Specificity of the Deoxyhypusine Hydroxylase-Eukaryotic Translation Initiation Factor (eIF5A) Interaction

IDENTIFICATION OF AMINO ACID RESIDUES OF THE ENZYME REQUIRED FOR BINDING OF ITS SUBSTRATE, DEOXYHYPUSINE-CONTAINING eIF5A

Received for publication, August 7, 2006, and in revised form, January 8, 2007 Published, JBC Papers in Press, January 9, 2007, DOI 10.1074/jbc.M607495200

Kee Ryeon Kang 1,2, Yeon Sook Kim 1, Edith C. Wolff, and Myung Hee Park 3

From the Oral and Pharyngeal Cancer Branch, NIDCR, National Institutes of Health, Bethesda, Maryland 20892

Deoxyhypusine hydroxylase (DOHH) is a novel metalloenzyme that catalyzes the final step of the post-translational synthesis of hypusine \( \text{N}^\text{H}-(4\text{-amino-2-hydroxybutyl})\text{lysine} \) in the eukaryotic translation initiation factor 5A (eIF5A). Hypusine synthesis is unique in that it occurs in only one protein, denoting the strict specificity of the modification enzymes toward the substrate protein. The specificity of the interaction between eIF5A and DOHH was investigated using human eIF5A (eIF5A-1 isoform) and human recombinant DOHH. DOHH displayed a strong preference for binding the deoxyhypusine-containing form of eIF5A, over the eIF5A precursor or the hypusine-containing eIF5A, indicating a role for the deoxyhypusine residue in binding. In addition to the deoxyhypusine residue, a large portion of the eIF5A polypeptide \((>20–90\text{ amino acids})\) is required for effective modification by DOHH. We have identified the amino acid residues of DOHH that are critical for substrate binding by alanine substitution of 36 conserved amino acid residues. Of these, alanine substitution at Glu57, Glu90, and Glu208 displayed a strong preference for binding the deoxyhypusine-containing eIF5A (Dhp), hydroxylates this intermediate to form the hypusine residue and mature eIF5A (4, 5).

eIF5A and its modification enzymes, DHS and DOHH, are essential for mammalian cell proliferation (1, 6–11). Various metal chelating inhibitors of DOHH, including mimosine and ciclopirox olamine, inhibit cell proliferation by causing cell cycle arrest at the G1/S boundary (12). Ciclopirox olamine, in particular, exerted strong inhibition of human umbilical vein endothelial cell (HUVEC) proliferation and angiogenesis in model assays, suggesting the potential utility of DOHH inhibitors as antitumor agents (13). Although DOHH has been proposed as a potential target of antitumor therapy (13) and anti-human immunodeficiency virus type 1 therapy (14), no specific inhibitors of DOHH are currently available.

The unique feature of hypusine formation is the strict substrate specificity of this protein modification. Hypusine synthesis occurs exclusively in one cellular protein, eIF5A precursor, at one specific lysine residue (Lys50 for the human protein) and thereby represents one of the most specific protein modifications known to date. eIF5A and its modifying enzymes, DHS and DOHH, are highly conserved in all eukaryotes (1, 8). The amino acid sequence conservation surrounding the hypusine site of eIF5A is especially high in eukaryotes, suggesting that the hypusine residue has an important basic function that has been preserved through evolution. Furthermore, the sequence conservation of eIF5A and its modifying enzymes reflects an important aspect of the substrate specificity of hypusine synthesis.

The basis of the specificity of the hypusine modification must reside in the selective interactions between the eIF5A substrate protein and its modification enzymes. For example, DHS and Lia1 (Lia, ligand of eIF5A, later identified as DOHH (5)) were the two proteins identified as eIF5A-binding proteins from a

*This work was supported by the Intramural Research Program of the National Institutes of Health (NIDCR). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Both authors contributed equally to this work.

2 Current address: Medical Research Center for Neural Dysfunction, Dept. of Biochemistry, School of Medicine and Institute of Health Sciences, Gyeongsang National University, 92 Chilam-Dong, Jinju 660-751, Korea.

3 To whom correspondence should be addressed: Bldg. 30, Rm. 211, OPCB, NIDCR, National Institutes of Health, Bethesda MD 20892-4340. Tel.: 301-496-5056; Fax: 301-402-0823; E-mail: mhpark@nih.gov.

4 The abbreviations used are: eIF5A, eukaryotic translation initiation factor 5A; eIF5A-1, primary isoform of eIF5A; eIF5A(Lys), eIF5A precursor; eIF5A(Dhp), eIF5A intermediate containing deoxyhypusine; eIF5A(Hpu), eIF5A mature form containing hypusine; DOHH, deoxyhypusine hydroxylase; GST, glutathione S-transferase; DTT, dithiothreitol; CD, circular dichroism; HEAT repeat, a protein structural motif found in Huntington,elongation factor 3 , a subunit of protein phosphatase 2A, and the Target of rapamycin; aa, amino acid; BisTris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; DHS, deoxyhypusine synthase.
yeast two-hybrid screening (15). Deoxyhypusine synthase is the major detectable eIF5A-binding protein in Saccharomyces cerevisiae (16) or in mammalian cells upon affinity purification using epitope-tagged eIF5A bait protein. Furthermore, stable complexes of eIF5A(Lys)/DHS and eIF5A(Dhp)/DHS are detectable by native gel electrophoresis (17). No other cellular proteins undergo deoxyhypusine modification in intact cells or in vitro (19), supporting the notion that the first step (deoxyhypusine synthesis) is a key element defining the specificity of the hypusine modification. The substrate specificity of DHS has been extensively studied, both for spermidine (20) and the eIF5A precursor (21). Neither free lysine, nor a short peptide containing the highly conserved region around the lysine to be modified, acts as a substrate of DHS. A long polypeptide of eIF5A(Lys) (larger than aa 30–80) is required for effective modification by DHS (21).

