Deoxyhypusine Hydroxylase Is an Fe(II)-dependent, Heat-repeat Enzyme
IDENTIFICATION OF AMINO ACID RESIDUES CRITICAL FOR Fe(II) BINDING AND CATALYSIS*

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Deoxyhypusine hydroxylase (DOHH) catalyzes the final step in the post-translational synthesis of hypusine (N^ε-(4-amino-2-hydroxybutyl)lysine) in eIF5A. DOHH is a heat-repeat protein with eight tandem helical hairpins in a symmetrical dyad. It contains two potential iron coordination sites (one on each dyad) composed of two strictly conserved His-Glu motifs. The purified human recombinant DOHH was a mixture of active holoenzyme containing 2 mol of iron/mol of DOHH and inactive metal-free apoenzyme. The two species could be distinguished by their different mobilities upon native gel electrophoresis. The DOHH apoenzyme exhibited markedly reduced levels of iron and activity. DOHH activity could be restored only by the addition of Fe^{2+} to the apoenzyme but not by other metals including Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}. The role of the strictly conserved His-Glu residues was evaluated by site-directed mutagenesis. Substitution of any single amino acid in the four His-Glu motifs with alanine abolished the enzyme activity. Of these eight alanine substitutions, six, including H56A, H89A, E90A, H207A, H240A, and E241A, caused a severe reduction in the iron content. Our results provide strong evidence that Fe(II) is the active-site-bound metal critical for DOHH catalysis and that the strictly conserved His-Glu motifs are essential for iron binding and catalysis. Furthermore, the iron to DOHH stoichiometry and dependence of iron binding on each of the four conserved His-Glu motifs suggest a binuclear iron mediated reaction mechanism, distinct from that of other Fe(II)-dependent protein hydroxylases, such as prolyl 4-hydroxylase or lysyl hydroxylases.

Eukaryotic translation initiation factor 5A (eIF5A)§ is the only protein in nature that contains an unusual amino acid, hypusine (N^ε-4-amino-2-hydroxybutyl)lysine) (for a recent review, see Ref. 1). Hypusine is derived from one specific lysine residue of the eIF5A precursor protein by a posttranslational modification reaction that involves two enzymes. In the first step deoxyhypusine synthase catalyzes the NAD-dependent cleavage and transfer of the aminobutyl moiety of the polyamine spermidine to the ε-amino group of the lysine residue to form an intermediate, deoxyhypusine (N^ε-4-aminobutyllysine) residue (2, 3). This intermediate is subsequently hydroxylated by deoxyhypusine hydroxylase (DOHH) (4, 5) to form the hypusine residue in mature eIF5A. eIF5A and its modification are essential for eukaryotic cell proliferation (1, 6–11).

eIF5A is a highly conserved protein that occurs in all eukaryotes. Its homolog is found in Archaea (12), and a distant homolog, elongation factor P, is found in bacteria (13, 14). The essential nature of eIF5A and deoxyhypusine synthase in eukaryotic cell proliferation was established from gene disruption studies in Saccharomyces cerevisiae (15–18). A DOHH homolog gene is found in all eukaryotic species. Although it is not an essential gene in S. cerevisiae (because the DOHH null strain is viable (4, 19)), inactivation of the DOHH gene is recessively lethal in multicellular eukaryotes, e.g. Caenorhabditis elegans (20) and Drosophila melanogaster (21), suggesting a requirement for the fully modified eIF5A in higher eukaryotes.

DOHH from mammalian cells and tissues shares similarities with other protein hydroxylases, such as prolyl hydroxylase and lysyl hydroxylases. Like these enzymes, DOHH is inhibited by a panel of metal chelators including iminos, 2,2′-dipyridyl, deferiprone, deferoxamine, and ciclopirox olamine (5, 22–24), and it recognizes a similar amino acid sequence surrounding the modification site, a GXG motif (where X is the deoxyhypusine, lysine, or proline residue that undergoes modification) in the substrate proteins. Because of these similarities, DOHH had been assumed to be a member of the super family of Fe(II) and 2-oxoacid-dependent dioxygenases. Enzymes of this family share a common β jelly roll structure, termed the double-stranded β-helix (DSBH) (25–27). DSBH enzymes display universal dependence on Fe(II) and contain a consensus Fe(II) coordination sequence. They catalyze a wide variety of reactions that include hydroxylation of amino acid residues, such as proline, lysine, aspartic acid, and asparagines, in the substrate proteins, repair of alkylated DNA/RNA, biosynthesis of antibiotics and plant products, and metabolism of small molecules (25, 26). Most representative hydroxylases utilize molecular oxygen as the source of the hydroxyl group and couple the oxidative decomposition of a co-substrate, α-ketoglutarate, to hydroxylation of the substrate, leading to formation of the hydroxylated product, CO_2 and succinate (25, 27, 28). In the case of DOHH, however, neither the addition of the three cofactors of DSBH enzymes, Fe^{2+}, ascorbic acid, and α-ketoglutarate, enhanced the activity of the partially purified rat testis enzyme nor could the release of ^14CO_2 from α-keto[^14C]glutarate be detected with the same enzyme (5). Although the inhibition of DOHH activity in cells by known iron chelators was shown to be overcome by the addition of either Fe^{2+}
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or Fe$^{3+}$ in HeLa cell extracts (22), there has been no definitive evidence on the identity of the metal involved in DOHH catalysis. Thus, the relationship between the DOHH and DSBH enzymes has remained obscure.

