Published in final edited form as:
J Biol Chem. 2007 March 16; 282(11): 8300–8308.

Specificity of the deoxyhypusine hydroxylase-eIF5A interaction: Identification of amino acid residues of the enzyme required for binding of its substrate, deoxyhypusine-containing eIF5A*

Kee Ryeon Kang[1,#], **Yeon Sook Kim**[#], **Edith C. Wolff**, and **Myung Hee Park**[2]
From the Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892

Abstract

Deoxyhypusine hydroxylase (DOHH) is a novel metalloenzyme that catalyzes the final step of the post-translational synthesis of hypusine [N^{ε}-(4-amino-2-hydroxybutyl)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 (>aa20-90) 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, Glu208, Glu241, Gly63 or Gly214 caused a severe impairment in eIF5A(Dhp) binding, with a complete loss of binding and activity in the E57A and E208A mutant enzymes. Only aspartate substitution mutants, G57D or G208D retained partial activity and substrate binding, whereas alanine, glutamine or asparagine mutants did not. These findings support a proposed model of DOHH-eIF5A binding in which the amino group(s) of the deoxyhypusine side chain of the substrate is primarily anchored by γ-carboxyl groups of Glu57 and Glu208 at the DOHH active site.

Hypusine [N^{ε}-(4-amino-2-hydroxybutyl)-lysine] is an unusual amino acid that occurs in all eukaryotes. It is formed post-translationally in eukaryotic translation initiation factor 5A (eIF5A) through the action of two enzymes (see a recent review (1)). The first enzyme, deoxyhypusine synthase (DHS), catalyzes the NAD-dependent cleavage of spermidine and transfer of its 4-aminobutyl moiety to the ε-amino group of a specific lysine residue to form the deoxyhypusine [N^{ε}-(4-aminobutyl)lysine] residue (2,3). The second enzyme, deoxyhypusine hydroxylase (DOHH), 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). Ciclolopirox olamine, in particular, exerted strong inhibition of HUVEC proliferation

*This research was supported by the Intramural Research Program of the National Institutes of Health (NIDCR, NIH).

[2]Address correspondence to Myung Hee Park, Bldg 30, Room 211, OPCB, NIDCR, NIH, Bethesda MD 20892-4340 Tel: 301-496-5056; FAX: 301-402-0823; email: mhpark@nih.gov.
[1]Current address: Medical Research Center for Neural Dysfunction, Department of Biochemistry, School of Medicine and Institute of Health Sciences, Gyeongsang National University,92 Chilam-Dong, Jinju, 660-751, South Korea
[#]These authors contributed equally to this work.

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-HIV-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 yeast two-hybrid screening (15). Deoxyhypusine synthase is the major detectable eIF5A-binding protein in *S. cerevisiae* (16) or in mammalian cells[4] 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 (18) 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 for 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 aa30-80) 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[5]. However, it was not known if deoxyhypusine hydroxylation can be catalyzed by other known protein hydroxylases of this family (22,23), 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 beta helix (DSBH)) 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 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 DSBH 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 two moles of iron per mole of DOHH holoenzyme, DOHH probably contains one binuclear (diiron) active center (24). In DOHH there are four strictly conserved His-Glu motifs (His56-Glu57,

[4]J. Kaevel and MHP, Unpublished results
[5]ECW, H.M. Hanauske-Abel, MHP, Unpublished results

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 in spite of 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 (aa20-90) 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 (GSH) was purchased from Sigma, Glutathione Sepharose 4B from Amersham Biosciences, Thrombin Cleavage Capture Kit from Novagen, and EDTA-free protease inhibitor cocktails from Sigma. [1,8-^{3}H]Spermidine.3HCl (20 Ci/mmol) was purchased from Dupont/NEN. Precast Tris-glycine and NuPAGE (Bis-Tris) gels, electrophoresis buffers and Simply Blue staining solution were from Invitrogen, the Quick Change Site-Directed Mutagenesis Kit from Stratagene. A monoclonal antibody to eIF5A was from Beckton Dickinson. The radiolabeled deoxyhypusine-containing protein, human eIF5A([^{3}H]Dhp) and the yeast eIF5A([^{3}H]Dhp (gene product of *TIF51A*) 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, I246A, 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 Quick Change 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 and 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 N- or C-terminal half and subcloning into BamHI/ SalI sites of pGEX-4T-3 vector. The entire open reading frame (ORF) 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 600nm) by addition of 1 mM isopropyl β-D-thiogalactoside (IPTG) 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 cocktail (EDTA free) and lysed by sonication using an Ultrasonic Processor. After centrifugation of the lysate at 15,000 x *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 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 Thrombin Cleavage Capture Kit (Novagen) to release free DOHH enzymes. The enzyme was equilibrated in buffer A for activity assays, in HPLC water for metal analysis, or 50 mM sodium phosphate buffer, pH 7.5 for analysis of CD spectra.

Deoxyhypusine hydroxylase (DOHH) assays—A typical DOHH reaction mixture contained 25 mM Tris.HCl, pH 7.5, 6 mM DTT, 25 μg BSA, 2 pmol (~$4x10^4$ dpm) of the radiolabeled protein substrate, human or *S. cerevisiae* eIF5A([^{3}H]Dhp), and indicated amounts of purified human or *S. cerevisiae* enzymes in 20 μl. After incubation at 37°C for 1h, 500 μg of carrier BSA was added to each sample, followed by precipitation with 10 % trichloroacetic acid (TCA), and the precipitates were hydrolysed in 0.4 ml of 6N 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 the radioactivity was measured using a Beckman LS6000IC scintillation counter.

