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A fully active catalytic domain of bovine aspartyl (asparaginyl) β-hydroxylase expressed in *Escherichia coli*: Characterization and evidence for the identification of an active-site region in vertebrate α-ketoglutarate-dependent dioxygenases

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ABSTRACT **The α-ketoglutarate-dependent dioxygenase aspartyl (asparaginyl) β-hydroxylase (EC 1.14.11.16) specifically hydroxylates one aspartic or asparagine residue in certain epidermal growth factor-like domains of a number of proteins. The expression in *Escherichia coli*, purification, characterization of a fully active catalytic domain, and evidence for the identification of an active-site region of this enzyme are described. Sequence alignment analyses among the vertebrate α-ketoglutarate-dependent dioxygenases and chemical modification studies were undertaken aimed at locating specific regions of 52-kDa recombinant aspartyl (asparaginyl) β-hydroxylase involved in substrate binding and/or catalysis. Based upon these studies, an alignment of the C-terminal regions of prolyl and lysyl hydroxylase and of aspartyl (asparaginyl) β-hydroxylase is proposed. When histidine-675, an invariant residue located in a region of homology within this alignment, was mutated to an alanine residue in aspartyl (asparaginyl) β-hydroxylase (H675A), no enzymatic activity was detected. Chemical modification studies show that the wild-type protein is protected from iodo[^{14}C]acetamide labeling by Fe^{2+}/α-ketoglutarate whereas the H675A mutant protein is not, suggesting that this mutant does not bind Fe^{2+}/α-ketoglutarate.**

Many proteins from both vertebrates and invertebrates that function extracellularly as either integral components of the plasma membrane or as soluble components of plasma possess discrete domains with a characteristic pattern of three disulfide bonds as found in epidermal growth factor (EGF) (1–5). There is increasing evidence that these EGF-like domains are involved in protein–protein interactions (6–10). In several cases, these intermolecular interactions have been shown to be Ca^{2+} dependent (9–15).

In many of the EGF-like domains that can potentially bind Ca^{2+}, a specific aspartic or asparagine residue has been shown to be posttranslationally hydroxylated (4). Despite the location of EGF domains within a region of their respective proteins that is likely to be required for biologic activity, the physiologic role of the β-hydroxyl modification has not been defined. One approach to addressing this question involves the specific inhibition of hydroxylation within these EGF domains without alterations in their primary sequences or changes in other posttranslational modifications. As a first step toward the development of specific hydroxylation inhibitors, we have sought to elucidate the enzymology involved in this modification. Following the demonstration of aspartyl hydroxylation in a cell-free system (16), this α-ketoglutarate-dependent dioxygenase (16, 17) was purified from bovine liver (18); its cDNA was cloned and its biologic activity was expressed in an *in vitro* translation system (19). The enzyme aspartyl (asparaginyl) β-hydroxylase [peptide–L-aspartate,2-oxoglutarate:oxygen oxidoreductase (3-hydroxylating)] contains a relatively compact C-terminal catalytic domain and an extended N terminus. The present studies describe the expression in *Escherichia coli*, purification, and characterization of a fully active catalytic domain of bovine Asp (Asn) β-hydroxylase. In addition, evidence for the identification of an active-site region is presented that may facilitate further studies for the entire family of vertebrate α-ketoglutarate-dependent dioxygenases.

