Deoxyhypusine Synthase Generates and Uses Bound NADH in a Transient Hydride Transfer Mechanism*

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Deoxyhypusine is a modified lysine residue. It is formed posttranslationally in the precursor of eukaryotic initiation factor 5A (eIF5A) by deoxyhypusine synthase, employing spermidine as a butylamine donor. In the initial step of this reaction, deoxyhypusine synthase catalyzes the production of NADH through dehydrogenation of spermidine. Fluorescence measurements of this reaction revealed a 22-nm blue shift in the emission peak of NADH and a ~15-fold increase in peak intensity, characteristics of tightly bound NADH that were not seen by simply mixing NADH and enzyme. The fluorescent properties of the bound NADH can be ascribed to a hydrophobic environment and a rigidly held, open conformation of NADH, features in accord with the known crystal structure of the enzyme. Considerable fluorescence resonance energy transfer from tryptophan 327 in the active site to the dihydronicotinamide ring of NADH was seen. Upon addition of the eIF5A precursor, utilization of the enzyme-bound NADH for reduction of the eIF5A-imine intermediate to deoxyhypusine was reflected by a rapid decrease in the NADH fluorescence, indicating a transient hydride transfer mechanism as an integral part of the reaction. The number of NADH molecules bound approached four/enzyme tetramer; not all of the bound NADH was available for reduction of the eIF5A-imine intermediate.

Deoxyhypusine synthase (EC 1.1.1.249) is responsible for the first reaction in the posttranslational synthesis of hypusine (N^2-(4-amino-2-hydroxybutyl)lysine) in a single eukaryotic protein, the eIF5A precursor. In this complex reaction, the amidobutyl moiety of spermidine is transferred to a specific lysine in the eIF5A precursor to form a deoxyhypusine (N^2-(4-amidobutyl)lysine) residue. The final reaction of hypusine synthesis is carried out by a specific deoxyhypusine hydroxylase. The fully modified eIF5A in its mature, hypusine-containing form is required for eukaryotic cell proliferation, although its mode of action is not yet clear (1–6). Expression of the gene for deoxyhypusine synthase, as well as for eIF5A, has been shown to be essential for yeast vitality (7).

Deoxyhypusine synthase is an NAD-dependent dehydrogenase. The overall reaction has been postulated to occur in four steps (Scheme I), including two imine intermediates in the transfer of the amidobutyl group from spermidine, as follows: 1) spermidine + NAD → dehydrosperrmidine + NADH; 2) dehydrosperrmidine + enzyme → enzyme-Lys-imine intermediate + diaminopropane; 3) enzyme-imine intermediate + eIF5A precursor → eIF5A-Lys-imine intermediate + enzyme; 4) eIF5A-imine intermediate + [H] → deoxyhypusine in the eIF5A intermediate.

Direct evidence has been presented for the first two steps (8, 9), but the details of the final two steps have remained elusive. Steps 1 and 2 occur in the absence of eIF5A precursor and can be measured by following the release of 1,3-diaminopropane and the formation of the enzyme-imine intermediate, which can be trapped with cyanoborohydride (9). In the presence of the eIF5A precursor the final two steps are rapidly completed. NADH generated in the initial dehydrogenation (step 1) has been presumed to be used in the final reduction step (step 4) because the purified enzyme does not seem to require addition of a reducing agent. However, there has been no direct evidence for the role of NADH in the final step, and it was not known whether NADH remains bound to the enzyme or first dissociates and reassociates with the enzyme at step 4.

The crystal structure of human deoxyhypusine synthase with bound NAD has been obtained at a resolution of 2.2 Å (10). The enzyme is a homotetramer of two pairs of identical, tightly associated dimers of the 41-kDa subunits. There are two active sites in each dimer interface running anti-parallel to each other for a total of four potential active sites, each capable of binding NAD in a specific orientation with respect to a presumed spermidine-binding cavity. Under the conditions of crystallization with excess NAD, the tetramer contains four molecules of NAD that are completely buried in the subunit interfaces. The arrangement of the NAD in a partly extended configuration is similar to that of many dehydrogenases (11, 12). The NAD-binding site of each subunit is a fairly typical “Rossman fold” (13), which places the NAD adjacent to the spermidine-binding site. The nicotinamide ring would be in close juxtaposition to the N=C bond of spermidine that is dehydrogenated. The access of solvent to the active site region, and the dissociation of bound nucleotide, although possible, would appear to be severely limited without conformational change.

