Human deoxyhypusine synthase: interrelationship between binding of NAD and substrates

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Deoxyhypusine synthase catalyses the NAD-dependent transfer of the butylamine moiety from the polyamine, spermidine, to a specific lysine residue of a single cellular protein, eukaryotic translation-initiation factor 5A (eIF5A) precursor. The native enzyme exists as a tetramer of four identical subunits and contains four binding sites for NAD. The binding of spermidine and NAD was studied by a filtration assay. [³H]Spermidine binding to the enzyme was not detectable alone or in the presence of the eIF5A precursor, but was detected only in the presence of NAD or NADH, suggesting that a NAD/NADH-induced conformational change is required for the binding of spermidine. A strong NAD-dependent binding was also observed with a spermidine analogue, *N*¹-guanyl-1,7-diamino[³H]heptane (GC₇),

but not with [¹⁴C]putrescine or [¹⁴C]spermine. Although [³H]NAD binding to the enzyme occurred in the absence of spermidine, its affinity for the enzyme was markedly enhanced by spermidine, GC₇, and also by the eIF5A precursor. The maximum binding for NAD and spermidine was estimated to be ≈ 4 molecules each/enzyme tetramer. The dependence of spermidine binding on NAD and the modulation of binding of NAD by spermidine and the eIF5A precursor suggest intricate relationships between the binding of cofactor and the substrates, and provide new insights into the reaction mechanism.

Key words: eIF5A, hypusine, spermidine.

INTRODUCTION

The post-translational synthesis of deoxyhypusine [*N*^ε-(4-amino-butyl)lysine] catalysed by deoxyhypusine synthase is the first step in the biosynthesis of hypusine [*N*^ε-(4-amino-2-hydroxybutyl)lysine] [1,2]. This reaction involves modification of the eukaryotic translation-initiation factor 5A (eIF5A) precursor protein by the NAD-dependent transfer of the butylamine moiety from the polyamine, spermidine, to the ϵ -amino group of a specific lysine residue of the substrate protein [3–6]. Hypusine synthesis represents the most specific post-translational modification known to date.

eIF5A and deoxyhypusine synthase are both highly conserved proteins that are ubiquitous in eukaryotes [1,2,7]. There is abundant evidence supporting the notion that hypusine is essential for eukaryotic cell proliferation. Inactivation of the two *eIF5A* genes [8,9] or the deoxyhypusine synthase gene [10,11] in *Saccharomyces cerevisiae* results in the loss of cell viability. Furthermore, inhibitors of deoxyhypusine synthase [12,13] or deoxyhypusine hydroxylase [14] cause the arrest of growth in various mammalian cells. Cytostasis was attributed to the deprivation of eIF5A in L1210 cells depleted of spermidine by an inhibitor of *S*-adenosylmethionine decarboxylase [15,16].

Human deoxyhypusine synthase in its native state exists as a tetramer of four identical subunits of 41 kDa. It catalyses a complex reaction involving a cofactor, NAD, and two substrates, spermidine and eIF5A precursor. In a complete reaction mixture containing spermidine, NAD and eIF5A precursor, the enzyme catalyses the cleavage of spermidine to generate 1,3-diaminopropane and the enzyme-bound 4-aminobutyl moiety, which is transferred to a lysine residue of the eIF5A precursor to form a deoxyhypusine residue [6]. In the absence of the eIF5A precursor, however, the enzyme generates 1,3-diaminopropane and Δ^1 -

pyrroline as cleavage products of spermidine [17]. Based on previous studies, we proposed [18] a mechanism of deoxyhypusine synthesis that occurs by way of four substeps: (i) NAD-dependent dehydrogenation of spermidine, (ii) formation of an enzyme-imine intermediate by transfer of the butylamine moiety of dehydrospemidinium 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 proposed pathway for the deoxyhypusine synthase reaction, including the current results, is shown in Scheme 1.

