Complex formation between deoxyhypusine synthase and its protein substrate, the eukaryotic translation initiation factor 5A (eIF5A) precursor

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Deoxyhypusine synthase catalyses the first step in the post-translational synthesis of hypusine [*N*^ε-(4-amino-2-hydroxybutyl) lysine] in a single cellular protein, the precursor of eukaryotic initiation factor 5A (eIF5A). Deoxyhypusine synthase exists as a tetramer with four potential active sites. The formation of a stable complex between human deoxyhypusine synthase and its protein substrate, human recombinant eIF5A precursor (ec-eIF5A), was examined by affinity chromatography using polyhistidine-tagged (His·Tag) ec-eIF5A, by a gel mobility-shift method, and by analytical ultracentrifugation. Deoxyhypusine synthase was selectively retained by His·Tag-ec-eIF5A immobilized on a resin. The complex of deoxyhypusine synthase and ec-eIF5A was separated from the free enzyme and protein substrate by electrophoresis under non-denaturing conditions.

The stoichiometry of the two components in the complex was estimated to be 1 deoxyhypusine synthase tetramer to 1 ec-eIF5A monomer by N-terminal amino acid sequencing of the complex. Equilibrium ultracentrifugation data further supported this 1:1 ratio and indicated a very strong interaction of the enzyme with ec-eIF5A ($K_d \leq 0.5$ nM). Formation of the complex was not dependent on NAD⁺ or spermidine and occurred at pH 7.0–9.2. An enzyme–product complex, as well as the deoxyhypusine-containing product (modified ec-eIF5A), was also detected at pH 7.0–9.2 in a complete reaction mixture containing 1 mM spermidine.

Key words: equilibrium ultracentrifugation, hypusine, protein–protein interactions, tetrameric enzyme.

INTRODUCTION

Deoxyhypusine synthase (EC 1.1.1.249) catalyses the first step in hypusine [*N*^ε-(4-amino-2-hydroxybutyl) lysine] biosynthesis, one of the most specific post-translational modifications known to date. It recognizes one specific lysine residue of a single substrate protein, the precursor of a putative eukaryotic translation initiation factor, eIF5A [1] (for reviews see [2–4]). Hypusine modification converts the inactive eIF5A precursor to a biologically active mature protein, eIF5A [5,6]. Hypusine and eIF5A are vital for eukaryotic cell proliferation [2,7–11] and thus deoxyhypusine synthase presents a unique target for intervention in cellular proliferation.

Deoxyhypusine synthase requires NAD⁺ as a cofactor [12,13] and mediates the transfer of a 4-aminobutyl moiety from the amine substrate, spermidine, to its protein substrate, the eIF5A precursor. The complex reaction consists of four steps: 1) NAD⁺-dependent dehydrogenation of spermidine, 2) formation of an enzyme–imine intermediate by transfer of the butylamine moiety of dehydrospermidine to lysine in the active site of the enzyme, 3) a second transamination resulting in the transfer of the same butylamine moiety from the enzyme intermediate to the ϵ -amino group of a specific lysine residue of the eIF5A precursor, and 4) enzyme-mediated reduction to form deoxyhypusine in the eIF5A precursor [2,4,13,14].

As is the case for eIF5A, the amino acid sequence of deoxyhypusine synthase is highly conserved among various eukaryotic

species [3,15–19]; both eIF5A and deoxyhypusine synthase also appear to be functionally conserved throughout eukaryotic evolution. Deoxyhypusine synthases from several species share similar physical and catalytic properties. Experimental evidence from gel filtration and/or ultracentrifugation studies suggests that the enzymes from rat, human, yeast (*Saccharomyces cerevisiae*) and *Neurospora crassa* exist as a homotetramer of identical subunits [13,15–18,20]. They exhibit narrow specificity toward spermidine and NAD⁺. One of the most striking features of deoxyhypusine synthase is its specificity toward the protein substrate. Its low K_m value (< 1 μ M [15–17,21]) suggests a high affinity between the enzyme and eIF5A precursor. Deoxyhypusine synthase does not modify free lysine or the lysine residue in a synthetic peptide with the sequence of 16 amino acids of the human precursor surrounding the lysine residue (Lys⁵⁰) that undergoes modification. Previous studies involving stepwise truncation of the eIF5A precursor protein from the N- or C-terminus, or both, provided evidence that a large portion of the eIF5A precursor molecule (a minimum of 50 amino acids) is required for modification by the enzyme [22]. Therefore, the recognition of the protein substrate by the enzyme presumably involves highly specific interactions dependent on the three-dimensional structure of the two proteins.

The crystal structure of human recombinant deoxyhypusine synthase in a complex with NAD⁺, recently determined [23], reveals a symmetrical tetrameric organization of the enzyme. Although the tetramer contains four potential active sites, it is

Abbreviations used: eIF5A, eukaryotic translation initiation factor 5A; GC₇, *N*¹-guanylyl-1,7-diaminoheptane; hypusine, *N*^ε-(4-amino-2-hydroxybutyl) lysine.

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not known whether or not all of these sites will engage in the binding of spermidine and the eIF5A precursor and function simultaneously as catalytic centres.

Deoxyhypusine synthase binds NAD^+ and spermidine in a manner that is independent of the protein substrate and can catalyse an NAD^+ -dependent cleavage of spermidine in the absence of eIF5A precursor [13,14,17]. Since modification of the eIF5A precursor occurs at a later step of the deoxyhypusine synthase reaction, i.e. after the formation of the enzyme intermediate, it was not known whether the eIF5A precursor can bind to the enzyme independently of NAD^+ and spermidine. In the case of the *N. crassa* enzyme, the binding of enzyme and substrate protein may be dependent on NAD^+ [24,25]. The structure of the enzyme/eIF5A precursor complex has not been reported yet and little was known about the requirements for binding and the stoichiometry of the deoxyhypusine synthase/eIF5A precursor complex. We have examined the interaction between enzyme and substrate and determined the stoichiometry of the complex and the dissociation constant. These studies also provide evidence for an enzyme-product complex in the mixture containing all the reaction components, and offer insight into the relationship between spermidine concentration and the pH dependence of the deoxyhypusine synthesis reaction.