Recently our group has identified and cloned the yeast DOHH gene (YJR070C) (by screening a S. cerevisiae GST open reading frame library (29)) and also the human DOHH gene (4). DOHH homolog exists in all eukaryotes as a single gene in each species, and the amino acid sequence is highly conserved. Examination of the sequence alignment of DOHH revealed that this enzyme belongs to a family of HEAT-repeat proteins, named for human huntingtin (H), elongation factor 3 (E), a subunit of protein phosphatase 2A (A), and the target of rapamycin (TOR), that are commonly involved in protein-protein interactions (30). In these proteins the HEAT motif, composed of an α-helical hairpin (a pair of α-helices) of roughly 50 amino acids, is tandemly repeated to form superhelical structures. Computer modeling of DOHH predicted a structure consisting of eight HEAT repeats in a symmetrical dyad of four HEAT motifs connected by a variable region, a structure that is totally different from the β jelly roll structures of Fe(II)- and 2-oxoacid-dependent dioxygenases. The model predicts that the N- and C-terminal halves of DOHH form a symmetric shell-like structure with four conserved His-Glu pairs potentially contributing to metal chelation (4). However, this homology-based model represents only an approximate structure for the protein and its possible metal interactions.

In an effort to validate the proposed model and to gain insight into the reaction mechanism of DOHH, we performed structural analyses of the wild type and mutant enzymes by CD spectroscopy and investigated the roles of iron and the conserved His-Glu residues in DOHH catalysis. Our results demonstrate that the four strictly conserved His-Glu pairs indeed constitute the iron coordinating active site for DOHH. Furthermore, we present definitive evidence that Fe(II), but not Fe(III) or other divalent transition metals, is the critical metal for DOHH catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials**

L-Glutathione (GSH) was purchased from Sigma, glutathione-Sepharose 4B from Amersham Biosciences, thrombin cleavage capture kit was from Novagen, and Complete protease inhibitor mixture from Roche Applied Science. [1,8-3H]Sperrmine-3HCl (~20 Ci/mmol) was purchased from PerkinElmer Life Sciences. Precast Tris-glycine and NuPAGE (Bis-Tris) gels, MOPS running buffer, and Simply Blue staining solution were purchased from Invitrogen. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. The bacterial expression vector (pGEX-4T-3/hDOHH) expressing human DOHH was from Novagen. The bacterial vector encoding human wild type DOHH as a GST fusion protein, human eIF5A ([3H]Dhp) was prepared in an expression vector (pGEX-4T-3/hDOHH) expressing human DOHH, directed mutagenesis kit was purchased from Stratagene. The bacterial expression vector was treated with thrombin cleavage capture kit to release free DOHH enzymes.

**Site-directed Mutagenesis—**Human recombinant mutant DOHH enzymes with a single amino acid replaced with alanine (H56A, E57A, H89A, E90A, H207A, E208A, H240A, and E241A) and four double mutant enzymes with His-Glu pair replaced with an Ala-Ala pair (H56A/E57A, H89A/E90A, H207A/E208A, and H240A/E241A) were generated using the QuikChange site-directed mutagenesis kit. The bacterial vector encoding human wild type DOHH as a GST fusion protein, pGEX-4T-3/hDOHH, was used as a template for PCR, and the primer sets were designed for substitution of His or Glu with alanine. The entire open reading frame of the mutated DOHH was sequenced for confirmation of the intended mutation. The mutated plasmids were introduced into BL21(DE3)-competent cells for overexpression of the mutant enzymes.