A remarkable feature of deoxyhypusine synthase is its specificity towards NAD, spermidine and the eIF5A precursor protein. Studies using compounds structurally related to spermidine or NAD as inhibitors of the reaction provided evidence for the basis of its high specificity towards spermidine [12] and NAD [6], respectively. The three-dimensional crystal structure of the NAD complex of human deoxyhypusine synthase was determined recently [19]. It reveals a symmetrical tetrameric organization of the enzyme with 4 NAD molecules bound at the interfaces of two subunits. The NAD-binding site of deoxyhypusine synthase displays a fairly typical 'Rossmann fold', a structure common to most NAD/NADH-binding proteins [20]. Although 1,7-diaminoheptane, a structural analogue of spermidine, was included in the crystallization mixture as a probe for the spermidine-binding site, it was not detected in the crystal structure. Nevertheless, based on the orientation of the nicotinamide ring of NAD bound to the enzyme and the active-site lysine residue (Lys-329), the mode of spermidine binding with its N-4–C-5 bond in juxtaposition with the nicotinamide ring could be predicted. Deoxyhypusine synthase catalyses an NAD-dependent cleavage of spermidine in the absence of the eIF5A precursor, suggesting

Abbreviations used: eIF5A, eukaryotic translation-initiation factor 5A; GC₇, *N*¹-guanyl-1,7-diaminoheptane; PEI, polyethylenimine.

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Scheme 1 Proposed mechanism of the deoxyhypusine synthase reaction

The four reaction steps proposed previously [18] are indicated by 1–4, along with a newly proposed parallel pathway that involves the initial binding of eIF5A. The proposed non-covalent complexes between enzyme, the cofactor and the substrates/products are shown in boxes. The postulated reaction intermediates are shown in shaded ovals. The enzyme-imine intermediate ($E = Bt \cdot NADH$) involving Lys-329 of the human enzyme has been identified [18]. An abortive cycle leading to cleavage of spermidine in the absence of eIF5A precursor is indicated by steps 1 and 2 and the broken arrows. Abbreviations: E, enzyme; Spd, spermidine; DhSpd, dehydrospemidine; Dap, 1,3-diaminopropane; Δ^1 -pyr, Δ^1 -pyrroline; Dhp, deoxyhypusine; Lys, lysine; 5A(Lys), eIF5A precursor with Lys residue at position 50; 5A(Dhp), eIF5A precursor with deoxyhypusine residue at position 50; $E = Bt$, the enzyme-imine intermediate with butylamine moiety attached to Lys-329 in an imine linkage; $5A = Bt$, eIF5A-imine intermediate with butylamine moiety attached to Lys-50 in an imine linkage.

that the binding of NAD and spermidine can occur independently of the eIF5A precursor. Furthermore, the enzyme can bind the eIF5A precursor in the absence of NAD and spermidine [21]. However, the relationship between the binding of NAD and spermidine is not known, e.g. whether binding at one site affects the binding of another and whether all four potential active sites participate in catalysis. The present paper provides evidence that (i) the binding of NAD and spermidine follows an ordered type of addition, (ii) all four potential active sites can be engaged simultaneously in the NAD-dependent dehydrogenation of spermidine and spermidine cleavage, and (iii) there is a complex interrelationship between the binding of NAD, spermidine and the eIF5A precursor.

EXPERIMENTAL

Materials

[1,8- 3H]Spermidine·3HCl (27.6 Ci/mmol) and ^{125}I -labelled Bolton–Hunter reagent [*N*-succinimidyl-3-(4-hydroxy-3- ^{125}I)iodophenyl]propionate; 2200 Ci/mmol} were purchased from Dupont NEN; [4- 3H]NAD (3.54 Ci/mmol) and [^{14}C]spermidine (111 mCi/mmol) were from Amersham. [^{14}C]Putrescine (110 mCi/mmol) was from American Radiolabelled Chemicals. *N*¹-Guanyl-1,7-diaminoheptane (GC₇) [12] and [3H]GC₇ [13] were synthesized in our laboratory. Glass-fibre filters (GF/B, 25 mm diameter) were obtained from Whatman and polyethylenimine (PEI) was from Sigma. The MonoQ column and Q-Sepharose (fast flow) resin were from Amersham Pharmacia Biotech.