Little was known about the structure or specificity of deoxyhypusine hydroxylase until our recent cloning of yeast and human DOHH (5). It had been presumed to be a non-heme iron enzyme belonging to the family of 2-oxoacid- and Fe(II)-dependent dioxygenases, in part because deoxyhypusine hydroxylase activity in mammalian cells and tissues is inhibited by iron chelators (4, 12, 13) and is dependent on molecular oxygen. However, it was not known if deoxyhypusine hydroxylation can be catalyzed by other known protein hydroxylases of this family, such as prolyl and lysyl hydroxylases or vice versa. In fact, the DOHH structure predicted from sequence analysis and computer modeling (5) is entirely unrelated to the β jelly roll structure (termed double-stranded β helix) of Fe(II)- and 2-oxoacid-dependent dioxygenases. DOHH consists of eight HEAT repeats, in a symmetrical dyad of four HEAT motifs connected by a variable region. The predicted α-helical structure of DOHH was validated experimentally by determination of the α-helical content of purified human recombinant DOHH by CD analysis (77 versus 76–78%, calculated from the HEAT repeat model) (24).

The iron-binding active site of DOHH, and mode of iron binding also differ from those of 2-oxoacid and Fe(II)-dependent dioxygenases, suggesting a distinct reaction mechanism (24). In many of the double-stranded β helix enzymes, iron is coordinated by His-X-Asp/Glu-Xn-His at three coordination sites, and by a 2-oxoacid and oxygen (22). Judging from the stoichiometry of 2 mol of iron per mol of DOHH holoenzyme, DOHH probably contains one binuclear (diiron) active center (24). In DOHH there are four strictly conserved His-Glu motifs (His56–Glu57, His89–Glu90, His207–Glu208, and His240–Glu241) predicted to be involved in the iron binding (5). Replacement of any one of these amino acids with alanine abolished DOHH activity (24). Of the eight alanine substitution mutant enzymes, six (H56A, H89A, E90A, H207A, H240A, and E241A) were deficient in iron binding, suggesting that His56, His89, Glu90, His207, His240, and Glu241 are involved in the coordination of iron (24). As for the two mutant enzymes E57A and E208A, which were totally inactive despite their normal iron content, the reason for their lack of activity was unknown.

In this study we have investigated the structural basis of the specificity of deoxyhypusine hydroxylation. Our results demonstrate that deoxyhypusine hydroxylase specifically recognizes the deoxyhypusine form, eIF5A(Dhp), but not the lysine form, eIF5A(Lys), and that a large portion (aa 20–90) of this 154-aa protein is required for effective hydroxylation by DOHH. Analysis of DOHH mutant enzymes provides evidence that Glu57 and Glu208 of the conserved His–Glu motifs of DOHH, although not involved in iron coordination, are required for the association with the substrate protein. Based on the current findings, we propose a model of eIF5A(Dhp)/DOHH binding in which the side chain carboxyl groups of Glu57 and Glu208 of DOHH form ionic bridges with the amino group(s) of the deoxyhypusine side chain of the substrate protein.

EXPERIMENTAL PROCEDURES

Materials

L-Glutathione was purchased from Sigma, glutathione-Sepharose 4B from Amersham Biosciences, Thrombin Cleavage Capture Kit from Novagen, and EDTA-free protease inhibitor mixtures from Sigma. [1,8-3H]Spermidine·3HCl (20 Ci/mmol) was purchased from DuPont. Precast Tris glycer-one NuPAGE (BisTris) gels, electrophoresis buffers, and Simply Blue staining solution were from Invitrogen, the QuikChange Site-directed Mutagenesis Kit was from Stratagene. A monoclonal antibody to eIF5A was from BD Biosciences. The radiolabeled deoxyhypusine-containing protein, human eIF5A([3H]Dhp) and the yeast eIF5A([3H]Dhp (gene product of TIFS1A) were prepared in an in vitro deoxyhypusine synthase reaction as described (25, 26). The truncated forms of eIF5A were generated as described previously (21). The oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc.

Methods

Generation of DOHH Mutant Enzymes and Truncated Enzymes—Human recombinant mutant DOHH enzymes with a single amino acid of the strictly conserved His-Glu motifs replaced with alanine (H56A, E57A, H89A, E90A, H207A, E208A, H240A, and E241A) were reported previously (24). 28 additional mutant enzymes with alanine substitution of all other conserved amino acids (R26A, L28A, K55A, G63A, Q64A, L74A, R88A, E93A, E120A, T121A, C122A, D148A, P149A, R175A, Y176A, R183A, S202A, V212A, G214A, Q215A, L225A, E234A, M237A, R239A, S246A, G247A, I258A, and S272A) and those with substitutions of Glu57 or Glu208 with aspartic acid, asparagine, or glutamine, respectively (E57D, E57N, E57Q, E208D, E208N, and E208Q), 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 (5), was used as a template for PCR and the primer sets were designed for substitution of an individual amino acid with alanine, aspartic acid, asparagine, or glutamine. Human DOHH fragments, N-terminal half (aa 1–151) and C-terminal half (aa 152–302), were generated as GST fusion proteins using the pGEX-4T-3 vector by PCR amplification of the N- or C-terminal halves and subcloning

5 J. Kaevel and M. H. Park, unpublished results.
6 E. C. Wolff, H. M. Hansaue-Abel, and M. H. Park, unpublished results.
into BamHI/Sall sites of pGEX-4T-3 vector. The entire open reading frame of the mutated or truncated DOHH was sequenced for confirmation of the intended mutation or truncation. The recombinant plasmids were introduced into BL21(DE3) competent cells for overexpression of the mutant enzymes.