Combined deoxyhypusine synthase/ deoxyhypusine hydroxylase assays—In order 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 condition optimized for the two purified human recombinant enzymes. The reaction mixture in 40 μl contained 0.125M Tris.HCl, pH 8.5, 6 mM DTT, 1 mM NAD, 6 μCi [^{3}H]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 BSA was added and the proteins were precipitated with 10% TCA containing polyamines (1 mM, putrescine, spermidine and spermine). After removal of [^{3}H]spermidine by repeated washing with 10 % TCA containing unlabeled polyamines, the TCA 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 IPTG for 3h. The cells were sonicated in 0.2 ml of Buffer A, containing protease inhibitor cocktail and clarified supernatant was obtained after centrifugation at 15,000 rpm 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, ^{3}H-labeled eIF5A(Dhp) peptides were prepared from BL21(DE3) lysates expressing fragments of eIF5A precursor, by the DHS reaction. The reaction mixture (40 μl) containing 0.2 M glycine-NaOH buffer (pH 9.5), 1mM DTT, 1 mM NAD, 6 μCi [^{3}H]spermidine, and 1 μl of protease inhibitor cocktail, and cell lysate containing 1–10 μg of intact or 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 [^{3}H] 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. Deoxyhypusine synthases from human, rat or yeast, while 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 Km values of the human enzyme were 0.065 μM 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. While the Vmax 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 protein for the human and yeast enzymes, respectively.).

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([^{3}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 (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 1–2), eIF5A(Dhp) (lanes 3–4) or eIF5A(Hpu) (lanes 5–6). 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 resins (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 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 to 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. In order to determine whether each domain could function independently as an active enzyme, we generated N-terminal (aa1-151) and C-terminal (aa152-302) 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). In order 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. Since the two enzymes have different pH and salt concentration optima, we carried out the combined DHS/DOHH reaction under compromise 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. 3D structure of eIF5A (31), modeled after the crystal structure of the archaeal homolog of eIF5A(32), predicts that the two domains are connected by a hinge region (surrounding Pro82 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. Since 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. 3B and 3C). When the N-terminal domain peptides, with intact N-terminal but with different degree of truncation from the C-terminal (three peptides aa1-90, 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 aa1-80 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 peptides aa10-90, aa20-90 and aa30-90. Whereas aa10-90 was hydroxylated almost as efficiently as aa1-90, a marked reduction of hydroxylation was observed in peptides with further N-terminal truncation. The peptide aa 20-90 was hydroxylated at ~30 % of the level of the intact protein and hydroxylation was negligible in the peptide aa 30-90. 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 aa20-90 is required for effective hydroxylation by DOHH.

In order 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, peptides aa1-90, aa10-90 and aa20-90 were effectively copurified with GST-DOHH (Fig. 3D). In contrast, little binding of aa1-80, aa1-70 and aa30-90 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)

In order 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). Since alanine or other amino acid substitution 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. 4, 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. 4, IB), while only six of them (H56A, H89A, E90A, H207A, H240A and E241A) are defective in the binding of iron (Fig. 4, IC) (24). The reason for the total inactivity of the two other mutant enzymes of this group (group I), E57A and E208A, is due to the defect in substrate binding. GST-fusion proteins of E57A and E208A failed to pull down any detectable amount of eIF5A(Dhp) (Fig. 4, IA), indicating the critical role of Glu57 and Glu208 in substrate binding. Considering that E57A and E208A mutant enzymes contained a normal level of iron (24) and were capable of binding iron and forming holoenzyme (Fig. 4, IC), the inactivity of these two enzymes is most likely due to their inability to bind eIF5A(Dhp). Substrate binding was also markedly reduced in two other Glu site mutant enzymes, E90A and E241A (Fig. 4, IA), suggesting additional contributions of the acidic residues Glu90 and Glu241 for substrate binding. Since these mutant enzymes are also defective in the binding of iron, Glu90 and Glu241 are likely to be involved in iron coordination as well as in eIF5A(Dhp) binding. In contrast to the Glu-site mutant enzymes, strong signals of eIF5A(Dhp) binding was observed in all the His-site mutant enzymes (Fig. 4, IA), an indication 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. 4, IC). Apparently, the association of eIF5A(Dhp) with DOHH does not depend on enzyme-bound iron, since iron-deficient enzymes, H56A, H89A, H207A and H240A, all showed strong substrate binding (Fig. 4, IA).

The critical roles of the deoxyhypusine residue of eIF5A(Dhp) and of 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. In order 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. 4, IIA) and enzyme activity (Fig. 4, IIB), 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. 4, IIA) and no activity was detectable with these mutant enzymes (Fig. 4, IIB). The finding that only aspartate, but not alanine, asparagines 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 I).

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 wild type enzyme, and were capable of binding both iron and the substrate protein (data not shown). Only four mutant enzymes, G63A, E93A, G214A and M237A, displayed a drastic reduction in enzyme activity (Fig. 4, IIIB). G63A and G214A contained a normal level of iron and holoenzyme (Fig. 4, IIIC), but showed diminished substrate binding (Fig. 4, IIIA), suggesting that defective substrate binding is responsible for the loss in the activity. As for M237, activity loss may be partly due to reduced iron binding (weak holoenzyme band in Fig. 4, IIIC). The cause of inactivity of E93A is not clear, since this mutant enzyme showed binding of both the substrate protein and iron.