**Overexpression and Purification of the Human Recombinant Wild Type and Mutant DOHH—**The selected clones were grown in 120 ml of LB medium containing 100 μg/ml ampicillin. Protein expression was induced at a density of 0.6 (OD$_{600}$ nm) by the addition of 1 mM isopropyl β-D-thiogalactoside for 2 h. Cells pellets were resuspended in 2.4 ml of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT)) containing a protease inhibitor mixture (EDTA-free) and lysed by sonication using an ultrasonic processor. After centrifugation of the lysate at 15,000 × g for 30 min, the clarified supernatant was rotated with 0.6 ml of GSH-Sepharose for 3 h at 4 °C. The resins were washed with buffer B (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM NaCl) 3 times and divided into two tubes. One-half was used for preparation of GST fusion enzymes by elution with buffer C (50 mM Tris-HCl, 1 mM DTT, 30 mM GSH, final pH 8.0). The other half of the resin was treated with thrombin using the thrombin cleavage capture kit to release free DOHH enzymes.

The enzyme was equilibrated in buffer A for activity assays, in HPLC metal-free water for metal analysis, or 50 mM sodium phosphate buffer, pH 7.5, for analysis of CD spectra.

**Analysis of Secondary Structures of DOHH by Circular Dichroism—**Circular dichroic spectra were measured in a Jasco J-715 spectropolarimeter using quartz cuvettes thermostatted at 25 °C. Far ultraviolet spectra were measured from 260 to 190 nm, with four scans at 50 nm/min, time constant of 1 s, bandwidth of 1 nm, and slit width of 500 μm using 200 μl of solution in a 1-mm path length cuvette. Protein concentrations were in the range 100–200 μg/ml. Visible spectra were measured from 600 to 250 nm with a 1-cm path length cuvette. The protein concentration was 2.7 mg/ml. The instrument was continually flushed with nitrogen and was calibrated every 2 weeks with a solution of ammonium (→)-10-camphorsulfonate. Protein concentrations were measured spectrophotometrically assuming that a 1 mg/ml solution has
an absorbance of 0.657 at 280 nm. Data were converted into mean residue ellipticities using a mean residue weight of 109. Estimates of secondary structure were made using the CONTIN program (32).

**DOHH Assay**—A typical DOHH reaction mixture contained 25 mM Tris-HCl, pH 7.5, 6 mM DTT, 25 μg of bovine serum albumin, 1–2 pmol of the radiolabeled protein substrate (2–4 × 10⁴ dpm), human eIF5A([³H]Dhp), and enzyme (cell lysate or 0.02–3 μg of purified enzyme) in 20 μl. After incubation at 37 °C for 1 h, 500 μg of carrier bovine serum albumin was added to each sample followed by precipitation with 10% trichloroacetic acid. The precipitates were hydrolyzed in 6 N HCl at 110 °C for 18 h. The content of [³H]hypusine and [³H]deoxyhypusine was determined after ion exchange chromatographic separation as described earlier (24, 33).

**Analysis of the Metal Content of DOHH**—The buffers for the wild type DOHH and the mutant enzymes were prepared using metal-free HPLC water. To remove extraneous metals, protein solutions were exchanged with either HPLC water or with 50 mM Tris-HCl buffer, pH 7.5, pretreated with CHELEX 100 resin. The HPLC water or the Tris buffer was also analyzed for any metal contamination. The protein samples were analyzed for metal content (Cadmium, Cobalt, Chromium, Copper, Iron, Magnesium, Manganese, Nickel, and Zinc) using inductively coupled plasma-high resolution mass spectrometry, Thermo Finnigan Element I or Element 2 (conducted by Dr. Ted Huston, W. M. Keck Elementary Geochemistry Laboratory, Department of Geological Sciences, University of Michigan).

**Separation of DOHH Apoenzyme and Holoenzyme by Native Gel Electrophoresis and Their Identification by Amino Acid Sequencing**—DOHH holo- and apoenzymes were separated by analytical native gel electrophoresis on precast 8–16% Tris-glycine polyacrylamide gels (Invitrogen) at 4 °C using a Tris-glycine buffer, pH 8.3, at 125 V. Samples were applied after 30 min of pre-electrophoresis. For amino acid sequencing, WT1 was separated as above except that a neutral Tris-glycine running buffer was also analyzed for any metal contamination. The protein samples were analyzed for metal content (Cadmium, Cobalt, Chromium, Copper, Iron, Magnesium, Manganese, Nickel, and Zinc) using inductively coupled plasma-high resolution mass spectrometry, Thermo Finnigan Element I or Element 2 (conducted by Dr. Ted Huston, W. M. Keck Elementary Geochemistry Laboratory, Department of Geological Sciences, University of Michigan).