Human eIF5A precursor protein was purified from *Escherichia coli* lysates after overexpression of the human eIF5A cDNA according to a published procedure [22]. Human deoxyhypusine synthase was purified from the lysates of *E. coli* BL21(DE3) overexpressing the human deoxyhypusine synthase cDNA to 90–95% homogeneity as described previously [18,23]. Human deoxyhypusine synthase was iodinated using Bolton–Hunter reagent according to a published procedure [24] with minor

modifications. NAD and NADH (100% pure) were purchased from Boehringer Mannheim. [3H]NADH was prepared from [3H]NAD with the use of alcohol dehydrogenase (Roche Molecular Biochemicals). The purity of [3H]NADH was determined by ion-exchange chromatography [17], and highly pure ($\approx 95\%$) [3H]NADH was used for binding studies. Other reagents were from commercial sources as indicated in the text.

Binding assays for spermidine and NAD

Of a variety of filters tested, glass-fibre filters (Whatman GF/B) presoaked in 0.3% (v/v) PEI for > 2 h were found to retain the enzyme effectively with low levels of background binding of [3H]spermidine or [3H]NAD to the filters. Therefore, these filters were used in a rapid-filtration assay [25,26]. A typical assay mixture for spermidine binding contained 2 μ g of enzyme, 0.5 mM dithiothreitol, 2 μ Ci (3.6 μ M) of [1,8- 3H]spermidine in 20 μ l, 0.2 M glycine/NaOH buffer, pH 9.5, and 0.5 mM NAD. Incubations were conducted at room temperature for 10 min. The mixtures were filtered under vacuum through PEI-treated filters and the filters were washed quickly three times with 5 ml of ice-cold glycine/NaOH buffer. The filters were left overnight after addition of scintillation fluid for complete release of radioactivity and the radioactivity was measured using a liquid-scintillation counter (Beckman). Specific binding of spermidine was calculated from the total bound radioactivity minus non-specific binding, which was measured in the absence of the enzyme with or without an equal amount of BSA or deoxyhypusine synthase K329R mutant enzyme, which is totally devoid of spermidine binding.

The NAD-binding assay was carried out using the same method as for spermidine binding but with 5 μ g of enzyme and 1 μ Ci (14.2 μ M) [3H]NAD. Unlabelled NAD and spermidine were added as indicated. Incubation time was 20 min. In each case the data shown in the Figures and Table are representative

of two or more experiments which, although conducted under slightly different conditions, displayed similar results.

Determination of spermidine bound to the enzyme in the presence of NAD

Spermidine bound under the reaction conditions (in the presence of NAD) was estimated after taking into account the fact that the specific activities of the spermidine cleavage products are half that of [^3H]spermidine, and after correction for distribution of radioactivity in spermidine/dehydrospermidine, the enzyme intermediate, Δ^1 -pyrroline and pyrrolidine, as described below (dehydrospermidine is postulated to be formed in the initial step of the deoxyhypusine synthase reaction by the NAD-dependent dehydrogenation of spermidine, but no physical evidence has been obtained for its existence, and since our ion-exchange chromatography system does not separate dehydrospermidine from spermidine, it is not possible to determine the relative amounts of enzyme-bound spermidine and dehydrospermidine before or after reduction). The [^3H]spermidine-binding assay filters were treated with 0.1 M NaBH_3CN for 40 min on ice. Carrier BSA (0.5 mg) was added and the proteins in the mixture were precipitated with 10% trichloroacetic acid. The amount of radioactivity remaining on the filters and in the trichloroacetic acid precipitates after thorough washes with 10% trichloroacetic acid containing 1 mM unlabelled polyamines was taken as a measure of the covalent enzyme intermediate [18]. This was confirmed by ion-exchange chromatographic identification of labelled deoxyhypusine in acid-hydrolysed samples. The radioactive materials in the trichloroacetic acid supernatant were analysed by ion-exchange chromatography using the three-buffer system described previously [18] and were identified as spermidine/dehydrospermidine, Δ^1 -pyrroline and pyrrolidine.

