Enzyme-Substrate Intermediate at a Specific Lysine Residue Is Required for Deoxyhypusine Synthesis

THE ROLE OF LYS329 IN HUMAN DEOXYHYPUSINE SYNTHASE

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Deoxyhypusine synthase catalyzes the first step in the post-translational synthesis of hypusine [N\(^\text{N}\)-[4-amino-2-hydroxybutyl]lysine] in eukaryotic translation initiation factor 5A. We recently reported biochemical evidence for a covalent enzyme-substrate intermediate involving a specific lysine residue (Lys329) in human deoxyhypusine synthase (Wolff, E. C., Folk, J. E., and Park, M. H. (1997) *J. Biol. Chem.* 272, 15865–15871). In an effort to evaluate the role of this enzyme-substrate intermediate in catalysis, we carried out site-directed mutagenesis (Lys to Arg and/or Ala) of the conserved lysine residues in human deoxyhypusine synthase. A drastic reduction in enzyme intermediate formation and enzymatic activities was observed with mutant proteins with substitution at Lys\(^\text{K287}\) but not with those with mutations at residues 141, 156, 205, 212, 226, 251, or 338. Lys to Ala or Lys to Arg substitution at Lys\(^\text{329}\) totally abolished covalent enzyme-substrate intermediate formation and deoxyhypusine synthesis activity, indicating that Lys\(^\text{329}\) is the unique site for the enzyme intermediate and that it is absolutely required for deoxyhypusine synthesis in the eukaryotic translation initiation factor 5A precursor. The R329A mutant showed spermidine cleavage activity (~6% of the wild type enzyme) suggesting that in contrast to deoxyhypusine synthesis, spermidine cleavage can occur without enzyme intermediate formation.

Hypusine [N\(^\text{N}\)-[4-amino-2-hydroxybutyl]lysine] is formed by a unique two-step post-translational modification that occurs only in one known cellular protein, the precursor of eukaryotic translation initiation factor 5A (eIF-5A)\(^\text{1}\) (for reviews see Refs. 2 and 3). In the first step, deoxyhypusine synthase catalyzes the NAD-dependent dehydrogenation of spermidine to generate 1,3-diaminopropane and \(\Delta^1\)-pyrroline (see Scheme 1, *thin arrows*) (19). Both pathways are initiated by NAD-dependent dehydrogenation of spermidine to generate a postulated dehydrosperrmidine intermediate.

Recently we have obtained evidence for a covalent enzyme-substrate intermediate with the 4-aminobutyl moiety from spermidine attached to the ε-amino group of a specific lysine residue of the enzyme in an imine linkage (20). This transient enzyme-imine intermediate, which accumulates in the absence of the protein substrate, was trapped in a stable form by reduction with \(\text{NaB}_3\text{H}_4\)-CN. \(\text{[H]}\)Deoxyhypusine was identified as the component of the reduced enzyme-imine intermediate radiolabeled from \(\text{[H]}\)spermidine, indicating that a lysine residue acts as an acceptor of the 4-aminobutyl moiety. The specific site of the intermediate formation was determined by amino acid sequencing of the labeled peptide to be Lys\(^\text{329}\) in the human enzyme. It was further shown that the 4-aminobutyl moiety of the enzyme-imine intermediate could be transferred to the protein substrate, the eIF-5A precursor, to produce a deoxyhypusine residue. However, it was not clear whether this enzyme-imine intermediate involving Lys\(^\text{329}\) is an obligatory catalytic intermediate in the normal course of the deoxyhypusine synthesis reaction or an artificial enzyme adduct that happened to form in vitro only under special circumstances, that is, in the absence of the natural acceptor of the butylamine.
Deoxyhypusine synthase reaction.

**Scheme 1**

**site-directed Mutagenesis of human deoxyhypusine synthase**

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**Experimental Procedures**

**Materials**

[1,8-3H]Spermidine-HCl (15–27.6 Ci/mmol) was purchased from NEN Life Science Products. NaBH3CN from Aldrich was recrystallized. D-[1,8-3H]Spermidine (15–27.6 Ci/mmol) was purchased from NEN Life Science Products. NaBH3CN from Aldrich was recrystallized.

