Structure–function studies of human deoxyhypusine synthase: identification of amino acid residues critical for the binding of spermidine and NAD

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Deoxyhypusine synthase catalyses the first step in the biosynthesis of hypusine [*N*^ε-(4-amino-2-hydroxybutyl)lysine]. The crystal structure of human deoxyhypusine synthase in complex with NAD revealed four NAD-binding sites per enzyme tetramer, and led to a prediction of the spermidine-binding pocket. We have replaced each of the seven amino acid residues at the predicted spermidine-binding site, and eleven residues that contact NAD, on an individual basis with alanine. Of the amino acid residues at the spermidine site, substitution of Asp-243, Trp-327, His-288, Asp-316 or Glu-323 with alanine caused an almost complete loss of spermidine binding and enzyme activity; only the mutation Tyr-305 → Ala showed partial binding and activity. His-288 → Ala was also deficient in terms of binding NAD. NAD binding

was significantly reduced in all of the NAD-site mutant enzymes, except for Glu-137 → Ala, which showed a normal binding of NAD, but was totally lacking in spermidine binding. Of the NAD-site mutant enzymes, Asp-342 → Ala, Asp-313 → Ala and Asp-238 → Ala displayed the lowest binding of NAD. These enzymes and His-288Ala also showed a reduced binding of spermidine, presumably because spermidine binding is dependent on NAD. These findings permit the positive identification of amino acid residues critical for binding of spermidine and NAD, and provide a new insight into the complex molecular interactions involved in the deoxyhypusine synthase reaction.

Key words: eIF5A, hypusine, site-directed mutagenesis.

INTRODUCTION

Deoxyhypusine [*N*^ε-(4-aminobutyl)lysine] is an intermediate in the post-translational synthesis of an unusual amino acid, hypusine [*N*^ε-(4-amino-2-hydroxybutyl)lysine] [1,2]. Hypusine is formed in a single cellular protein in two enzymic steps. In the first step, deoxyhypusine synthase catalyses the NAD-dependent transfer of the butylamine moiety from the polyamine spermidine to the ε-amino group of a specific lysine residue of the precursor of eukaryotic translation initiation factor 5A (eIF5A) to form a deoxyhypusine residue [3–6]. In the second step, deoxyhypusine hydroxylase catalyses hydroxylation of this residue to complete the synthesis of hypusine [7]. Hypusine biosynthesis presents one of the most specific post-translational modifications known to date.

There is substantial evidence supporting the essential role of eIF5A and its hypusine modification in eukaryotic cell proliferation. The highly conserved nature of both eIF5A and deoxyhypusine synthase in the eukaryotic kingdom attests to their fundamental role in cells [1,2,8]. Suppression of the expression of the two eIF5A genes [9,10] or a single deoxyhypusine synthase gene [11,12] results in a loss of cell viability in *Saccharomyces cerevisiae*. Furthermore, inhibitors of deoxyhypusine synthase [13] or deoxyhypusine hydroxylase [14] exert anti-proliferative effects in various mammalian cells. The arrest of growth of L1210 cells depleted of the polyamine spermidine by inhibitors of polyamine biosynthesis was attributed to the deprivation of eIF5A and hypusine [15,16]. Thus deoxyhypusine synthase occupies a pivotal position in the role of polyamines in eukaryotic cell proliferation.

The complex deoxyhypusine synthase reaction involves NAD, spermidine and eIF5A precursor, and is proposed to occur in

four steps: (i) NAD-dependent dehydrogenation of spermidine [17]; (ii) formation of an enzyme-imine intermediate by transfer of the butylamine moiety from dehydrospermidine to the active site lysine residue (Lys-329 for the human enzyme) [18]; (iii) transfer of the same butylamine moiety from the enzyme intermediate to the eIF5A precursor; and (iv) reduction of the eIF5A-imine intermediate to form a deoxyhypusine residue. A remarkable feature of this enzyme is its narrow specificity towards its cofactor and substrates. The basis of this specificity has been studied using compounds structurally related to NAD [6] and spermidine [19], and truncated eIF5A peptides [20].

The three-dimensional structure of the NAD complex of human deoxyhypusine synthase reveals a symmetrical tetrameric organization of the enzyme with four NAD molecules, two bound at each interface of two subunits [21]. Although a spermidine analogue, 1,7-diaminoheptane, was included in the initial crystallization mixture as a probe for the spermidine-binding site, it was not detected in the crystal structure, possibly because the low pH of the buffer precluded binding of this analogue. However, on the basis of the involvement of the active-site residue, Lys-329, and also on the role of NAD as a hydrogen acceptor from spermidine, a spermidine binding mode that involves ionic and hydrophobic interactions with certain amino acid residues of the enzyme could be predicted [21].

In a recent study, we have developed assays for the binding of NAD and spermidine to human deoxyhypusine synthase [22]. The data from the binding studies indicate that the enzyme tetramer can bind up to four molecules each of NAD and spermidine, and that there are close interactions between the binding of NAD and spermidine. The binding of spermidine is dependent on NAD, suggesting that NAD binding induces a conformational change in the enzyme to permit subsequent

Abbreviations used: DTT, dithiothreitol; eIF5A, eukaryotic translation initiation factor 5A; ec-eIF5A, recombinant human eIF5A precursor; TCA, trichloroacetic acid.