**Fe(II) Requirement of Deoxyhypusine Hydroxylase**

![Fe(II) Requirement of Deoxyhypusine Hydroxylase](image)

**RESULTS**

**The Helical Structure of DOHH Wild Type and Mutant Enzymes**—We have previously proposed that the DOHH structure would consist of eight HEAT repeats in a symmetrical dyad of four HEAT motifs connected by a variable region (1, 4). In an effort to validate the super helical structure of DOHH, its secondary structure was determined by CD spectroscopy (Fig. 1). The purified human recombinant wild type enzyme (WT1) displayed a CD spectra typical of an α-helix-rich protein (Fig. 1) and was found to contain 77 ± 1% α-helix, 0 ± 2% β-sheet, 5 ± 1% turn, and 18 ± 1% remainder. This experimentally determined α-helical content is in close agreement with the calculated value obtained from the predicted HEAT-repeat repeat model (76–78%) of DOHH.

DOHH contains four characteristic, strictly conserved His-Glu motifs that were proposed as the metal binding sites. To investigate the role of these residues in structure, metal binding, and catalysis, we generated mutant enzymes with substitution of the His-Glu pairs with Ala-Ala pairs and also mutants with the eight His or Glu residues individually replaced with alanine. Alanine substitution was chosen because it effectively negates side-chain effects and was predicted not to alter the secondary structure of main chains. As expected, all of the mutant enzymes with alanine substitution of the conserved His-Glu residues gave CD spectra similar to that of the wild type DOHH (data not shown). Furthermore, the CD spectrum of the wild type apoenzyme, WTa1, was also similar to that of the iron-containing enzyme WT1 (data not shown). These findings suggest that the α-helical secondary structure of DOHH does not depend on the conserved His-Glu motifs or metal binding.

**Activities and Metal Content of the Wild Type DOHH and Mutant Enzymes**—Identification of Fe(II) as the Catalytic Metal for DOHH—Purified human recombinant DOHH catalyzed the conversion of deoxyhypusine-containing eIF5A, eIF5A(Dhp), to the hypusine-containing protein in a time- and concentration-dependent manner (Fig. 2, A and B). The pH dependence activity measurement indicates that the purified recombinant enzyme has optimal activity at pH 8–8.5 (Fig. 2C).

When the metal content of the wild type DOHH (purified by one-step affinity chromatography on GSH-Sepharose followed by cleavage of the GST tag) was measured by inductively coupled plasma-high resolution mass spectrometry, iron was found to be the major metal. In the early preparations of wild type enzyme, its content varied from 0.7 to 1.3 mol/mol (not shown). The variation in the iron content in different preparations of wild type enzyme appears to be due to a gradual dissociation of iron during purification and due to the differences in the extent of buffer exchange or dialysis before the metal analysis. Although a low level of Zn²⁺ was detected in some DOHH preparations, of the nine metals analyzed (Cadmium, Cobalt, Chromium, Copper, Iron, Magnesium, Manganese, Nickel, and Zinc), no metal other than iron was consistently found to be associated with DOHH at a significant level (>0.07 mol/mol).

The importance of iron in DOHH activity is evident from the reduction or loss of activity upon removal of enzyme-bound iron. Compared
with the activity of WT1 (the wild type enzyme purified by one-step affinity chromatography on GSH-Sepharose). WT1a, prepared using a lysis buffer containing 4 mM EDTA followed by gel filtration, and WT1a, prepared from WT1 by removal of iron by extensive dialysis in acidic sodium citrate buffer, pH 4.5, were found to contain a very low level of iron (0.02 mol of iron/mol of DOHH) (Fig. 3A). Both preparations (WT1a and WT1a) showed a marked reduction in DOHH activity (Fig. 3B). The addition of ferrous ion (2 μM) increased the activity of a normal wild type DOHH preparation (WT1) and restored the activities of WT1a and WT1a enzymes to a large extent, indicating that the lack of iron was the cause for the reduced activities of the two apoenzyme preparations.

In contrast to the wild type enzyme (WT1), all the mutant enzymes with alanine substitutions of the conserved His-Glu residues at the predicted metal chelation sites (including eight single mutants (M1, H56A; M2, E57A; M3, H89A; M4, E90A; M5, H207A; M6, E208A; M7, H240A; M8, E241A) and four double mutants (DM1, H56A/E57A; DM2, H89A/E90A; DM3, H207A/E208A; DM4, H240A/E241A) were assayd at 0.3- and 3.0-μg levels (bars shown are for 3.0 μg only). hDOHH, human DOHH.

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FIGURE 3. Iron content (A) and DOHH activities (B) of the wild type and mutant enzymes. A, the iron content was determined for WT1, WTa1, and WTa2 and 12 mutant enzymes using ~100 μg of purified proteins. B, the DOHH reaction was carried out as described under “Experimental Procedures.” Wild type enzymes (WT1, WTa1, and WTa2) were used at 0.1 μg with or without 2 μM ferrous ammonium sulfate. The 12 mutant enzymes (M1, H56A; M2, E57A; M3, H89A; M4, E90A; M5, H207A; M6, E208A; M7, H240A; M8, E241A; DM1, H56A/E57A; DM2, H89A/E90A; DM3, H207A/E208A; DM4, H240A/E241A) were assayed at 0.3- and 3.0-μg levels (bars shown are for 3.0 μg only).