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 (eEF2), which is the target site of ADP ribosylation by diphtheria toxin (33). Whereas diphthamide formation in eEF2 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 *S. 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 (>aa20-90), for binding as well as for catalysis. From a 3D model of human eIF5A (31), eIF5A consists of two beta-sheet domains, N-terminal domain and the C-terminal domain connected by a proline(31) 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 N-terminal domain of 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 mutant 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 Glu57 and Glu208, but not the His residues, are critical for binding of eIF5A(Dhp). In this regard, it is tempting to speculate that Glu57 and Glu208 are primarily responsible for anchoring the deoxyhypusine side chain of eIF5A(Dhp). Side chain carboxyl groups of Glu57 and Glu208 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 this hypothesis. It is curious that alanine substitution of Glu90 and Glu241 (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 Glu90 and Glu241 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, IIIA and IIIB). Since iron binding seems to be normal for these two enzymes (Fig. 4, IIIC), 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 residues Gly63 and Gly214 (each located at the end of HEAT repeats 2 and 6) are predicted to cap the alpha-helices, and their substitution with alanine would cause a distortion in the local helical structure and thereby affect the substrate protein binding. M237A (Fig. 4, group III) showed strong substrate binding, but its iron binding was significantly reduced. Thus the activity loss in M237A may be at least partly due to impaired iron binding. The basis of activity loss in E93A is not clear. Although E93A is capable of binding both iron and substrate protein, slight alteration in the local conformation of the mutant enzyme may cause imperfect coordination of iron or distorted binding of substrate protein, thereby preventing productive reaction. The full explanation of loss of activities in these mutant enzymes will await the determination of crystal structures of the wild type and mutant enzymes.

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, since 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, since the iron-deficient enzymes (including H56A, H89A, H207A and H240A) showed strong signals of eIF5A(Dhp) binding.

In spite of 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). Since 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 (EFP) (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 HIV-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 Glu57 and Glu208 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.

Acknowledgements

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

References

1. Park MH. J Biochem (Japan) 2006;139:161–169. [PubMed: 16452303]
2. Wolff EC, Lee YB, Chung SI, Folk JE, Park MH. J Biol Chem 1995;270(15):8660–8660. [PubMed: 7721768]
3. Joe YA, Wolff EC, Park MH. J Biol Chem 1995;270(38):22386–22386. [PubMed: 7673224]
4. Abbruzzese A, Park MH, Folk JE. J Biol Chem 1986;261(7):3085–3085. [PubMed: 3949761]
5. Park JH, Aravind L, Wolff EC, Kaevel J, Kim YS, Park MH. Proc Natl Acad Sci U S A 2006;103(1):51–51. [PubMed: 16371467]
6. Gerner EW, Mamont PS, Bernhardt A, Siat M. Biochem J 1986;239(2):379–379. [PubMed: 3101665]
7. Byers TL, Lakanen JR, Coward JK, Pegg AE. Biochem J 1994;303(Pt 2):363–363. [PubMed: 7980394]
8. Chen KY, Liu AY. Biol Signals 1997;6(3):105–105. [PubMed: 9285092]
9. Chattopadhyay MK, Tabor CW, Tabor H. Proc Natl Acad Sci U S A 2003;100(24):13869–13869. [PubMed: 14617780]
10. Caraglia M, Marra M, Giuberti G, D'Alessandro AM, Baldi A, Tassone P, Venuta S, Tagliaferri P, Abbruzzese A. J Biochem (Tokyo) 2003;133(6):757–757. [PubMed: 12869532]
11. Nishimura K, Murozumi K, Shirahata A, Park MH, Kashiwagi K, Igarashi K. Biochem J 2005;385(Pt 3):779–779. [PubMed: 15377278]