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binding of spermidine. Furthermore, the binding of NAD was strongly enhanced by spermidine. These findings prompted us to assess the role of each amino acid residue predicted to be involved in the binding of NAD and of spermidine, and to verify the proposed model of spermidine binding. Thus we targeted the amino acid residues in the predicted spermidine-binding site and the NAD-binding site for site-directed mutagenesis, and characterized the mutant enzymes for their ability to bind NAD, spermidine and eIF5A precursor, and also for their activities in spermidine cleavage, enzyme-intermediate formation and deoxyhypusine synthesis.

EXPERIMENTAL

Materials

[1,8-³H]Spermidine·3 HCl (27.6 Ci/mmol) was purchased from Dupont New England Nuclear (Boston, MA, U.S.A.); [4-³H]NAD (3.54 Ci/mmol) was from Amersham (Arlington, IL, U.S.A.). Precast polyacrylamide gels, sample buffer, running buffer and molecular-mass standards (Mark 12) were from Novex (San Diego, CA, U.S.A.). Glass-fibre filters (GF/B; 25 mm diam.) were obtained from Whatman, and polyethylenimine was purchased from Sigma (St. Louis, MO, U.S.A.). Oligonucleotide primers were synthesized by the Biosynthesis Company (Lewisville, TX, U.S.A.). QuickChange Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA, U.S.A.). The pET-11a expression vector and the host BL21(DE3) competent cells were from Novagen (Madison, WI, U.S.A.). MonoQ column and Q-Sepharose (Fast flow) resin were from Pharmacia Biotech Inc. (Piscataway, NJ, U.S.A.). Recombinant human eIF5A precursor, ec-eIF5A, was purified from *Escherichia coli* lysates after overexpression of the human eIF5A cDNA, as described previously [20]. The human wild-type enzyme was purified from a lysate of *E. coli* BL21(DE3) overexpressing human deoxyhypusine synthase cDNA, as described in [18].

Methods

Site-directed mutagenesis

Mutant enzymes with amino acid residues individually replaced with alanine or arginine were generated by using high-fidelity *pfu Turbo* DNA polymerase and the QuickChange Site-Directed Mutagenesis kit, according to the manufacturer's instructions. The pET-11a vector containing human deoxyhypusine synthase cDNA was employed as the template, and primer sets were designed for substitution of each amino acid. After the *Pfu Turbo* polymerase reaction, the parental template DNA was removed by digestion with *DpnI*, and the resulting mixture was used for transformation of XL1-Blue-competent cells. The entire open reading frame of the inserted DNA was sequenced for confirmation of the intended mutation and the fidelity of the polymerase reaction. The selected mutated plasmids were introduced into *E. coli* BL21(DE3) cells for overexpression of the desired proteins.

Overexpression and purification of the mutant proteins

The selected clones were grown in 10 litres of Luria–Bertani medium, supplemented with 100 µg/ml ampicillin; protein expression was induced with 1 mM isopropyl β-D-thiogalactoside for 2 h. Purification of the mutant proteins was achieved following a protocol similar to that described for purification of the wild-type enzyme [18] and other mutant enzymes [23]. Cell pellets

were resuspended in 150 ml of buffer A [50 mM Tris/HCl (pH 6.8)/0.1 mM EDTA/1 mM dithiothreitol (DTT)/1 mM PMSF], and sonicated using a Branson sonifier Model 350 in an ice bath. The clear supernatant of the lysate, after centrifugation at 15000 g for 1 h, was loaded on to a Q-Sepharose column (40 ml) equilibrated with buffer A. After washing the column with buffer A (150 ml), a stepwise elution with buffer A containing 0.1, 0.2, 0.3, 0.4 or 0.5 M NaCl (50 ml each step) was performed. Each mutant enzyme was identified by SDS/PAGE of the fractions, and was found to be eluted in the range of 0.25–0.4 M NaCl, close to the ionic strength required for the elution of the wild-type enzyme. The fractions containing the mutant protein were pooled and precipitated with ammonium sulphate (45–70% -satd). Ammonium-sulphate-precipitated fractions were dialysed against buffer A, applied to a MonoQ HR 10/10 column equilibrated with the same buffer, and the enzyme was eluted with a linear gradient of NaCl (0.1–0.5 M) in buffer A. The fractions containing the desired protein were subjected to another round of chromatography on a MonoQ column for further purification. The mutant proteins thus obtained were of > 50% purity.

Binding assays for NAD and spermidine

Binding of [³H]NAD and [³H]spermidine to human deoxyhypusine synthase wild-type and mutant enzymes was measured by a rapid filtration assay using glass-fibre filters (Whatman GF/B) pre-soaked in 0.3% (v/v) polyethylenimine [24,25], as described previously [22]. The radioactivity on washed filters was measured using a liquid scintillation counter (Beckman, Fullerton, CA, U.S.A.), after having been left standing overnight to allow the release of the radioactivity into the scintillation fluid, Cytosint (ICN; Aurora, OH, U.S.A.). Because the enzyme catalyses the NAD-dependent dehydrogenation and cleavage of spermidine, in a mixture containing both NAD and spermidine, the enzyme-bound radioactivity from [³H]spermidine was identified as a mixture of spermidine, dehydrosperrmidine, enzyme intermediate and Δ¹-pyrroline; that from [³H]NAD was a mixture of NAD and NADH [22].