MATERIALS AND METHODS

Materials

[1,8- ^3H]Spermidine trihydrochloride (15 and 27 Ci/mmol) and [adenine-2,8- ^3H]NAD $^+$ (34.9 Ci/mmol) were purchased from DuPont NEN; Ni(II) immobilized resin (His-Bind resin) and buffer kit, pET-11a, pET-24b expression vector and host *Escherichia coli* BL21(DE3) competent cells from Novagen; T4 DNA ligase and *E. coli* DH5 α competent cells from Life Technologies, Inc.; AmpliTaq DNA polymerase from Perkin-Elmer; restriction enzymes from Boehringer-Mannheim; precast polyacrylamide gels, wide range protein standards (Mark 12) and sample buffers from Novex; molecular mass standard proteins (α -lactalbumin, bovine erythrocyte carbonic anhydrase, chicken egg albumin, BSA and jack bean urease) for non-denaturing gel electrophoresis from Sigma; polyvinylidene difluoride 0.2 μm membranes from BioRad. Oligonucleotide primers were synthesized by the Midland Certified Reagent Co. pKK233-2::5A-2 was kindly provided by Dr. John W. B. Hershey (University of California School of Medicine, Davis, CA, U.S.A.). ec-eIF5A was purified from *E. coli* lysates after overexpression of human eIF5A cDNA as described previously [22]. Human recombinant deoxyhypusine synthase was purified by successive chromatography on Q-Sepharose, Mono Q (Pharmacia) and butyl-Sepharose columns (to > 95% purity) as described previously [14,17,23].

Preparation of polyhistidine-tagged ec-eIF5A

A recombinant plasmid encoding ec-eIF5A tagged with 6 histidine residues at the C-terminus was constructed by PCR, using the vector pET-24b (Novagen) which has a His-Tag sequence (CACCACCACCACCAC) located C-terminal to the site of insertion, and a kanamycin resistance selectable marker. pKK233-2::5A-2 was used as template DNA with primers A [CTTCCAGTATGCTCATATGGCAGATGACTTGGACTTCGAGACA (*NdeI* site underlined)] and B [CTTCTATGGATCCGTAAGCTTTGCCATGGCTTGATTGCAACAGC (*BamHI* site underlined)] to obtain the coding region of eIF5A. The PCR product was digested with *NdeI* and *BamHI* and ligated to the linearized pET-24b vector. The ligation mixture

was used to transform *E. coli* DH5 α . After confirming the sequence, the recombinant plasmid was used for transformation of *E. coli* BL21(DE3) for protein expression. The selected transformants were grown in Luria-Bertani medium supplemented with kanamycin (30 $\mu\text{g}/\text{ml}$) and expression of the polyhistidine-tagged protein was induced by 1 mM isopropyl- β -D-thiogalactoside (IPTG).

Pelleted cells from 900 ml of culture were sonicated (60 s at 70 watts) in 30 ml buffer A (50 mM Tris/HCl, pH 8.0/0.5 M NaCl) containing 5 mM imidazole and centrifuged (30 min at 15000 g). The supernatant solution was loaded at a slow flow rate on to a column (2 cm $\times$ 15 cm) of His-Bind resin (50 ml) which had been activated with Ni(II) (according to the manufacturer's recommendations) and equilibrated with buffer A containing 5 mM imidazole. The column was washed with 52 ml of Buffer A containing 5 mM imidazole and 45 ml buffer A containing 60 mM imidazole. His-Tag-ec-eIF5A protein was eluted with a high concentration of imidazole (1 M) in buffer A (40 ml). The eluted protein was concentrated by ultrafiltration (Amicon, YM10 membrane) and equilibrated with 50 mM Tris/HCl, pH 7.5.

Protein affinity columns of His-Tag-ec-eIF5A immobilized on Ni(II) resin were made by incubating (per column) 20 μl of Ni(II) resin (His-Bind) with 27 μg of purified His-Tag-ec-eIF5A protein in 50 mM Tris/HCl buffer, pH 8.0, for 1 h at 4 $^{\circ}\text{C}$. The resin was washed 2 $\times$ with 60 μl buffer A containing 5 mM imidazole to remove excess His-Tag-ec-eIF5A.

Partially purified (approx. 30% pure) recombinant enzyme (10 μg of total protein, prepared by ion exchange chromatography on a MonoQ column, as described previously [17]) was loaded on to a column (approx. 20 μl) of resin with immobilized His-Tag-ec-eIF5A at 4 $^{\circ}\text{C}$. No NAD^+ or spermidine was added. The washing and elution steps were carried out the same as those for chromatography on Ni(II) resin as described above.

Co-expression of deoxyhypusine synthase and His-Tag-ec-eIF5A in *E. coli*

BL21(DE3) cells were co-transformed with a recombinant pET-11a plasmid containing the human deoxyhypusine synthase cDNA sequence [17] and with a recombinant pET-24b plasmid containing the human eIF5A cDNA sequence. Co-transformants were selected on Luria-Bertani plates containing kanamycin (30 $\mu\text{g}/\text{ml}$) and ampicillin (50 $\mu\text{g}/\text{ml}$), and examined for their expression of deoxyhypusine synthase and ec-eIF5A upon a 2–3 h induction with isopropyl- β -D-thiogalactoside.

Detection of complex formation by non-denaturing gel electrophoresis and determination of the ratio of the two proteins in the complex

Mixtures of highly purified recombinant human deoxyhypusine synthase and ec-eIF5A, in the presence or absence of NAD^+ , spermidine, or N^1 -guanyl-1,7-diaminoheptane (GC_7), were incubated in 200 mM Tris/HCl, pH 8.3, at room temperature for 20 min. After the addition of native gel sample buffer (Novex), the proteins were separated under non-denaturing conditions on a 8–16% (w/v) gradient polyacrylamide gel (Novex) in 25 mM Tris base/192 mM glycine running buffer at 4 $^{\circ}\text{C}$. The enzyme/ec-eIF5A complex was transferred on to a polyvinylidene difluoride membrane (0.2 μm pore size), as described previously [15], and the molar ratio of enzyme to ec-eIF5A was determined by N-terminal amino acid sequencing (carried out by Joseph Leykam, The Macromolecular Structure Facility, Biochemistry Department, Michigan State University, MI, U.S.A.). Alternatively, its composition was determined after SDS/PAGE of the complex as

follows: each band corresponding to the complex of deoxyhypusine synthase and ec-eIF5A, detected by staining with Coomassie Brilliant Blue R-250, was excised from the native gel, cut into fine pieces, incubated with an equal volume of equilibration buffer [62.4 mM Tris/HCl, pH 6.8, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS and 10% (v/v) glycerol] for 15 min and loaded into the well of a 14% (w/v) polyacrylamide gel. The gel was subjected to SDS/PAGE under denaturing conditions in 25 mM Tris base/192 mM glycine buffer. The quantity of proteins in each enzyme or ec-eIF5A band was estimated by densitometry scanning, using calibration curves determined for each protein.