Determination of enzyme-bound NAD and NADH

Radioactive materials from [^3H]NAD-binding assay filters were eluted by treatment with 20 mM triethanolamine buffer, pH 7.7, at 50 °C. After removal of enzyme by ultrafiltration (Centricon10, Amicon), the labelled nucleotides with 50 nmol each of unlabelled NAD and NADH were separated by chromatography on a MonoQ (5/5) column as described in [17]. Elution positions of NAD and NADH were identified by measurement of absorption at 260 and 340 nm, and radioactivity was measured.

RESULTS

A filtration assay, developed for the determination of hormone binding to receptors and utilized for ligand binding of other proteins [25–27], offered a simple and convenient method for measurement of the binding of spermidine and NAD to human deoxyhypusine synthase. Efficacy of binding of the enzyme to the PEI-treated glass filter was determined using ^{125}I -labelled enzyme (results not shown). Owing to the acidic nature of the enzyme (pI 5.1), its binding to the filter coated with positively charged PEI was nearly complete in the range of 1–10 μg at pH 6–10.

Binding of NAD to deoxyhypusine synthase

Binding of [^3H]NAD to the enzyme could be demonstrated in the absence of spermidine. Under the experimental conditions of Figure 1(A), the binding of NAD reached a maximum level after 20 min at room temperature. NAD showed binding at a broad range of pH (pH 6–9.5), with its optimum binding at pH 9.5. The

Figure 1 Time and pH dependence of binding of NAD (A) and spermidine (B) to deoxyhypusine synthase

(A) NAD binding. For time dependence, the mixture containing 5 μg (≈ 125 pmol of monomer) of the enzyme and 1 μCi of [^3H]NAD (14.2 μM) in pH 9.5 buffer was incubated for 2–30 min. For pH dependence, the mixture containing 5 μg of enzyme and 1 μCi of [^3H]NAD in 0.2 M glycine/NaOH buffer at the indicated pH was incubated for 20 min. (B) Spermidine (Spd) binding. For time dependence, the mixture containing 2 μg (≈ 50 pmol of monomer) of deoxyhypusine synthase protein, 0.5 mM NAD and 2 μCi of [^3H]spermidine (3.6 μM) in 0.2 M glycine/NaOH buffer, pH 9.5, was incubated for the indicated time. For pH dependence, spermidine binding was determined as above, except that the pH of the 0.2 M glycine/NaOH buffer was varied from pH 6 to 11. Incubation time was 10 min. The experiment was repeated twice with similar results.

enzyme-bound radioactivity increased linearly with the amount of the enzyme in the range of 1–10 μg (results not shown).

Dependence of spermidine binding on NAD/NADH

In a mixture containing human deoxyhypusine synthase and [^3H]spermidine, binding of radioactive spermidine to the enzyme was not detectable regardless of the pH of the binding buffer, or spermidine concentration. When NAD was included in the mixture, rapid binding of radioactivity from [^3H]spermidine was observed, suggesting that binding of NAD to the enzyme induces the binding of spermidine. However, in the presence of NAD, spermidine undergoes an enzyme-mediated cleavage (Scheme 1). Therefore, in this case, the enzyme-bound radioactivity would be a mixture of spermidine and the reaction intermediates, dehydrospermidine and the enzyme-imine intermediate.

As shown in Figure 1(B), spermidine binding to the enzyme measured in the presence of NAD reached a maximum level within 10 min at room temperature. The pH-dependence plot shows optimum binding of spermidine at pH 9–9.5. The pH optimum of spermidine binding suggests that the enzyme indeed binds the N^1,N^8 -diprotonated form of spermidine, which is most abundant at pH 9–9.5 [28] and which has been predicted to be the reactive form [12,21]. The pattern of pH dependence is similar to that of the deoxyhypusine synthesis reaction [3],

Figure 2 Dependence of spermidine binding on NAD/NADH (A, B) and spermidine (C) concentration

(A) The mixture contained 2 μg of the enzyme, 1 μCi (1.8 μM) of [^3H]spermidine (Spd) and various concentrations of NAD ($\blacktriangledown$) or NADH ($\blacksquare$). (B) The same as in (A) except that the total concentration of spermidine ([^3H]spermidine plus unlabelled spermidine) was 100 μM . (C) The mixture contained 2 μg of the enzyme, and the indicated total concentration of spermidine without ($\bullet$) or with 500 μM NAD ($\blacktriangledown$) or NADH ($\blacksquare$). The experiment was repeated three times with similar results.