Assay for deoxyhypusine synthesis

The enzyme activity was assayed as described previously [17] by measuring the incorporation of radioactivity from [1,8-³H]spermidine into the eIF5A precursor protein as [³H]-deoxyhypusine. A typical reaction mixture contained, in 20 µl: 0.2 M glycine/NaOH buffer, pH 9.5, 1 mM DTT, 5 µg of BSA, 0.5 mM NAD, 5 µM [³H]spermidine, 9 µM ec-eIF5A and wild-type enzyme or mutant protein (0.01–1 µg). After incubation at 37 °C for 30 min, 0.5 mg of BSA was added, and the proteins were precipitated with 10% (w/v) trichloroacetic acid (TCA). The precipitates, after washing thoroughly with 10% (w/v) TCA containing 1 mM each of putrescine, spermidine and spermine, were subjected to acid hydrolysis in 6 M HCl for 16 h. [³H]-Deoxyhypusine released by acid hydrolysis was measured after its separation by ion-exchange chromatography [17]. For determination of the kinetic parameters, the assay conditions were modified as indicated in the legend to Table 1.

Assay for spermidine cleavage

Partial activity was measured in the absence of the protein substrate, ec-eIF5A, as described previously [17]. After incubation of the reaction mixture containing all the components

Table 1 Kinetic parameters for wild type and mutant enzymes

Kinetic constants were determined as described previously [17]. Separate determinations of the parameters for NAD and spermidine were made using ec-eIF5A as the protein substrate. The fixed concentrations of NAD, spermidine and ec-eIF5A were 0.5 mM, 30 μ M and 9 μ M, respectively. The varied concentrations in most cases were 1–200 μ M for NAD and 1–50 μ M for spermidine. For Asn-106 and Ser-109, with a high K_m for spermidine, the fixed concentration of spermidine for the determination of the K_m for NAD was 200 μ M. The amount of enzyme used was approx. 20 ng. The incubations were performed for 30 min. V'_{max} is the apparent V_{max} when NAD was varied and does not represent a true V_{max} , since the spermidine concentration was not saturating. The experiments were repeated twice, yielding similar results.

Enzyme	NAD		Spermidine		
	K_m^{NAD} (μ M)	V'_{max} (μ M $\cdot$ h $^{-1}$ $\cdot$ μ g $^{-1}$)	$K_m^{spermidine}$ (μ M)	V_{max} (μ M $\cdot$ h $^{-1}$ $\cdot$ μ g $^{-1}$)	$V_{max}/K_m^{spermidine}$
Wild-type	7.0 $\pm$ 0.2	112.5 $\pm$ 2.0	5.4 $\pm$ 0.21	154.0 $\pm$ 7.7	28.5
Tyr-305Ala	100 $\pm$ 1.8	108.8 $\pm$ 4.0	9.2 $\pm$ 0.17	132.0 $\pm$ 4.9	14.3
Asn-106Ala	90.9 $\pm$ 0.91	65.8 $\pm$ 4.7	20.0 $\pm$ 0.14	75.0 $\pm$ 3.2	3.8
Thr-308Ala	5.0 $\pm$ 0.15	118.2 $\pm$ 4.7	8.0 $\pm$ 0.25	159.0 $\pm$ 6.7	19.9
Gly-283Ala	12.7 $\pm$ 0.38	108.3 $\pm$ 9.3	7.6 $\pm$ 0.31	109.2 $\pm$ 6.0	14.3
Ser-317Ala	200 $\pm$ 10.8	123.3 $\pm$ 10.2	7.6 $\pm$ 0.4	144.0 $\pm$ 9.0	18.9
Ser-105Ala	4.1 $\pm$ 0.14	12.3 $\pm$ 0.53	3.9 $\pm$ 0.09	16.0 $\pm$ 0.19	4.1
Thr-131Ala	10.1 $\pm$ 0.59	13.2 $\pm$ 0.62	3.8 $\pm$ 0.27	13.4 $\pm$ 0.32	3.5
Ser-109Ala	6.3 $\pm$ 0.13	9.0 $\pm$ 0.06	101 $\pm$ 8.4	37.5 $\pm$ 0.16	0.37

described above for deoxyhypusine synthesis (except for ec-eIF5A), the reaction mixture was treated with 10% (w/v) TCA, and the TCA supernatant was analysed for the degradation products of spermidine by ion-exchange chromatography. The estimated activity was on the basis of the quantity of 1,3-diamino[3 H]propane produced from [1,8- 3 H]spermidine.

Assay for enzyme-intermediate formation

The covalent enzyme intermediate formed in the reaction mixture in the absence of ec-eIF5A was trapped by reduction with NaBH₃CN as a stable adduct, as described previously [18]. The degree of enzyme-intermediate formation was determined by measurement of [3 H]deoxyhypusine in the acid hydrolysate of TCA-precipitated enzyme protein, and also by detection of the labelled enzyme intermediate by fluorography after SDS/PAGE [18], as shown in Figure 4.

RESULTS

Although the binding sites of NAD and spermidine are depicted separately in Figures 1 and 2, the two binding sites overlap in three-dimensional structure and are inseparable. Thus it is possible that a mutation at the spermidine site affects NAD binding, and vice versa. Furthermore, a single mutation at either binding site could result in a change in the global conformation of the protein. Thus, in evaluating the role of each amino acid, we examined whether the mutation altered the ability of the enzyme to form a tetramer, its ability to bind spermidine, NAD or ec-eIF5A (the human eIF5A precursor), and how the alterations in binding related to its activity in spermidine cleavage, enzyme-intermediate formation and overall deoxyhypusine synthesis.