12. Hanauske-Abel HM, Park MH, Hanauske AR, Popowicz AM, Lalande M, Folk JE. Biochim Biophys Acta 1994;1221(2):115–115. [PubMed: 8148388]
13. Clement PM, Hanauske-Abel HM, Wolff EC, Kleinman HK, Park MH. Int J Cancer 2002;100(4): 491–491. [PubMed: 12115536]
14. Andrus L, Szabo P, Grady RW, Hanauske AR, Huima-Byron T, Slowinska B, Zagulska S, Hanauske-Abel HM. Biochemical Pharmacology 1998;55:1807–1818. [PubMed: 9714299]
15. Thompson GM, Cano VS, Valentini SR. FEBS Lett 2003;555(3):464–464. [PubMed: 14675757]
16. Jao DL, Chen KY. J Cell Biochem 2006;97(3):583–583. [PubMed: 16215987]
17. Lee YB, Joe YA, Wolff EC, Dimitriadis EK, Park MH. Biochem J 1999;340 (Pt 1):273–273. [PubMed: 10229683]
18. Park MH, Cooper HL, Folk JE. Proc Natl Acad Sci U S A 1981;78(5):2869–2869. [PubMed: 6789324]
19. Park MH, Wolff EC. J Biol Chem 1988;263(30):15264–15264. [PubMed: 3139668]
20. Jakus J, Wolff EC, Park MH, Folk JE. J Biol Chem 1993;268(18):13151–13151. [PubMed: 8514754]
21. Joe YA, Park MH. J Biol Chem 1994;269(41):25916–25916. [PubMed: 7929297]
22. Hausinger RP. Crit Rev Biochem Mol Biol 2004;39(1):21–21. [PubMed: 15121720]
23. Hanauske-Abel HM, Popowicz AM. Curr Med Chem 2003;10(12):1005–1005. [PubMed: 12678673]
24. Kim YS, Kang KR, Wolff EC, Bell JK, McPhie P, Park MH. J Biol Chem 2006;281(19):13217–13217. [PubMed: 16533814]
25. Park JH, Wolff EC, Folk JE, Park MH. J Biol Chem 2003;278(35):32683–32683. [PubMed: 12788913]
26. Kang KR, Wolff EC, Park MH, Folk JE, Chung SI. J Biol Chem 1995;270(31):18408–18408. [PubMed: 7629166]
27. Park MH, Liberato DJ, Yergey AL, Folk JE. J Biol Chem 1984;259(19):12123–12123. [PubMed: 6434537]
28. Schwelberger HG, Kang HA, Hershey JW. J Biol Chem 1993;268(19):14018–14018. [PubMed: 8314769]
29. Clement PM, Johansson HE, Wolff EC, Park MH. FEBS J 2006;273(6):1102–1102. [PubMed: 16519677]
30. Wolff EC, Park MH, Folk JE. J Biol Chem 1990;265(9):4793–4793. [PubMed: 2108161]
31. Facchiano AM, Stiuso P, Chiusano ML, Caraglia M, Giuberti G, Marra M, Abbruzzese A, Colonna G. Protein Eng 2001;14(11):881–881. [PubMed: 11742107]
32. Kim KK, Hung LW, Yokota H, Kim R, Kim SH. Proc Natl Acad Sci U S A 1998;95(18):10419–10419. [PubMed: 9724718]
33. Jorgensen R, Merrill AR, Andersen GR. Biochem Soc Trans 2006;34(Pt 1):1–1. [PubMed: 16246167]
34. Kimata Y, Kohno K. J Biol Chem 1994;269(18):13497–13497. [PubMed: 8175783]
35. Schnier J, Schwelberger HG, Smit-McBride Z, Kang HA, Hershey JW. Mol Cell Biol 1991;11(6): 3105–3114. [PubMed: 1903841]
36. Wöhl T, Klier H, Ammer H. Mol Gen Genet 1993;241(34):305–305. [PubMed: 8246884]
37. Sasaki K, Abid MR, Miyazaki M. FEBS Lett 1996;384(4):151–151. [PubMed: 8612813]
38. Park MH, Joe YA, Kang KR. J Biol Chem 1998;273(3):1677–1677. [PubMed: 9430712]
39. Weir BA, Yaffe MP. Mol Biol Cell 2004;15(4):1656–1656. [PubMed: 14767070]
40. Sugimoto A. Differentiation 2004;72(2–3):81–81. [PubMed: 15066188]
41. Spradling AC, Stern D, Beaton A, Rhem EJ, Laverty T, Mozden N, Misra S, Rubin GM. Genetics 1999;153(1):135–135. [PubMed: 10471706]
42. Benne R, Brown-Luedi ML, Hershey JW. J Biol Chem 1978;253(9):3070–3070. [PubMed: 641055]
43. Kang HA, Hershey JW. J Biol Chem 1994;269(6):3934–3934. [PubMed: 8307948]
44. Hanauske-Abel HM, Slowinska B, Zagulska S, Wilson RC, Staiano-Coico L, Hanauske AR, McCaffrey T, Szabo P. FEBS Lett 1995;366(2–3):92–92. [PubMed: 7789538]
45. Hanawa-Suetsugu K, Sekine S, Sakai H, Hori-Takemoto C, Terada T, Unzai S, Tame JR, Kuramitsu S, Shirouzu M, Yokoyama S. Proc Natl Acad Sci USA 2004;101(26):9595–9600. [PubMed: 15210970]

46. Glick BR, Ganoza MC. Proc Natl Acad Sci U S A 1975;72(11):4257–4257. [PubMed: 1105576]
47. Ruhl M, Himmelspach M, Bahr GM, Hammerschmid F, Jaksche H, Wolff B, Aschauer H, Farrington GK, Probst H, Bevec D, Hauber J. J Cell Biol 1993;123(6):1309–1309. [PubMed: 8253832]
48. Lipowsky G, Bischoff FR, Schwarzmaier P, Kraft R, Kostka S, Hartmann E, Kutay U, Görlich D. EMBO J 2000;19(16):4362–4362. [PubMed: 10944119]
49. Schatz O, Oft M, Dascher C, Schebesta M, Rosorius O, Jaksche H, Dobrovnik M, Bevec D, Hauber J. Proc Natl Acad Sci U S A 1998;95(4):1607–1607. [PubMed: 9465063]
50. Hofmann W, Reichart B, Ewald A, Muller E, Schmitt I, Stauber RH, Lottspeich F, Jockusch BM, Scheer U, Hauber J, Dabauvalle MC. J Cell Biol 2001;152(5):895–895. [PubMed: 11238447]
51. Xu A, Jao DL, Chen KY. Biochem J 2004;384(Pt 3):585–585. [PubMed: 15303967]
52. Zuk D, Jacobson A. Embo J 1998;17(10):2914–2914. [PubMed: 9582285]
53. Valentini SR, Casolari JM, Oliveira CC, Silver PA, McBride AE. Genetics 2002;160(2):393–393. [PubMed: 11861547]
54. Zanelli CF, Valentini SR. Genetics 2005;171(4):1571–1571. [PubMed: 16157662]
55. Chatterjee I, Gross SR, Kinzy TG, Chen KY. Mol Genet Genomics 2006;275(3):264–264. [PubMed: 16408210]