The characteristic absorption of the dihydronicotinamide ring of NADH, and the fluorescence emission of this reduced nucleotide, have been useful in studies of dehydrogenases. NADH in water or buffer emits a strong fluorescence with a peak at 457–465 nm when excited in the region of 340 nm. However, if NADH is bound to one of several dehydrogenases or in the presence of nonpolar solvents, the fluorescence emission peak is blue-shifted (~20 ± 5 nm), and its intensity is markedly enhanced (11). These facts, plus the increased sensitivity of fluorescence methods, prompted us to follow the for-
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Experimental Procedures

Materials—NAD and NADH were obtained from Roche Molecular Biochemicals, CalBiochem, and Sigma; the concentration of stock solutions was verified by the absorbance at 260 nm for NAD or at 340 and 260 nm for NADH using molar extinction coefficients of 18 × 10^3 M⁻¹ cm⁻¹ (NAD) and 15 × 10^3 M⁻¹ cm⁻¹ (NADH) at 260 nm and 6.2 × 10^3 M⁻¹ cm⁻¹ at 340 nm (NADH).

Spermidine-3HCl was from Sigma; tetrahydrofuran (with peroxide-inhibitor) was from Aldrich; BCA protein assay reagents were from Pierce; N°-guanyl-1,7-diaminoheptane (GC 7) was synthesized as described previously (14). Deoxyhypusine synthase (40,970-Da monomer mass) expressed in Escherichia coli from a human deoxyhypusine synthase cDNA was purified (≥95%) according to a published procedure (10, 15). Mutant enzyme proteins, generated by site-directed mutagenesis were expressed and purified by a single round of the procedure; the enzyme was prepared and purified as described previously (16), with minor modifications. Protein concentrations were determined by absorbance at 280 nm (theoretical E (molar) = 38360) and by the BCA method (17), which gave ~95–98% of the A_{280} value with pure proteins; purity was estimated by densitometry after SDS-polyacrylamide gel electrophoresis.

Methods—Fluorescence data were generated with a Perkin-Elmer spectrofluorometer (model MPP-66) operating in ratio mode, with a Perkin-Elmer 7300 Professional Computer, software version 1.8 for data analysis. Uncorrected spectra were collected (for increased sensitivity) at ambient temperature in 3 × 3-mm light path masked quartz cells. In most cases the slit widths were 5 nm for both excitation and emission, scan speed was 120 nm/min, and the response time was 2 s. For the emission spectra of NADH, excitation was at 340 nm (similar results were obtained with excitation at 340, 350, or 355 for the enzyme reactions). The reaction mixtures were maintained at ambient temperature during the measurements and stored at 4 °C for the longer time intervals (2–24 h) between measurements. Typical assay conditions were 0.2 M glycine-NaOH buffer, pH 9.2, 200 μM NAD⁺, 40–100 μM spermidine, and 2–5 μM (monomer)² deoxyhypusine synthase in 100 μL. Generally, a background emission scan was collected on the buffer, enzyme, and either NAD or spermidine, separately and together. The first emission spectrum was collected as soon as possible (10–15 s after mixing) after addition of the final component directly into the fluorescence cell, and subsequent scans were obtained at intervals depending on the speed of the reaction. Both peak wavelength and intensity (in arbitrary units (A.U.)) were recorded. Time courses were followed by measuring the fluorescence intensity at 441 nm. Corrections were made for dilution effects (usually 2–5%) for comparison with initial values.

Protein (tryptophan) fluorescence emission from 300 to 600 nm was observed after excitation at 295 nm to minimize end absorption of NAD or NADH and contribution from tryptophan. Even so, as noted in the text, some activation of bound NADH (350-nm peak) and, potentially, of the phenolate form of tyrosine (peak at 293 nm) could occur. Because the intensity was much greater than for NADH fluorescence, the slit widths were reduced to 4 nm each.

For the measurement of NADH fluorescence in a nonpolar solvent, tetrahydrofuran (THF) (from a freshly opened bottle) was first transferred to small vials sealed with teflon-lined caps for mixing with NADH, using an argon-flushed syringe and precautions to prevent peroxide formation. The vials were weighed to determine the amount of tetrahydrofuran added; a small volume (1–10 μL) of a concentrated NADH solution was added by syringe. Subsequently, the NADH/THF mixtures were transferred to capped cuvettes under argon. The polarity of mixtures of THF and water (90–100% THF) was estimated by comparison with published values obtained with the solvatochromatic dye pentaphenyl pyridinium phenolate and expressed as molar electronic transition energies (E_P(30)) values, in kcal/mol at 25 °C (18). Correction for nonlinearity in mixed solvents was made according to Dawber et al. (19). Graphic fitting was done with the program Kaleidagraph (Synergy Software) version 3.0.8, fitting the data to the equation for a hyperbola, y = a + b/(x + c) or for linear least squares.