Tetramer formation and binding of ec-eIF5A by the wild-type and mutant enzymes

If the substitution of a single amino acid residue at the NAD or spermidine-binding site causes changes in the local structure of the binding pocket, but not in the overall conformation of the enzyme, the resulting mutant enzyme should retain its ability to form a tetramer and a stable complex with ec-eIF5A. The mutant enzymes alone, or in a mixture with ec-eIF5A, were subjected to

Figure 1 Proposed model of the spermidine-binding site of human deoxyhypusine synthase

The active site residue Lys-329, and amino acid residues most likely to be involved in the binding of spermidine, are shown. His-288 was included because of its proposed role as proton acceptor during the hydride transfer reaction. Asp-243 and the principal binding site of NAD are on a neighbouring subunit, as indicated by the more darkly grey-shaded background. Potential hydrogen bonds are shown by dotted lines. The figure has been designed on the basis of results published previously [21].

electrophoresis under non-denaturing conditions (Figure 3, upper panel). In the absence of ec-eIF5A, all the mutant enzymes migrated close to the position of the wild-type enzyme, suggesting that they exist in a similar tetrameric form. In the mixtures of each enzyme (except for Asp-313Ala) and excess ec-eIF5A, no band relating to the presence of free enzyme was detected. Instead, a band of protein denoted by 'E·S', with a lower mobility than the enzyme tetramer, was observed. The E·S bands derived from Asp-243Ala and Lys-329Ala showed a greater mobility shift than any of the others. When the E·S bands were excised and subjected to SDS/PAGE (Figure 3, lower panels), they dissociated into 41 kDa and 18 kDa proteins, corresponding to human deoxyhypusine synthase monomer and ec-eIF5A monomer respectively, suggesting that E·S bands are indeed complexes of the mutant enzymes and ec-eIF5A. For all the mutant enzymes but one, ec-eIF5A was detected in the E·S bands, providing evidence that each of these mutant enzymes can form a stable complex with ec-eIF5A. Asp-313Ala appeared to be defective in the formation of a stable complex with ec-eIF5A,

Figure 2 Schematic diagram of the NAD-binding site of human deoxyhypusine synthase

Only the residues in direct contact with NAD are shown. All the residues except for Ala-309 were targeted for alanine substitution. Hydrogen bonds are shown as dashed lines. Most of the amino acid residues are derived from one subunit of the enzyme (open boxes). The residues in the shaded boxes come from the other tightly associated subunit. The figure has been designed on the basis of results published previously [21].

since ec-eIF5A was hardly detectable in the E·S band of Asp-313Ala. For the wild-type enzyme, the stoichiometry of the complex was determined to be one enzyme tetramer:one ec-eIF5A monomer [26]. From a comparison of the relative intensity of the two bands, most mutant enzymes appeared to form a complex similar to that of the wild-type enzyme. Interestingly, the relative contents of ec-eIF5A in the E·S complexes of Asp-243Ala and Lys-329Ala were much higher than those of the wild-type enzyme and other mutant enzymes. From densitometric analysis of staining intensity, the ratio of enzyme tetramer to ec-eIF5A monomer was estimated to be approximately 1:4 for these two complexes.

Enzyme-imine intermediate formation by the mutant enzymes

The complete reaction catalysed by deoxyhypusine synthase results in the formation of a deoxyhypusine residue in the substrate protein. However, in the absence of eIF5A precursor, accumulation of a covalent enzyme-imine intermediate occurs [18]. This intermediate can be trapped by reduction with NaBH_3CN or NaBH_4 , generating a modified enzyme containing a deoxyhypusine residue in place of Lys-329. Using this technique, a number of the mutant enzymes, when incubated with NAD and labelled spermidine, were also found to form the enzyme

Figure 3 Detection of enzyme tetramer and enzyme-ec-eIF5A complex for the wild-type and mutant enzymes by non-denaturing PAGE and SDS/PAGE

Each enzyme (5 μg), alone or in a mixture with 10 μg of ec-eIF5A, was incubated in 0.1 M glycine/NaOH buffer, pH 9.5, for 20 min at room temperature, and then subjected to electrophoresis under non-denaturing conditions as described previously [26] (upper panel). The band of shifted mobility, denoted by E·S, was excised from the stained native gel and subjected to SDS/PAGE (lower panels). The experiment was repeated three times, yielding similar results. Abbreviations used are: E, enzyme; E·S, complex of enzyme tetramer with ec-eIF5A; 5A, ec-eIF5A; Spd, spermidine; WT, wild type.

Figure 4 Enzyme-intermediate formation by the wild-type and mutant enzymes

Each enzyme (6 μ g) was incubated with 0.5 mM NAD and 3 μ Ci (5 μ M) of [3 H]spermidine in the absence of ec-eIF5A at 37 $^{\circ}$ C for 2 min. The reaction mixture was treated with NaBH₃CN (0.03 mM) on ice for 30 min and precipitated with TCA after addition of carrier BSA (100 μ g). Two-thirds of the TCA precipitates were used for SDS/PAGE. The rest was used for quantification of [3 H]deoxyhypusine, as described previously [18]. The position of the enzyme is indicated by E and the arrow. The experiment was repeated twice, yielding similar results.

intermediates, which were converted into labelled, modified enzymes upon subsequent reduction (Figure 4). Of the spermidine-site mutant enzymes, only Tyr-305Ala was radio-labelled. All of the NAD-site mutant enzymes were able to form intermediates, even though enzyme labelling was barely detectable for Glu-137Ala, Asp-238Ala and Asp-342Ala (labelling at 1%, 1.8% and 6.0% of the wild-type enzyme level respectively). The labelled protein in each case was confirmed as the reduced enzyme intermediate by ion-exchange chromatographic identification of radioactive deoxyhypusine following acid hydrolysis.