The abbreviations used are

eIF5A
: eukaryotic 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

GSH
: glutathione

DTT
: dithiothreitol

CD
: circular dichroism. DSBH, double stranded beta helix

HEAT-repeat
: a protein structural motif found in **H**untingtin, **E**longation factor 3, a subunit of protein phosphatase 2**A**, and the **T**arget of rapamycin

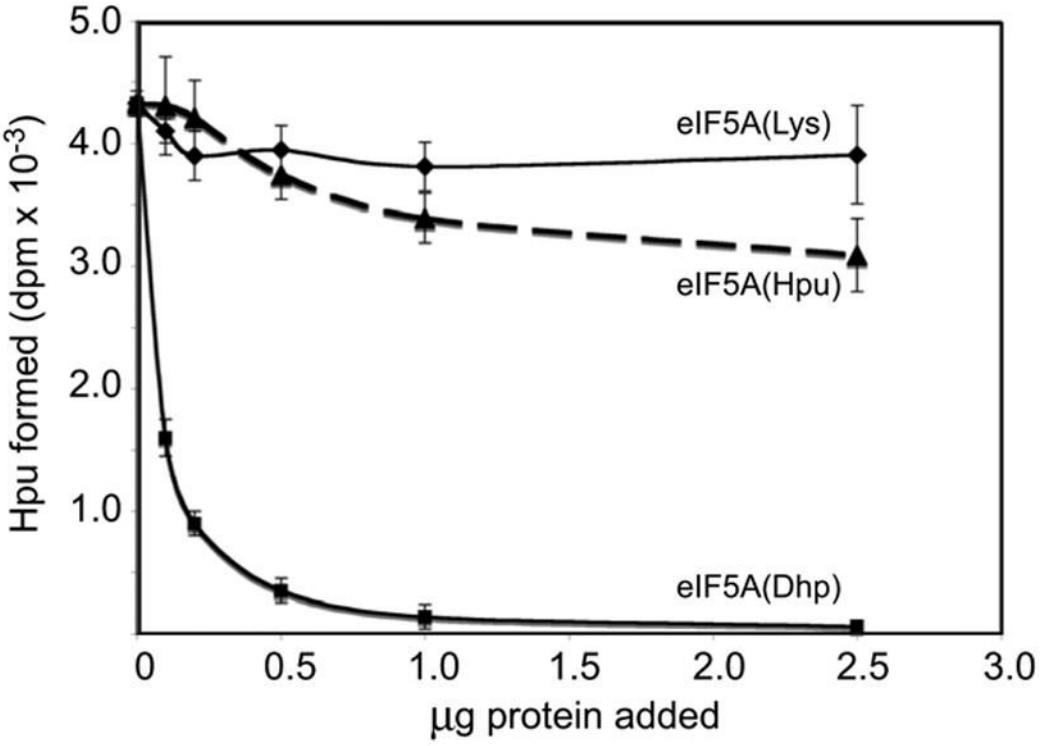


FIGURE 1. Effects of unlabeled eIF5A(Lys), eIF5A(Dhp) and eIF5A(Hpu) on hydroxylation of eIF5A([^{3}H]Dhp)

The reaction was conducted in a typical mixture (see" Experimental Procedures") using 2 pmol of human eIF5A([^{3}H]Dhp) (33 ng, 0.1 μM) and 0.1 μg 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.