Figure 3 Specificity of spermidine binding to deoxyhypusine synthase

(A) Binding of polyamines. The mixture contained 10 μg of the enzyme, and [^3H]spermidine (Spd), [^{14}C]putrescine (Ptc) or [^{14}C]spermine (Spm), each at 100 μM , without (open bar) or with (solid bar) 0.5 mM NAD. (B) The inhibition of spermidine binding by GC_7 . The binding mixture contained 2 μg of enzyme, and 3.6 μM [^3H]spermidine without NAD (bar 1) or with 0.5 mM NAD (bars 2–5) together with GC_7 as indicated: none (bar 1), 10 μM (bar 3), 100 μM (bar 4) and 1 mM (bar 5). (C) The inhibition of GC_7 binding by spermidine. The binding mixture contained 2 μg of enzyme and 2.7 μM [^3H]GC $_7$ without (bar 1) or with NAD (bars 2–5) together with spermidine as indicated: none (bar 2), 10 μM (bar 3), 100 μM (bar 4) and 1 mM (bar 5). The experiment was repeated twice with similar results.

indicating that spermidine binding is the determining factor underlying pH dependence of the enzyme reaction. A linear relationship was observed between the amount of enzyme used (1–10 μg) and the amount of radioactivity bound (results not shown).

The binding of spermidine to deoxyhypusine synthase was also induced by NADH (Figure 2A and 2B), but not by NADP or NADPH (results not shown). NADH cannot support dehydrogenation and cleavage of spermidine by deoxyhypusine synthase. Thus [^3H]spermidine bound to the enzyme in the presence of NADH must remain as spermidine. At a low concentration of [^3H]spermidine (1.8 μM), NADH was much weaker than NAD in induction of spermidine binding, especially at low concentrations (Figure 2A). This is probably because the affinity for NADH is much lower than that for NAD (see Figure 4 below). However, at high concentrations of spermidine (100 μM ; Figure 2B) or NAD/NADH (500 μM ; Figure 2C), the differential effects of the two nucleotides were less apparent. At 100 μM spermidine (Figure 2B), half-maximal binding of spermidine was observed at ≈ 7 μM NAD. At a fixed concentration (500 μM) of NAD or NADH, the maximum binding of spermidine was observed at > 20 μM spermidine (Figure 2C). At these concentrations of spermidine and NADH, the maximum amount of spermidine bound was calculated to be ≈ 3.7 molecules/enzyme

tetramer, suggesting that all four sites can be occupied simultaneously by spermidine. A similar estimate was obtained when binding was measured in the presence of NAD. From a Scatchard analysis of spermidine binding in the presence of NADH, the K_d value for spermidine binding was estimated to be ≈ 1 μM . This value is much lower than the K_m (7.2 μM) reported for spermidine for the deoxyhypusine synthesis reaction [23], suggesting that binding of spermidine is not a rate-limiting step in the deoxyhypusine synthesis reaction.

Specificity of spermidine binding to deoxyhypusine synthase

The features of the spermidine-binding site of deoxyhypusine synthase and the mode of spermidine binding were predicted from inhibitor studies [12] and the X-ray structure [19]. The development of a binding assay permits direct assessment of the specificity of spermidine binding to the enzyme. Putrescine and spermine, even at 100 μM , displayed little binding to deoxyhypusine synthase, regardless of the presence of NAD (Figure 3A). This is consistent with the poor inhibition of the enzyme reaction by these compounds [12]. On the other hand, GC_7 , a potent inhibitor of the enzyme, exhibited a strong and NAD-dependent binding (Figure 3C, bars 1 and 2). Moreover, this compound caused an effective inhibition of the binding of

Table 1 Identification of enzyme-bound materials derived from (A) [1,8-³H]spermidine and (B) [³H]NAD

The amounts of enzyme used for spermidine and NAD binding were 2 and 5 μ g, respectively. Incubation time was 30 min. In the case of spermidine binding, radioactivity in the trichloroacetic acid (TCA) precipitate represents the covalent enzyme-imine intermediate; radioactivity in the TCA supernatant represents spermidine/dehydrospemidine or its cleavage product, non-covalently associated with the enzyme. The experiment was repeated twice with similar results.