MATERIALS AND METHODS

Construction of a Plasmid (p52) for Expression of a 52,000-Da Asp (Asn) β-Hydroxylase (P52). The cDNA coding for P52 is located between nt 931 and 2265 (18, 19). A cDNA fragment (nt 1–1033) was deleted from bovine Asp (Asn) β-hydroxylase cDNA by digestion with *Pst* I and ligated to form pNC3d-1. A 1.4-kb cDNA insert [nt 1034–2498, containing a 233-bp 3′ untranslated region (19)] resulting from *Xba* I/*Xho* I digestion was isolated and then cloned into an *E. coli* expression vector, pFLAG-1 (IBI) (20), to form p52-1. Since this 1.4-kb cDNA insert lacked nt 931–1033, a synthetic double-stranded DNA fragment containing these nucleotides, a *Hin*dIII site at the 5′ end, and a *Pst* I site at the 3′ end was inserted into p52-1 to form the expression plasmid p52. The cDNA reading frame (nt 931–2265) of p52 was confirmed as follows: p52 was then used to transform *E. coli* DH5αF′IQ (BRL); it was then purified and the 5′ ends of the cDNA insert were sequenced. Purified p52 was introduced into both a normal *E. coli* strain and a protease-deficient strain (BL21; Novagen).

Expression of P52. A p52 transformant was grown at 37°C in 3× LB medium (30 g of tryptone, 15 g of yeast extract, and 5 g of NaCl per liter, pH 7.5) to an OD_{595} of 0.4–0.6. The culture was diluted 1:40 with 3× LB and incubated at 37°C until an OD_{595} of 1.5–1.8 was attained. Expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside for 4 hr at 28°C.

For the purification of P52 (Table 1), 300 g of frozen cells were resuspended in 1.2 liters of ice-cold lysis buffer (50 mM Tris·HCl, pH 7.5/0.1% Nonidet P-40/3 mM EDTA with protease inhibitors) and lysed. Protease inhibitor concentrations were as follows: aprotinin, 3.0 μg/ml; leupeptin, 1.5 μg/ml; pepstatin, 2.1 μg/ml; phenylmethylsulfonyl fluoride, 0.6 mM; soybean trypsin inhibitor, 0.01%; benzamidine, 10

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Abbreviations: EGF, epidermal growth factor; P52, 52-kDa recombinant Asp (Asn) β-hydroxylase.

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Table 1. Purification of recombinant Asp (Asn) β-hydroxylase

Enzyme fraction	Protein, mg	Activity,* μmol	Yield, %	Specific activity,* μmol/mg	Purification
Cell lysate	36,000	3100	100	0.087	1.0
S-Sepharose	650	780	25	1.2	14
Mono S (no. 1)	94	440	14	4.7	54
Mono S (no. 2)	38	190	6.1	5.1	59
Gel filtration	15	130	4.2	8.9†	102
Mono S	7.5	110	3.5	14‡§	160

*$^{14}CO_2$ release from α-keto[1-^{14}C]glutaric acid, and direct *erythro*-β-hydroxyaspartate determination (18, 21, 22). The substrate for specific activity determination was the EGF-like domain of human Factor X where Asn has replaced Asp at position 18 (18).
†Represents specific activity of the purified fusion protein.
‡Represents specific activity of purified P52.
§When this fraction was assayed for the formation of *erythro*-β-hydroxyaspartate, the specific activity was 13 μmol/mg (93% coupling), where coupling is defined as the ratio of moles of *erythro*-β-hydroxyaspartate to the moles of CO_2 produced.

mM. The lysate was centrifuged at 105,000 × *g* for 35 min. The resulting supernatant was applied at 10 ml/min to an S-Sepharose fast flow column (5 cm × 10 cm; Pharmacia) equilibrated at 4°C with 50 mM Tris·HCl, pH 7.5/0.1% Nonidet P-40 with the protease inhibitors at one-third of the above concentrations, except for soybean trypsin inhibitor and benzamidine, which remained at their respective concentrations. The column was washed with 800 ml of equilibration buffer without the detergent (buffer A) and the enzyme was eluted at 5 ml/min with a 600-ml linear gradient from 0 to 1 M NaCl in buffer A. Peak activity fractions were dialyzed at 4°C against 4 liters of buffer A containing 1 mM EDTA. In three separate chromatographic runs (45 ml per run) the retentate was applied at 2 ml/min at ambient temperature to a Mono S FPLC column (HR 10/10; Pharmacia) in buffer A. The column was washed with 24 ml of buffer A and the enzyme was eluted with an 80-ml linear gradient of 0–0.75 M NaCl in buffer A. The combined pools of enzymatic activity (18 ml) were dialyzed at 4°C against buffer A and then were applied in two separate runs at ambient temperature at 1 ml/min to a Mono S FPLC column (HR 5/5; Pharmacia). The column was washed with buffer B (buffer A without soybean trypsin inhibitor and benzamidine). The bound enzyme was eluted with a 60-ml linear gradient of 0–0.75 M NaCl in buffer B. Eight milliliters of the peak activity pool was concentrated to ≈4 ml and applied to an HPLC gel filtration column (Protein-Pak 125, 7.8 mm × 30 cm; Waters) in eight separate runs. The activity was eluted with 50 mM Tris·HCl, pH 7.5/250 mM NaCl at 0.5 ml/min.