Deoxyhypusine synthase assay

The activity of deoxyhypusine synthase was determined as described previously [13–17].

Analytical ultracentrifugation

Equilibrium ultracentrifugation was carried out in a Beckman-XLA analytical ultracentrifuge with either enzyme alone or the same enzyme concentrations in a 1:1 or 1:2 (enzyme tetramer to ec-eIF5A monomer) mixture with ec-eIF5A in 180 μ l of 50 mM Tris/HCl, pH 7.2/0.5 mM dithiothreitol, at a rotor speed of 5500 g. Scans were taken at 280 nm when equilibrium was reached at temperatures increasing, in steps of 4 °C, from 2 to 38 °C. In a separate experiment three different concentrations of ec-eIF5A alone (A_{280} 0.235, 0.125 and 0.065 respectively) in a 20 mM Tris/HCl, pH 6.8/1 mM dithiothreitol buffer were run under similar conditions, except that the rotor speed was 31 000 g.

To analyse the data for the association of enzyme with ec-eIF5A, we used an equilibrium model similar to those described earlier [26,27], which expresses the total monomer concentration distribution as a function of the radial position r ,

$$c_T(r) = c_{b,H} \exp(A_H M_H \delta r^2) + c_{b,F} \exp(A_F M_F \delta r^2) + c_{b,H}^n c_{b,F} \exp[\ln K_{H_4F_n} - \ln E_{H_4F_n,280} + (4A_H M_H + nA_F M_F) \delta r^2] + \epsilon \quad (1)$$

where $c_{b,X}$ is the monomer concentration, in absorbance scale, at the cell base, r_b , of the species 'X', which is specified by the subscripts H and F, referring to deoxyhypusine synthase and ec-eIF5A respectively. $\ln K_X$ is the natural logarithm of the association constant of the species 'X' indicated by the corresponding subscripts. M_X is the molar mass of the monomer indicated by the subscript, while buoyancy and the effects of the centrifugal field are represented in the term $A_X = (1 - \bar{v}_X \rho) \omega^2 / 2RT$ where $\bar{v}_X$ is the compositional partial specific volume of the solute molecule 'X' at the temperature T , ρ is the specific mass of the buffer at that temperature, ω is the rotational speed used in $\text{rad} \cdot \text{s}^{-1}$, R is the gas constant and T the absolute temperature; ϵ is the baseline offset correction for the finite absorbance of the buffer. $E_{X,280}$ is the molar absorption coefficient of species 'X' at 280 nm (E_F 4620 $\text{M}^{-1} \cdot \text{cm}^{-1}$, E_H 38880 $\text{M}^{-1} \cdot \text{cm}^{-1}$) and $\delta r^2 = r^2 - r_b^2$. Also, n indicates the number of ec-eIF5A monomers bound to the enzyme tetramer and its value may be determined by the best statistical fit of the data. This model was used in a non-linear regression scheme to fit the collected data.

For each temperature at which an apparent association/equilibrium constant, K , was reliably computed we proceeded to calculate the corresponding Gibbs free energy of association, $\Delta G^0 = -RT \ln(K)$. The data points were fitted by the thermodynamic equation

$$\Delta G^0(T) = \Delta H^0(T_0) - T \Delta S^0(T_0) + \Delta C_p^0 [(T - T_0) - T \ln(T/T_0)] \quad (2)$$

where $T_0 = 273.15$ K is a reference temperature and ΔH^0 , ΔS^0 and ΔC_p^0 are the standard enthalpic, entropic and heat capacity (at constant pressure) changes associated with the complex formation; their values were determined by the fitting procedure.

RESULTS

His-Tag-ec-eIF5A was constructed with an extra amino acid sequence, including six histidine residues, at the C-terminus of the human eIF5A precursor. The His-Tag-ec-eIF5A expressed in *E. coli* could be readily purified by affinity binding to a Ni(II) resin column, as shown in Figure 1(A). It serves as a good substrate for human deoxyhypusine synthase with a K_m of 3.4 μ M (compared with 0.86 μ M for ec-eIF5A measured under the same conditions; results not shown), suggesting that His-Tag-ec-eIF5A can readily bind to the human enzyme.

Figure 1(B) presents evidence for the physical association of His-Tag-ec-eIF5A with human deoxyhypusine synthase in an extract of *E. coli* BL21(DE3) cells co-expressing the enzyme and substrate. Whereas the expression level of His-Tag-ec-eIF5A was quite high in the clarified cell lysate (Figure 1B, lane 1), overexpression of the enzyme protein was not clearly visible because of contaminating bacterial proteins that co-migrate with the enzyme on SDS polyacrylamide gels. However, the presence of enzyme protein was evident from the high deoxyhypusine synthase activity measured in the lysate (results not shown). When the lysate from cells expressing both enzyme and substrate proteins was applied to a Ni(II) resin column, enzyme that had been retained even after extensive washing was released, together with His-Tag-ec-eIF5A, only upon elution with 1 M imidazole buffer (Figure 1B, lane 6). This enzyme band is not present in the eluate of the cell extract expressing His-Tag-ec-eIF5A alone (compare lane 6 in Figure 1A with lane 6 in Figure 1B). Since the enzyme by itself, either in a pure form or in the crude lysate, does not bind to the Ni(II) resin in the absence of His-Tag-ec-eIF5A (results not shown), it must be bound as a complex with the His-Tag-ec-eIF5A.