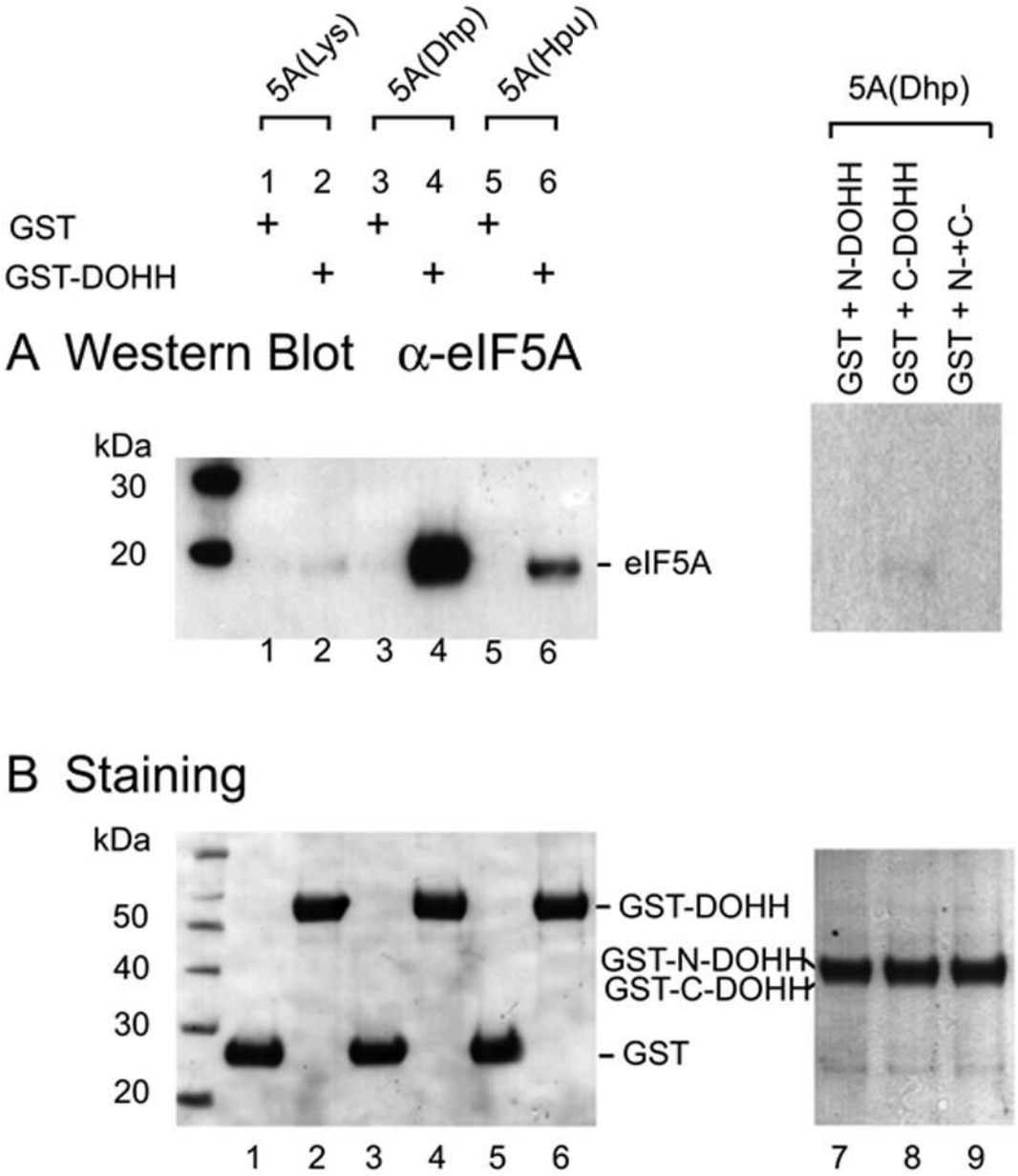


FIGURE 2. Co-purification of eIF5A(Dhp) with GST-DOHH

A), Detection of co-purified eIF5A by western blot using an eIF5A monoclonal antibody and B), *Ponceau S* staining of the same membrane (before western) showing the input of close to equal amounts of GST or GST-fusion proteins applied. BL21(DE3) lysates expressing GST or GST fusion proteins of human DOHH (intact enzyme or N- or C- terminal domain) were mixed either with human eIF5A(Lys), eIF5A(Dhp) or eIF5A(Hpu) prior to GSH-Sepharose affinity purification as described under “Experimental Procedures”. The experiment was carried out twice with virtually the same results.

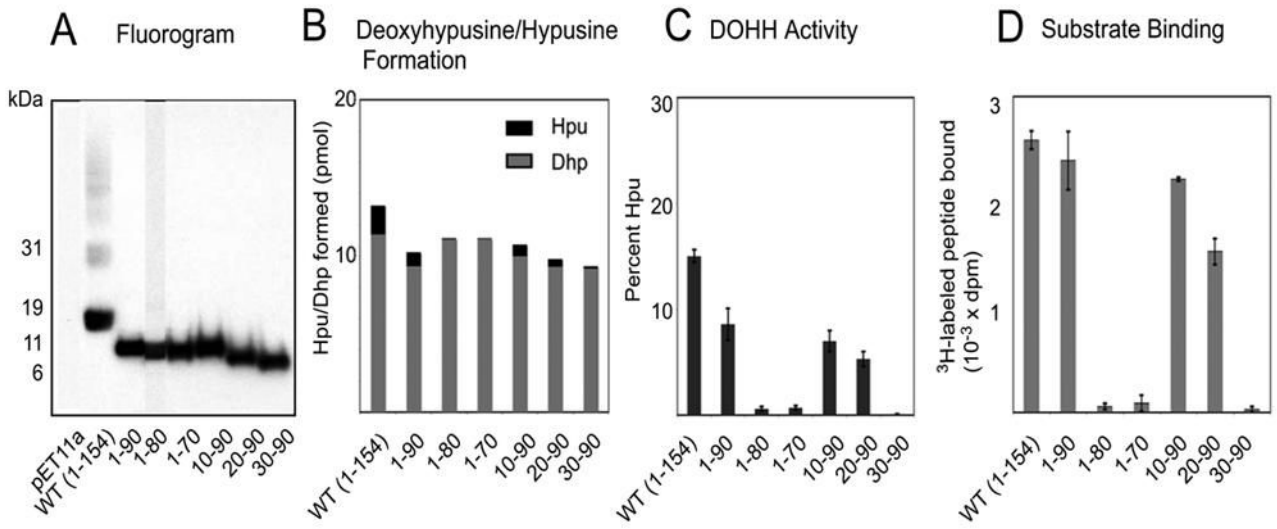


FIGURE 3. Comparison of intact and various truncated eIF5A polypeptides as substrates for DOHH (A–C) and of their binding to GST-DOHH (D)

Clarified lysates of BL21(DE3) cells expressing human eIF5A or eIF5A peptides with a truncation from the N- or C-terminal were used for a DHS assay or for combined DHS/DOHH assay as described under “Experimental Procedures”. A) Fluorogram of SDS gel of a portion of the combined DHS/DOHH reaction mixtures. B) The amounts of radiolabeled hypusine and deoxyhypusine formed in the combined DHS/DOHH reaction, as determined by ion exchange chromatography of acid hydrolysates as described under “Experimental Procedures”. C) Radioactive hypusine as a percent of the total of radioactive hypusine plus deoxyhypusine formed in the combined DHS/DOHH reaction. D) Binding of radiolabeled eIF5A(Dhp) peptides to GST-DOHH from GST-pull down assays. The data (C and D) represent the averages of duplicate experiments.