Comparison of enzymic activities, spermidine binding and NAD binding of spermidine-site mutant enzymes

Residues proposed to be involved in the spermidine binding and/or in the deoxyhypusine synthase reaction are shown in Figure 1. The activities of spermidine-site mutant enzymes were examined in the partial reactions of spermidine cleavage and enzyme-intermediate formation, and in the complete reaction of deoxyhypusine synthesis in ec-eIF5A (Figure 5A). Of the spermidine-site mutant enzymes, Lys-305Ala was the only one that retained a significant level of the three enzymic activities and spermidine binding. Spermidine binding was totally dependent on NAD in the Lys-305Ala mutant, as in the wild-type enzyme (Figure 5B). For all the rest of the mutants, deoxyhypusine synthesis activity and enzyme-intermediate formation were almost completely abolished. Trp-327Ala, Lys-329Ala, and Asp-316Ala displayed only minimal spermidine cleavage activity (1–6% of the wild-type enzyme), but no detectable enzyme-intermediate formation or deoxyhypusine synthesis activity. In most of these mutant enzymes, the lack of enzymic activities could be attributed to the loss of spermidine binding. For Asp-243Ala, Trp-327Ala, His-288Ala, Asp-316Ala and Glu-323Ala, spermidine binding was less than 2% of the wild-type enzyme at

two different concentrations of spermidine (3.6 μ M and 100 μ M), thus providing evidence that each of these spermidine-site residues is crucial for binding of spermidine, and, therefore, for enzyme activity. Lys-329Ala bound spermidine at 18–24% of the level of the wild-type enzyme, and was able to perform spermidine cleavage, albeit at a low rate. It was, however, totally devoid of deoxyhypusine synthesis activity, because it lacks the active site Lys-329. Lys-329Arg, on the other hand, showed neither spermidine binding nor enzymic activity.

NAD binding to the mutant enzymes was measured at two concentrations, 14.2 and 300 μ M, in the presence or absence of 5 μ M spermidine. Conditions of 300 μ M NAD and 5 μ M spermidine were chosen to simulate the activity assay conditions. NAD binding was not dependent on spermidine. However, an enhancement of NAD binding by spermidine was observed in Lys-305Ala, Lys-329Ala and the wild-type enzyme, each of which is capable of spermidine binding (Figure 5C), but not in any of the other enzymes that are deficient in spermidine binding. This enhancement was more pronounced at 14 μ M than at 300 μ M NAD, and at higher concentrations of spermidine (results not shown). Some alteration in NAD binding was apparent in the spermidine-site mutant enzymes, but these changes were not as dramatic as those of spermidine binding, except for His-288Ala and Lys-305Ala. The level of NAD binding of His-288Ala was found to be < 5% of that of the wild-type enzyme at both low and high concentrations, regardless of the presence of spermidine. Thus the loss of NAD binding in His-288Ala probably contributes to its loss of spermidine binding. The decrease in NAD binding also seemed to be responsible for the reduction of spermidine binding by Lys-305Ala. This enzyme showed a much greater dependence of NAD binding on spermidine than did the wild-type enzyme. NAD binding by both Trp-327Ala and Lys-329Arg was < 4% of the wild-type enzyme at 14 μ M NAD, but reached a significant level (> 40% of the wild-type enzyme) at 300 μ M, suggesting a reduced affinity for

Figure 5 Comparison of enzymic activities (A), spermidine binding (B) and NAD binding (C) of spermidine-site mutant enzymes

(A) Activities in spermidine (Spd) cleavage, enzyme-intermediate formation (Enz-intermediate) and deoxyhypusine (Dhp) synthesis were measured as described in the Experimental section, and as reported previously [17,18,23]. Dhp synthesis was measured in a mixture containing 20 ng of wild-type enzyme or Lys-305Ala, and up to 1 μ g of enzyme for those mutants with extremely low activity ($< 1\%$ of the wild-type activity), 5 μ M [3 H]Spd, 0.5 mM NAD and 9 μ M ec-eIF5A in 20 μ l. Incubations were at 37 $^{\circ}$ C for 30 min. Spd cleavage was measured as above (omitting ec-eIF5A) with 0.4 μ g for the wild-type enzyme and Lys-305Ala, and 5 μ g for other mutants with low activities. Enzyme-intermediate formation was measured as described previously [18]. The levels of activity are expressed as percentages of the wild-type activities, which were 23 pmol/30 min per μ g, 3.7 pmol/2 min per μ g and 1050 pmol/60 min per μ g, respectively, for spermidine cleavage, enzyme-intermediate formation and deoxyhypusine synthesis. (B) Binding of [3 H]spermidine was measured as described previously [22] using 2 μ g of each enzyme, with or without 0.5 mM NAD, in 20 μ l of 0.2 M glycine/NaOH buffer, pH 9.5, 0.5 mM DTT, at 3.6 μ M or 100 μ M [3 H]spermidine. (C) Binding of [3 H]NAD was measured as described previously [22] using 5 μ g of each enzyme at 14.2 μ M or 300 μ M [3 H]NAD, with or without 5 μ M spermidine. The data shown are the averages of three experiments with $\pm$ S.D. indicated by error bars, except where these would be too small to be visible. WT, wild type.