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FIGURE 4. Native gel electrophoresis of the wild type and mutant DOHH. A, the wild type enzyme preparations with low iron content, WTa1 and WTa2, migrated at the position of Band 2, indicating that this second band represented the iron-free apoenzyme. Upon incubation of WTa1 with 0.2 μM ferrous ammonium sulfate for 2 h, a portion of Band 2 protein (apoenzyme) was converted to Band 1, indicating a partial reconstitution of apoenzyme with iron to form the holoenzyme (Fig. 4B, lane 1); the first, a well focused, fast moving band (Band 1, solid arrowhead), and the second, a more diffuse, slow moving band (Band 2, open arrowhead). With the wild type enzyme preparations, iron free WT1a and WT1a2, migrated at the position of Band 2, indicating that this second diffuse band represents the iron-free apoenzyme. Upon incubation of WT1a1 with 0.2 μM ferrous ammonium sulfate for 2 h, a portion of Band 2 protein (apo-enzyme) was converted to Band 1, indicating a partial reconstitution of apoenzyme with iron to form the holoenzyme (Fig. 4B, compare lanes 3 and 3’). When the lane containing the WT1 enzyme was sliced and proteins were extracted from the gel slices for the DOHH

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FIGURE 5. pH dependence of the DOHH reaction. The separation of iron-containing holoenzyme and iron-free apoenzyme of the wild type and mutant DOHH by native gel electrophoresis and gel filtration—The three preparations of the wild type enzyme (WT1, WTa1, and WTa2) and mutant enzymes appeared similarly pure and displayed one predominant band of 32 kDa upon SDS-PAGE (Fig. 4A, Coomassie Blue staining). However, upon electrophoresis under non-denaturing conditions, heterogeneity was observed. The wild type enzyme, WT1, resolved into two major species (Fig. 4B, lane 1); the first, a well focused, fast moving band (Band 1, solid arrowhead), and the second, a more diffuse, slow moving band (Band 2, open arrowhead). The wild type enzyme preparations with low iron content, WTa1 and WTa2, migrated at the position of Band 2, indicating that this second diffuse band represents the iron-free apoenzyme. Upon incubation of WT1a1 with 0.2 μM ferrous ammonium sulfate for 2 h, a portion of Band 2 protein (apo-enzyme) was converted to Band 1, indicating a partial reconstitution of apoenzyme with iron to form the holoenzyme (Fig. 4B, compare lanes 3 and 3’). When the lane containing the WT1 enzyme was sliced and proteins were extracted from the gel slices for the DOHH
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assay, activity was found at the position of Band 1 (holoenzyme monomer), but no activity was detectable at the position of Band 2 (apoenzyme monomer) (data not shown). To confirm that the two bands of the WT1 proteins are indeed DOHH holo- and apoenzymes, the proteins were transferred to a polyvinylidene difluoride membrane after native gel electrophoresis and subjected to amino acid sequencing. After nine cycles of Edman degradation, both bands yielded the identical amino acid sequence, GSMVTEQEV, in which MVTEQEV is the first seven amino acids of human DOHH (the leading sequence, Gly-Ser, is derived from the pGEX-4T-3 vector after thrombin cleavage of the R-G bond to remove the GST tag). Furthermore, when the WTa2 enzyme was incubated with various transition metals for reconstitution, only Fe\(^{2+}\) and Fe\(^{3+}\) were found to convert a portion of the Band 2 form to the Band 1 form (Fig. 4C, lanes 7 and 8), suggesting that iron is the only metal that effectively binds to the active site of DOHH.

All the mutant enzymes with reduced iron content (H56A, H89A, E90A, H207A, H240A, and E241A) migrated mainly as the slow moving diffuse band (Band 2), consistent with the wild type apoenzyme bands of WT1 and WTa2. The two mutant enzymes that contained iron at the level of the wild type enzyme (Band 1) and apoenzyme (Band 2), like the wild type DOHH.