His-Tag-ec-eIF5A affinity column chromatography was used to characterize the enzyme–substrate interaction *in vitro*, and to determine the requirements for binding. Figure 1(C) shows evidence that deoxyhypusine synthase is selectively retained by His-Tag-ec-eIF5A immobilized on the Ni(II) resin, and is released from the resin together with His-Tag-ec-eIF5A upon elution with 1 M imidazole buffer. Under the specific chromatography conditions employed approx. 50% of the enzyme in the partially purified preparation was adsorbed to the affinity resin. (The band just above the enzyme band in Figure 1C, lanes 2–6, is the contaminating bacterial protein that co-purifies with the enzyme during ion-exchange chromatography.) The same pattern of adsorption and elution was observed in the absence (as shown in Figure 1C) or in the presence of 1 mM each of NAD^+ and spermidine (results not shown).

Definitive evidence for the formation of a stable complex of the enzyme and the native substrate, ec-eIF5A, under various conditions was obtained from non-denaturing gel electrophoresis (Figure 2A). Since the pI of the enzyme (4.8–5.1) is close to that of ec-eIF5A (pI 5.1) molecular size should be the major factor determining the electrophoretic mobility of these proteins in the native state. As expected, the tetrameric enzyme (164 kDa) was separated from the much smaller protein substrate (16.7 kDa) upon native gel electrophoresis (Figure 2A, lane 2 versus lane 8). In a mixture containing enzyme (E) and ec-eIF5A (S) at a ratio of 1:4 (molar basis) in 200 mM Tris/HCl, pH 8.3, a new band of protein (denoted by E–X), migrating more slowly than the enzyme, was observed instead of the enzyme tetramer (Figure

Figure 1 Complex formation between His-Tag-ec-elf5A and deoxyhypusine synthase

(A) Binding of His-Tag-ec-elf5A to Ni(II) resin; (B) binding of His-Tag-ec-elf5A/human deoxyhypusine synthase complex to Ni(II) resin; (C) binding of human deoxyhypusine synthase to His-Tag-ec-elf5A immobilized on Ni(II) resin. Chromatography was carried out as described in the Materials and methods section, but on a smaller scale using a cell lysate of *E. coli* BL21(DE3) cells over expressing His-Tag-ec-elf5A alone (A), or both His-Tag-ec-elf5A and human deoxyhypusine synthase (B). A protein affinity column of immobilized His-Tag-ec-elf5A (C) was prepared as described in the Materials and methods section using purified His-Tag-ec-elf5A protein. The His-Tag-ec-elf5A-charged resin was washed $2 \times$ with buffer A containing 5 mM imidazole to remove excess His-Tag-ec-elf5A. Partially purified (approx. 30% pure) recombinant enzyme was loaded on to the affinity column. The washing and elution steps were as described in the Materials and methods section. A portion of each fraction from the various columns was subjected to SDS/PAGE on 14% (w/v) polyacrylamide gels. (A) lane 1, (B) lane 7, and (C) lane 1 contain protein standards. The positions of human deoxyhypusine synthase (E) and His-Tag-ec-elf5A (S) on each gel are indicated by solid arrows. The dashed arrow next to lane 6 of panel (A) indicates the expected position of the enzyme, and demonstrates the absence of enzyme in this case.

Figure 2 Detection of the deoxyhypusine synthase/ec-elf5A complex formation by (A) non-denaturing PAGE and (B) SDS/PAGE

(A) Mixtures of highly purified recombinant human deoxyhypusine synthase (5.6 μ g, 0.034 nmol tetramer), and ec-elf5A (2.5 μ g, 0.15 nmol) in 200 mM Tris/HCl buffer, pH 8.3, in a total volume of 10 μ l were incubated at ambient temperature for 20 min in the presence or in the absence of 1 mM NAD^+ , 1 mM spermidine, or 0.1 mM GC_7 . Lane 1, molecular mass standards for non-denaturing PAGE (Sigma); lane 2, enzyme alone; lane 3, enzyme plus ec-elf5A; lane 4, enzyme plus ec-elf5A and NAD^+ ; lane 5, enzyme plus ec-elf5A and spermidine; lane 6, enzyme plus ec-elf5A, NAD^+ and spermidine (complete reaction); lane 7, enzyme plus ec-elf5A, NAD^+ and GC_7 ; lane 8, ec-elf5A alone. (B) SDS/PAGE of the enzyme complex (E-X) bands (lanes 3–7) excised from the gel shown in (A). Lane 1, molecular mass standards; lane 2, ec-elf5A with carrier BSA (66 kDa); lanes 3–7, the E-X complexes from the corresponding lanes of the gel shown in (A); lane 8, enzyme alone. E, enzyme (deoxyhypusine synthase); S, substrate (ec-elf5A); P, product (deoxyhypusine-containing intermediate); E-X, enzyme complex with S or P.

2A, lanes 3–7). The identity of this band as a complex was determined by electroelution and electrophoresis under denaturing conditions, as shown in Figure 2(B). The proteins from the E-X bands in Figure 2(A), lanes 3–5 and 7 dissociated into the enzyme subunit (41 kDa) and ec-elf5A (16.7 kDa) upon SDS/PAGE (Figure 2B), providing direct proof that these bands contain a complex (E-S) of enzyme and protein substrate. The enzyme appeared to be saturated with ec-elf5A at the ratio used,

since a large portion of substrate, but little free enzyme, was detected in Figure 2(A), lanes 3–5 and 7. The ratios of intensity of E and S after SDS/PAGE were similar for all the complexes, and approached close to 10:1, corresponding to a molar ratio of 1:1 for enzyme tetramer:substrate monomer (see below). The formation of the E-S complex was not dependent on spermidine, NAD^+ or GC_7 (a spermidine analogue inhibitor of deoxyhypusine synthase [28]), suggesting that binding of NAD^+ or spermidine to

Figure 3 Identification of ^3H -labelled deoxyhypusine-containing product (P) and its complex with the enzyme (E-P) by (A) non-denaturing PAGE and (B) fluorography

