with BSA as standard (Bradford, 1976), and immunoblotting procedures were performed according to methods previously outlined (Wang *et al.*, 1991; Jia *et al.*, 1994).

Table 1: Enzymatic Activity of Mutants Derived from Site-Directed Mutagenesis of Residues within or near the His-2 Motif of Asp (Asn) β-Hydroxylase[a]

| mutant | % wild-type activity | % coupling[b] |
|---|---|---|
| wildtype | 100 | 104 |
| H675L | <1 | ---[c] |
| H675R | <1 | --- |
| H675F | <1 | --- |
| H675Q | <1 | --- |
| H675D | 20 | 117 |
| H675E | 12 | 131 |
| H667L | 106 | 92 |
| H671L | 16 | 104 |
| H686L | 9 | 140 |
| R684K | 87 | 100 |
| R684A | 8 | 104 |
| R684D | <1 | --- |
| R684E | 2 | --- |
| R682A | 10 | 64 |
| P678L | 1 | --- |
| P678V | 90 | 101 |
| G659A | 21 | 85 |
| G669A | 90 | 93 |

[a] Activity is expressed as picomoles of $CO_2$ released per microgram of protein. Mutant enzyme activities were compared to wild-type activity (WT, 100% = 10 pmol $min^{-1}$ $\mu g^{-1}$), and were corrected for the amount of Asp β-hydroxylase using scanning densitometry. All values shown are the means of two or more determinations with standard deviations no greater than 10%. The level of detection for the mutant enzymes was 1% WT activity. [b] [(pmol of β-Hya)/(pmol of $CO_2$)] × 100; Hya was determined by HPLC (Przysiecki *et al.*, 1987; Gronke *et al.*, 1990). [c] (---) low activity precluded β-hydroxyaspartic acid analysis.

## RESULTS AND DISCUSSION

To evaluate the role of the His-2 motif in Asp (Asn) β-hydroxylase catalysis and/or substrate binding, a site-directed mutational analysis of several residues within or adjacent to the His-2 motif was conducted. Candidate positions were chosen for mutagenesis based upon the sequence alignment shown in Figure 1. Myllylä *et al.* (1992) found that prolyl-4 and lysyl hydroxylases were inactiviated by diethyl pyrocarbonate and that this inactivation could be prevented by the presence of cosubstrates. These results suggest that histidine residues are present in the active site of α-ketoglutarate-dependent dioxygenases and that they are probably involved in α-ketoglutarate or $Fe^{2+}$ binding. Therefore, all of the histidines in the vicinity of the His-2 motif (H667, H671, H675, and H686) were initially investigated. Each was individually replaced with leucine. The effect of these substitutions on enzymatic activity was determined (Table 1). H675L was the only histidine to

Table 2: $K_m$ and $V_{max}$ Values of Wild-Type and Mutant Aspartyl $\beta$-Hydroxylases[a]

| P52 enzyme | $K_m(Fe^{2+})$ (at 600 $\mu$M α-KG) ($\mu$M) | $K_m$(α-KG) (at 1 mM $Fe^{2+}$) ($\mu$M) | $V_{max}$ (pmol $min^{-1}$ $\mu g^{-1}$) | $V_{max}/K_m$ |
|---|---|---|---|---|
| wild type | 13 ± 4 | 102 ± 1 | 8.6 ± 1 | 0.66 |
| H675D | 93 ± 16 | 98 ± 17 | 18.6 ± 4 | 0.20 |
| H675E | 52 ± 1 | 125 ± 10 | 6.5 ± 0.6 | 0.13 |

[a] Values are the average of duplicate determinations (±range).

Table 3: Protection from Iodo[$^{14}$C]acetamide Labeling by α-KG/$Fe^{2+}$ of Wild-Type and His-675 to Glu Mutant Proteins[a]

| | [$^{14}$C]IA only (dpm) | [$^{14}$C]IA + α-KG/$Fe^{2+}$ (dpm) | % protection by α-KG/$Fe^{2+}$ |
|---|---|---|---|
| wild type | 2348 | 423 | 82[b] |
| H675E | 2737 | 2271 | 17[c] |

[a] See Experimental Procedures for details. [b] The mean of five similar experiments was 77 ± 4%. [c] The result of a duplicate experiment was 28%.

leucine mutant that did not have detectable activity. This histidine is invariant among the vertebrate α-KG-dependent dioxygenases. As previously reported (Jia *et al.*, 1994), substitution of this residue with alanine (H675A) also resulted in no detectable enzyme activity. To determine whether the loss of enzymatic activity was due to measurable structural changes in the mutant enzyme, the H675L protein was purified to homogeneity by published procedures (Jia *et al.*, 1994) and analyzed by CD spectroscopy. Like the H675A mutant (Jia *et al.*, 1994), the CD spectrum for H675L is identical to the wild-type recombinant protein (data not shown), indicating that no major structural changes had occurred as a result of these mutations.