**Methods**

**Site-directed Mutagenesis—Altered enzymes in which an Arg or an Ala residue was substituted for the specified lysine residue were produced by PCR-directed mutagenesis (22) using primers in which the Lys codon (AAG) was replaced by an Arg codon (AGG) for Lys → Arg mutations with the exception of those at Lys287 and Lys292, where CGC was used) or an Ala codon (GCT) at Lys 329 or Lys 287 (Table I). Full-length PCR products were obtained by two rounds of PCR reactions. Terminal primers A and B were designed to hybridize with the N-terminal region and the C-terminal region, respectively, with the introduction of restriction sites (NdeI in primer A and BamHI in primer B) to facilitate ligation and cloning. For each mutation a set of internal primers were designed to hybridize with the regions flanking the modification site. Internal reverse primers (B series) have a mutated codon sequence, and half of the sequence of this primer is complementary to the forward internal primer (A series). In the first reactions, either primer A and internal primer B or primer B and internal primer A were used as templates for PCR. In the second reactions, the PCR products were used as templates for the production of a single annealed PCR product. The conditions for PCR were: 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension reaction at 74 °C for 2 min for 35 cycles and a final extension for 10 min. The final PCR product was digested with NdeI and BamHI and ligated to the pET11a vector linearized with NdeI and BamHI and dephosphorylated by calf intestinal alkaline phosphatase. This ligation mixture was used to transform E. coli DH5α. After confirming the mutation sites by sequencing, the recombinant plasmid isolated from the DH5α transformant was used for transformation of E. coli BL21 (DE3) for protein expression.

**Overexpression and Partial Purification of the Mutated Proteins**

The selected transformants overexpressing the desired mutant protein were grown in 500 ml of LB medium supplemented with 100 μg/ml ampicillin. When the cell density reached an optical density of 0.6 at 600 nm, isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM, and the cells were harvested after culture for 2–3 h. Cell pellets were suspended in 10 ml of buffer A (50 mM Tris-Cl, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), sonicated at the maximum intensity, and pulsed at 70% intervals (Sonicor model W-375, Ultrasonics Inc.) for 5 min in an ice bath. The supernatant from centrifugation at 15,000 × g for 1 h was loaded on a MonoQ column (HR 10/10). After washing the column with buffer A, the proteins were eluted with a KCl gradient (0–0.6 M KCl in buffer A, over 60 min). Fractions of 2 ml were collected, and the proteins were analyzed by SDS-PAGE and assayed for activity. For the mutant enzymes with little or no deoxyhypusine synthetic activity, K329R, K329A, K287R, and K287A, extreme precautions were taken to prevent any cross-contamination with the wild type or any of the other mutant proteins. These precautions included thorough washing of the sonication probe, strict separation of samples, and purification of each mutant protein on an individually prepared column of new Q-Sepharose (Fast-flow) resin in place of the MonoQ column. The clarified cell lysate (5 ml) prepared as described above was applied to a column (~10 ml) of Q-Sepharose that had been equilibrated in buffer A. After washing the column with buffer A, the mutant protein was eluted by the stepwise addition of buffer A containing 0.2, 0.3, 0.4, or 0.5 M NaCl, and 1-ml fractions were collected and pooled, concentrated, and desalted by centrifugation in Centricron 30 filter devices (Amicon). A culture of a transformant with the pET11a vector without any insert DNA was processed in parallel as a negative control, and bacterial proteins eluting at the same ionic strength (0.3–0.4 M NaCl) as the enzyme were pooled and concentrated. Total protein concentration was determined by the bicinchoninic acid method using bovine serum albumin (Fierce) as a standard, and by measuring the absorbance at 280 nm.
RESULTS

The mutant proteins were expressed in *E. coli* at approximately the same level as the wild type enzyme. Partially purified preparations (10–40% purity, Fig. 1A) of the mutant proteins were obtained by one-step ion exchange chromatography.

In each case, the elution pattern from the ion exchange column of the mutant protein (not shown) was quite similar to that of the wild type enzyme. Thus it appears that the mutations did not cause gross changes in conformation or stability of the proteins.