(A) Highly purified deoxyhypusine synthase (5.6 μg , 0.034 nmol tetramer) was incubated with a large excess of ec-eIF5A (10 μg , 0.6 nmol) in 200 mM Tris/HCl buffer, pH 8.3, in a total volume of 10 μl for 20 min at ambient temperature with additions as indicated below, before electrophoresis under non-denaturing conditions. The gel was stained with Coomassie Brilliant Blue R-250. A fluorogram (B) was prepared by soaking the gel shown in (A) in 1 M sodium salicylate for 1 h before drying it and exposing it to Kodak XAR-5 film at -70°C . Lane 1, enzyme alone; lane 2, mixture of enzyme and ec-eIF5A with NAD^+ (1 mM), spermidine (1 mM) and ^3H -spermidine (1 μCi); lane 3, mixture of enzyme and ec-eIF5A with NAD^+ (1 mM) and ^3H - NAD^+ (0.1 μCi , 0.28 μM); lane 4, mixture of enzyme and ec-eIF5A with spermidine and ^3H -spermidine; lane 5, mixture of enzyme and ec-eIF5A with spermidine and ^3H - NAD^+ ; lane 6, ec-eIF5A alone. The radiolabelled bands from lane 2 in (B) were excised, hydrolysed in 6 M HCl and analysed by ion exchange chromatography, as described previously [13,15,17]. ^3H -Deoxyhypusine was recovered from both the positions of P (23 000 c.p.m.) and E-P (983 c.p.m.).

the enzyme is not a prerequisite for its association with the protein substrate, which is consistent with the results shown in Figure 1(C).

Interestingly, however, in the presence of all the components of the complete reaction, including 1 mM of spermidine and NAD^+ , (Figure 2A, lane 6), the complex band (E-X) appeared somewhat broader, and the substrate was replaced by another new band (P), which migrated more slowly than the protein substrate (S) in native gel electrophoresis. This new band was presumed to be the modified protein product (containing deoxyhypusine) since the addition of the 4-aminobutyl moiety from spermidine to one lysine residue of ec-eIF5A will reduce its overall negative charge and its electrophoretic mobility at pH 8.3. The results shown in Figure 3 demonstrate that band P is the product of the enzymic reaction, i.e. the deoxyhypusine-containing form of eIF5A. In this experiment, the two proteins were incubated with NAD^+ and spermidine (1 mM each) together with $[1,8\text{-}^3\text{H}]$ spermidine (Figure 3A and Figure 3B, lane 2). Radioactivity from the latter was incorporated into the protein substrate to form a labelled product, which migrated more slowly than the unmodified substrate. When the labelled protein band was excised and the protein hydrolysed in 6 M HCl, radioactive deoxyhypusine was released as the single labelled component, indicating that the labelled protein was the deoxyhypusine-containing eIF5A intermediate. Some radioactivity was also detected in the upper band (Figure 3B, lane 2), suggesting the presence of the complex E-P. Hydrolysis of this band yielded radioactivity in ^3H -deoxyhypusine as the sole labelled component, confirming that this band contained a

Figure 4 Complex formation from mixtures of enzyme and substrate in varying molar ratios at pH 7.0–9.2

Non-denaturing PAGE after incubation of the enzyme and substrate in (A) 200 mM sodium phosphate buffer, pH 7.0, (B) 200 mM Tris/HCl buffer, pH 8.3, and (C) 200 mM glycine/NaOH buffer, pH 9.2. The pH of the gel electrophoresis running buffer was also adjusted to the indicated value. Deoxyhypusine synthase, 5.6 μg (0.034 nmol tetramer) and varying amounts of ec-eIF5A, 4.6 μg (0.27 nmol), 2.3 μg (0.136 nmol), or 1.1 μg (0.07 nmol) were incubated as before. (A) Lane 1, (B) lane 9, and (C) lane 5 are protein standards; (A) lane 5, (B) lane 1, and (C) lane 9 are enzyme; (A) lane 9, (B) lane 5, and (C) lane 1 are ec-eIF-5; all panels, lanes 2–4, no NAD^+ or spermidine; all panels, lanes 6–8, NAD^+ and spermidine (Spd, 1 mM each). The molar ratios of enzyme tetramer to ec-eIF5A monomer in all panels were 1:8 (lanes 2 and 6), 1:4 (lanes 3 and 7), and 1:2 (lanes 4 and 8). The positions of E, S, P, the E-S and E-P complexes are indicated. Abbreviations are as described in the legend to Figure 2.

complex of the enzyme and the protein product, the deoxyhypusine-containing eIF5A intermediate. Control experiments with ^3H -spermidine, but without NAD^+ , or ^3H - NAD^+ , with or without spermidine, showed no labelling at the positions of P or E-P bands (Figure 3B, lanes 3–5).

The data presented in Figure 4 confirm the formation of product and E-P complex in the complete reaction mixture. They further show the effects of varying the molar ratios of E and S and of varying the pH on the formation of the E-S complex (Figures 4A, 4B and 4C, lanes 2–4) and of P and the E-P complex (Figures 4A, 4B and 4C, lanes 6–8). The composition of the E-S complexes did not seem to vary when they were isolated from mixtures containing different E to S molar ratios (enzyme tetramer to ec-eIF5A monomer), i.e. 1:8 (lanes 2), 1:4 (lanes 3) or 1:2 (lanes 4). The ratio in the complex in each case approached close to 1:1 (enzyme tetramer to ec-eIF5A monomer) when assessed by densitometric scanning of Coomassie Blue-stained polypeptide bands of E and S after

Figure 5 Effect of pH on (A) the deoxyhypusine synthase reaction at two different spermidine concentrations, and on (B) the K_m values and the fraction of spermidine in the diprotonated form