**Purification of Wild Type and Mutant DOHH Enzymes**—The wild type and mutant DOHH enzymes were purified as described previously (5, 24). The selected clones of BL21(DE3) cells were grown in 120 ml of LB medium containing 100 µg/ml of ampicillin. Protein expression was induced at a density of 0.6 (OD$_{600}$ nm) by addition of 1 mM isopropyl-β-D-thiogalactoside for 3 h. Cell 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 $\times$ g for 30 min, the clarified supernatant was rotated with 0.6 ml of GSH-Sepharose for 3 h at 4 °C. The lysates were washed with buffer B (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 M NaCl) three times using spin modules (Q-Biogene) 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 (Novagen) to release free DOHH enzymes. The enzyme was equilibrated in buffer A for activity assays, in high performance liquid chromatography water for metal analysis, or 50 mM sodium phosphate buffer, pH 7.5, for analysis of CD spectra.

**DOHH Assays**—A typical DOHH reaction mixture contained 25 mM Tris-HCl, pH 7.5, 6 mM DTT, 25 µg of bovine serum albumin, 2 pmol ($\sim$4 $\times$ 10$^4$ dpm) of the radiolabeled protein substrate, human or *S. cerevisiae* eIF5A([³H]Dhp), and the indicated amounts of purified human or *S. cerevisiae* enzymes 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, and the precipitates were hydrolyzed in 0.4 ml of 6 M HCl for 18 h at 110 °C. The radiolabeled deoxyhypusine and hypusine in the acid hydrolysate were separated by ion-exchange chromatography as described (13, 27) and radioactivity was measured using a Beckman LS6000IC scintillation counter.

**Combined Deoxyhypusine Synthase/Deoxyhypusine Hydroxylase Assays**—To test truncated eIF5A polypeptides as substrates for DOHH, the BL21(DE3) lysates expressing fragments of eIF5A precursor, eIF5A(Lys), were used as substrates for DHS/DOHH assays under conditions optimized for the two purified human recombinant enzymes. The reaction mixture in 40 µl contained 0.125 M Tris-HCl, pH 8.5, 6 mM DTT, 1 mM NAD, 6 µCi of [³H]spermidine, 0.2 µg of DHS, and 0.5 µg of DOHH and BL21(DE3) lysate containing 1–10 µg of intact or truncated forms of eIF5A(Lys). After incubation for 2 h at 37 °C, an aliquot of the reaction mixture was used for SDS-PAGE for fluorographic detection of radiolabeled peptides. To the rest, 500 µg of carrier bovine serum albumin was added and the proteins were precipitated with 10% trichloroacetic acid containing polyamines (1 mM putrescine, spermidine, and spermine). After removal of [³H]spermidine by repeated washing with 10% trichloroacetic acid containing unlabeled polyamines, the trichloroacetic acid-precipitated proteins were hydrolyzed and the radiolabeled hypusine and deoxyhypusine were measured as described above.

**GST Pulldown Assays**—BL21(DE3) cells (10 ml) transformed with pGEX-4T-3 empty vector (encoding GST), or pGEX-4T-3 vectors encoding human DOHH wild type or mutant enzymes as GST fusion proteins were cultured and the protein expression was induced by 1 mM isopropyl-β-D-thiogalactoside for 3 h. The cells were sonicated in 0.2 ml of Buffer A containing protease inhibitor mixture, and the clarified supernatant was obtained after centrifugation at 15,000 $\times$ g for 30 min. To the clarified supernatant (0.1 ml) containing ~100 µg of GST, or GST-DOHH (wild type enzyme or mutant enzymes), 25 µl of washed GSH-Sepharose beads were added and the mixture rotated for >1 h at 4 °C. Then 1 µg of eIF5A(Lys), eIF5A(Dhp), or eIF5A(Hpu) was added to the mixture and rotated for 2 h at 4 °C. After adsorption, the beads were washed with Buffer B three times using spin modules. The GSH-Sepharose-bound proteins were eluted with 40 µl of Buffer C. An aliquot was used for SDS-PAGE and Western blotting for detection of co-purified eIF5A.

For binding assays of the truncated eIF5A peptides, [³H]-labeled eIF5A(Dhp) peptides were prepared from BL21(DE3) lysates expressing fragments of the eIF5A precursor, by the DHS reaction. The reaction mixture (40 µl) containing 0.2 M glycine-NaOH buffer, pH 9.5, 1 mM DTT, 1 mM NAD, 6 µCi of [³H]spermidine, and 1 µl of protease inhibitor mixture, and cell lysate containing 1–10 µg of intact or the truncated forms of eIF5A(Lys) and 0.4 µg of DHS was incubated at 37 °C for 2 h. An aliquot of the reaction mixture was used for estimation of [³H]deoxyhypusine formed. A reaction mixture containing 100,000 dpm (~5 pmol) of each labeled peptide was used for the GST pulldown assay as described above and the amount of bound labeled peptide was estimated.