Results

Fluorometric Characterization of NADH Generated during the Deoxyhypusine Synthase Reaction—The reduction of NAD to NADH associated with the dehydrogenation of spermidine was readily observed, in a mixture containing deoxyhypusine synthase, NAD, and spermidine, by the fluorescence of the newly formed NADH. Fig. 1 shows a comparison of the fluorescent properties of enzyme-generated NADH (curve 1) with those of NADH in solution (curve 2) at the same pH. It is clear that both the excitation (Fig. 1A) and emission (Fig. 1B) peaks are shifted. The blue shift of the emission peak of NADH, from 463 to 441 nm for the enzyme-generated NADH (curve 1) is demonstrated more clearly in the comparison of peak-normalized curves (Fig. 1B, inset). A shift of this magnitude is characteristic of the fluorescence emission of NADH bound to other dehydrogenases (11, 20–23). To assess the polarity of the environment of NADH in the protein, we examined the fluorescence of free NADH in nonpolar solvents, which are known to induce a blue shift in peak wavelength, e.g. Scott et al. (20). We found that a λ_max of 443–442 nm was attained in 92–95% peroxide-free tetrahydrofuran in H_2O, approaching the 22-nm blue shift seen with deoxyhypusine synthase. Calculation of the extent of nonpolar character of this solvent yielded a value of 40–41 E_P(30), approximately equivalent to a dielectric constant of 9–12 (18).

The excitation maximum of enzymatically generated NADH is red shifted to 350 nm compared with 340 nm for the free NADH (Fig. 1A, curve 1 versus curve 2). The higher energy excitation peak seen at 285 nm for the enzyme-generated NADH indicates a contribution of efficient resonance energy transfer from a tryptophan of the enzyme to the reduced dinucleotide, as will be explored in a later section. The intensity of the emission peak of enzyme-generated NADH is much greater than that of free NADH. The peak intensity of the generated NADH was 71.8 A.U. with 1.2 μM enzyme (theoretically equivalent to 1.2 μM NAD binding sites) (Fig. 1B, curve 1).

² C. H. Lee, P. Um, and M. H. Park, submitted for publication.
³ The enzyme concentration is given throughout in terms of the monomer, which equals the number of presumptive active sites, even though the tetrameric form is the active form. In the crystal there are four apparently identical active sites/tetramer.
whereas the dashed line dine, is shown in Fig. 2.

NADH (for 3–4 h, with only a modest drop after 24 h at 4 °C. There was
an initial rapid phase followed by a gradual increase. Upon prolonged incubation in the presence of NAD and spermidine, the NADH fluorescence intensity stayed fairly stable (Fig. 2A), when the emission peak was at 441 nm, at all other times the peak was at 441 ± 1.5 nm (Fig. 1C), even after 16–24 h at 4 °C (data not shown) or ultrafiltration.

The formation of enzyme-associated NADH under various conditions in the absence of the eIF5A precursor is illustrated in Fig. 2. The addition of NAD (which has no intrinsic fluorescence at 441 nm) to the enzyme did not change its low background fluorescence emission. However, the further addition of spermidine elicited a rapid increase in fluorescence (λ<sub>max</sub> = 441 nm) as the reaction progressed (Fig. 2A). Essentially the same was true when the reaction was initiated by the addition of NAD to a mixture of enzyme and spermidine (Fig. 2B). In the presence of excess spermidine and NAD, both the initial rate and the final level of fluorescence intensity were directly proportional to the amount of enzyme added (Fig. 2C). The initial rate of NADH formation was estimated to be ~3 μmol/min/μmol enzyme monomer, at ~25 °C and 500 μM NAD. A dependence of NADH formation on spermidine concentration, as well as that of NAD and enzyme, was also observed (data not shown). In the case of very low NAD concentration, e.g., 4 μM (Fig. 2B, curve 2) or less, both the rate and extent of reaction were reduced, because of substoichiometric amounts of NAD. The effect of the spermidine analog, GC7, a competitive inhibitor for spermidine, is shown in Fig. 2A. GC7 at 1.5 μM almost completely prevented the generation of NADH in a reaction mixture containing 17 μM spermidine and 10 μM NAD. These results are consistent with the potent inhibition of deoxyhypusine synthase by GC7 (the K<sub>i</sub> for GC7 in the complete reaction is 10 nM, whereas the K<sub>i</sub> for spermidine is 7.2 μM) (15) and support the assumption that deoxyhypusine synthase activity is being measured.

The increase in fluorescence appeared to be biphasic, comprising an initial rapid phase followed by a gradual increase. Upon prolonged incubation in the presence of NAD and spermidine, the NADH fluorescence intensity stayed fairly stable for 3–4 h, with only a modest drop after 24 h at 4 °C. There was no indication of a shift of the fluorescence peak toward free NADH (λ<sub>max</sub> ≈ 460 nm), even as a shoulder on the 441-nm peak. Thus, a steady state pool of NADH generated by the reaction and bound to the enzyme seems to be maintained. The data shown in Fig. 3 suggest that there is no association of exogenously added NADH with deoxyhypusine synthase. In this experiment increments of enzyme of 1–7.7 μM were added to 10 μM NADH, and there was no change in position or magnitude of the emission peak of NADH at 463 nm. More importantly, there was no increase in 441-nm fluorescence. Incubations of enzyme up to 24 μM enzyme also did not change the intensity at 463 nm (data not shown).