DOHH is an acidic protein with a calculated pl of 4.74. Because the binding of Fe\(^{2+}\) or Fe\(^{3+}\) will reduce the negative charge of the enzyme, the iron-containing holoenzyme is expected to migrate slower than the apoenzyme if there is no conformational difference. That the electrophoretic mobility of the iron-containing holoenzyme (Band 1) is faster than that of the iron-free apoenzyme (Band 2) suggests that the binding of iron to the DOHH active site causes a significant conformational change, giving rise to a more compact form. An indication of a larger hydrodynamic size of the apoenzyme than the holoenzyme was also given from their differential migration on gel filtration (Fig. 5). DOHH wild type enzyme (purified by affinity chromatography) eluted as one major peak (at a position expected for 32-kDa monomer) with a leading edge shoulder (Fig. 5A). When the protein in each fraction was analyzed by SDS-PAGE (Fig. 5B) and native gel electrophoresis (Fig. 5C), the early shoulder fractions (fractions 22–24) were found to contain the apoenzyme (Band 2 form) and the center peak fractions (fractions 25–28), largely the holoenzyme (Band 1 form) with a small amount of apoenzyme. DOHH activity in the fractions corresponded to the intensity of the holoenzyme band; there was no detectable activity in the apoenzyme fractions (Fig. 5D).

To obtain a homogenous DOHH preparation and to determine the iron stoichiometry of the holoenzyme, we performed preparative gel electrophoresis under non-denaturing conditions using the Mini Prep Cell (Bio-Rad). The DOHH wild type enzyme (WT1) resolved into two peaks, peak 1 (fractions 17–21) and peak 2 (fractions 30–36), each of which migrated as a single band of 32 kDa upon SDS-PAGE (Fig. 6A). Upon native gel electrophoresis, fractions in the first peak contained mainly Band 1 and only trace amounts of Band 2. The second peak contained largely Band 2 (Fig. 6B), with a small amount of Band 1 in later fractions (fractions 33–35). The iron content of peak 1 was determined by the iron content of peak 1 protein is indeed the holoenzyme, optimally containing 2 mol of iron/mol of enzyme monomer. Little iron was found in early fractions of peak 2 (fractions 30–32), confirming that the diffuse Band 2 (open arrowhead) is the apoenzyme. DOHH activity was found mainly in the first peak (DOHH holoenzyme monomer) (Fig. 6C), in accordance with the data from assays of gel slices and gel filtration fractions (described above). DOHH activity was undetectable in fractions 30–32 from peak 2, a confirmation that the apoenzyme is inactive. A low level of activity in fractions 33 and 34 is likely due to a small contamination with the holoenzyme (Fig. 6B, seen as faint bands in fractions 33 and 34), which presumably derived from a third band (gray arrowhead, Fig. 6B, first lane) that has DOHH activity and DOHH sequence (data not shown) and is most likely a dimer of the DOHH holoenzyme. This third band was not consistently detected in different native gels, but its level seemed to increase at high DOHH concentrations and in the absence of DTT. Overall, these results provide strong evidence that human recombinant DOHH is an iron-dependent enzyme optimally containing 2 mol of iron/mol of protein.

The Effects of Various Metal Ions on DOHH Activity—The specificity of metal-induced changes in the electrophoretic mobility of DOHH apoenzymes (Fig. 4C) suggests that the human enzyme binds only Fe\(^{2+}\) or Fe\(^{3+}\) with high affinity at the active site. Therefore, we examined the effects of various metal salts (CaCl\(_2\), CoCl\(_2\), CuSO\(_4\), NiCl\(_2\), CrCl\(_2\), CuSO\(_4\), Fe(NO\(_3\))\(_2\), FeCl\(_3\), MgCl\(_2\), MnCl\(_2\), ZnCl\(_2\), H\(_2\)O, CuCl\(_2\), MnCl\(_2\), ZnCl\(_2\), H\(_2\)O, and ZnCl\(_2\)) at 1–1000 μM concentration; it caused inhibition at high concentrations (100 μM). A relatively small increase in the activity by Fe\(^{3+}\) at 100 μM may be due to a conversion of a small portion of Fe\(^{3+}\) to Fe\(^{2+}\) in the reaction mixture containing 6 mM DTT. These results indicate the oxidation state-specific catalytic function of Fe\(^{3+}\) in DOHH catalysis. When the same panel of metal ions was added to the iron-containing DOHH (WT1), only Fe\(^{3+}\) stimulated the activity at 1–3 μM concentration; it caused inhibition at high concentrations (>100 μM). Other metal ions including Cu\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), and Cr\(^{3+}\),...
DISCUSSION