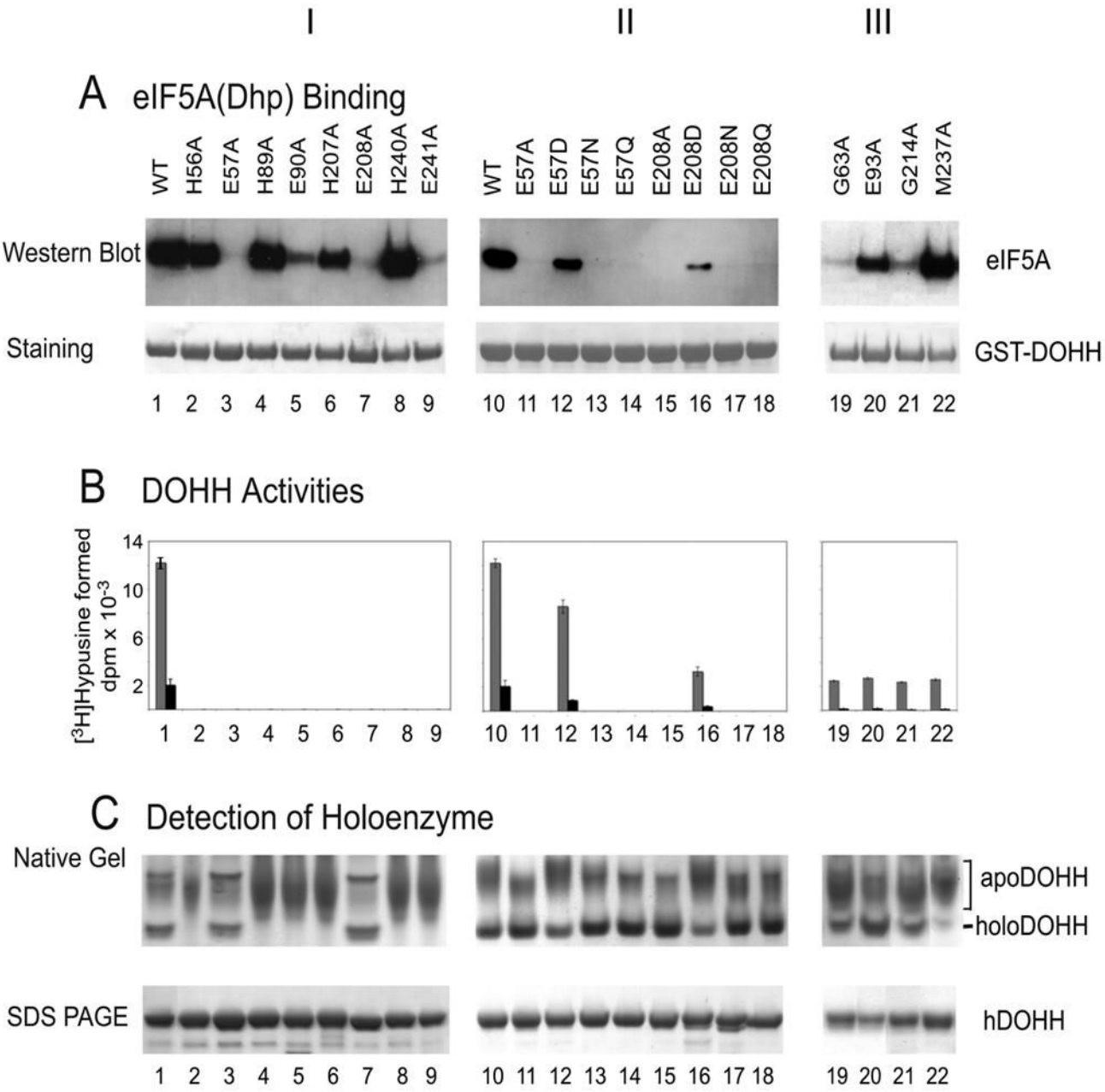


FIGURE 4. Comparison of substrate binding (A), activities (B) and holoenzyme content (C) of DOHH wild type and mutant enzymes