**Tetramer Formation by Mutant Enzymes**—Deoxyhypusine synthase exists as a homotetramer of 41-kDa subunits in the native state, as evidenced by sedimentation rates upon ultracentrifugation, mobility in gel filtration, and migration upon electrophoresis in nondenaturing gels (11, 12, 16–18). This structure appears to be important for stability, for binding of substrates, and for catalysis. In fact, a recent x-ray crystallographic study reveals a tetrameric organization of enzyme subunits. To determine if any of the mutations altered the ability of the proteins to form the tetramer, we compared the mobilities of the mutant enzymes with wild type enzyme in nondenaturing as well as SDS gels (Fig. 1). The 41-kDa subunit of all the mutant enzymes migrated the same distance as the wild type enzyme upon SDS-PAGE (Fig. 1A). Furthermore, the pattern of migration on nondenaturing gels (Fig. 1B) suggests that each of the mutant enzymes exists in tetrameric form, similar to that of the wild type enzyme. A very slight difference in mobility on a nondenaturing gel was observed with K156R and K287R, compared with wild type or other mutant proteins (Fig. 1B).

**Enzyme-Imine Intermediate Formation**—Our initial interest was to identify the lysine residue uniquely involved in the enzyme intermediate formation. If the enzyme intermediate plays a vital role in catalysis, the site of its formation should be conserved. Of the 17 lysines in human deoxyhypusine synthase, nine that are conserved between the human and the yeast enzyme, *i.e.*, the residues at 141, 156, 165, 205, 212, 226, 251, 287, 329, and 339 (of the human enzyme), were targeted for site-directed mutagenesis in *E. coli* and *N. crassa* (in press).

In *E. coli*, the amount of spermidine cleavage activity of the enzyme was measured after ion exchange chromatographic separations on SDS-PAGE and by the bicinchoninic acid method noted above. The enzyme activity was based on the enzyme or mutant protein concentration of 1 pmol h⁻¹ of deoxyhypusine formed. The calculation of the specific activity was based on the enzyme or mutant protein concentration estimated by the densitometric analysis of Coomassie Blue-stained bands on SDS-PAGE and by the bicinchoninic acid method noted above.

**Enzyme Intermediate Formation**—Labeling of the enzyme by [³H]spermidine was carried after NaBH₃CN reduction as described previously (20) and in the legend to Fig. 2. The degree of labeling in [³H]deoxyhypusine was measured after ion exchange chromatographic separation of the reduced enzyme intermediate, acid hydrolysis, and ion exchange chromatography of the acid hydrolysate as in the deoxyhypusine synthesis assay (above).

In *N. crassa*, the amount of deoxyhypusine formed was measured after the partial purification of the reduced enzyme intermediate, acid hydrolysis, and ion exchange chromatography of the acid hydrolysate as described previously (19) and as noted in the legend to Table II. After incubation of the reaction mixture at 37 °C for 60 min unless noted otherwise. After trichloroacetic acid precipitation, the precipitate was washed three times with 10% trichloroacetic acid containing putrescine, spermidine, spermine, and protein was removed by one-step ion exchange chromatography, as described previously (19). After incubation of the reaction mixture at 37 °C for 30 min, 1 mM NAD, 7–9 mM [³H]spermidine was carried after NaBH₃CN reduction as described previously (19) and as noted in the legend to Table II. After incubation of the reaction mixture at 37 °C for 30 min, 1 mM NAD, 7–9 mM [³H]spermidine was carried after NaBH₃CN reduction as described previously (19) and as noted in the legend to Table II.

**Restriction sites in primers A and B are underlined. The mutated codon in the reverse primers are indicated as bold and underlined.**

| Restriction site in primers A and B | The mutated codon in the reverse primers |
|------------------------------------|------------------------------------------|
| BamHI                              | K329R                                    |
| KpnI                               | K287R                                    |
| SmaI                               | K156R                                    |
| KpnI                               | K205R                                    |
| SmaI                               | K141R                                    |
| KpnI                               | K251R                                    |
| SmaI                               | K101R                                    |
| KpnI                               | K141R                                    |
| SmaI                               | K287R                                    |
| KpnI                               | K239R                                    |

| Nucleotide sequence of primers used in PCR |
|-------------------------------------------|
| Forward primers                           |
| Primer A 1–20                             |
| Primer B 1110–1084                        |

| Reverse primers                           |
| Primer A 1–20                             |
| Primer B 1110–1084                        |
mutagenesis (Table I). The resulting mutant proteins were examined for their ability to form the enzyme intermediate.