Deoxyhypusine hydroxylase belongs to a family of proteins that contain tandem repeats of α-helical hairpins known as HEAT motifs. Sequence analysis of DOHH showed that it is composed of two symmetrical domains (N- and C-terminal), each containing four HEAT repeats connected by a variable loop (4). Furthermore, it has four strictly conserved His-Glu pairs, two on each dyad, that were proposed as metal coordination sites. A homology model of DOHH based on the known crystal structure of a HEAT-repeat protein, the *E. coli* protein YibA (Protein Data Bank code 1OYZ), presents a solenoid structure with a concave inner surface (4). However, this model did not predict a precise distance between the presumed metal binding His-Glu pairs on the two dyad arms nor the stoichiometry of bound metal to protein. In an effort to experimentally validate the proposed DOHH model and/or to refine it, we investigated the role of iron and the strictly conserved His-Glu residues in DOHH structure and catalysis. We have identified Fe(II) as the active site metal of DOHH and His-56, His-89, Glu-90, His-207, His-240, and Glu-241 as the iron coordination sites. Furthermore, we provide strong evidence for the critical role of each residue of the His-Glu motifs in DOHH catalysis. Thus, our results support a super helical DOHH model in which the two metal binding sites are closely interactive for binuclear iron binding and catalysis (Scheme 1A).

The binding of iron does not affect the secondary structure of DOHH and its α-helical content since there is no clear distinction between the CD spectra of iron-containing DOHH and the iron-deficient forms. Despite similarities in the α-helical content between the two forms of DOHH, the iron binding does have a profound effect on the tertiary structure of DOHH, as a difference in the hydrodynamic size was clearly noted for the apo- and holoenzymes. Electrophoretic mobility on native gel depends on both protein charge and hydrodynamic size. Based on the charge effects alone, an iron-containing holoenzyme is expected to migrate more slowly than the iron-free apoenzyme. On the contrary, the DOHH holoenzyme migrates faster than the apoenzyme, indicating that the iron-containing DOHH is in a more compact conformation than the apoenzyme (Figs. 4B and 6B). Consistent with this notion, the apoenzyme was found to elute in earlier fractions than the holoenzyme upon gel filtration (larger hydrodynamic size) (Fig. 5).
observation the DOHH model, the two arms of the apoenzyme would be further separated than in the holoenzyme (Scheme 1B). In addition, unlike the holoenzyme, which migrates as a well focused band on native gels, the apoenzyme migrates as a diffuse band suggesting a heterogeneous mixture of different conformations. This mixture may represent apoenzyme molecules with varying distances between the two dyad arms, probably due to a greater flexibility of the dyad arms around the variable hinge loop in the absence of bound metal. It seems likely that the binding of iron bridges the two dyad arms of the DOHH monomer, resulting in a homogeneous population with a compact, concave structure, as depicted in Scheme 1A.

The separation of holoenzyme from apoenzyme enabled us to determine the stoichiometry of iron binding to DOHH and to identify the active form of DOHH. Because the iron content of early preparations of the wild type DOHH (mixtures of apo- and holoenzymes) ranged from 0.7 to 1.3, it was not clear whether a DOHH monomer could bind one atom of iron only or two atoms only or both one and two atoms. Attempts to reconstitute DOHH to a fully charged holoenzyme were not successful, since incubation of DOHH (WT1) with iron (0.1–1.0 mM) only slightly increased the holoenzyme fraction (not shown), and iron at this high concentration inhibits DOHH activity. By preparative gel electrophoresis, the holoenzyme, containing close to 2 mol of iron/mol of protein, was isolated and was identified as the active form of DOHH. No DOHH peak with 1 mol/mol of iron was identified. Although the DOHH monomer contains two iron coordination sites (one on each dyad arm), it is likely that binuclear iron binding occurs coordinately to form one active center. If the two iron coordination sites (the two sites formed by His-56, His-89, and Glu-90 for the N-terminal dyad and His-207, His-240, and Glu-241 for the C-terminal dyad) were to operate as two separate active centers, one would expect a 50% reduction in iron binding or in DOHH activity by a single alanine substitution at either site. However, a single alanine substitution at any of the His or Glu residues in the four His-Glu motifs abolished DOHH activity. Furthermore, substitution of alanine for any one of the six residues His-56, His-89, Glu-90, His-207, His-240, or Glu-241 caused a 70–90% reduction in the iron content. A tempting model to explain our overall data is a DOHH active center with a diiron coordinated by the six His and Glu residues from both dyad arms (Scheme 1A).

**FIGURE 7.** The effects of various metals on DOHH apoenzyme (WTa2) (A) and iron-containing enzyme WT1 (B). The activity assays were carried out as described under “Experimental Procedures” using 0.15 μg each of WTa2 (A) or WT1. The metal ions were added at 1, 3, 10, 100, and 1000 μM to the reaction mixture. The y axis represents the percentage of control (no addition).
Fe(II) Requirement of Deoxyhypusine Hydroxylase

Binuclear iron binding has been characterized in several non-heme enzymes and iron-binding proteins such as hemerythrin and ferritin (34, 35). A well studied group of diiron enzymes, including the ribonucleotide reductase R2 subunit, methane monooxygenase, and Δ2-desaturases, have an active site diiron, located in a four-helix bundle, typically liganded with two His and four Glu residues. Two of the glutamate/aspartate residues form a bidentate carboxyate bridge to the two iron atoms. Upon reaction with dioxygen, an oxo/peroxo link may be formed (34, 36, 37). It would be interesting to determine how a binuclear center in DOHH would be configured and to examine how it is related to other known diiron enzymes. Further site-directed mutagenesis of other conserved amino acid residues of DOHH is under way to identify additional amino acid residues that may be critical for iron binding and catalysis and to gain further insights into the reaction mechanism of DOHH.