**RESULTS**

**Cross-reactivity of Human and Yeast Deoxyhypusine Hydroxylase with Heterologous Substrates**—In the *S. cerevisiae* strain in which both of the yeast eIF5A genes (TIF51A and TIF51B) are disrupted, human eIF5A (both isoforms, eIF5A-1 and eIF5A-2) can complement the yeast protein (28, 29), suggesting a functional conservation of eIF5A throughout eukaryotic evolution. Deoxyhypusynase syntheses from human, rat, or yeast, whereas being strictly specific for eIF5A, displayed cross-reactivity toward heterologous substrates in *vitro* (3, 26). Likewise, human and *S. cerevisiae* deoxyhypusine hydroxylases also exhibit cross-reactivity with heterologous substrate proteins. As shown in Table 1, The $K_m$ values of the human enzyme were 0.065 and 0.376 µM toward human and yeast eIF5A(Dhp), respectively; those for the yeast DOHH were 0.022 and 0.054 µM, respectively, for the human and yeast substrate. Whereas the $V_{max}$ values were comparable for the two enzymes with either of the two substrates, the specific activities of the purified recombinant enzymes were quite low (~23.7 and 29.4 of pmol/h/µg of protein for the human and yeast enzymes, respectively).
TABLE 1
Kinetic constants for human and yeast deoxyhypusine hydroxylases

| Enzyme          | Substrate     | $K_p$ (μM) | $V_{max}$ (nM/h) |
|-----------------|---------------|-----------|------------------|
| Human DOHH      | Human 5A(Dhp) | 0.065 ± 0.0074 | 56.8 ± 1.2      |
| Human DOHH      | Yeast 5A(Dhp) | 0.376 ± 0.090 | 61.5 ± 5.1      |
| Yeast DOHH      | Human 5A(Dhp) | 0.022 ± 0.0029 | 38.9 ± 0.76     |
| Yeast DOHH      | Yeast 5A(Dhp) | 0.054 ± 0.00086 | 49.2 ± 0.20     |

FIGURE 1. Effects of unlabeled eIF5A(Lys), eIF5A(Dhp), and eIF5A(Hpu) on hydroxylation of eIF5A($^{13}$H)Dhp. The reaction was conducted in a typical mixture (see “Experimental Procedures”) using 2 pmol of human eIF5A($^{13}$H)Dhp (33 ng, 0.1 μM) and 0.1 μM of human DOHH. Unlabeled human eIF5A(Lys), eIF5A(Dhp), and eIF5A(Hpu) were added at 0.1, 0.2, 0.5, 1.0, and 2.0 μg and incubation was 45 min at 37 °C. The data represent the averages of duplicate experiments.

The Specific Recognition of eIF5A(Dhp) by DOHH Revealed by Activity Assays—We first determined if unmodified precursor, eIF5A(Lys), or fully modified hypusine-containing protein, eIF5A(Hpu), could interfere with hydroxylation of the radiolabeled deoxyhypusine protein, eIF5A($^{13}$H)Dhp, by DOHH. When eIF5A(Lys) or eIF5A(Hpu) was added to the reaction mixture containing 2 pmol (34 ng) of radiolabeled eIF5A(Dhp), little or no inhibition was observed with eIF5A(Lys) even at 2.5 μg (75-fold excess). This finding indicates that the eIF5A precursor does not inhibit binding of the substrate protein to DOHH. Mature eIF5A, the hypusine-containing protein, caused a small inhibition of deoxyhypusine hydroxylation, suggesting that eIF5A(Hpu), the product of the DOHH reaction, does not effectively compete with eIF5A(Dhp) for binding to DOHH. In contrast, addition of unlabeled eIF5A(Dhp) sharply reduced the level of radioactive hypusine formed, obviously by dilution of the specific radioactivity of the substrate protein. The results demonstrate the importance of the deoxyhypusine side chain of eIF5A(Dhp) for recognition and modification by DOHH.

Specificity of the Interaction between eIF5A and DOHH Revealed by GST Pulldown Assays—The specificity of interaction between eIF5A(Dhp) and DOHH was also examined by pulldown experiments using GST-DOHH (Fig. 2). Clarified lysates from BL21(DE3) cells overexpressing GST-DOHH (Fig. 2, lanes 1, 3, and 5), or GST-DOHH wild type enzyme (Fig. 2, lanes 2, 4, and 6), were used for the pulldown of eIF5A(Lys) (lanes 1 and 2), eIF5A(Dhp) (lanes 3 and 4), or eIF5A(Hpu) (lanes 5 and 6). The bottom panel (Ponceau S staining of the blotted membrane) shows that almost equal amounts of GST or GST-DOHH enzymes were applied for Western analysis of co-purified eIF5A (top panel) using a monoclonal antibody that reacts with all three forms of eIF5A equally well. Whereas there was no eIF5A associated with the GST purified by GSH affinity resin (negative control, lanes 1, 3, and 5), a strong eIF5A signal was associated with the GST-DOHH pulled down in the presence of the deoxyhypusine-containing form, eIF5A(Dhp) (lane 4), indicating a high affinity binding of the two proteins. In contrast, eIF5A(Lys) was barely detectable in the GST-DOHH purified in a parallel manner (lanes 2). In accordance with the lack of inhibition of the DOHH reaction by eIF5A(Lys) (Fig. 1), the failure of the eIF5A precursor to associate with GST-DOHH suggests a requirement for the 4-aminobutyl side chain of the deoxyhypusine residue for effective binding to DOHH. In the case of the hypusine containing form, the signal was much weaker than that of the deoxyhypusine form (compare lane 6 with lane 4). The relatively low copurification yield of the hypusine form, compared with eIF5A(Dhp), is also consistent with the weak inhibition of deoxyhypusine hydroxylation by eIF5A(Hpu) (Fig. 1).

DOHH Enzyme with Both N- and C-terminal Dyad Arms Is Required for Binding of the Substrate Protein—DOHH is a symmetrical protein, with highly similar N- and C-terminal domains connected by a variable loop. To determine whether each domain could function independently as an active enzyme, we generated N-terminal (aa 1–151) and C-terminal (aa 152–302) fragments as two separate GST fusion proteins. When assayed for DOHH activity, these truncated enzymes were totally inactive alone or in combination (data not shown). Thus the two DOHH domains must be joined together to form one active center. The two domains were also incapable of binding eIF5A(Dhp), alone (Fig. 2, lanes 7 and 8) or in combination (Fig. 2, lane 9). Furthermore, the iron content of the purified DOHH N- and C-terminal domains (GST fusion proteins or free proteins) was negligible (less than 5% of the wild type enzyme). These findings provide evidence that both the N- and C-terminal dyad arms, linked by the variable loop, are required for binding of both the substrate protein and iron, and for catalysis.