(A)							
[³ H]Spermidine (μ M)	NAD (μ M)	NADH (μ M)	Total bound (pmol)	TCA supernatant (% of total bound)			TCA precipitate (% of total bound) Deoxyhypusine
				Δ^1 -Pyrroline	Pyrrolidine	Spermidine	
3.6	500	0	27	5.3	36.8	7.5	50.4
100	500	0	48	5.6	27.2	39	28.2
3.6	0	100	16	< 0.5	< 1	> 95	< 1.5
100	0	100	46	< 0.5	< 0.5	> 95	< 1.5

(B)					
[³ H]NAD (μ M)	Spermidine (μ M)	Total bound (pmol)	NAD (% of total bound)	NADH (% of total bound)	
100	100	115	5.1	94.9	
100	0	68	> 95	< 2	

spermidine in a concentration-dependent manner (Figure 3B, bars 3–5). The binding of GC₇ was also inhibited by spermidine (Figure 3C, bars 3–5). Spermidine was not as strong an inhibitor of GC₇ binding as GC₇ was an inhibitor of spermidine binding, consistent with the finding that the K_i value for GC₇ (≈ 10 nM) as an inhibitor of deoxyhypusine synthase is much lower than the K_m of spermidine. These data indicate that GC₇ indeed competes with spermidine for binding at the active sites of deoxyhypusine synthase.

Identification of enzyme-bound radioactive material derived from [³H]spermidine

Since spermidine undergoes the reactions shown in Scheme 1 in the presence of NAD and deoxyhypusine synthase, the enzyme-bound radioactive materials derived from [³H]spermidine were analysed (Table 1A). The relative amounts of enzyme-bound spermidine cleavage products varied with time of incubation and spermidine concentration. For samples containing 3.6 μ M spermidine, after a 30-min incubation, the enzyme-bound radioactivity was mostly in the form of the spermidine cleavage products, enzyme-imine intermediate (50.4%) and enzyme-bound Δ^1 -pyrroline/pyrrolidine (≈ 42.1 %). Only 7.5% of radioactivity was in the position of spermidine/dehydrospemidine. The percentage of enzyme-bound radioactivity in the spermidine cleavage products was lower at 100 μ M spermidine than at 3.6 μ M. In a mixture containing NADH instead of NAD, essentially all the radioactivity associated with the enzyme was found in spermidine.

Identification of enzyme-bound NAD and NADH

In the presence of spermidine, NAD rapidly undergoes reduction to NADH in the initial step of the reaction and stays as enzyme-bound NADH in the absence of the eIF5A precursor (Scheme 1) [29]. In a mixture containing both [³H]NAD (100 μ M) and spermidine (100 μ M), most of the enzyme-bound radioactivity was found in NADH (95%) and only a small fraction (5%) in NAD (Table 1B). On the other hand, in a mixture without spermidine (Table 1B) or in a mixture containing GC₇ in place of spermidine (results not shown), only radiolabelled NAD was found as the enzyme-bound nucleotide.

Figure 4 Enhancement of binding of NAD (A) and NADH (B) by spermidine and GC₇

(A) The mixture contained 5 μ g of enzyme protein and increasing total concentrations of NAD ([³H]NAD plus NAD), without (●) or with 100 μ M spermidine (■) or GC₇ (△). (B) The same as in (A) except for the addition of [³H]NADH instead of [³H]NAD. The experiment was repeated twice with similar results.