Utilization of NADH in the Final Step of Deoxyhypusine Synthesis—The foregoing results demonstrate that the fluorescence observed (in the absence of the eIF5A precursor) is due to enzyme-bound NADH that is formed via the enzymatic reduction of NAD in the active site. The data presented in Fig. 4 provide evidence that the NADH generated in step 1 is used in the final step of deoxyhypusine synthesis. A rapid, although incomplete, decrease in emission at 441 nm was observed when eIF5A precursor was added after the NADH had been allowed to build up in a reaction mixture that contained enzyme, NAD, and spermidine (Fig. 4A, curves 2 and 3). This finding suggests that a rapid reoxidation of NADH occurs upon addition of eIF5A precursor, because of steps 3 and 4. The level of fluorescence was restored, however, as eIF5A precursor was consumed. The recovery rate was inversely related to the amount of precursor added. To assess the extent of net consumption of the enzyme-bound NADH in the absence of new formation of NADH, we removed NAD and spermidine by ultrafiltration before the addition of eIF5A. The fluorescence changes following ultrafiltration are shown in Fig. 4B. The enzyme retained bound NADH after ultrafiltration, as shown by the initial point in Fig. 4B. Upon addition of an excess of ec-eIF5A, there was a rapid decrease in the intensity of fluorescence at 441 nm, which remained at a low level. The small rise after the first addition is most likely due to a very small amount of residual NAD and spermidine. In several experiments with different amounts of enzyme the residual final fluorescence intensity after eIF5A precursor addition amounted to ~33–50% of the initial value. There was no condition in which all of the NADH fluorescence could be abolished even when a large excess of eIF5A precursor was added. The eIF5A precursor, per se, had no effect on the fluorescence of free NADH in solution or on the low background
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Fluorescence emission intensity after excitation at 340 nm. The final component of the reaction mixture was added in the fluorescence cell at time 0, and emission scans (A and B) or emission at the fixed wavelength, 441 nm (C), was followed over the indicated time course. The peak intensity and wavelength were recorded for the fixed wavelength, 441 nm (A). The emission peaks in A were at 437 nm for enzyme alone, 450–466 nm after NAD, 446 nm at 25 s after spermidine addition, and 442–441 nm for all subsequent time points. The emission peaks in B were 438 nm at 0 time, 452 nm at 10 s, and 441–439 nm for all subsequent time points, as shown in Fig. 1C. Reaction mixtures (100 μl) were as follows. A. 3.6 μM deoxyhypusine synthase (monomer), 19 μM NAD, 17 μM spermidine, and 1.5 μM GC7 (final concentrations). B. 10 μM deoxyhypusine synthase, 50 μM spermidine, and 40 μM NAD. C. deoxyhypusine synthase (curve 1, ▲, 1.4 μM; curve 2, ×, 0.7 μM; curve 3, ●, 0.42 μM; curve 4, ○, 0.28 μM; curve 5, ●, 0.14 μM), 50 μM NAD, and 100 μM spermidine. Other conditions were as described under “Experimental Procedures.”

fluorescence of the enzyme-NAD complex.

In a complete reaction mixture containing eIF5A precursor, the level of enzyme-bound NADH represents a dynamic balance between its formation and reoxidation. The effect of eIF5A precursor concentration on the accumulation of NADH was examined. Both the initial rate of NADH formation and the final level reached decreased in a manner proportional to the amount of eIF5A precursor initially present in the reaction mixture before the addition of spermidine, as shown in Fig. 5. The inset shows the individual reactions. However, it is clear that the accumulation of NADH was not totally prevented even in the presence of a relatively high level of eIF5A precursor. This observation may mean that a certain fraction of the NADH bound on the enzyme is not readily accessible for reduction of the eIF5A-imine intermediate.

The Stoichiometry of NADH Associated with Deoxyhypusine Synthase—In view of these considerations on the accessibility of enzyme-bound NADH, it became crucial to know the actual number of NADH molecules generated on the tetrameric enzyme as reflected by its fluorescence. As a first step, the yield of fluorescence intensity per unit of NADH formed was determined under conditions where all of the NAD present could be assumed to be reduced, i.e., very low NAD concentration (1 μM) and an excess of enzyme (2–12 μM monomer) and spermidine (100 μM). Fluorescence emission intensities were plotted as A.U. (at 441 nm) against enzyme amount (Fig. 6A) after the reaction had been allowed to proceed. From the limiting value at the highest enzyme concentrations, 1 μM NADH bound to the enzyme was calculated to equal 68 fluorescence units (in this instrument with 5-nm excitation and emission slit widths and a cell of 3-mm pathlength) as an average of four separate experiments. Because the peak intensity of 1 μM free NADH is ~4.55 A.U., under the same instrument conditions, an estimate of the increase in intensity of NADH when bound to deoxyhypusine synthase is ~15-fold.