Structural Features of eIF5A(Dhp) Polypeptide Required for Binding to and as Substrate for DOHH—DOHH does not hydroxylate deoxyhypusine as the free amino acid and free deoxyhypusine does not inhibit deoxyhypusine hydroxylation in the substrate protein (data not shown). To determine which portion of the eIF5A(Dhp) polypeptide is required for the DOHH reaction, we used truncated eIF5A(Lys) peptides in a combined DHS/DOHH reaction. Because the two enzymes have different pH and salt concentration optima, we carried out the combined DHS/DOHH reaction under compromised conditions using high levels of both enzymes and using clarified lysates of BL21(DE3) cells overexpressing intact eIF5A(Lys), or truncated polypeptides.

eIF5A (154 aa for the human eIF5A) is composed of two domains, a basic N-terminal domain and an acidic C-terminal
domain. A three-dimensional structure of eIF5A (31), modeled after the crystal structure of the archael homolog of eIF5A (32), predicts that the two domains are connected by a hinge region (surrounding Pro^{82} for the human eIF5A) and that the hypusine site is located in an exposed loop in the N-terminal domain. Truncated N-terminal domain peptides of human eIF5A were tested as substrates for DOHH. Because the purpose of this experiment was to distinguish the efficiency of eIF5A peptides as substrates for DOHH, and not for DHS, we used a large excess of DHS to attain a comparable level of labeling of all eIF5A(Dhp) peptides (Fig. 3A). A pronounced difference was observed in the extent of hydroxylation of these peptides (Fig. 3, B and C). When the N-terminal domain peptides, with intact N-terminal but with a different degree of truncation from the C-terminal (three peptides aa 1–90, 1–80, and 1–70) were compared, the peptide aa 1–90 was hydroxylated effectively (60% of the intact eIF5A(Dhp)). However, further C-terminal truncation (peptides aa 1–80 and 1–70) caused a sharp decline in the substrate activity. Starting with aa 1–90, we also examined the effects of stepwise N-terminal truncation using the aa 10–90, 20–90, and 30–90 peptides. Whereas aa 10–90 was hydroxylated almost as efficiently as aa 1–90, a marked reduction of hydroxylation was observed in peptides with further N-terminal truncation. The aa 20–90 peptide was hydroxylated at ~30% of the level of the intact protein and hydroxylation was negligible in the aa 30–90 peptide. These results suggest that truncation of 30 amino acids or more from the N terminus and 74 amino acids or more from the C terminus sharply diminishes substrate activity and that a eIF5A(Dhp) polypeptide larger than aa 20–90 is required for effective hydroxylation by DOHH.

To determine whether the reduced substrate activity of the truncated peptides is due to their decreased affinity for the enzyme, we measured the binding of these peptides by GST-DOHH pulldown assays. Upon incubation of the radiolabeled eIF5A(Dhp)-truncated peptides with GST-DOHH, aa 1–90, 10–90, and 20–90 peptides were effectively copurified with GST-DOHH (Fig. 3D). In contrast, little binding of aa 1–80, 1–70, and 30–90 was detected, in accordance with their ineffectiveness as DOHH substrates (Fig. 3C).

Identification of Amino Acid Residues of DOHH Critical for Binding of eIF5A(Dhp)—To identify the amino acid residues of DOHH that are involved in the binding of the protein substrate, we tested DOHH mutant enzymes with a single amino acid substitution for their ability to bind eIF5A(Dhp) (Fig. 4A). Because alanine or other amino acid substitutions may cause a disruption in the DOHH polypeptide backbone structure, we measured the α-helical content of the mutant enzymes by CD analysis. There were no significant changes in the α-helical content of the mutant enzymes from that of the wild type enzyme (data not shown).

In the first group (Fig. 4A, group I) are shown the wild type enzyme and those mutant enzymes with alanine substitution at the conserved His-Glu motifs. All these mutant enzymes are totally inactive (Fig. 4B, group I), whereas only six of them (H56A, H89A, E90A, H207A, H240A, and E241A) are defective in
that the His residues of the His-Glu motifs are not directly involved in substrate binding. Therefore, the inactivity of these His-site mutant enzymes must be due to the lack of iron binding (Fig. 4C, group I). Apparently, the association of eIF5A(Dhp) with DOHH does not depend on enzyme-bound iron, because iron-deficient enzymes, H56A, H89A, H207A, and H240A, all showed strong substrate binding (Fig. 4A, group I).

The critical roles of the deoxyhypusine residue of eIF5A(Dhp) and Glu57 and Glu208 of DOHH in the eIF5A(Dhp)/DOHH interaction suggest an ionic interaction between the amino group(s) of the deoxyhypusine side chain of the substrate protein and the side chain carboxyl groups of the Glu residues of the enzyme. To test this possibility, we generated additional mutant enzymes with aspartate, asparagine, or glutamine substitution for Glu57 or Glu208 (Fig. 4, group II). Only the enzymes with aspartate substitution (E57D and E208D) partially retained substrate binding (Fig. 4A, group II) and enzyme activity (Fig. 4B, group II), E57D exhibiting higher substrate binding and enzyme activity than E208D. Reduced substrate binding and enzyme activity in the Asp substitution enzymes may reflect a weaker ionic interaction due to the shorter side chain length of Asp than that of Glu. In contrast, substrate binding was undetectable for those with alanine, asparagine, or glutamine substitutions (Fig. 4A, group II) and no activity was detectable with these mutant enzymes (Fig. 4B, group II). The finding that only aspartate, but not alanine, asparagine, and glutamine could partially substitute for Glu57 and Glu208 underscores the importance of the acidic carboxyl group of Glu57 and Glu208 of the DOHH active site in anchoring amino group(s) of the deoxyhypusine side chain of the substrate protein (Scheme 1).