Two milligrams of HPLC-purified hydroxylase was digested at 25°C for 3 hr with 20 units of porcine enterokinase (Sigma) in 8 ml of 10 mM Tris·HCl, pH 8.0/10 mM $CaCl_2$. The P52 was isolated immediately by using a Mono S FPLC column (HR 5/5) as described above but in the absence of protease inhibitors.

Site-Directed Mutagenesis. The *Hin*dIII–*Xho* I fragment from p52 was subcloned into the *Hin*dIII/*Sal* I sites of pALTER-1 and the Altered Sites *in vitro* mutagenesis system (Promega) was employed. Histidine-675 (codon CAC) was changed to alanine (codon GCC) by using a 31-base synthetic oligonucleotide. The mutation was identified through DNA sequencing using Sequenase version 2.0 (United States Biochemical) (23) and the entire H675A-P52 gene was sequenced to confirm that there were no additional mutations. The H675A DNA *Hin*dIII–*Xba* I insert from the pALTER-1 mutagenesis plasmid was then subcloned back into the expression vector pFLAG-1 and used to transform *E. coli* strain BL21. The cell pellet from a 1-liter culture was suspended in 50 mM Tris·HCl (pH 7.0), lysed by addition of lysozyme (1 mg/ml), and frozen at −70°C for 20 min. DNase I (BRL) was added to the thawed lysates. After centrifugation at 12,400 × *g* for 10 min, the supernatant was applied to an Anti-FLAG M_2 affinity gel column as per instructions from IBI, except that 50 mM Tris·HCl (pH 7.0) was substituted for phosphate-buffered saline. The protein was eluted from the column with FLAG peptide at 150 μg/ml in 50 mM Tris·HCl (pH 8.0). Column fractions containing mutant P52 were identified by Western blot analysis (18, 19) and were assessed for purity by SDS/PAGE. Mutant P52 fractions were ≈30% pure and were assayed for Asp (Asn) β-hydroxylase activity (18) using the second EGF-like domain of bovine protein S as substrate (24). Wild-type P52 enzyme was purified and assayed in parallel.

Inactivation and Labeling of P52 with Iodo[^{14}C]acetamide. All reactions were carried out in foil-shielded test tubes at 37°C. Each tube contained 350 μl of 50 mM Tris·HCl, pH 8.0/50 mM NaCl/1 mM iodo[^{14}C]acetamide (5.3 mCi/mmol; 1 mCi = 37 MBq). The reaction was initiated by the addition of P52 to a final concentration of 0.25 mg/ml. Immediately and at the indicated times, three 8-μl aliquots were removed. One aliquot was added to 500 μl of 50 mM Tris·HCl, pH 7.0/200 mM NaCl/0.1% bovine serum albumin. Eight microliters of this diluted enzyme was then immediately assayed for hydroxylase activity (18, 24). The other two aliquots, made 20 mM in unlabeled iodoacetamide, were applied to Whatman 3MM filter papers to determine incorporation of radioactivity into P52. The filters were processed by standard procedures with cold 10% (wt/vol) trichloroacetic acid, cold 5% trichloroacetic acid, cold ethanol/diethyl ether (1:1, vol/vol), and diethyl ether. Hydroxylase activity and incorporation of radioactivity at the initial time point served as the control for 100% hydroxylase activity and 0% incorporation, respectively.