We determined the stoichiometry of NADH molecules/tetramer in two ways, using the value of 68 A.U. for the fluorescence equivalent of bound NADH. In the first, increasing amounts of NAD (10–500 μM) were added to a relatively small amount of enzyme (1–2 μM), with an excess of spermidine (100 μM). This determination yielded a ratio of 0.88 molecule of NADH per monomer of enzyme (Fig. 6B). In the second approach, increasing amounts of enzyme (0.14–1.4 μM) were added to a constant intermediate amount of NAD (50 μM), and the reaction was initiated with excess spermidine (100 μM) and allowed to approach a plateau, as shown in Fig. 2C. The final corrected values, plotted against enzyme amount showed a linear relationship (Fig. 6C). In this case 0.87 μM NADH corresponded to 1 μM enzyme active sites, equivalent to 3.49 molecules of NADH/enzyme tetramer.

**NADH Fluorescence Studies with K329A and W327A Mutant Enzymes**—According to the proposed reaction sequence, the cleavage of spermidine follows its initial dehydrogenation by deoxyhypusine synthase. There should be a correlation be-
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Fig. 4. Addition of eIF5A precursor to the enzyme-NADH complex. A, before ultrafiltration. B, after ultrafiltration. Fluorescence was followed at 441 nm after excitation at 340 nm. ec-eIF5A (eIF5A precursor) was added at the times indicated by the arrowheads. A, spermidine was added before spermidine. Inset, initial concentrations of ec-eIF5A were as described in the legend for Fig. 4A, except that ec-eIF5A was added at 42 min 12 μM ec-eIF5A was added. The curves were normalized to the same enzyme amount (4.8 μM, monomer). B, to reaction mix 1 from A, after taking initial readings, ec-eIF5A was added in two portions, 18 μM (time 0) and 12 μM (after 30 min), expressed as final concentrations; the observed fluorescence was corrected for dilution after addition of spermidine or ec-eIF5A and after ultrafiltration. The relative fluorescence of sample 1, with the value immediately after ultrafiltration set = 100, is shown as the right-hand axis of B. For the ultrafiltration experiments, the reaction mixtures (−100 μl) after NADH had accumulated were transferred to ultrafiltration devices with a molecular mass cutoff of 30 kDa (Microcon 30 (Amicon) prewashed with 0.2 M glycine-NaOH buffer, pH 9.2) and centrifuged at 5,400 × g for 10 min at 4 °C; the retained enzyme-NADH complex (−10 μl) was diluted with 100 μl of the same buffer, recentrifuged, and then reconstituted with 80 μl of buffer before collection in 89–95 μl. The samples were transferred to the fluorescence cuvettes, and fluorescence emission was followed with additions as indicated.

Fig. 5. NADH accumulation in the presence of eIF5A precursor. Fluorescence emission at 441 nm (λex = 340) was followed over time (as shown in the inset), and the initial rate or the final level attained is plotted against the ec-eIF5A concentration. The conditions were as described in the legend for Fig. 4A, except that ec-eIF5A was added before spermidine. Inset, initial concentrations of ec-eIF5A were as follows: curve 1, none; curve 2, 6 μM; curve 3, 12 μM; curve 4, 24 μM; curve 5, 36 μM. A duplicate without ec-eIF5A is included in the main plot.

tween spermidine cleavage and NADH generation. We therefore examined certain mutant enzymes deficient in spermidine cleavage activity. It had been reported earlier that replacement of the active site lysine residue (Lys327) with arginine abolished all enzyme activity, including spermidine cleavage as well as deoxyhypusine synthesis (24). The mutant enzyme, K329R, was totally inactive in the generation of NADH fluorescence. On the other hand K329A retains a low level (2–5%) of spermidine cleavage activity (24), although its contribution to NADH fluorescence is 20% of the wild-type enzyme. ec-eIF5A, displayed low activity (1–3% of the wild type enzyme) in forming NADH with typical excitation and emission spectra (data not shown). The other mutant of interest involves substitution of the Trp327 residue, which lies in the spermidine-binding site. Trp327 is close to the postulated position of the secondary nitrogen and the aminopropyl moiety of spermidine and may stabilize its binding. W327A, which exhibits very low spermidine cleavage activity (−2% of the wild type) had low but detectable activity in forming NADH (1.2 × 10−5 μkat/min/μM enzyme or <1% of the wild type). The emission peak in this case, although small, was characteristic of bound NADH with a peak at 441 nm (see below).