Based upon these results, we investigated the role in catalysis of this essential histidine. Replacement of H675 with arginine, phenylalanine, or glutamine resulted in no detectable activity (Table 1). Two other His-675 mutants involving Asp and Glu substitutions gave 20% and 12% wild-type activity, respectively (Table 1). The partial hydroxylase activity found in the H675D and H675E mutant enzymes was confirmed by the presence of $\beta$-hydroxyaspartic acid in the hydrolysates of reaction mixtures (Table 1), indicating that decarboxylation of α-KG and hydroxylation of the peptide substrate are coupled in these mutants. These results strongly suggest that activity resulting from substitutions at position 675 correlates with replacement residues that can act as potential metal binding ligands or as general bases (Higaki *et al.*, 1992). There appears to be no correlation with respect to charge, size, and hydropathy. To address this further, the mutants that gave appreciable activity (H675D and H675E) were characterized. By assaying these mutant enzymes at varying concentrations of $Fe^{2+}$ and α-KG, the $K_m$ values for these substrates were determined (Table 2). The $K_m(Fe^{2+})$ values for the H675D and H675E mutants are increased approximately 8- and 4-fold, respectively, with a 2-fold increase in $V_{max}$ for H675D, no significant change in $V_{max}$ for H675E, and no significant change in $K_m$(α-KG), at saturating $Fe^{2+}$, for either mutant compared to the WT P52 enzyme. The resulting differences in $V_{max}/K_m$ values can account for the relative activities of these proteins (Tables 1 and 2). Furthermore, in support of these data, the partially active H675D and H675E mutants also showed a 5-fold increase in $K_m^{app}(Fe^{2+})$ relative to wild type at a lower α-KG concentration (137 $\mu$M) with no change in $V_{max}^{app}$ (data not shown). The findings that activity correlates with the potential to coordinate metals at position 675 and that partial activity can be largely accounted for by an increase in the $K_m$ of $Fe^{2+}$ suggests that the catalytic role of H675 is to bind $Fe^{2+}$. Since the $K_m$ of $Fe^{2+}$ is increased and the $V_{max}$ not significantly decreased in those H675 mutants with partial activity (H675D and H675E), it is likely that substitutions at position 675 affect the affinity for $Fe^{2+}$ rather than result in a nonproductive binding mode. These results support the conclusion that H675 is specifically involved in $Fe^{2+}$ coordination and do not support an alternative explanation that the loss of enzymatic activity resulting from amino acid replacements at position 675 is simply due to nonspecific perturbations in the protein structure.

The binding of α-KG/$Fe^{2+}$ to wild-type and mutant P52 enzymes can be analyzed by monitoring the ability of α-KG/$Fe^{2+}$ to prevent incorporation of radioactivity from [$^{14}$C]IA into the protein (Jia *et al.*, 1994). Previous experiments (Jia *et al.*, 1994) showed that wild-type recombinant aspartyl $\beta$-hydroxylase is protected against [$^{14}$C]IA inactivation and labeling when incubated in the presence of both α-KG and $Fe^{2+}$. The H675A mutant protein is not protected against [$^{14}$C]IA labeling by α-KG/$Fe^{2+}$, suggesting that this mutant does not bind either α-KG or $Fe^{2+}$ or both (Jia *et al.*, 1994). In the absence of α-KG or $Fe^{2+}$, the amount of incorporated radioactivity from [$^{14}$C]IA was approximately the same for the wild-type and mutant proteins (Table 3). Preincubation of WT P52 protein with α-KG (200 $\mu$M) and $Fe^{2+}$ (50 $\mu$M) consistently gives ~80% protection against incorporation of [$^{14}$C]IA, while the H675E mutant protein shows much less protection from [$^{14}$C]IA incorporation in the presence of α-KG/$Fe^{2+}$ (Table 3). The partially active H675E mutant shows some protection (~23%, the average of duplicate experiments), suggesting that H675E binds α-KG/$Fe^{2+}$, less effectively than wild type, but better than the inactive H675A mutant protein. This result is consistent with the elevated $K_m(Fe^{2+})$ observed for H675E relative to the wild-type protein, since as discussed above the $K_m(Fe^{2+})$ is likely to reflect binding affinity. Therefore, while the wild type is saturated at this $Fe^{2+}$ concentration, the H675E mutant enzyme is not saturated [Table 2, $K_m(Fe^{2+})$]. Thus, the lower level of protection found in the H675E mutant protein (as well as the H675A mutant enzyme) is most likely due to a decreased ability of these mutant proteins to bind $Fe^{2+}$ under conditions where the wild-type enzyme is effectively saturated.