RESULTS AND DISCUSSION

Previous studies concerned with the purification of Asp (Asn) β-hydroxylase from bovine liver (18) indicated that a 52-kDa proteolytic fragment of the intact enzyme with an N terminus at lysine-310 (19) possessed full *in vitro* catalytic activity. The cDNA corresponding to this form of Asp (Asn) β-hydroxylase was inserted into the pFLAG-1 expression vector and cloned into *E. coli* BL21. After induction with isopropyl β-D-thiogalactopyranoside, ≈70% of the putative fusion protein in the total cell lysate was soluble and this accounted for 0.5–1.0% of the total soluble protein.

A conventional purification procedure (Table 1) was developed. After HPLC gel filtration, an apparent single species >90% pure by Coomassie blue staining and positive by immunoblot analysis was obtained. Two amino termini resulting from cleavages within the OmpA signal peptide of the fusion protein were present. Importantly, however, the en-

terokinase cleavage site remained intact. The HPLC gel-filtered fusion protein was then digested with enterokinase to 70–80% completion. P52 was generated and purified by Mono S chromatography. Based upon the specificity of enterokinase, only the expected N-terminal sequence was detected (the first amino acid, leucine, is derived from the cloning site), indicating a purity of >98%. Upon analysis with carboxypeptidase Y, the expected C-terminal isoleucine and the penultimate alanine were evident with no indication of heterogeneity. A molecular mass of 51,034 ± 51 Da for P52 was obtained from laser desorption time-of-flight mass spectroscopy, in excellent agreement with the value of 51,040 Da calculated from the deduced amino acid sequence. Furthermore, equilibrium sedimentation studies indicate that P52, like its native counterpart (18), exists as a monomer of 50,700 ± 1100 Da.

K_m and V_{max} values were determined for both the 52-kDa bovine native enzyme and P52 using EGF-X_{1H}-Asn as a substrate (18). These values were 19 ± 6 μM and 0.51 ± 0.10 μmol/min per mg of protein for the native enzyme and 24 ± 2 μM and 0.46 ± 0.04 μmol/min per mg of protein for P52, demonstrating that the carbohydrate found in the native enzyme is not required for hydroxylase activity *in vitro*.

Chemical modification studies and sequence alignment analysis among the various vertebrate α-ketoglutarate-dependent dioxygenases were undertaken, aimed at locating specific regions of P52 involved in substrate binding and/or catalysis. Initial studies indicated that native Asp (Asn) β-hydroxylase could be inactivated by *N*-ethylmaleimide or iodoacetamide, and the loss of enzymatic activity with either reagent could be prevented by the substrate Fe^{2+}/α-ketoglutarate. Incubation of P52 with 1 mM iodoacetamide resulted in a time-dependent loss of enzymatic activity, with ≈15% of the activity remaining after 30 min. No enzymatic activity significantly above control levels generated in the absence of peptidyl substrate or enzyme was detected after 40 or 60 min, whereas in the absence of iodoacetamide, enzymatic activity was stable. The incorporation of radioactivity from iodo[^{14}C]acetamide into P52 was proportional to the loss of enzymatic activity. Incubation for 1 hr longer than required for >95% inactivation with 5 times more iodo[^{14}C]acetamide resulted in no further increase in incorporated radioactivity. From these data, the stoichiometry of labeling was estimated to be 5.4 mol of -CH_2CONH_2 incorporated per mol of P52. In a similar experiment, mass spectral analysis of a sample that was 93% inactivated indicated that 4.5 mol of -CH_2CONH_2 was incorporated per mol of P52. These results suggest that five residues were modified concomitant with inactivation. Consistent with these results, several radiolabeled peptides were resolved following trypsin digestion of P52 inactivated with iodo[^{14}C]acetamide (Fig. 1). The structures of these peptides were determined by N-terminal sequence analysis and mass spectral analysis and corroborated by monitoring the release of radioactivity at each cycle of Edman degradation (Table 2). In all cases the release of radioactivity corresponded to the release of *S*-acetamidocysteine. The results indicated that all seven cysteine residues were modified to a similar extent. The finding that the number of sites labeled exceeds the stoichiometry of iodoacetamide modification (7 versus 5) suggests that after modification of five cysteine residues, the reactivity of the two remaining potential sites becomes reduced.