NAD. Other mutants, including Asp-243Ala, Lys-329Ala, Asp-316Ala and Glu-323Ala, displayed a level of NAD binding similar to that of the wild-type enzyme, especially at a high concentration of NAD (300 μ M).

Comparison of enzymic activities, NAD binding and spermidine binding of NAD-site mutant enzymes

Figure 2 shows amino acid residues in the binding site for NAD that were identified from the crystal structure of human deoxyhypusine synthase in a complex with NAD [21]. Enzymic activities of the NAD-site mutant enzymes are shown in Figure 6(A). Thr-308Ala, Gly-283Ala and Ser-317Ala exhibited activities comparable with those of the wild-type enzyme. In contrast, the enzymic activity of Asp-342Ala, Asp-238Ala and Glu-137Ala were $< 5\%$ of the wild-type enzyme. The loss of activity of these

Figure 6 Comparison of enzymic activities (A), NAD binding (B) and spermidine binding (C) of NAD-site mutant enzymes

The activities and the binding of NAD and spermidine were measured in the same way as described in the legend for Figure 5. The values are the averages of three experiments, with $\pm$ S.D. indicated by error bars, except where too small to be visible. WT, wild type.

three enzymes can be explained by their loss of spermidine binding (Figure 6B). Other mutant enzymes showed various degrees of reduction in spermidine binding and activity. In general, the levels of spermidine cleavage activity and enzyme-intermediate formation were similar, and could be related to the degree of spermidine binding [the binding condition for NAD indicated by the black bar in Figure 6(C), and that for spermidine indicated by the grey bar in Figure 6(B), approximate to the levels of NAD (0.5 mM) and spermidine (5 μ M) used in the activity assays]. However, deoxyhypusine synthesis activity was reduced differentially to an even lower level than spermidine cleavage or enzyme-intermediate formation in Asp-313Ala, Asn-106Ala, Ser-105Ala, Thr-131Ala and Ser-109Ala. The lack of deoxyhypusine synthesis by Asp-313Ala is presumably due to its inability to bind ec-eIF5A (Figure 3). The disproportionate decrease in the ability to synthesize deoxyhypusine by the other four mutant enzymes suggests that the substitutions not only affected binding of NAD and/or spermidine, but also influenced later steps of the deoxyhypusine synthesis reaction.

All but one of the NAD-site mutant enzymes showed a reduced level of NAD binding (1–30% of that of the wild-type at 14.2 μ M; Figure 6C). Most of the NAD-site enzymes, including Asn-106Ala, Thr-308Ala, Gly-283Ala, Ser-317Ala, Ser-105Ala, Thr-131Ala and Ser-109Ala, also displayed a stronger dependence of NAD binding on spermidine than the wild-type enzyme (7–50-fold enhancement by spermidine compared with 2.5-fold for the wild-type enzyme at 14.2 μ M). Low NAD binding by

these mutant enzymes appeared to be either partly or fully overcome at high concentrations of NAD in the presence of spermidine (black bars in Figure 6C). In contrast with other NAD-site mutant enzymes, Glu-137Ala exhibited even greater NAD binding than the wild-type enzyme (at 14 μ M). Interestingly, this enzyme was totally defective in spermidine binding, suggesting that Glu-137 is critical for the binding of spermidine. Mutations involving Asp-342, which is predicted to interact with the adenine ring of NAD, caused a drastic reduction in NAD binding, suggesting that this residue is important for nucleotide anchoring. This loss of NAD binding is likely to be responsible for the loss of spermidine binding and enzyme activities of Asp-342Ala. On the other hand, Asp-238Ala appeared to be defective in the binding of both NAD and spermidine, since the binding of spermidine (< 1 % of the wild-type enzyme; Figure 6B) was even lower than could be accounted for by the reduced NAD binding (14 % of the wild-type enzyme; Figure 6C).

Kinetic parameters of the mutant enzymes

Direct estimation of the association constants for NAD and spermidine was not possible, because the binding step cannot be dissociated from the enzymic reaction in the presence of NAD and spermidine. Thus we compared the kinetic parameters of the mutant enzymes in the deoxyhypusine synthesis reaction. K_m and V_{max} values of NAD and spermidine for deoxyhypusine synthesis were determined with the mutant enzymes (Table 1). Those enzymes that showed no activity, such as Lys-329Ala and Lys-329Arg, and those with activities too low to make a reliable estimation (< 0.1 % of the wild-type activity), were excluded. Since the spermidine concentration employed was less than saturation for technical reasons, the apparent V_{max} values obtained upon varying the NAD concentration are not 'true' maximum velocities. The V_{max} values obtained at a fixed concentration of NAD with varying spermidine concentrations are better approximations for these parameters. Large increases in the K_m values were observed for NAD (K_m^{NAD}) in Lys-305Ala, Asn-106Ala and Ser-317Ala mutants, and for spermidine ($K_m^{Spermidine}$) in Asn-106Ala and Ser-109Ala. The mutant enzymes Ser-105Ala, Thr-131Ala and Ser-109Ala displayed a marked reduction in V_{max} . The values for the apparent catalytic efficiency, calculated here as V_{max}/K_m for spermidine, were > 50 % of the wild-type value for Lys-305Ala, Thr-308Ala, Gly-283Ala and Ser-317Ala, but were reduced by more than 7-fold for Asn-106Ala, Ser-105Ala, Thr-131Ala and Ser-109Ala.