The effects of site-directed mutagenesis of conserved residues in the vicinity of position 675 and within the His-2 motif on Asp (Asn) $\beta$-hydroxylase activity were studied to determine the importance of this region in catalysis. As stated previously, all of the histidine residues within or near the His-2 motif were mutated to leucine. As shown, H675L was the only histidine to leucine mutant that showed no detectable activity. H667, H671, and H686 each retained activity when substituted with leucine. However, H671L and H686L showed significantly reduced activity relative to the wild-type enzyme (16% and 9% of wild-type activity respectively, Table 1), indicating that they may play important roles in catalysis and/or substrate binding or serve in a structural function. The conserved basic residue (Arg) in the His-2 motif (Arg in lysyl hydroxylase, Lys in prolyl hydroxylase), at position 684 in aspartyl hydroxylase (Figure

1), can be substituted with a similarly charged lysine with little loss in enzymatic activity (87% of wild-type activity, Table 1). However, other substitutions at this position resulted in substantial (8% of WT for R684A and 2% of WT for R684E) or complete (<1% of WT for R684D) loss in enzymatic activity (Table 1). Thus, although a basic residue is conserved at this position, and is likely to be important for catalysis, it appears that at least for aspartyl hydroxylase this functional group is not essential, since the R684A mutant possesses appreciable activity. The importance of several additional residues within the His-2 motif (P678 and R682), the dibasic-glycine region (G659), and the MHPG sequence (G669) was also determined by site-directed mutagenesis (Figure 1). The proline 678 to leucine mutation was inactive, while P678V showed essentially no loss in activity (90% of WT activity, Table 1), indicating the importance of steric bulk to this position. This result suggests a role for this residue in maintaining the conformation of the active site. The fact that this proline residue is highly conserved suggests a structural role for this residue throughout the α-KG-dependent dioxygenase family. Substitution of the two invariant glycine residues (G659 and G669) with alanine resulted in 21% and 90% wild-type activity, respectively (Table 1). Replacement of R682 (located in the His-2 motif, but not conserved) with alanine resulted in loss of approximately 90% enzymatic activity (Table 1). In all of the partially active mutants, the release of $CO_2$ from α-KG remained coupled to the hydroxylation of the peptidyl substrate (% coupling, Table 1). Coupling is defined as the ratio of moles of hydroxylated product formed (β-hydroxyaspartic acid), as determined by isolation of β-Hya by HPLC, to the moles of $CO_2$ released from α-KG, as determined by $^{14}CO_2$ release assays (see Experimental Procedures). Therefore, % coupling reflects the extent to which these two reactions are coupled. This result is consistent with previous studies with this enzyme in which, in the absence of peptidyl substrate, no appreciable release of $CO_2$ from α-KG has been observed (Gronke *et al.*, 1989). Since peptide-dependent decarboxylation of α-KG occurs in wild-type Asp (Asn) β-hydroxylase and all of the active mutants presented here, it remains to be determined whether or not other regions of the enzyme can be mutated and result in an uncoupled release of $CO_2$.