Fig. 2 shows the pattern of radiolabeling of wild type and mutant enzyme proteins after their incubation with [1,8-3H]spermidine and NAD in the absence of the protein substrate, eIF-5A precursor, followed by NaBH₃CN reduction. Using approximately the same amount of each enzyme protein (~2 µg), intense labeling was observed with the mutant proteins K141R, K156R, K205R, K212R, K226R, and K251R as well as with the wild type. Radiolabeling of K338R was reduced to ~38% of the wild type. The labeling of the mutant K287R was too low to be clearly visible at the exposure time of the fluorogram of Fig. 2B. However, when a large amount (50 µg) was used, the incorporation of radioactivity into the mutant protein was clearly detectable and was estimated to be ~0.7% of wild type enzyme (Table II). A similarly low but definite labeling (0.6% of wild type enzyme) was also observed with the K287A mutant protein (Table II). In contrast, no radiolabeling was detectable (Table II) with K329R or with K329A even when the incubation was scaled up with 130 µg of mutant protein. (With 130 µg of wild type enzyme the level of labeling would be ~10 × 10⁴ dpm.)

Radiolabeled deoxyhypusine was released upon acid hydrolysis from all the proteins that were labeled. Upon digestion of the labeled mutant enzymes with trypsin and chymotrypsin only a single labeled peptide was detected in each case, as shown for K287R (Fig. 3). The labeled peptide from each of the mutant proteins showed the same mobility on a two-dimensional peptide map (Fig. 3) as the peptide (identified previously as Gly-deoxyhypusine-Ile-Arg (20)) corresponding to Gly₃₂₈-Arg₃₃₁ of the labeled wild type enzyme. These findings indicate that in the mutant proteins, as well as in the wild type enzyme, the enzyme intermediate is formed exclusively with the lysine at residue 329.

Activities of Mutant Proteins in Deoxyhypusine Synthesis and Spermidine Cleavage—The deoxyhypusine synthase reaction involves interactions of the enzyme with the three substrates, spermidine, NAD, and eIF-5A precursor protein, and consists of at least four proposed sequential events (Scheme 1), namely: (I) NAD-dependent dehydrogenation of spermidine, (II) tran-
Table II
Comparison of specific activities of deoxyhypusine synthase mutants for deoxyhypusine synthesis in eIF-5A, for spermidine cleavage, and for enzyme labeling

| Enzyme mutant | Deoxyhypusine synthesis in eIF-5A | Spermidine cleavage | Enzyme labeling |
|---------------|----------------------------------|--------------------|-----------------|
| K287R         | 1.1                              | 5.3                | 0.7             |
| K287A         | 0.03                             | 2.4                | 0.6             |
| K329R         | 0.00                             | 0.00               | 0.00            |
| K329A         | 0.00                             | 5.8                | 0.00            |

*These values are below the detection limit (<0.001%).

Discussion

Two-dimensional peptide maps prepared from a trypsin + chymotrypsin digest of wild type (A) or mutant K287R (B) enzyme after radiolabeling. Radiolabeling of the enzymes was carried out as described previously (20), using 5 μg of the wild type enzyme or 31 μg of K287R mutant enzyme. The radiolabeled enzyme band was excised from the SDS gel and thoroughly washed to remove SDS. The labeled protein in the gel slice was digested with trypsin (Worthington), tosylphenylalanlanyl chloromethyl ketone-treated, 20 μg/ml) and chymotrypsin (Worthington, Nα-p-tosyl-L-lysine chloromethyl ketone-treated, 120 μg/ml) in 0.15 ml of 50 mM ammonium bicarbonate buffer, pH 8.0, for 22 h. The lyophilized digest was separated on silica gel-coated plastic sheets (EM Science) as described (20). DNP, dinitrophenyl.