In an effort to identify additional residues of DOHH that might be involved in eIF5A(Dhp) binding, we generated 28 additional mutant enzymes with alanine substitution at all the other conserved amino acids. In contrast to the Ala substitution mutants of the His-Glu motifs, none of the Ala mutant enzymes outside of the His-Glu motifs were totally inactive. Eleven of these mutant enzymes with a significant reduction in activity were tested for their ability to bind iron and the substrate. Seven mutant enzymes, R26A, R88A, R175A, R183A, S202A, Q215A, and G247A, showed enzyme activities 20–50% of that of the
**eIF5A(Dhp) Binding Site of Deoxyhypusine Hydroxylase**

![Image of the proposed model for the binding of eIF5A(Dhp) to DOHH.

The active site His-Glu residues important for binding of iron and the substrate protein are numbered. Side chain carboxyl groups of Glu and Glu may also contribute to substrate binding by interaction with the deoxyhypusine residue or other basic residues surrounding it. The six residues, His, His, Glu, His, and Glu, implicated in iron binding are in blue. Iron atoms are not included in the diagram, because substrate protein binding does not depend on iron binding.]

DISCUSSION

The post-translational synthesis of hypusine in the eIF5A precursor represents one of the two most specific protein modifications known to date, in that it occurs only at one specific lysine residue in one protein. The other protein modification known to occur in a single cellular protein is the synthesis of diphthamide in the eukaryotic elongation factor 2, which is the target site of ADP-ribosylation by diphtheria toxin (33). Whereas diphthamide formation in eukaryotic elongation factor 2 does not appear to be essential for cell growth (34), the deoxyhypusine/hypusine modification is vital for eukaryotic cell proliferation and survival. From gene disruption studies in *S. cerevisiae* (35–38), it is well established that eIF5A and deoxyhypusine synthase are essential for eukaryotic cell viability and proliferation. Whereas DOHH is not essential in *S. cerevisiae* (5, 15) or *Schizosaccharomyces pombe* (39), inactivation of the DOHH gene is recessively lethal in multicellular eukaryotes, e.g. *Caenorhabditis elegans* (40) and *Drosophila melanogaster* (41), suggesting a requirement for a fully modified eIF5A in higher eukaryotes.

The essential nature of the deoxyhypusine/hypusine synthesis led us to investigate the basis of the exquisite specificity of this protein modification. The absolute selectivity of the first step enzyme, DHS, is evident from the incorporation of radioactivity from [1,8-^3^H]spermidine into only one cellular protein, eIF5A, in intact mammalian cells (18) or *in vitro* cell-free lysates (19). Although it is not possible to directly assess DOHH specificity in a similar manner as that of DHS, our results demonstrate that DOHH is specific for the deoxyhypusine residue and that it recognizes a large peptide (aa >20–90), for binding as well as for catalysis. From a three-dimensional model of human eIF5A (31), eIF5A consists of two β-sheet domains, the N-terminal domain and the C-terminal domain connected by a proline hinge (P82 for the human eIF5A). Thus, almost the entire N-terminal domain of the eIF5A polypeptide seems to be required to serve as effective substrates for both DHS and DOHH. The exclusive selectivity of DOHH for eIF5A can also be inferred from the fact that no other cellular protein contains a deoxyhypusine residue or the amino acid sequence closely related to eIF5A. Both DHS and DOHH appear to exert a similar degree of specificity with respect to the protein substrate, in that they both depend on a large portion of the N-terminal domain of the eIF5A substrate protein for effective modification.

We further explored structural features of DOHH required for its substrate binding and catalysis. Although DOHH consists of symmetrical N- and C-terminal domains, the two halves as fragments (alone or in combination) do not exhibit any activity (data not shown), and are not capable of binding either eIF5A(Dhp) (Fig. 2) or iron. Analysis of alanine substitution mutants enzymes offers new insights into eIF5A/DOHH binding. Total inactivity of the eight mutant enzymes with a single alanine substitution in the His-Glu motifs and lack of iron binding in six of these mutant enzymes confirms that the two dyad arms cooperate to bind iron. Likewise, the impaired substrate binding of each of the mutant enzymes E57A, E90A, E208A, and E241A provides further evidence for the involvement of both N- and C-terminal domains for substrate binding and catalysis.

It is interesting to note that the Glu residues of the His-Glu motifs, especially Glu and Glu, but not the His residues, are critical for binding of eIF5A(Dhp). In this regard, it is tempting to speculate that Glu and Glu are primarily responsible for anchoring the deoxyhypusine side chain of eIF5A(Dhp). Side chain carboxyl groups of Glu and Glu may form ionic bridges with the terminal amino group and secondary amino group of the deoxyhypusine residue (Scheme 1). The finding that only aspartate substitution mutants of these Glu residues (but not those with Ala, Asn, or Gln substitution) exert partial substrate binding and activity renders substantial support for

---

**SCHEME 1. Proposed model for the binding of eIF5A(Dhp) to DOHH.** The active site His-Glu residues important for binding of iron and the substrate protein are numbered. Side chain carboxyl groups of Glu and Glu may also contribute to substrate binding by interaction with the deoxyhypusine residue or other basic residues surrounding it. The six residues, His, His, Glu, His, His, and Glu, implicated in iron binding are in blue. Iron atoms are not included in the diagram, because substrate protein binding does not depend on iron binding. This scheme represents a simplified hypothetical diagram of the DOHH-eIF5A(Dhp) complex, indicating the key residues involved in binding without specific indication of orientation of the two proteins.
this hypothesis. It is curious that alanine substitution of Glu\(^{20}\) and Glu\(^{241}\) (two other Glu residues of the His-Glu pairs) also caused marked reduction in substrate binding. In addition to their involvement in iron coordination, these residues Glu\(^{20}\) and Glu\(^{241}\) may further strengthen ionic interactions to the deoxyhypusine side chains or to its neighboring basic amino acid residues of eIF5A(Dhp).