The enzymatic activity of the partially active H675 and R684 mutants on another EGF substrate, EGF-$X_{1H}$, a peptide whose structure is based on the first EGF-like domain in human factor X (Gronke *et al.*, 1990), was determined (Table 4). The wild-type enzyme is 2-fold more active with EGF-$S_{2B}$ as a substrate than with EGF-$X_{1H}$. For EGF-$S_{2B}$, an asparagine residue is hydroxylated, whereas for EGF-$X_{1H}$ an aspartic acid residue becomes modified. Although H675D and H675E mutants have appreciable activity with EGF-$S_{2B}$ (Table 1), no activity was detected with EGF-$X_{1H}$ (Table 4). Thus, whereas the glutamate and aspartate residues are able to support catalysis by coordinating $Fe^{2+}$, the net charge in the immediate vicinity of the $Fe^{2+}$ may be important for determining substrate selectivity. One possibility is that the negative charge on the Asp residue of EGF-$X_{1H}$ interacts unfavorably with the H675D and H675E mutants. This unfavorable interaction would not occur in the case of the wild-type enzyme or when the EGF-like domain contains Asn as the amino acid that undergoes hydroxylation. Selectivity with the R684K and R684A mutants also supports the importance of the His-2 motif in catalysis. This motif appears to be necessary for establishing a critical binding relationship among all of the substrates of Asp (Asn) β-hydroxylase.

Table 4: Enzymatic Activity of Wild-Type and Mutant P52 Enzymes on EGF-$X_{1H}$ and Comparison to EGF-$S_{2B}$

| P52 enzyme | % wild-type activity[a] | selectivity[c] |
|---|---|---|
| wild type | 100[b] | 2 |
| H675D | <1 | >20 |
| H675E | <1 | >12 |
| R684K | 25 | 7 |
| R684A | 1 | 16 |

[a] Activity was determined using the $CO_2$ release assay and is expressed as a percent of wild-type activity using EGF-$X_{1H}$ as substrate. Reported values are the means of three or more determinations with standard deviations no greater than 10%. Enzymatic activity was measured as picomoles of $CO_2$ released per microgram of protein and was corrected for the amount of Asp-hydroxylase using scanning densitometry. The concentration of the EGF peptides used was 50 μM, which is the approximate $K_m$ concentration (Wang *et al.*, 1991; Jia *et al.*, 1994). EGF-$X_{1H}$ contains an aspartic acid and EGF-$S_{2B}$ an asparagine at the site of hydroxylation. [b] Activity of WT on EGF-$X_{1H}$ is 50% of the WT activity on EGF-$S_{2B}$. [c] Defined as ratio of activity with EGF-$S_{2B}$ to activity with EGF-$X_{1H}$.

The results of this mutational analysis support our hypothesis and provide strong evidence that the region of the primary structure in the vicinity of the His-2 motif is important for enzymatic activity, containing functionally critical residues. The substantial loss of enzymatic activity resulting from substitution of several residues within this highly conserved region suggests that this group of residues constitutes an active site. Based on the studies, a significant function is indicated for the the highly conserved basic residue (R684) and the highly conserved proline residue (P678). Furthermore, it appears that His-675 is essential for enzymatic activity in Asp (Asn) β-hydroxylase and is directly involved in $Fe^{2+}$ binding. Since the α-KG-dependent dioxygenases have identical cofactor requirements including $Fe^{2+}$ and α-KG (Kivirikko & Myllylä, 1980), this invariant residue probably plays an identical role throughout this class of enzymes. Lamberg *et al.* (1995) recently reported that His-483, the invariant histidine residue in the His-2 motif of the α subunit of human prolyl hydroxylase, is essential for catalytic activity. In addition, mutation of two other conserved histidine residues, His-412 and His-501 (located in the His-2 motif), completely inactivated the enzyme (H412S) or reduced its activity to about 5% (H501S) (Lamberg *et al.*, 1995). The authors speculate that the most likely role for all three of these critical histidines is to provide the three ligands involved in $Fe^{2+}$ coordination (Lamberg *et al.*, 1995). These results support our findings and substantiate the hypothesis that these conserved residues may play identical roles throughout this class of enzymes. Asp (Asn) β-hydroxylase may therefore serve as a model for elucidating the catalytic mechanism of α-KG-dependent dioxygenases, especially since other members such as proly-4 and lysyl hydroxylases are much more complex structurally than bovine aspartyl hydroxylase (Kivirikko & Myllylä, 1980; Berg *et al.*, 1979; Turpeenniemi-Hujanen *et al.*, 1980, 1981).

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