The most striking effects were seen with mutations at Lys329. With K329A and K329R, which cannot form the enzyme intermediate (Fig. 2 and Table II), absolutely no deoxyhypusine synthesis activity could be detected (Table II) even when 20 μg of the mutant protein was used. (The equivalent amount of wild type enzyme would yield 3.7 × 10^5 dpm of [3H]deoxyhypusine.) The K329R mutant enzyme was also devoid of any spermidine cleavage activity. Interestingly, the K329A mutant enzyme exhibited spermidine cleavage activity at 5.8% of the wild type enzyme, suggesting that spermidine cleavage can occur independently of enzyme intermediate formation in this case.

**Discussion**

The results reported here complement our previous study, in which the site of enzyme intermediate formation was deter-
mined by amino acid sequencing (20). Together these studies provide definitive evidence that Lys329 is the sole site of the covalent enzyme-substrate intermediate formation in human deoxyhypusine synthase and that the enzyme-substrate complex at Lys329 is an obligatory catalytic intermediate in the course of deoxyhypusine synthesis. Furthermore, the catalytic properties of some mutant proteins with Lys → Ala or Lys → Arg substitution offer new insights into structure-function relationships and the complex reaction mechanism of deoxyhypusine synthase (Scheme 1).

Even though the mutant enzymes used in this study were partially pure, the observed activities of deoxyhypusine synthesis and spermidine cleavage must be intrinsic to the recombinant proteins and are not due to contaminating E. coli proteins. E. coli does not contain genes for either deoxyhypusine synthase or eIF-5A and cannot contribute any deoxyhypusine synthetic activity. However, contamination with spermidine cleavage activity from a bacterial source was a concern because certain bacteria, e.g. Serratia marcescens and Micrococcus rubens, are known to contain spermidine dehydrogenase/oxi- dases that cleave spermidine to Δ1-pyrolone and diaminopropane in a reaction that requires an electron acceptor other than NAD (23, 24). The spermidine cleavage, as well as enzyme intermediate formation and deoxyhypusine synthesis activities, observed in the current experiments was strictly dependent on NAD, supporting the assumption that this cleavage results from catalysis by deoxyhypusine synthase. Furthermore, no spermidine cleavage or deoxyhypusine synthesis activity was detectable from a parallel protein preparation from E. coli transformed with the pET11a vector alone without any insert DNA under the same assay conditions.

Comparison of the activities of all the mutant proteins in enzyme intermediate formation, spermidine cleavage, and deoxyhypusine synthesis (Fig. 4) shows a pattern of general correlation between the three aspects of enzyme activity. This would be expected if spermidine cleavage and deoxyhypusine synthesis occur by way of the enzyme intermediate, as proposed in Scheme 1. Defects in enzyme intermediate formation, observed in several of these mutants, caused an almost equal or greater reduction in deoxyhypusine synthesis. Spermidine cleavage activity seemed to correlate generally with enzyme intermediate formation. In the case of the K287R, K287A, and K329A mutants, the relative activity of spermidine cleavage is greater than that of enzyme intermediate formation, suggesting that in special circumstances, spermidine cleavage can occur independently of the formation of the enzyme intermediate, as will be discussed below.

The mutant proteins K329A and K329R offered a key opportunity to assess the role of Lys329 in enzyme intermediate formation and in the three aspects of catalysis. Even with the biochemical evidence for the enzyme intermediate at Lys329 in vitro presented in the preceding paper (20), in the case of the mutant enzymes lacking this residue, K329A and/or K329R, it was conceivable that an alternate lysine residue near the active site could be recruited for enzyme intermediate formation. However, no labeling of the enzyme was observed with these two mutant enzymes, indicating that there is a stringent requirement for the orientation of Lys329 for it to carry out the transamination involving dehydraspermidine and to function as an acceptor of the butylamine moiety (Scheme 1) (20). The total lack of deoxyhypusine synthase activity of the K329R and K329A mutant enzymes leads us to conclude that the enzyme intermediate at Lys329 is critical for the catalysis as the mediator of butylamine transfer to the eIF-5A precursor.