Modulation of binding of NAD/NADH by spermidine and GC₇

Although binding of NAD to deoxyhypusine synthase occurs in the absence of spermidine, spermidine and its analogue, GC₇, cause a marked enhancement of binding of NAD (Figure 4A). In the absence of spermidine, even at a high concentration of NAD (100 μ M), only 2–2.5 molecules of NAD were bound/enzyme tetramer. However, the number of NAD molecules bound at this concentration increased to close to 4 molecules/enzyme tetramer upon addition of spermidine (50 μ M; results not shown). Binding of NADH was barely detectable in the absence of spermidine or GC₇ (Figure 4B). Like NAD, NADH binding was drastically enhanced by spermidine and GC₇. Parenthetically, this effective binding of [³H]NADH to the enzyme in the presence of spermidine is consistent with the inhibition of [³H]NAD binding by NADH under the same conditions (results not shown). The stimulatory effects were more pronounced for NADH than for

Figure 5 Effects of eIF5A precursor on the binding of NAD (A) and spermidine (B)

(A) The mixture contained 5 μg of enzyme and 1 μCi of [³H]NAD (14.2 μM) with or without the indicated concentrations of eIF5A precursor (5A) and spermidine (Spd). (B) The mixture contained 2 μg of enzyme and 2 μCi of [³H]spermidine with or without eIF5A precursor and NADH. The experiment was repeated twice with similar results.

NAD and at low concentrations of NAD or NADH (Figure 4). GC₇, which binds at the spermidine-binding site with a higher affinity than spermidine, exerted stronger stimulation of binding of NAD and NADH than spermidine. From Scatchard plot analysis, the K_d value for NAD binding alone in the absence of spermidine was estimated to be $\approx 30 \mu\text{M}$. The dissociation constant for the binding of NAD was markedly reduced, to $0.5 \mu\text{M}$, in the presence of GC₇. The maximum amount of NAD/NADH bound in the presence of GC₇ or spermidine was estimated to be 3.7–3.8 molecules/enzyme tetramer.

Effect of eIF5A precursor on the binding of NAD and spermidine

Since deoxyhypusine synthase can catalyse a partial reaction, the NAD-dependent cleavage of spermidine in the absence of eIF5A precursor, it is evident that binding of NAD and spermidine to the enzyme does not require eIF5A precursor. However, eIF5A precursor was shown to bind the enzyme in the absence of NAD or spermidine [21]. Therefore, it was of interest to examine the effects of eIF5A precursor on the binding of NAD and spermidine to the enzyme. As shown in Figure 5(A), addition of eIF5A precursor caused an enhancement of NAD binding, suggesting an increase in NAD affinity in the presence of eIF5A precursor. The stimulation of NAD binding was less pronounced with eIF5A precursor than with spermidine. Spermidine (50 μM) caused an additional increase in NAD binding over that with eIF5A precursor alone. Binding of spermidine was still dependent on NAD/NADH in the presence of eIF5A precursor, and eIF5A precursor did not induce the binding of spermidine to the enzyme (Figure 5B). Instead, eIF5A precursor (10–50 μM) caused a reduction in the NADH-induced binding of spermidine (3.6 μM), suggesting an inhibition of spermidine binding by high concentrations of eIF5A precursor. This inhibition of spermidine binding by a high concentration of eIF5A precursor may reflect interference with spermidine binding by the side chain of Lys-50 of the substrate protein, which must bind at the spermidine-binding groove prior to its modification.

DISCUSSION

From the crystal structure of human deoxyhypusine synthase [19], spermidine-binding sites and its mode of binding were predicted in an orientation with the N-4–C-5 bond of spermidine in close juxtaposition to the nicotinamide ring of bound NAD. In the crystals, which were formed at low pH, the entrance to the proposed spermidine-binding pocket appears to be blocked by an

α-helix from another subunit. This observation underscores the fact that, whereas allowing predictions as to substrate and cofactor binding, the currently available crystal structure of deoxyhypusine synthase provides little information on the reaction dynamics of the enzyme in solution or on the conformational changes required for its multi-step reaction. The data presented here provide new insights into the complex interactions between the binding sites for NAD, spermidine and eIF5A precursor, and thus further our understanding of the reaction mechanism of deoxyhypusine synthase.