DISCUSSION

Deoxyhypusine synthase is highly conserved in all eukaryotes [8]. Deoxyhypusine and deoxyhypusine synthase-like sequences have also been found in several strains of Archaea, although the sequences are not as highly conserved. The amino acids at the spermidine site (Asp-243, Trp-327, His-288, Lys-329, Asp-316, Glu-323 and Tyr-305) are totally conserved in all eukaryotes. Of these, the active-site residue Lys329, as well as Trp-327 and Glu-323, are also conserved in all Archaea reported so far. Of the NAD-site residues, Asp-313, Ser-105, Asp-238, Ser-109 and Glu-137 are strictly conserved in all eukaryotes, and Asp-342 is either conserved or replaced with glutamate. Substitution of Asp-313, Asp-238 and Asp-342 with alanine caused an almost complete loss of NAD binding and enzymic activity. The effect of substitution of Ser-105 and Ser-109 with alanine was less drastic, presumably because of the structural similarity between serine and alanine. With Ser-105Ala and Ser-109Ala, the decrease in

deoxyhypusine synthesis activity was greater than that which could be accounted for by the decrease in the binding of NAD and spermidine, suggesting that these residues are critical for the enzymic reaction. Thus the importance of the residues derived from this site-directed mutagenesis study appears to be consistent with the conservation of amino acid sequences.

The substitution of a single amino acid residue in each mutant protein did not appear to cause a gross change either in protein folding or in the global conformation. The mutant enzymes displayed a similar elution profile upon ion-exchange chromatography, and appeared to form a tetrameric structure, like the wild-type enzyme. Their ability to form stable complexes with eIF5A (with the exception of Asp-313Ala) supports further their structural resemblance. It seems likely, therefore, that the complex changes in NAD and spermidine binding and in the enzyme activities, in most cases, result from changes in the local structure surrounding the active site. The only mutant enzyme that might have minor structural changes beyond the active site is Asp-313Ala, which does not form a stable complex with eIF5A.

Substitutions at the proposed spermidine-binding site had striking effects on spermidine binding and enzymic activities. Except for Lys-305Ala, substitution in each case caused an almost complete loss of spermidine binding and activity, suggesting that each of the amino acid residues is crucial for binding and activity. On the other hand, most substitutions at the NAD site, with the exception of Asp-342Ala, Asp-313Ala and Asp-238Ala, did not abolish NAD binding, but appeared to reduce the affinity of NAD binding in an incremental fashion. This contrast may be due, at least in part, to the fact that the number of amino acids involved in anchoring NAD is greater than that involved in spermidine binding. In addition, substitution of spermidine-site amino acids with alanine, in every case, led to a drastic change in the charge, hydrophobicity or size of the residue. However, mutation of the NAD-site residues is limited by the fact that some of the substituted residues are not substantially different from alanine.

The related binding study with the wild-type enzyme suggests that the initial binding of NAD induces a conformational change in the enzyme, which allows subsequent binding of spermidine [22]. Quenching of the intrinsic tryptophan fluorescence of the enzyme was observed upon addition of NAD to the enzyme. This was followed by additional quenching of tryptophan fluorescence upon addition of spermidine [27] or GC₇ (E. C. Wolff, personal communication), suggesting a conformational change during the course of the ternary complex formation. Most of the mutant enzymes studied here appear to follow an ordered addition of NAD and spermidine, since the binding of spermidine is dependent on NAD. For the wild-type and most of the mutant enzymes, NAD binding does not require spermidine, but is strongly enhanced by its presence. There are other cases, e.g. Clostridial glutamate dehydrogenase, in which a substrate or its analogue increases the affinity of cofactor binding [28]. The stimulatory effect of spermidine on NAD binding appeared to be more pronounced in certain of the mutant enzymes, i.e. those with reduced affinity for NAD.

The binding pattern of the mutant enzymes can be interpreted in terms of the three-dimensional structure of the enzyme [21]. The loss of spermidine binding (with retention of NAD binding) in the mutant enzymes Asp-243Ala, Asp-316Ala and Glu-323Ala confirms the importance of the carboxyl groups of Asp-243, Asp-316 and Glu-323 in the anchoring of spermidine via salt bridges to the terminal-end amino groups of spermidine. For other spermidine-site mutant enzymes, substitution of Trp-327, His-88 and, to a lesser extent, Tyr-05, with alanine resulted in decreased binding of both spermidine and NAD, suggesting that replace-