Tryptophan Fluorescence Studies with Wild Type and Mutant W327A Enzymes—The tryptophan emission peak of 331 nm, found with deoxyhypusine synthase (Fig. 7), shows considerable overlap with the excitation spectrum of NADH (Fig. 1). Therefore the appearance of an excitation peak at 285 nm, shown in Fig. 1, suggested that fluorescence resonance energy transfer from a nearby tryptophan or tyrosine residue to the dihydronicotinamide ring of NADH might be taking place. Energy transfer to NADH has been observed with other dehydrogenases, e.g. lactate dehydrogenase (21). Addition of NAD to the enzyme in the absence of spermidine partially quenched tryptophan fluorescence by a mechanism other than resonance energy transfer (data not shown). We therefore chose as the baseline for the tryptophan fluorescence the emission spectrum of the enzyme-NAD complex at the relatively high concentration of NAD (200 μM), as shown in Fig. 7A. The excitation was at 295 nm to exclude contributions from tyrosine. Reduction of the NAD in the presence of spermidine (curve 2 versus curve 1) led to additional quenching of tryptophan fluorescence by 44 ± 7% calculated from the area under the peak at 331 nm (Fig. 7, legend). Simultaneously, an emission peak at 441 nm appeared. This peak is mainly due to fluorescence resonance energy transfer (Fig. 7A, curve 2), although a portion of the peak at 441 nm is a result of direct excitation of NADH at 295 nm. From the ratio of the absorbance at 295 and 340 nm (or the same ratio in the excitation spectrum shown in Fig. 1), the contribution to 441-nm emission from this source can be estimated to be ~20–25% of the total emission. The excitation spectrum of the new 441-nm emission shows peaks at 285 and 350 nm (data not shown), a pattern that is similar to that shown in Fig. 1A, thus indicating identity with bound NADH. Excitation at 295 nm yields at least 50% greater emission intensity in the 441-nm peak than seen after excitation at 350 nm, further suggesting that there is very efficient resonance energy transfer, presumably from a nearby tryptophan residue, most likely Trp327. Denaturation in 3 M guanidinium chloride abolishes this resonance energy transfer, indicating a requirement for the intact conformation of the protein for this transfer.
It also implicates Trp$^{327}$ as the most likely tryptophan involved, because although Trp$^{327}$ is in the active site, not far from the NAD, it is on the adjacent monomer, not the same monomer.

Confirmation of the role of Trp$^{327}$ comes from the W327A mutant, from which essentially no resonance energy transfer is observed (Fig. 7B, curve 2 versus curve 1). Nevertheless, the small amount of NADH generation by this mutant is accompanied by a small (7.8%) decrease in emission at 331 nm, as well as a very small increase in emission at 441 nm after excitation at 295 nm. This is detailed by the difference spectrum between curves 1 and 2 of Fig. 7B (Fig. 7C, inset). This peak is much smaller than the corresponding peak after 350 excitation, in contrast to the case with the wild type enzyme. Rather than invoking a leaky mutant, this peak can be explained in approximately equal parts by contributions from direct excitation of NADH and from other sources of energy transfer (see “Discussion”). The source of this additional resonance energy transfer, possibly from one or more of the three other tryptophan residues, is not certain at present. In any case, the efficiency of the energy transfer from Trp$^{327}$ is consistent with its close proximity to NADH in the active site, and it seems reasonable to assume that most of the resonance energy transfer is coming from Trp$^{327}$.

**DISCUSSION**

In the present study we have addressed the role of NAD and NADH in the reaction catalyzed by deoxyhypusine synthase. Specifically, we have described the dehydrogenation of spermidine with reduction of NAD to NADH and the use of NADH for reduction of the eIF5A-imine intermediate in the final step. The proposed overall reaction is shown in Scheme I; the shaded ovals indicate enzyme forms to which NADH is bound. By utilizing the known fluorescent properties of NADH, we found that: 1) NADH is generated by in situ reduction of enzyme-bound NAD and remains associated with the enzyme; 2) a

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4 Because the excitation was at 295 nm, the contribution of tyrosine residues would be expected to be minimal (the absorption is <1.2% of tryptophan at that wavelength); however, the possibility exists that the phenolate ion of tyrosine, which has an absorption peak at 293, could be a factor, even though these reactions are carried out at pH 9.2. The $pK_a$ of tyrosine residues buried in a protein, (e.g. Tyr$^{200}$ and Tyr$^{276}$ (on opposite monomers)) is often much higher (>pH 11) than that of free tyrosine in solution (9.8), hence the contribution from any tyrosinates is likely to be small.

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**Fig. 6. Calibration of NADH bound on deoxyhypusine synthase.** A, titration for maximum A.U. at 441 nm, per limiting NAD (1 $\mu$M) formed, by increasing the amount of enzyme. Deoxyhypusine synthase was incubated with 1 $\mu$M NAD and 100 $\mu$M spermidine until the reaction was essentially complete. The fit of the experimental points to a hyperbola gave a maximum value of 68.15 in this case. B, determination of the maximum NADH formed on a limited amount of enzyme. Deoxyhypusine synthase (1.3 $\mu$M) was incubated with 100 $\mu$M spermidine and various concentrations of NAD. The A.U. after the reaction was divided by 68 to give the equivalent NADH concentration in $\mu$M. In this experiment the ratio of NADH formed to enzyme tetramer was 0.88. C, titration of NADH formed versus amount of enzyme. The fluorescence obtained with increasing amounts of enzyme were calculated in terms of equivalent NADH concentration, by dividing the A.U. by 68, and the straight line was determined by least squares calculation. The dashed line drawn at the equivalent of 4 $\mu$M enzyme (monomer), i.e. 1 $\mu$M tetramer, intersects the line at 3.49 $\mu$M NADH.