Since deoxyhypusine synthase could be retained effectively on PEI-treated glass filters, this filtration assay offered a rapid and simple method to measure binding of NAD and spermidine to the enzyme. The binding of deoxyhypusine synthase to the positively charged PEI-coated glass filters was nearly quantitative in the range of the enzyme used. The effective binding of the enzyme must be due to its highly acidic nature (pI 5.1) [23]. It was not possible to determine dissociation constants for NAD and for spermidine from assay mixtures containing both because the binding step could not be dissociated from the ensuing enzymic reaction. Apparently there is strong mutual enhancement of binding between NAD and spermidine. The increased binding may be driven by the reaction as well as by conformational effects. The enhancement of spermidine binding by NADH, in effect the unreactive NAD analogue, and the enhancement of NAD binding by the unreactive spermidine analogue, GC₇, may reflect conformational effects in the absence of reaction. The thermodynamic stability of the inactive ternary complexes, enzyme–NADH–spermidine or enzyme–NAD–GC₇, may also contribute to the enhanced binding.

The results presented in Figure 2 suggest that spermidine binding occurs following a conformational change induced by NAD binding. In an independent experiment, quenching of the tryptophan fluorescence of deoxyhypusine synthase was observed upon addition of NAD (E. C. Wolff, unpublished work), consistent with conformational change of the enzyme upon cofactor binding. Change in the intrinsic tryptophan fluorescence of the protein has been observed upon binding of the cofactors NAD or NADH in a number of enzymes, including glyceraldehyde-phosphate dehydrogenase [30,31], liver alcohol dehydrogenase [32] and UDP-galactose 4-epimerase [33]. For certain enzymes, e.g. horse liver alcohol dehydrogenase [34–36], the conformational change could be visualized by direct comparison of X-ray structures of the apoenzyme and the holoenzyme containing NAD/NADH or both the cofactor and a substrate analogue. Since the three-dimensional structure of deoxyhypusine synthase without NAD has not yet been resolved, the precise conformational change that promotes the binding of spermidine cannot be predicted. It may involve opening of the entrance to the active site and/or bringing amino acid residues together in a proper arrangement and orientation to form a tight binding site for spermidine.

The dependence of spermidine binding on NAD suggests an ordered type of addition of NAD and spermidine in the deoxyhypusine synthase reaction (Scheme 1). Previously, we proposed a mechanism involving four steps (Scheme 1, 1–4; [18]). This proposal was based on the fact that an abortive partial reaction, the NAD-dependent cleavage of spermidine (Scheme 1, steps 1, 2 and broken arrows), can be dissociated from eIF5A modification in the absence of the eIF5A precursor. Furthermore, addition of the eIF5A precursor to the preformed enzyme-imine intermediate (Scheme 1, E = Bt·NADH) led to rapid modification of the eIF5A precursor [18], thus demonstrating that the deoxyhypusine synthesis reaction can proceed in this sequence (Scheme 1, steps 1–4). However, in a complete reaction mixture

containing NAD, spermidine and eIF5A precursor, the binding of NAD and eIF5A precursor to the enzyme presumably occurs in random order because eIF5A precursor binding to the enzyme occurs in the absence of NAD and vice versa [21]. Thus binding of the eIF5A precursor may precede binding of NAD. Addition of [³H]spermidine and NAD to the eIF5A precursor–enzyme complex leads to deoxyhypusine synthesis (not shown), suggesting that the complex of eIF5A precursor–enzyme can proceed with the binding of NAD and spermidine and subsequent steps. Since spermidine binding is dependent on NAD even in the presence of eIF5A precursor, an ordered type of addition of NAD and spermidine appears to apply to this case as well, leading to a sequence of parallel reactions. The relative significance of either proposed pathway may depend on the association constants as well as the concentrations of NAD and eIF5A precursor in the reaction mixture. This hypothetical mechanism needs to be verified with kinetic experiments. Under saturating concentrations of NAD and spermidine, deoxyhypusine synthase can catalyse NAD-dependent cleavage of spermidine at all four active sites of the enzyme tetramer. This finding is consistent with the estimate of ≈ 3.5 NADH molecules/enzyme tetramer obtained from our independent experiments employing NADH fluorescence as a measure of the initial reaction of deoxyhypusine synthase [29]. The present findings from NAD/spermidine-binding studies complement the NADH fluorescence results and reveal further complexity in the proposed pathway of the deoxyhypusine synthase reaction.

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