Based on previous studies it was proposed that spermidine cleavage by deoxyhypusine synthase in the absence of the eIF-5A precursor involves the nucleophilic attack by the e-amino group of Lys329 of the enzyme on the carbon of the N=C bond of dehydraspermidine, resulting in the release of diaminopropane with concomitant formation of an enzyme-imine intermediate (20). The butylamine side chain of this intermediate, in turn, can undergo cyclization to release Δ1-pyrolone (Scheme 1, plain arrow) in the absence of eIF-5A precursor, the ultimate acceptor of the four carbon moiety. With K329A, where Lys329 is missing, NAD-dependent cleavage of spermidine was still detectable (5.8% of wild type enzyme). In this case, spermidine cleavage and cyclization of the butylamine moiety to Δ1-pyrolone could occur by the nucleophilic attack by the terminal amino group of dehydraspermidine on the C of its own N=C bond (19, 25, 26) (see Scheme 1, dashed arrow). In contrast to K329A, no spermidine cleavage activity was detectable with K329R. The difference in spermidine cleavage activities between the two mutant proteins may reflect an interesting feature of the spermidine-binding pocket of the enzyme. Previous studies, in which the spermidine-binding site of the enzyme was probed by the use of various spermidine analogues as inhibitors (27), suggested that the spermidine-binding site is a narrow groove, especially around the secondary nitrogen. It is tempting to speculate that replacement of Lys329 with a bulky Arg residue introduces steric hindrance that prevents spermidine binding or the cyclization of the butylamine moiety of dehydraspermidine, whereas substitution with Ala does not.

The catalytic properties of the mutant proteins permit us to make certain predictions on the role of these residues in substrate binding, catalytic reactions, and the conformational integrity of the enzyme. The interpretations drawn from these findings are consistent with x-ray crystallographic data obtained for human deoxyhypusine synthase in a complex with NAD.5 Amino acid residues in contact with NAD were identified and residues involved in spermidine binding can be predicted from the crystallographic data and molecular modeling.5 Because the Lys to Arg substitution at several sites, i.e. 156, 205, 212, 226, and 251, did not cause a significant change in enzyme intermediate formation, spermidine cleavage, or deoxyhypusine synthase in the eIF-5A precursor, these residues probably are not directly involved in tetramer assembly, substrate binding, or catalysis. Indeed, residues 141, 156, 205, 212, 226, 251, and 338 are not located near the NAD-binding site or the predicted spermidine-binding site at the active center of the enzyme, and the mutations at some of these sites may influence the activity indirectly by causing a slight alteration in the binding of substrate or in enzyme conformation. Replacement of Lys267 with either Arg or Ala caused a drastic reduction in the enzyme activities as measured by spermidine cleavage, enzyme intermediate formation, and deoxyhypusine synthesis. Unlike Lys329, this residue (Lys267) does not participate directly in the catalytic reaction, nor does it appear to serve in the binding of NAD or spermidine. Lys267 is next to His268, which is predicted to play a key role in the NAD-dependent dehydrogenation of spermidine.5 It is possible that substitution of Lys267 with either Ala or Arg could cause a distortion in the orientation of His268 or in the conformation of the active site such that the ability of the enzyme to interact with spermidine and NAD and/or to catalyze the NAD-dependent dehydrogenation would be seriously compromised.

Most importantly, Lys329 is located at the active center of the enzyme near the NAD-binding site and in the middle of the predicted spermidine-binding pocket in an orientation that would enable its role as the acceptor of the butylamine moiety

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from the dehydrospermidine intermediate. It is not one of the anchoring sites for NAD or spermidine. The K329R mutant enzyme forms a tetramer and also can associate with the eIF-5A precursor protein. Judging from the spermidine cleavage activity of K329A, it is clear that the K329A mutant enzyme can carry out NAD-dependent dehydrogenation of spermidine. The lack of deoxyhypusine synthesis activity by both the K329A and K329R mutant enzymes is most likely attributable to their inability to form the enzyme-imine intermediate, which is essential for the butylamine transfer from dehydrospermidine to the eIF-5A precursor. X-ray crystallographic studies of the mutant enzymes should provide further insights into the mechanism and the structure-function relationships of this unusual enzyme.

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