**Fig. 7. Comparison of tryptophan fluorescence of the wild type (A) and mutant W327A (B) enzymes, before (curve 1) and after (curve 2) the reaction with NAD and spermidine.** Emission scans (excitation at 295 nm, slits 5 nm). Incubation conditions for both curve 1 and mutant W327A after the partial reaction gives a very small peak at 285 nm, consistent with excitation of tryptophan residue(s), as well as a slightly larger peak at 348 nm (for bound NADH) (data not shown). The two tryptophan emission peaks, with and without NADH, were compared by peak intensity and area from 305 to 560 nm. The peak after NADH generation was corrected at the trailing red edge by normalization of the control curve and addition. The NADH emission peak area (391–560 nm) was corrected by subtracting the red edge of the normalized control curve.
fluorescence resonance energy transfer from this tryptophan to NADH in the active site.

This study presents the first experimental evidence for direct reutilization of the hydrid ion of NADH in deoxyhypusine synthesis. This type of internal transfer mechanism involving NAD and NADH in a single protein is rare but not unprecedented, as shown for enzymes that have tightly bound NAD/NADH such as \textit{E. coli} UDP-glucose-4-epimerase (26, 27), “nicotinoprotein” alcohol dehydrogenases (28, 29), and several others (27), including homospermidine synthases from plants (30, 31). Frey first used the term “transient hydride transfer,” which we have adopted here, for the mechanism in which a critical hydride ion is transferred from one intermediate to another \textit{via} NAD/NADH (27, 32). The mechanism employed by deoxyhypusine synthase appears to be similar in that there is an NAD-NADH cycle in which hydride transfer from the first substrate to the last intermediate takes place \textit{via} bound nucleotides. It differs in that NAD does not stay tightly associated with the enzyme, because NAD addition is required for activity (33, 34).

Fluorescence offers a unique opportunity to study NADH binding in the protein, without requiring separation techniques and to gain information on its environment in terms of hydrophobicity, immobilization, and interaction with nearby residues. The region around the nicotinamide ring of NAD in the crystal structure of the complex with deoxyhypusine synthase (10) has substantial nonpolar characteristics. The closest hydrophobic amino acid residues and the distance of closest contact with the nicotinamide ring of NAD are Ile\textsuperscript{166} (3.70 Å), Ala\textsuperscript{132} (5.12 and 5.31 Å), Trp\textsuperscript{327} (5.49 Å), Tyr\textsuperscript{176} (5.49 Å), Phe\textsuperscript{64} (6.52Å), and Val\textsuperscript{285} (7.8 Å). In addition, the \( \beta \) carbons of Asp\textsuperscript{316} and Ser\textsuperscript{105} and the \( \alpha \) carbons of Gly\textsuperscript{131} and Gly\textsuperscript{239} are also in close contact (\(-4\) Å). The residues above in \textit{italics} are on the monomer that has the spermidine binding pocket, which is otherwise largely hydrophilic; this arrangement is in accord with the generalization of Rossman that the nicotinamide ring is usually buried with one face toward hydrophobic residues and the other toward hydrophilic ones (13). The blue shift of the bound NADH or of NADH in tetrahydrofuran is consistent with such an environment. Compared with the \(-15\)-fold increase in fluorescence of NADH bound to deoxyhypusine synthase, the increase in peak intensity in 90% tetrahydrofuran was only 3-fold that of the same concentration of NADH in buffer, suggesting that there may be additional factors affecting the enhancement of fluorescence emission. One is the release of both static and collisional quenching between the adenine and dihydronicotinamide moieties that occurs in the “closed” conformation of NADH in aqueous solution, which has been cited by Weber and others (20, 21, 35, 36). When bound to an enzyme, the NADH generally assumes a more “open” conformation, increasing the distance between the two ring systems and thus increasing the fluorescence emission (20). In the crystal structure of NAD bound to deoxyhypusine synthase, the NAD exhibits a moderately extended conformation, with approximately 12 Å between the adenine and nicotinamide rings (10). Further stabilizing the open configuration is the fact that the NAD is deeply buried in the dimer interface between two identical monomers, in a binding site where the adenine and ribose moieties have hydrogen bond contacts with at least 9 residues, mostly on one monomer. This fixation also implies that the whole NADH molecule will be immobilized, which also contributes to enhanced fluorescence, as has been observed with other ligands (37), with NADH by increasing the viscosity (22) and with NADH in enzyme-bound forms (20, 21). A combination of these factors may explain the relatively high fluorescence intensity seen with NADH bound to deoxyhypusine synthase.