Since the cysteine residues were labeled proportionally to the loss of enzymatic activity, it was difficult to discern which cysteine modifications contributed the most to enzyme inactivation. The presence of micromolar α-ketoglutarate protected P52 against iodoacetamide inactivation (Table 3) and prevented the incorporation of radioactivity from iodo[^{14}C]acetamide at all sites of modification (Fig. 1*B*). The concentrations of α-ketoglutarate required for intermediate levels of protection are in the range of its K_m value of 20 μM

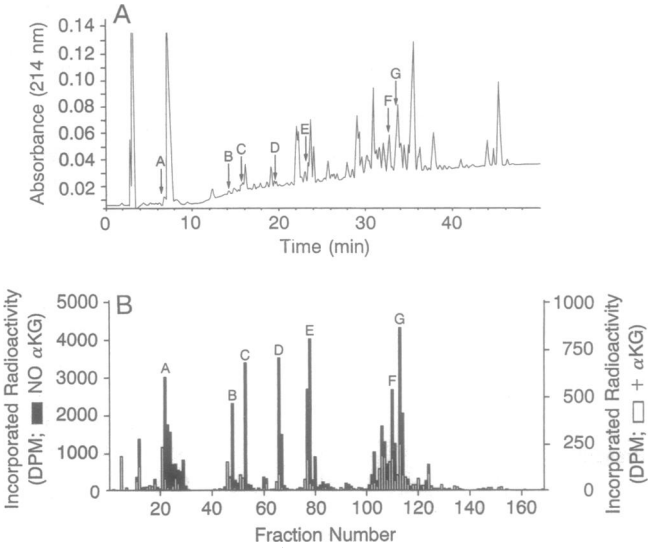

FIG. 1. Identification of the sites in P52 that were modified with iodo[^{14}C]acetamide. (*A*) HPLC resolution of peptides derived from tryptic digestion of P52. Fifty micrograms of P52 was added to 50 μl of 50 mM Tris·HCl, pH 8.0/50 mM NaCl/1 mM iodo[^{14}C]acetamide (24.1 mCi/mmol), incubated at 37°C for 1 hr, and then precipitated with 20% trichloroacetic acid. The resulting pellet was washed with trichloroacetic acid and then with acetone. The protein was then dissolved in 50 μl of 0.1 M NH_4HCO_3 (pH 7.8) and digested with 2 μg of trypsin at 37°C for 20 hr. The peptides were resolved on a C_{18} HPLC column (Waters; 0.39 cm × 30 cm) with a linear gradient of 0–50% solvent B over 50 min at 1 ml/min. Solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile. (*B*) Corresponding radioactivity elution profile for P52 treated with iodo[^{14}C]acetamide in the absence (■) and presence (□) of 50 μM Fe^{2+} plus 200 μM α-ketoglutarate (αKG). Regions A–G containing fractions with the major amounts of radioactivity were submitted for N-terminal sequence analysis and mass spectral analysis (Table 2).