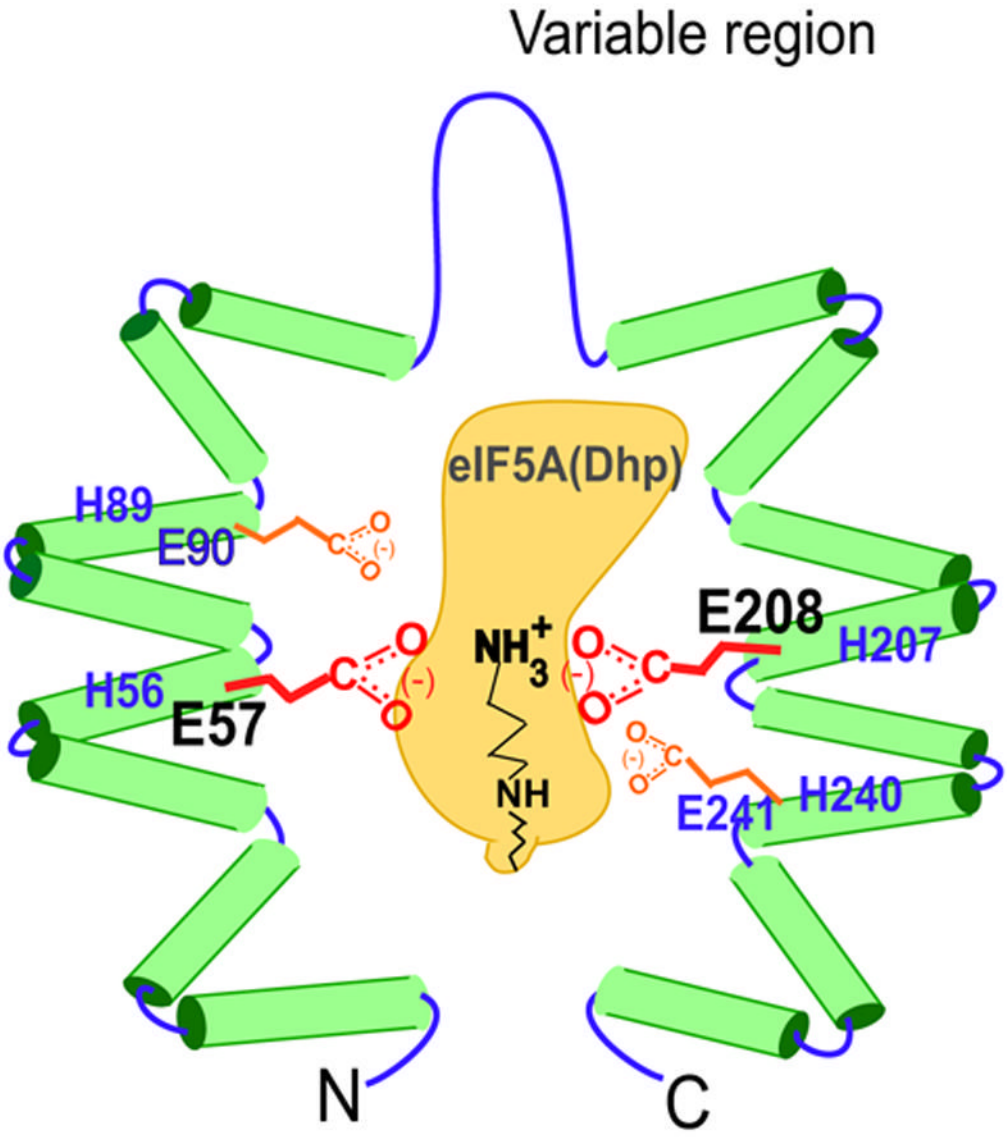
Groups I and II, mutant enzymes of the His-Glu motifs, group III, those outside of the His-Glu motifs.

A) Binding of eIF5A(Dhp) to the GST-DOHH wild type and mutant enzymes was assessed as described under "Experimental Procedures", using *E. coli* lysates expressing human GST-DOHH and purified human eIF5A(Dhp). Western blot with eIF5A antibody (top panels) and Ponceau S- stained membrane (before exposure to antibody) to show the amounts of GST-DOHH applied (bottom panels).

B) The activities of purified human DOHH mutant enzymes (after cleavage of GST) were determined using radiolabeled eIF5A([^{3}H]Dhp), 0.1 and 0.01 μg of enzymes as described under

"Experimental, Procedures" and the activities are expressed at two levels 0.1 (light grey bar) and 0.01μg (dark grey bar). The data represent the averages of duplicate experiments. All the mutant enzymes of group I and E57N, E57Q, E208N, E208Q were totally inactive with no detectable activity even at 1.0 μg.

C) Purified DOHH enzymes were electrophoresed under non-denaturing conditions (top panels) for detection of the holo- and apoenzyme, and under denaturing conditions (bottom panels). As described previously (24), purified DOHH enzymes (one major band on SDS-PAGE) resolve into the holoenzyme (lower bands) and the diffused apoenzyme (brackets) upon native gel electrophoresis, suggesting a more compact structure of the former than the latter.



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 Glu57 and Glu208 are proposed to interact with the amino group(s) of the deoxyhypusine side chain of eIF5A(Dhp). The γ-carboxyl groups of Glu90 and Glu241 may also contribute to the substrate binding by interaction with the deoxyhypusine residue or other basic residues surrounding it. The six residues, His56, His89, Glu90, His207, His240 and Glu241, implicated in iron binding are in blue. Iron atoms are not included in the diagram, since 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 the binding without specific indication of orientation of the two proteins.

TABLE I

Kinetic constants for human and yeast deoxyhypusine hydroxylases

Kinetic constants were determined as described previously (30). The reaction mixtures in 20 μl contained 50 mM Tris HCl, pH 7.5, 6 mM DTT, and 25 μg of BSA. The substrate protein eIF5A([^{3}H]Dhp) varied from 0.02–2.0 μM and human (0.05 μg) or yeast enzyme (0.03 μg) was added as indicated. Incubations were 30 min at 37°C, during which time less than 20 % of the substrate was converted to the product.

Enzyme	Substrate	Km μM	Vmax 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