ment of each of these amino acids caused some alteration in the topology of the NAD-binding site, as well as loss in spermidine binding. The imidazole ring of His-288 lines the spermidine-binding site, with N(ϵ 2) pointing towards the binding site, lying approximately in parallel with the nicotinamide ring of NAD. His-288 can potentially form a salt bridge or hydrogen-bond with Asp-238 of the adjacent NAD molecule, which forms an hydrogen-bond with the nicotinamide ribose. Furthermore, His-288 is in a helix that follows one of the NAD-binding loops of the classic Rossmann fold [29] (Gly-282, Gly-283 and Gly-284). Thus the His-288Ala mutation might perturb NAD binding by distorting the Rossmann fold, or by abolishing the interaction of His-288 with either the nicotinamide ring or Asp-238. Tyr-305 is located at the bottom of the spermidine-binding groove, and it is not likely to interact directly with spermidine; nor does it make a direct contact with NAD. Its contribution to NAD binding may lie in its position neighbouring a strand that leads into an NAD-binding loop, and that interacts with the adenine ring of NAD. In the case of Lys-329Arg and Trp-327Ala, a partial reduction in NAD binding cannot account for the total lack of spermidine binding. Indeed, these mutants appear to be defective in the binding of both NAD and spermidine. Trp-327 is located opposite both the nicotinamide ring of NAD and the imidazole ring of His-288, and may stabilize NAD binding. More importantly, Trp-327 may contribute to spermidine binding via hydrophobic interactions with the aminopropyl chain of spermidine, or by π -cation interaction [30]. For Lys-329Arg, the bulky guanidine moiety of arginine probably crowds the narrow neck of the spermidine-binding groove and prevents spermidine binding. The crowding around His-288 caused by this substitution with arginine might also contribute to the reduced NAD binding of Lys-329Arg. In contrast, the Lys-329Ala mutation would create a hole in the active site instead of crowding. Binding of NAD is normal, or even increased, in Lys-329Ala, whereas its binding of spermidine is weakened, possibly due to the extra space in the pocket. Of the NAD-site mutant enzymes, Asp-342Ala, Asp-238Ala and Asp-313Ala were defective in the binding of NAD, and consequently of spermidine, suggesting the critical importance of anchoring at the adenine ring and the nicotinamide ribose for binding of NAD in a correct conformation. Most of the NAD-site mutant enzymes showed a weakened binding of NAD, as expected, and a stronger dependence of its binding on spermidine. Somewhat unexpected results were obtained with Glu-137Ala. Glu-137 was predicted to be involved in hydrogen-bonding to N7 of the nicotinamide ring of NAD via an appreciable distance [4.7 Å (1 Å $\equiv$ 0.1 nm)]. However, NAD binding by Glu-137Ala was higher than that by the wild-type enzyme, whereas its spermidine binding was totally abolished, suggesting that Glu-137 is vital for the binding of spermidine, but not for that of NAD. Its role in spermidine binding might be explained from the fact that Glu-137 is hydrogen-bonded to the hydroxy group of Tyr-176, which forms a portion of the entrance of the spermidine-binding pocket. Thus it may strengthen spermidine binding by stabilizing the side chain conformation of Tyr-176, or it may contribute by adding overall negative charge to the spermidine-binding site, even though there are no direct salt bridges to spermidine.

In a previous paper [26], we have presented evidence for a stable complex between the wild-type enzyme and ec-eIF5A with a stoichiometry of 1:1 (enzyme tetramer:ec-eIF5A monomer). All the mutant enzymes except for Asp-243Ala and Lys-329Ala appear to form a complex similar to that of the wild-type enzyme. This is an intriguing phenomenon, since the enzyme tetramer contains four binding sites for both NAD and spermidine at the dimer interfaces. The crystal structure of eIF5A analogues from

two thermophilic Archaea species [31,32] reveals a long molecule, with a length of 63 Å and a width of 26 Å. It is conceivable that binding a molecule of this size to the enzyme could preclude the binding of a second molecule. The binding of the first ec-eIF5A molecule may cause a conformational change in the enzyme that prevents further binding. In the case of the two mutant enzymes, Asp-243Ala and Lys-329Ala binding of the first ec-eIF5A molecule apparently does hinder the binding of additional molecules. Efforts are at present underway to determine the X-ray structures of the ec-eIF5A complexes of both the wild-type enzyme and the two mutant enzymes.

The X-ray structure of another spermidine-binding protein, a bacterial polyamine transporter protein (PotD), in a complex with spermidine has been determined [33], and the roles of a number of amino acid residues at the spermidine-binding site have been evaluated by site-directed mutagenesis [34]. There are some similarities to the binding of spermidine to deoxyhypusine synthase, in that the two terminal amino groups of spermidine are anchored by acidic residues. However, clear differences exist between the spermidine-binding sites of the two proteins. In the case of the PotD protein, the residues most critical for spermidine binding are those interacting with the propylamine portion of spermidine, and those at its N1 and secondary amino groups. Several tryptophan and tyrosine residues lining the spermidine pocket are also important. Whereas deoxyhypusine synthase has no acidic residue anchoring spermidine at the secondary amino group, and is therefore unable to accommodate the binding of putrescine, the PotD protein is able to manage this. Thus the strict specificity of deoxyhypusine synthase can be explained, in part, by the critical importance of Asp-243, Asp-316 and Glu-323 in anchoring spermidine via its terminal amino groups.

The present study validates the topography of the spermidine binding site proposed previously [21], and demonstrates that Asp-243, Trp-327, Asp-316, Glu-323 and Glu-137 are critical for the binding of spermidine. It also permits the assessment of the relative importance of the amino acid residues in the NAD-binding site, and points to a role of His-288, Asp-342, Asp-313 and Asp-238 in the binding of NAD. Furthermore, it suggests that Trp-327, His-288 and Asp-238 are also important for the proper binding of both NAD and spermidine. Although supporting, in general, the previously proposed modes of binding of NAD and spermidine, it provides new insights into the role of certain individual amino acids, e.g. His-288, Glu-137 and Asp-313, that could not be predicted from the crystal structure. Furthermore, the binding properties and catalytic activities of the individual mutant enzymes unravel further complexities in the molecular interactions involved in the multi-step deoxyhypusine synthase reaction.

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