(A) Enzyme activity was determined as described previously [13–17] except that 200 mM Tris/HCl was used as the buffer at the indicated pH (at 24 °C), and the spermidine (Spd) concentration was varied. The reaction mixtures in (A) contained 1 mM dithiothreitol, 25 μg BSA, 6 μM ec-eIF5A and 20 ng of highly purified human deoxyhypusine synthase in a final volume of 20 μl. The 7 μM spermidine reaction mixture contained 2 μCi of [3 H]spermidine (15 Ci/mmol) alone, and the 500 μM spermidine reaction mixture contained 10 μCi [3 H]spermidine plus 2 μl of 4.65 mM unlabelled spermidine. After a 1 h incubation at 37 °C, precipitation of the proteins with 10% (w/v) trichloroacetic acid and acid hydrolysis, the radioactivity was determined by counting a portion of the fractions obtained by ion-exchange chromatography. The picomole quantities of product were calculated after correction for the specific radio-activity of spermidine in each case. (B) The reaction mixtures and assay procedures used were similar to (A) except that the incubation was for 30 min and less enzyme (7 ng/20 μl) was used in order to keep the total product formed to less than 10% of the starting amount of each substrate. The total spermidine concentration was varied by diluting the [3 H]spermidine with unlabelled spermidine to achieve concentrations that bracketed the K_m range. K_m (▲) is the observed value (apparent K_m) for total spermidine; K_m' (○) is the K_m for the diprotonated form of spermidine, calculated by multiplying the apparent K_m for spermidine by the fraction in the diprotonated form. The curve for the fraction of total spermidine present as the diprotonated form (—) was calculated using the pK_a values determined by Baillon et al. [31] (8.40 for the secondary nitrogen, N^4 , 9.98 for N^1 and 10.81 for N^6) and correcting the pH to 37 °C using the temperature coefficient of Tris (−0.028/°C).

SDS/PAGE (results not shown). The E–S complex formed from mixtures containing a 1:4 molar ratio of enzyme tetramer to ec-eIF5A was also subjected to N-terminal amino acid sequencing after its transfer from a native gel to a polyvinylidene difluoride membrane. In the first five cycles of Edman degradation of the complex, the signals for the enzyme-derived N-terminal sequence, MEGLS, compared with the signals for the substrate-derived N-terminal sequence, ADDLD (the N-terminal methionine is missing in ec-eIF5A, as is often the case for other recombinant proteins expressed in *E. coli*), corresponded to 262 and 70 pmol respectively, and 621 and 155 pmol respectively, in two separate experiments. These ratios are consistent with the stoichiometry for a complex in which each enzyme tetramer binds only one substrate molecule. It is clear from comparing lanes 2–4 of the

three panels, A, B, and C, of Figure 4 that the formation of the E–S complex was essentially complete at pH 7.0, 8.3 and 9.2. This association presumably is a catalytically productive one because, as shown in lanes 6–8 of panels A, B and C, in the presence of 1 mM NAD^+ and 1 mM spermidine, almost all of the protein substrate was converted into product at all three pH values (see also Figure 2A, lane 6, Figures 3A and 3B, lane 2). As discussed above, in these cases the broader enzyme–complex band presumably consists largely of the enzyme–product complex as there is little substrate left in the mixture.

Effective conversion of S to P at pH 7 (Figure 4, panel A, lanes 6–8) might be considered unexpected because in the regular *in vitro* deoxyhypusine synthase assay, where the concentration of [$1,8\text{-}^3\text{H}$]spermidine is 1–10 μM [13,14,16–21,25,29,30], the rate of the reaction is optimum at pH 9–9.5 and is barely detectable at neutral pH [15–17,25,29,30]. Accordingly, we compared the effects of pH in the deoxyhypusine synthase assay using low and high concentrations of spermidine (Figure 5A). At a low concentration of spermidine (7 μM) the deoxyhypusine synthesis rate decreased sharply as the pH was changed from pH 9.5 to 7.5. However, at a higher concentration (500 μM), the rate at pH 7.5 was as high as 80% of that at pH 9.5, suggesting that the effect of lowering pH can be overcome by increasing the spermidine concentration. The K_m for spermidine was determined and found to increase with decreasing pH, from 6.8 μM at pH 9.2 to 302 μM at pH 7.0 (Figure 5B). Spermidine can exist in three different charged forms at these pH values [31]. The fraction of total spermidine in the diprotonated form, the presumed active substrate [28], can be calculated from the pK_a values [31] and is shown in Figure 5(B). When the observed K_m for total spermidine was corrected by the fraction that is in the diprotonated form, the resultant K_m' is essentially constant as a function of pH (Figure 5B).

The associative behaviour, in solution, of ec-eIF5A and deoxyhypusine synthase, alone and together, was studied in more detail by equilibrium ultracentrifugation. ec-eIF5A exhibited interesting self-associative behaviour, consistent with the previous ultracentrifugation analysis of mature human eIF5A [32]. At low temperatures (≤ 14 °C) there was a clear indication of a relatively weak dimer formation with an association constant of approx. $5 \times 10^3 \text{ M}^{-1}$ or K_d of approx. 0.2 mM. At higher temperatures, and especially at higher concentrations, higher oligomers, such as trimers and tetramers, tended to form.

Deoxyhypusine synthase alone, analysed at three concentrations (A_{280} 0.25, 0.17 and 0.09, results not shown), exhibited strong self-association into tetramers, in agreement with earlier observations of the enzyme from various species [13,15–18,20]. A monomer–tetramer model fits the data from two concentrations of enzyme (A_{280} 0.25, 0.17) very well, which is consistent with the known crystal structure of the enzyme [23] and indicated an apparent binding constant of about $0.3 \times 10^5 \text{ M}^{-1}$ or K_d of approx. 35 μM for the addition of each monomer. This experiment also allowed the calculation of a binding constant for the monomer–tetramer association of the enzyme of approx. $1.3 \times 10^{17} \text{ M}^{-3}$, (similar to that for the rat [15] and yeast [16] enzymes), confirming that the enzyme exists predominantly as a tetramer. At the concentrations used in these experiments, the tetramer tended to aggregate at temperatures > 26 °C and complete thermodynamic analysis of the association was not possible. Parenthetically, this aggregative behaviour of the enzyme would not occur in physiological conditions, since the concentration of the enzyme in cells is quite low [15]. Interestingly, the addition of eIF5A precursor to the enzyme tetramer provided extra thermal stability, and the higher aggregation of enzyme tetramer or the complex (see below) did not occur until > 34 °C.