(18). Although 80% protection (remaining activity) was observed under these conditions (Table 3), higher levels of protection were not attained even when millimolar α-ketoglutarate was used (data not shown). Fe^{2+} alone offered no protection but in the presence of α-ketoglutarate, complete protection was observed (Table 3). These results suggested that binding of α-ketoglutarate to the enzyme was mediated by a metal ion [as has been proposed for other α-ketoglutarate-dependent dioxygenases on the basis of kinetic studies (25)], and, in the absence of added Fe^{2+}, limiting adventitious metal ion was utilized. Consistent with this explanation, in

Table 2. N-terminal sequence and mass spectral analysis of HPLC regions A–G from tryptic digest of P52

Region	Radiolabeled peptide	Mass, Da	
		Predicted	Observed
A	EGC*(696)(K)	493.6	*
B	KNENAC(637)K	864.0	865.0
C	C*(700)ANETR	749.7	749.4
D	(cyclized)C(700)ANETR†	733.7	733.9
E	AQC*(380)EDDLAEK	1179.2	1179.7
F	YSIMHPGTHVWPHTGPTNC*(681)R	2349.6	2352.2
G	TC*(644)SLLDKFPETTGC*(656)R	1785.9	1786.3

In all cases, the release of radioactivity monitored at each cycle of the Edman degradation corresponded to the presence of *S*-acetamidocysteine (C*). These residues are designated by their position in the native sequence (19).

*No mass spectral signal was observed for this sample.

†No sequence was observed and all radioactivity remained on the disc.

Table 3. α-Ketoglutarate (αKG) protection of P52 activity during iodoacetamide (IA) inactivation with or without EDTA

EDTA (0.5 M)	αKG, μM	Added Fe^{2+}, μM	IA, mM	Activity,* %
Absent	0	0	0	100
	0	50	0	100
	200	0	0	100
	0	50	1	<5
	0	0	1	<5
	2.5	0	1	17
	5	0	1	22
	10	0	1	50
	20	0	1	63
	60	0	1	78
	200	0	1	80
	200	50	1	105
Present	0	0	0	100
	200	0	0	100
	0	0	1	20†
	200	0	1	18

*Mean of duplicates (range, <10%).
†Apparent limited protection by EDTA.

the presence of EDTA, α-ketoglutarate did not protect against iodoacetamide inactivation (Table 3). In a control experiment, EDTA alone did not enhance the rate of iodoacetamide inactivation of P52. Thus, the radiolabeling of P52 by iodo[^{14}C]acetamide is specific in the sense that it quantitatively leads to enzyme inactivation that can be completely prevented by the substrate Fe^{2+}/α-ketoglutarate.

The results of the chemical modification studies are not uniquely consistent with the cysteine residues having a direct role in catalysis. However, since protection from both enzyme inactivation and cysteine modification was observed with a substrate common to all of the α-ketoglutarate-dependent dioxygenases, the primary structure in the immediate vicinity of each of the labeled cysteines of Asp (Asn) β-hydroxylase was compared with the primary structures within this family of enzymes. Initial inspection of the region between cysteine-656 and cysteine-681 suggested homology between residues 657–684 of Asp (Asn) β-hydroxylase and residues 693–718 of human lysyl hydroxylase that was independently corroborated (see below). Since these sequences are located within the C-terminal region of their respective proteins, we focused upon this region within the vertebrate α-ketoglutarate-dependent dioxygenases. A pairwise alignment of the C-terminal regions of human prolyl (α-subunit) hydroxylase (26) and lysyl hydroxylase (27) and a pairwise alignment of the C-terminal regions of lysyl hydroxylase and bovine aspartyl hydroxylase (19) could be generated and corroborated by a multiple sequence alignment that includes the C-terminal regions from chicken prolyl (28) and lysyl (29) hydroxylases (Fig. 2). This resulted in the alignment of the "His-2" motifs previously proposed to exist in prolyl and lysyl hydroxylase (31) and the corresponding His-2 motif of aspartyl hydroxylase identified by this analysis. This motif in vertebrates is characterized in part by an invariant histidine, proline, and conserved basic residue (lysine or arginine). Other regions of interest include the MHPG sequence containing an invariant glycine residue and the highly conserved dibasic–glycine region (Fig. 2).