Interestingly, the concentrated solutions of the wild type enzyme purified in the absence of EDTA displayed a characteristic blue color, and a broad absorption peak centered at 628 nm was detected (supplemental Fig. 1). In contrast, neither blue color nor any absorption peak above 300 nm was observed for the apoenzyme at the same protein concentration and in the same buffer. Thus, the blue color of DOHH solutions appears to be due to the diiron binding. Analogously, another blue non-heme iron protein, neelaredoxin (with absorption peak at 666 nm) also contains a biniron center, proposed to be coordinated by specific His and Glu residues (38).

In this report we have identified Fe(II) as the required metal for DOHH. Whereas both Fe2+ and Fe3+ can bind to DOHH apoenzyme (Fig. 4C) to induce a conformational change, only Fe2+ could restore the activity of the apoenzyme at 1–10 μM (Fig. 7A). In this concentration range, Fe3+ was without effect. These findings suggest that the iron binding and the conformational change per se are necessary but are not sufficient for DOHH catalysis and that only Fe2+ can fulfill the catalytic function. Other metals including Cd2+, Co3+, Mg2+, Mn2+, Ni2+, and Zn2+ did not convert the apoenzyme (slow migration form, band 2) to the faster moving form (band 1); presumably these metals also do not bind to the active site of DOHH. Consistent with the lack of conversion of DOHH apoenzyme to holoenzyme monomer (band 1) by these other metals (Fig. 4C), it is their failure to enhance the activities of the apoenzyme (Fig. 7B).

DOHH is predicted to fold into a HEAT-repeat structure completely unrelated to the DSBB structure of other protein hydroxylases that catalyze the hydroxylation of proline, lysine, aspartic acid, or asparagine (25, 26). Nonetheless, DOHH and DSBB enzymes share common features such as a Fe(II)-dependent reaction mechanism. Like DSBB enzymes, DOHH is dependent on molecular oxygen for hydroxylation (data not shown). However, there seems to be a fine distinction between DOHH and DSBB enzymes in details of mechanism, mode of iron binding, the consensus sequence of the coordination sites, and metal specificity. For example, DSBB enzymes contain a consensus ligand sequence, His1-X-Asp/Glu-Xn-His2, one site per catalytic subunit for coordination of one iron atom (25). On the other hand, in the case of DOHH monomer, two iron atoms likely form a binuclear center coordinated by a pair of His1-Glu1-X-Xn-His2-Glu2 sequence (n = 32) (one from each dyad arm and in which His1, His2, and Glu2 are three chelating residues) (4). Furthermore, the two iron binding sites of the dyad arms seem to closely cooperate for binuclear iron binding and catalysis.

DOHH catalyzes the final step in the maturation of elF5A, an essential protein in eukaryotic cell proliferation. DOHH as a key component of this maturation pathway and its product, elF5A, have been implicated in the regulation of mammalian cell proliferation (1.6–11.23). The metal chelating compounds that effectively inhibit deoxyhypusine hydroxylation in elF5A arrest cell cycle progression at the boundary of G1/S in mammalian cells, including human cancer cells and human umbilical vein endothelial cells (23, 24). Furthermore, these compounds caused a strong inhibition of angiogenesis in model assays, presumably by concomitant inhibition of DOHH and collagen prolyl-4-hydroxylase (24). elF5A and DOHH have also been implicated in human immunodeficiency virus-1 viral replication (39–43). Furthermore, metal chelating inhibitors of DOHH, e.g. deferiprone and ciclopirox olamine, have been reported to inhibit replication of human immunodeficiency virus-1 (44). Taken together, the ability to limit elF5A hypusination may provide a useful tool for targeted control of cell growth and expansion in disease states.

We are actively pursuing determination of the crystal structures of DOHH apoenzymes and a DOHH complex with its protein substrate. The crystal structures will offer the ultimate validation of DOHH model structure and the proposed iron binding mode and will provide further insights into the DOHH reaction mechanism and the specificity of enzyme-substrate interactions. Characterization of the reaction mechanism and determination of the crystal structure of DOHH will pave the way to the development of specific inhibitors of DOHH.

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