Figure 6 Analysis of the association of enzyme tetramer with ec-eIF5A by equilibrium ultracentrifugation

(Upper panel) Representative global least squares fit of the absorbance data at 280 nm at one temperature (22 °C). Inset, error (in absorbance units) for the same data. The samples, in 180 μ l of 50 mM Tris/HCl buffer, pH 7.2, containing 0.5 mM dithiothreitol, included three different concentrations of deoxyhypusine synthase in mixtures with ec-eIF5A. The molar concentration ratio of enzyme tetramer to substrate monomer was 1:1 in three cells (total protein, A_{280} 0.28, 0.2 and 0.12, for curves 1, 2 and 3 respectively) while another cell was loaded with a 1:2 enzyme tetramer to substrate monomer mixture (A_{280} 0.23, curve 4). The rotor speed was set at 8400 rev./min (5500 g). The data shown were chosen because at lower temperatures (≤ 14 °C) the data were mathematically ill-conditioned and at higher temperatures (> 34 °C) aggregates tended to form. (Lower panel) Gibbs free energy of association and standard thermodynamic parameter changes as functions of temperature for 1:1 complex formation at temperatures between 18 °C and 34 °C. The data points for ΔG° are shown along with a fitted regression curve (see the Materials and methods section, eqn. 2). The error bars for the fitted data points were smaller than the symbols shown in the figure. The temperature variations of these parameters were computed according to the method of Chun [47].

For the association of ec-eIF5A with the enzyme, the analysis of the collected sets of data at all temperatures pointed strongly to a complex of enzyme tetramer to ec-eIF5A monomer of 1:1 since $n = 1$ (for ec-eIF5A) gave the best statistical fit in the non-linear-regression analysis based on the equilibrium model given by eqn. (1) in the Materials and methods section. This was clear, in spite of the tendency of the precursor alone to form a loosely associated dimer, (K_d approx. 0.2 mM), mentioned above. Figure 6(upper panel) shows the fit of the absorbance equilibrium data for four different mixtures at one temperature (22 °C) and is representative of the data at other temperatures. The estimated association constants at 5 different temperatures, from 18 °C to 34 °C, were used to compute Gibbs free energy of association.

Figure 6(lower panel) illustrates the thermodynamic data fit for these 5 sets of data using eqn. (2). The calculated values for changes in enthalpy, entropy and heat capacity, were $\Delta H^\circ = 43.6$ kcal·deg⁻¹·mol⁻¹, $\Delta S^\circ = 0.2$ kcal·deg⁻¹·mol⁻¹ and $\Delta C_{p,T}^\circ = -1.5$ kcal·deg⁻¹·mol⁻¹ with respect to 0 °C. The fit of ΔG° versus T to eqn. (2) indicates the thermodynamic consistency of the data and supports the validity of the model. The thermodynamic parameter changes near physiological temperatures point to binding with significant ionic contributions in addition to hydrophobic effects. It was found that the binding of the protein substrate to enzyme tetramer was very strong, of the order of 2×10^9 M⁻¹ or $K_d \leq 500$ pM. This is consistent with the fact that the analysis failed to detect any free enzyme tetramer. The mean molecular mass of the complex was calculated to be 171–174 kDa.

DISCUSSION

Hypusine biosynthesis at one locus of a single cellular protein must depend on the very selective recognition of the substrate protein by deoxyhypusine synthase. In view of this specificity, the essential role of the unique hypusine modification in eukaryotic cell proliferation, and the strong affinity between the enzyme and its protein substrate, deoxyhypusine synthase/eIF5A precursor binding represents an interesting model for the study of protein–protein interactions. Elucidation of the crystal structure of human deoxyhypusine synthase [23] and, very recently, that of the *Methanococcus jannaschii* and *Pyrobaculum aerophilum* eIF5A precursor analogues [33,34] provides the basis for some speculation on their molecular interaction. However, prediction of the structure of the E–S complex by a molecular modeling approach, using the known crystal structures of the two molecules, has not been successful to date. Furthermore, attempts to determine the crystal structure of the E–S complex directly have been hampered by lack of knowledge about the stoichiometry of the E–S complex, its stability and a means of isolation. Employing protein affinity chromatography, non-denaturing gel electrophoresis and equilibrium ultracentrifugation, we demonstrated that the enzyme and the eIF5A precursor form a stable complex of 1 enzyme tetramer:1 substrate monomer of high affinity ($K_d \leq 0.5$ nM).

Native gel electrophoresis proved to be a simple and effective technique in this instance, especially for the isolation of the complex and determination of its stoichiometry. The mobility shift assay, successfully employed in the detection of other protein–protein complexes [35–38], is primarily suited to studying complexes of high affinity, such as the one between deoxyhypusine synthase and ec-eIF5A. It has several advantages over conventional gel filtration, which suffers from low resolution, especially when the difference in molecular mass between the complex and a component protein is small, as is the case for the complex (181 kDa) of deoxyhypusine synthase (164 kDa) with ec-eIF5A (~17 kDa). The gel mobility shift assay has also been found useful as a sensitive method to detect the binding of several mutant forms of deoxyhypusine synthase with the substrate protein (results not shown).

The complex observed between ec-eIF5A and human deoxyhypusine synthase does not require NAD⁺ or spermidine (see the data in Figures 2 and 4). Thus the human enzyme appears to be different from the *N. crassa* enzyme, which was reported to depend on the presence of NAD⁺ for binding to its substrate protein [24,25]. This may be due to structural differences in deoxyhypusine synthases from these species with respect to the NAD⁺ binding site, or may result from the effect of NAD⁺ binding on the conformation of the enzyme. Although the enzymes from diverse eukaryotes share basic common features,

such as substrate specificity, the imine intermediate formation [14,19], and tetrameric molecular organization, certain differences have been noted regarding their affinity for NAD^+ [16,17] and susceptibility to thiol-reactive reagents [20]. Conversely, the binding of NAD^+ and spermidine to the enzymes does not depend on the presence of the eIF5A precursor, as the enzymes from several species can catalyse the NAD^+ -dependent cleavage of spermidine in the absence of the eIF5A precursor [13–17]. Although the postulated reaction mechanism of deoxyhypusine synthase involves sequential participation of NAD^+ , spermidine and the protein substrate, the results from this study suggest that the binding of NAD^+ , spermidine and ec-eIF5A to the enzyme can occur in a random order. There are precedents for such behavior, for example with protein farnesyltransferase [39,40].