The alignment of these conserved regions suggests that they may function in an aspect of the catalytic mechanism common to these enzymes. When the invariant histidine-675 of the His-2 motif of Asp (Asn) β-hydroxylase was mutated to an alanine (H675A), no enzymatic activity was detected in the resulting mutant Asp (Asn) β-hydroxylase. (In these assays, the protein levels were increased >10-fold from standard assay conditions so that 1% of the wild-type activity could be detected.) This result supports the hypothesis based upon the sequence alignments that the region of the primary structure in the vicinity of the His-2 motif is important for enzymatic activity. Although the H675A mutant does not possess detectable enzymatic activity, the binding of Fe^{2+}/α-ketoglutarate to this mutant can be determined by monitoring the ability of Fe^{2+}/α-ketoglutarate to prevent the incorporation of radioactivity from iodo[^{14}C]acetamide into the mutant protein, since protection from labeling was observed for the wild type (Fig. 1; Table 3). The wild-type and mutant P52 proteins were incubated with iodo[^{14}C]acetamide in the presence and absence of Fe^{2+}/α-ketoglutarate and subjected to SDS/PAGE (Fig. 3). In the absence of Fe^{2+}/α-ketoglutarate, the amount of incorporated radioactivity (as well as the rate of incorporation; data not shown) was the same for the wild-type and mutant proteins (Fig. 3 legend). However, in the presence of Fe^{2+}/α-ketoglutarate, 79% protection from labeling was observed for the wild type, in