Two additional mutant enzymes, G63A and G214A, also displayed a striking reduction in substrate binding and in activity (Fig. 4, A and B, group III). Because iron binding seems to be normal for these two enzymes (Fig. 4C, third group), the impaired substrate binding by G63A and G214A is probably the cause for their reduced activity. However, they may not be directly involved in eIF5A(Dhp) binding. Glycine probably the cause for their reduced activity. However, they may not be directly involved in eIF5A(Dhp) binding. Glycine residues Gly\(^{63}\) and Gly\(^{214}\) (each located at the end of HEAT may not be directly involved in eIF5A(Dhp) binding. Glycine probably the cause for their reduced activity. However, they may not be directly involved in eIF5A(Dhp) binding. Glycine residues Gly\(^{63}\) and Gly\(^{214}\) (each located at the end of HEAT group(s) of the deoxyhypusine side chain of the substrate protein and side chain carboxyl groups of Glu\(^{27}\) and Glu\(^{208}\) of the DOHH active site have been identified (Scheme 1), the exact mode of coordination of iron or of the substrate binding at the DOHH active site and other interactions between the two proteins remain to be resolved. Efforts are underway to determine the crystal structures of DOHH and the eIF5A(Dhp)-DOHH complex. The crystal structures will offer ultimate validation of the DOHH model structure and the proposed mode of eIF5A(Dhp) binding and will pave the way to the development of structure-based, specific inhibitors of DOHH.

We previously reported evidence that the hydrodynamic size of the holo-DOHH is smaller than that of the apo-DOHH and proposed a DOHH model in which the diiron at the DOHH active center bridges the His-Glu motifs from the two dyad arms (24). The binding of eIF5A(Dhp) at the active site pocket of DOHH is also expected to involve both arms of DOHH, because the substrate binding is almost completely lost by alanine substitution of any of the Glu residues of the His-Glu motifs from either arm. As in the case of iron binding, anchoring of the deoxyhypusine side chain of eIF5A(Dhp) by these Glu residues may bring the two arms into close proximity. However, eIF5A(Dhp) binding to DOHH apparently does not depend on the bound iron, because the iron-deficient enzymes (including H56A, H89A, H207A, and H240A) showed strong signals of eIF5A(Dhp) binding.

Despite the indispensable nature of the hypusine/deoxyhypusine modification in eukaryotic cell proliferation, the precise cellular function of eIF5A remains to be elucidated. eIF5A is a small acidic protein that partially associates with ribosomes and that stimulates methionyl-puromycin synthesis in a model assay for translation initiation (42). Because its depletion caused relatively small reduction in global protein synthesis, it has been proposed to be a specific initiation factor for a subset of mRNAs (43, 44). The crystal structure of the archaeal homolog, aIF5A, is closely related to a bacterial ortholog of eIF5A, elongation factor P (45), an essential ribosomal protein that stimulates peptidyl transferase activity (46), suggesting a conserved role of eIF5A in translation. Several proteins have been reported as candidate eIF5A-binding proteins, including human immunodeficiency virus type 1 transactivator Rev (47), exportin 4 (48), ribosomal protein L5 (49), and nuclear actin (50). From differential display analysis of eIF5A-associated mRNAs, a number of mRNAs that are potential targets of eIF5A regulation have been reported (51). Genetic studies using S. cerevisiae harboring eIF5A temperature-sensitive mutants suggest a direct or indirect role of eIF5A in cell wall integrity, mRNA decay, actin polarization, and cell cycle progression (52–55). However, it is not clear if or how the previously reported candidate eIF5A binding partners (proteins and mRNA) could be involved in the expression of the pleiotropic phenotypes of the temperature-sensitive S. cerevisiae strains.

The proposed model of DOHH structure (5) and the currently proposed model of eIF5A(Dhp)/DOHH binding (Scheme 1) represent only an approximation of its actual structure. Although focal points of interaction involving the amino group(s) of the deoxyhypusine side chain of the substrate protein and side chain carboxyl groups of Glu\(^{27}\) and Glu\(^{208}\) of the DOHH active site have been identified (Scheme 1), the exact mode of coordination of iron or of the substrate binding at the DOHH active site and other interactions between the two proteins remain to be resolved. Efforts are underway to determine the crystal structures of DOHH and the eIF5A(Dhp)-DOHH complex. The crystal structures will offer ultimate validation of the DOHH model structure and the proposed mode of eIF5A(Dhp) binding and will pave the way to the development of structure-based, specific inhibitors of DOHH.