A further insight into the interactions in the active site derives from nonradiative energy transfer from the excited state of tryptophan(s) to the dihydronicotinamide ring of NADH, assuming that the position of NADH in the protein is similar to that of NAD in the crystal structure (10). Of the four tryptophan residues in each monomer, three on the same monomer as the NAD binding site (Trp\textsuperscript{182}, Trp\textsuperscript{201}, and Trp\textsuperscript{223}) are close enough for fluorescence resonance energy transfer, because the indole rings are 14.81, 20.19, and 20.75 Å, respectively, from the nicotinamide ring of NAD. However, the fourth tryptophan, Trp\textsuperscript{327}, is part of the active site, and the indole ring of Trp\textsuperscript{327} is only 5.42 Å from the nicotinamide ring of NAD in the crystal structure, although it is across the dimer interface. Despite the relatively unfavorable 70° angle between the two ring systems, Trp\textsuperscript{327} appears to contribute the bulk of the substantial fluorescence resonance energy transfer observed with deoxyhypusine synthase. This conclusion is confirmed by use of the W327A mutant, in which the energy transfer was largely abolished. The small residual energy transfer (\(-8\%) may come from the more distal tryptophan residues.

A four-step pathway of the deoxyhypusine synthase reaction was proposed on the basis of earlier results (8, 9). Reduction of NAD to NADH in step 1 was demonstrated in experiments in which \(^{3}H\) from [5-\(^{3}H\)]spermidine was transferred stereospecifically to NAD in the \( \pro-R \) position to generate [4R-NADH] (8). Thus deoxyhypusine synthase is an \( R \) (or \( A \)-specific dehydrogenase. The successive transimations of steps 2 and 3 were postulated on the basis of the identification of a specific enzyme-imine intermediate (9). The current NADH fluorescence data complement the earlier studies and provide further insights into the mechanism and dynamics of the deoxyhypusine synthesis reaction (Scheme I). The steady state level of enzyme-bound NADH achieved either in the presence or absence of eIF5A precursor represents a balanced state of all the intermediate reactions. At present, the relative rates of the individual steps and the rate-limiting steps are not known for the complete reaction. In the case of the partial reaction in the absence of eIF5A precursor (steps 1 and 2), the rapid initial increase in NADH fluorescence (Fig. 2) corresponds to the kinetics of rapid spermidine cleavage, as measured by appearance of 1,3-diaminopropane, and accumulation of enzyme-imine intermediate in the complex II, detected by reduction with NaBH\textsubscript{4}CN (9). The NADH presumably exists primarily in the complex with enzyme-imine intermediate (II), with very little as complex I, even at the early time points, as the maximal enzyme intermediate was found at 2 min (9). The enzyme-imine intermediate undergoes a slow, abortive reaction to generate \( \Delta^{1} \)-pyrroline (8, 9). The maintenance of a steady NADH fluorescence after the initial burst indicates that the total amount of NADH bound to the enzyme does not change during the slow degradation of the enzyme-imine intermediate in II. This finding suggests that after cleavage of \( \Delta^{1} \)-pyrroline from the imine intermediate, the enzyme still retains NADH as the complex E-NH. However, the continuing slow release of 1,3-diaminopropane must require an equally slow dissociation of NADH from E-NH to regenerate free enzyme and therefore to initiate a new cycle.

When eIF5A precursor is added to allow completion of the reaction, the pool of available complex II is rapidly depleted (Fig. 4), although it is clear that not all of the NADH fluorescence disappears. Whereas NADH in the complex II is avail-
able for the complete reaction, upon addition of eIF5A, the NADH from E-NH is not. The fact that not all of the NADH was reoxidized could be due to nonutilizable NADH in this E-NH pool, due to lack of access of eIF5A precursor to all the usable NADH (in intermediate form II), or due to steric hindrance, for example. In this regard, it is intriguing to note that the predominant species of the deoxyhypusine synthase-eIF5A precursor complex was identified as the complex of one enzyme tetramer and one eIF5A monomer (38). Note that the extent of the rapid decrease in NADH fluorescence upon eIF5A precursor addition was dependent on the amount of eIF5A and was less pronounced at a later time point (Fig. 4, compare curves 1 and 2). This observation suggests that the relative amount of E-NH versus complex II increased with longer incubation, i.e. that the pool of unavailable NADH was increasing with time, in the absence of the eIF5A precursor. Even under conditions where the enzyme is turning over rapidly, a significant fraction of NADH could be sequestered in E-NH, as well as in complexes I, II, and III, because NADH fluorescence was still observed in the complete reaction mixture containing eIF5A precursor.

In conclusion, fluorescence analysis has permitted the partial characterization of individual steps in the complex sequence catalyzed by deoxyhypusine synthase. The findings reported here are in agreement with, and extend, earlier enzymatic data obtained by different methods (8, 9, 14). Fluorescence parameters that were consistent with the binding of Trp327 are consistent with the location of the amino acid residues in the active site and with the observed open conformation of NAD. They provide evidence for the tightly bound nature of the NADH and reutilization of its hydride ion. This analysis also suggests an interrelationship between the NADH-enzyme complex forms that depends on the relative rate constants of the individual steps and on the availability of the eIF5A precursor.

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