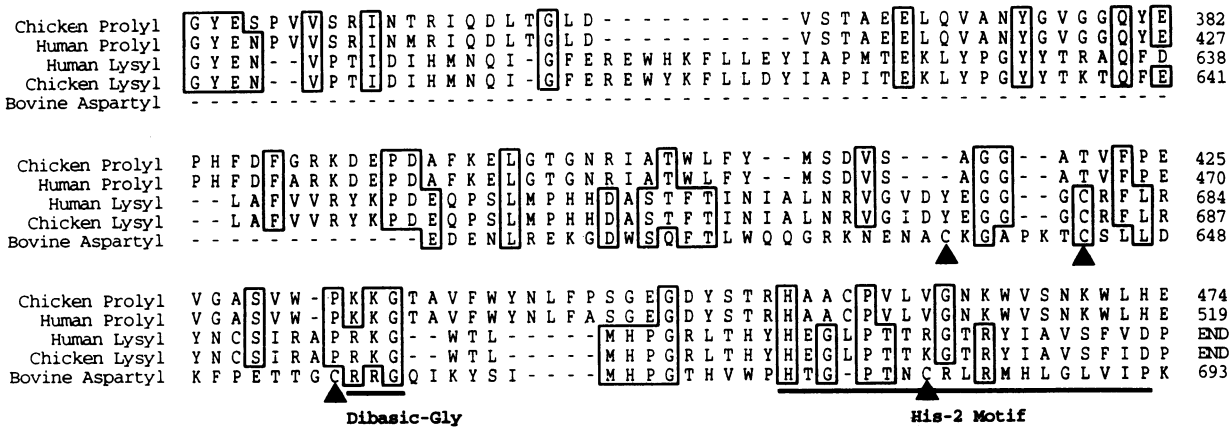

FIG. 2. Multiple sequence alignment of the C-terminal regions of vertebrate α-ketoglutarate-dependent dioxygenases. A multiple sequence alignment of the C-terminal regions of the human prolyl (26) and lysyl (27) and chicken prolyl (28) and lysyl (29) hydroxylase sequences was generated using the Genetics Computer Group program PILEUP (30). A pairwise alignment of the C-terminal regions of the bovine Asp (Asn) β-hydroxylase sequence and the human lysyl hydroxylase sequence was generated by the GAP program using default parameters and the Dayhoff mutation matrix as implemented in the Genetics Computer Group package (30). This alignment was used to align the bovine Asp (Asn) β-hydroxylase sequence by means of the program LINEUP (30). Additionally, a gap was introduced to align proline-678 of Asp (Asn) β-hydroxylase with prolines in the other four sequences. Sequence identities observed in three or more sequences are boxed. The dibasic-glycine region and the His-2 motif are underlined, and cysteines at positions 637, 644, 656, and 681 of the Asp (Asn) hydroxylase protected from labeling by α-ketoglutarate are indicated; see text for discussion.

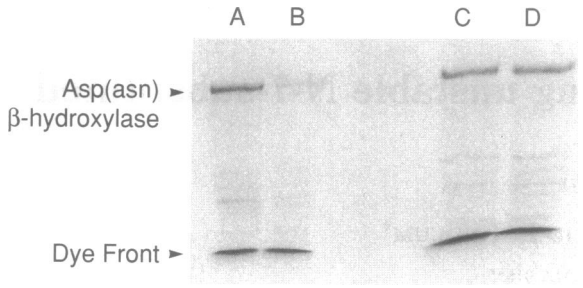

FIG. 3. Autoradiogram of iodo[^{14}C]acetamide-labeled wild-type and H675A Asp (Asn) β-hydroxylase. Partially purified wild-type P52 or H675A mutant protein (100 μg) was reacted with 1 mM iodo[^{14}C]acetamide (IA) with or without 200 μM α-ketoglutarate (αKG) and 50 μM Fe^{2+} in 104 μl as described in *Materials and Methods* and Fig. 1 legend. The acetone-washed protein pellets were dissolved in SDS/PAGE solubilization buffer. After SDS/PAGE and autoradiography the bands were excised from the gel and solubilized, and the amount of incorporated radioactivity was determined. Lanes: A, wild type plus IA (2703 dpm); B, wild type plus IA/αKG/Fe^{2+} (579 dpm); C, H675A plus IA (2554 dpm); D, H675A plus IA/αKG/Fe^{2+} (2347 dpm). Note: The dpm values were corrected for the actual amount of protein used, determined by scanning densitometry of the Coomassie blue-stained SDS/polyacrylamide gel prior to autoradiography. The relative mobilities of wild type and H675A were determined by Western blot analysis using monospecific antibodies.

agreement with the aforementioned results (Fig. 1; Table 3), while no appreciable protection from labeling was observed for the mutant. The most direct explanation for this result is that the mutant protein does not bind Fe^{2+}/α-ketoglutarate. The CD or intrinsic fluorescence spectra of the wild-type and H675A mutant proteins (purified to homogeneity) show no significant differences (data not shown). Furthermore, the thermal melt of both proteins as monitored by CD at 222 nm gave midpoint values of 50 ± 1°C and 49 ± 1°C, which are not significantly different. Although these results indicate that no major structural changes have taken place as a result of the H675A mutation, relatively small changes specific to the microenvironment of position 675 could have occurred that would not be detected by these methods. Therefore, in the absence of more precise structural data such as an x-ray structure, such changes cannot be excluded as a possible reason for the loss of enzymatic activity and Fe^{2+}/α-ketoglutarate binding.

Since we have shown that for the wild-type protein, Fe^{2+} alone does not protect and α-ketoglutarate in the absence of Fe^{2+} (presence of EDTA) does not protect, we cannot formally determine whether the H675A mutation directly affects Fe^{2+} or α-ketoglutarate binding or both. Nevertheless, these studies strongly suggest a role for the His-2 motif (31) in catalysis and indicate several additional residues that would be likely candidates for site-directed mutagenesis to probe the catalytic mechanism of α-ketoglutarate-dependent dioxygenases.

S.J. and K.M. contributed equally to this work and should be considered co-first authors.

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