**Acknowledgments—We thank Drs. Hans E. Johansson (Biosearch Technologies Inc.), Larry Fisher (NIDCR, National Institutes of Health), John Thompson (NIDCR, National Institutes of Health) L. Aravind (NCBI, NLM, National Institutes of Health), and J. E. Folk (NIDCR, National Institutes of Health) for critical reading of the manuscript and helpful discussions.**

**REFERENCES**

1. Park, M. H. (2006) *J. Biochem.* (Tokyo) 139, 161–169
2. Wolff, E. C., Lee, Y. B., Chung, S. I., Folk, J. E., and Park, M. H. (1995) *J. Biol. Chem.* 270, 8660–8666
3. Joo, Y. A., Wolff, E. C., and Park, M. H. (1995) *J. Biol. Chem.* 270, 22386–22392
4. Abbruzzese, A., Park, M. H., and Folk, J. E. (1986) *J. Biol. Chem.* 261, 3085–3089
5. Park, J. H., Aravind, L., Wolff, E. C., Kaevel, J., Kim, Y. S., and Park, M. H. (2006) *Proc. Natl. Acad. Sci. U. S. A.* 103, 51–56
6. Gerner, E. W., Mamont, P. S., Bernhardt, A., and Siat, M. (1986) *Biochem. J.* 239, 379–386
7. Byers, T. L., Lakanen, J. R., Coward, J. K., and Pegg, A. E. (1994) *Biochem. J.* 303, 363–368
8. Chen, K. Y., and Liu, A. Y. (1997) *Biol. Signals* 6, 105–109
9. Chattopadhyay, M. K., Tabor, C. W., and Tabor, H. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 13869–13874
10. Caraglia, M., Marra, M., Giuberti, G., D’Alessandro, A. M., Baldi, A., Tassone, P., Venuta, S., Tagliaferri, P., and Abbruzzese, A. (2003) *J. Biochem.* (Tokyo) 133, 757–765
11. Nishimura, K., Murozumi, K., Shrirahata, A., Park, M. H., Kashiwagi, K., and Igarashi, K. (2005) *Biochem. J.* 385, 779–785
12. Hanauske-Abel, H. M., Park, M. H., Hanauske, A. R., Popowicz, A. M., Lalande, M., and Folk, J. E. (1994) *Biochem. Biophys. Acta* 1221, 115–124
13. Clement, P. M., Hanauske-Abel, H. M., Wolff, E. C., Kleinman, H. K., and Park, M. H. (2002) *Int. J. Cancer* 100, 491–498
eIF5A(Dhp) Binding Site of Deoxyhypusine Hydroxylase

14. Andrus, L., Szabo, P., Grady, R. W., Hanauske, A. R., Huima-Byron, T., Slowinska, B., Zagul ska, S., and Hanauske-Abel, H. M. (1998) Biochem. Pharmacol. 55, 1807–1818

15. Thompson, G. M., Cano, V. S., and Valentini, S. R. (2003) FEBS Lett. 555, 464–468

16. Jao, D. L., and Chen, K. Y. (2006) J. Biol. Chem. 281, 1309–1310

17. Lee, Y. B., Joe, Y. A., Wolff, E. C., Dimitriadis, E. K., and Park, M. H. (1998) J. Biol. Chem. 273, 1677–1683

18. Hanauske-Abel, H. M., and Popowicz, A. M. (2003) J. Biol. Chem. 278, 151–154

19. Hanauske-Abel, H. M., Slowinska, B., Zagul ska, S., and Hanauske-Abel, H. M. (1998) J. Cell. Biochem. 72, 9–18

20. Hofmann, W., Reichart, B., Ewald, A., Muller, E., Schmitt, I., Stauber, R. H., Schatz, O., Oft, M., Dascher, C., Schebesta, M., Rosorius, O., Jaksche, H., Wohl, B., Aschauer, H., Farrington, G. K., Probst, H., Bevec, D., and Hauber, J. (1993) J. Biol. Chem. 268, 10419–10424

21. Facchiano, A. M., Stiuso, P., Chiusano, M. L., Caraglia, M., Giuberti, G., Marra, M., Abbuzzese, A., and Colonna, G. (2001) Protein Eng. 14, 881–890

22. Kim, K. K., Hung, L. W., Yokota, H., Kim, R., and Kim, S. H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10419–10424

23. Jorgensen, R., Merrill, A. R., and Andersen, G. R. (2006) Biochem. Soc. Trans. 34, 1–6

24. Kimata, Y., and Kohno, K. (1994) J. Biol. Chem. 269, 13497–13501

25. Schnier, J., Schwelberger, H. G., Smit-McBride, Z., Kang, H. A., and Hershey, J. W. (1991) Mol. Cell. Biol. 11, 3105–3114

26. Andrus, L., Szabo, P., Grady, R. W., Hanauske, A. R., Huima-Byron, T., Slowinska, B., Zagul ska, S., and Hanauske-Abel, H. M. (1998) Biochem. Pharmacol. 55, 1807–1818

27. Thompson, G. M., Cano, V. S., and Valentini, S. R. (2003) FEBS Lett. 555, 464–468

28. Jao, D. L., and Chen, K. Y. (2006) J. Biol. Chem. 281, 1309–1310

29. Hanauske-Abel, H. M., Slowinska, B., Zagul ska, S., and Hanauske-Abel, H. M. (1998) J. Cell. Biochem. 72, 9–18

30. Hofmann, W., Reichart, B., Ewald, A., Muller, E., Schmitt, I., Stauber, R. H., Schatz, O., Oft, M., Dascher, C., Schebesta, M., Rosorius, O., Jaksche, H., Wohl, B., Aschauer, H., Farrington, G. K., Probst, H., Bevec, D., and Hauber, J. (1993) J. Biol. Chem. 268, 10419–10424

31. Facchiano, A. M., Stiuso, P., Chiusano, M. L., Caraglia, M., Giuberti, G., Marra, M., Abbuzzese, A., and Colonna, G. (2001) Protein Eng. 14, 881–890

32. Kim, K. K., Hung, L. W., Yokota, H., Kim, R., and Kim, S. H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10419–10424

33. Jorgensen, R., Merrill, A. R., and Andersen, G. R. (2006) Biochem. Soc. Trans. 34, 1–6

34. Kimata, Y., and Kohno, K. (1994) J. Biol. Chem. 269, 13497–13501