An additional interesting aspect of this binding study is the detection of a stable complex between deoxyhypusine synthase and the modified protein product in the complete reaction mixture (Figures 2–4). This finding suggests that even after modification, the affinity of the protein product for the enzyme is strong enough to be sustained as an E–P complex. An association of enzyme and eIF5A in cells has been suggested from yeast two-hybrid analysis [3]. A strong association of enzyme and protein product (but only for the product formed catalytically) was also reported for protein farnesyltransferase [40]. In that case the dissociation of modified protein or peptide product from the enzyme only occurs after the binding of additional substrate, either farnesyl diphosphate or protein, implying a conformational change in the enzyme. A similar scenario may apply to the deoxyhypusine synthase reaction.

The data of Figures 4 and 5 clarify the relationship between the pH optimum of the reaction and spermidine concentration. It is apparent that the deoxyhypusine synthesis reaction can proceed effectively at both neutral pH and at basic pH when the spermidine concentration is high. It is in contrast with the routinely used assay, which utilizes low concentrations (1–10 μM) of radiolabelled spermidine of high specific radioactivity, and in which the reaction is optimal at pH 9.0 to 9.5 and marginally detectable at neutral pH [15–17,25,29,30]. An explanation for this pH optimum derives, in large part, from consideration of the charge states of spermidine. The pK_a values of the three amine groups of spermidine are 9.94, 8.40 and 10.81, for N^1 , N^4 and N^8 respectively, [31]. At pH 9 to 9.5 the major charge form of spermidine is the diprotonated form, with the two terminal amino groups positively charged and the secondary amino group uncharged (Figure 5B). The pH optimum (9–9.5) of the *in vitro* deoxyhypusine synthase reaction suggests that the diprotonated form is the active amine substrate [28]. Spermidine exists mainly in the triprotonated form at neutral pH and the percentage of the diprotonated form is very low (e.g. < 3.7% at pH 7.0). Therefore, when only 1–10 μM labelled spermidine is used for the assay at neutral pH the concentration of the diprotonated form is very low and it becomes a limiting factor in view of the K_m of spermidine (see Figure 5B). However, when the concentration of spermidine is raised, the concentration of the diprotonated form would be high enough even at neutral pH (e.g. approx. 40 μM of the diprotonated form at pH 7.0 in the case of 1 mM spermidine) to permit efficient deoxyhypusine synthesis.

The assignment of the diprotonated spermidine as the active substrate is also reasonable in view of the shape and arrangement of the spermidine binding site predicted from the crystal structure of the enzyme [23] and is consistent with studies on spermidine analogue inhibitors [28]. According to the proposed model, spermidine is bound in a long narrow groove, and anchored to acidic residues near the ends of this pocket through its two terminal amino groups. The ϵ -amino group of the active

site residue Lys³²⁹ is oriented toward the middle of this groove, and must approach the secondary nitrogen of dehydrosperridine in order to serve as an acceptor of the butylamine moiety. The ϵ -amino group of this lysine residue (pK_a 10.8) would be protonated at pH < 10 and the triprotonated form of spermidine is unlikely to bind at this site because of the electrostatic repulsion between the two positive charges. However, the diprotonated spermidine, with an uncharged secondary amine, could bind, and, presumably, act as the reactive substrate.

All of the techniques that we have employed point to a complex of one eIF-5A precursor molecule per tetramer of enzyme as the predominant species. Based on this stoichiometry, it appears that an enzyme tetramer binds one molecule of ec-eIF5A, and that one of the four potential active sites engages in substrate modification at one time. This is especially intriguing, since the enzyme has four apparently equal active sites, which encompass the NAD^+ and spermidine binding sites. These sites are arranged in a pair at a dimer interface, with access from opposite sides of the enzyme surface [23]. The eIF5A precursor is a relative long molecule, estimated as 63 Å long by 26 Å wide, with two distinct domains, in the crystal structure of the analogue from the thermophile *M. jannaschii* [33]; it lacks 10 N-terminal amino acids of human eIF5A, but has significant sequence similarity, and can be modified by the human enzyme (M. -H. Park, unpublished work). It has a tendency to form dimers, as one crystal structure analysed was an end-to-end dimer involving hydrogen-bonded association between identical domains on different molecules [33]. The other thermophilic eIF5A precursor analogue shows a similar structure and interactions between individual molecules [34]. The analytical ultracentrifugation data confirm this tendency, but they also show that the dimer, which is only loosely associated, dissociates in the presence of the enzyme. It is conceivable that binding a molecule of this size could preclude binding of a second one on the same face of the enzyme, as is the case for the complex of transthyretin and retinol binding protein [41]. However, it is hard to imagine the inability of a second precursor molecule to bind on the opposite face of the enzyme. If more than one eIF5A precursor binding site exists on the symmetrical enzyme tetramer, the binding of the first molecule of substrate may induce a conformational change in the enzyme subunits and prevent subsequent binding of an additional molecule(s). There are examples of dimeric enzymes that exhibit 'half of the sites' reactivity, some tetrameric proteins that also use only two of four potential active sites, and a few of six and eight subunits that exhibit similar behaviour [41–46], but no examples, to our knowledge, of the preferential binding of only one of four potential partners. Negative co-operativity, in which the affinity of binding for successive ligands decreases, has been demonstrated, for example, for the binding of NAD^+ to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase [44], but in most cases of protein-ligand binding to a tetrameric enzyme (for exception, see reference [41]), the stoichiometry of binding has not been well characterized.

It is not feasible at present to predict the precise mode of binding of ec-eIF5A and the enzyme. The structure of the crystals, prepared at pH 4.5, of the human enzyme complexed with NAD^+ does show an interesting feature, namely that the entrance to the active site pocket, to which spermidine and the eIF5A precursor must bind, is apparently blocked by a 'ball and chain' peptide arm from another subunit. This conformation thus probably represents the enzyme in an inactive state; in the active state this flexible loop must move away in order for the spermidine, and the protein substrate, to gain access to the active site. Crystals of the enzyme formed at neutral or basic pH may yield a structure of the enzyme in its active conformation. In

particular, structural determination of the human deoxyhypusine synthase/eIF5A precursor complex should provide a molecular basis for the unique specificity of the interaction between the two proteins.

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