Deoxyhypusine synthase catalyzes the first step in the two-step post-translational synthesis of hypusine, which is uniquely present in eukaryotic initiation factor 5A (eIF5A). Deoxyhypusine synthase and eIF5A are conserved throughout the eukaryotic kingdom, and both are essential for cell proliferation and survival. A previous study (Liao, D. I., Wolff, E. C., Park, M. H., and Davies, D. R. (1998) Structure 6, 23–32) of human deoxyhypusine synthase revealed four active sites of the homotetrameric enzyme located within deep tunnels. These Form I crystals were obtained under conditions of acidic pH and high ionic strength and likely contain an inactive enzyme. Each active-site entrance is blocked by a ball-and-chain motif composed of a region of extended structure capped by a two-turn α-helix. We report here at 2.2 Å a new Form II crystal of the deoxyhypusine synthase:NAD holoenzyme grown at low ionic strength and pH 8.0, near the optimal pH for enzymatic activity. The ball-and-chain motif could not be detected in the electron density, suggesting that it swings freely and thus it no longer obstructs the active-site entrance. The deoxyhypusine synthase competitive inhibitor N^1-guanyl-1,7-diaminoheptane (GC7) is observed bound within the putative active site of the enzyme in the new crystal form (Form II) after exposure to the inhibitor. This first structure of a deoxyhypusine synthase:NAD inhibitor ternary complex under physiological conditions now provides a structural context to discuss the results of previous biochemical investigations of the deoxyhypusine synthase reaction mechanism. This structure also provides a basis for the development of improved inhibitors and antiproliferative agents.

Deoxyhypusine synthase (DHS) catalyzes the first of two steps in the post-translational modification of a single lysine residue of the precursor eukaryotic initiation factor 5A (eIF5A(Lys)) to the unique amino acid hypusine (N^1-(4-amino-4-carboxy-butyryl)lysine) (1). DHS converts lysine to deoxyhypusine (N^1-(4-amino-4-carboxy-butyryl)lysine) by a NAD-dependent transfer of the butylamine moiety of spermidine. In the second step of the modification, deoxyhypusine hydroxylase catalyzes the hydroxylation of deoxyhypusine, yielding hypusine. A highly interesting aspect of this post-translational modification is the extreme specificity of it. The only known endogenous occurrence of hypusine is a single modified lysine in the mature form of eIF5A. The importance of this post-translational modification is underscored by the conservation of DHS and eIF5A in all eukaryotes and archaebacteria (2–4) and the requirement of active DHS and eIF5A for cell proliferation. eIF5A(Lys) is likely modified directly following translation, as there is little or no accumulation of eIF5A(Lys) in cultured cells (1). Specific inhibitors of DHS or deoxyhypusine hydroxylase result in cellular growth arrest and alter tumor cell proliferation and differentiation (5–9). Furthermore, the hypusine residue of eIF5A has been reported to be critical for in vitro RNA binding (10) and the interaction with exportin 4 (11).

The multistep reaction catalyzed by DHS requires two substrates, spermidine and eIF5A(Lys), plus the cofactor NAD. It is proposed to proceed in four steps, employing two imine intermediates and a transient hydroxide (12–14): 1) spermidine + NAD → dehydrospermidine + NADH, 2) dehydrospermidine + enzyme → enzyme(Lys)-imine intermediate + diaminopropane, 3) enzyme(Lys)-imine intermediate + eIF5A(Lys) → eIF5A(Lys)-imine intermediate + enzyme, and 4) eIF5A-imine intermediate + NADH → eIF5A-deoxyhypusine intermediate + NAD. Evidence for each of these steps has been reported based on biochemical and fluorescence studies (1, 12–14). The first two steps can proceed in the absence of eIF5A(Lys) yielding diaminopropane and Δ1-pyrroline.

The structure of human DHS complexed with NAD was reported previously by this laboratory (15) and will be referred to as the Form I crystal. These DHS crystals were grown in the presence of high ionic strength (1.7 M sodium/potassium phosphate) and at an acidic pH (∼4.5) at which DHS is inactive in vitro. This structure revealed that the entrance to each of the four tunnel-like active sites of the DHS homotetramer was sealed from the external milieu by a ball-and-chain motif composed of a two-turn α-helix (residues Ala11–Val17) attached to an extended loop. This compact helix obstructs the active-site entrance with the helical axis approximately perpendicular to the pathway down the tunnel. Furthermore, in the previous study the competitive DHS inhibitor 1,7-diaminoheptane was included in the crystallization mother liquor, but it was not observed in the refined crystal structure. The failure to observe bound 1,7-diaminoheptane was attributed to the obstructed active-site entryway, the acidic...
Structure of GC7-inhibited Deoxyhypusine Synthase

Table I

|                      | DHS/NAD (Form I - this study) | DHS/NAD (Form II) | DHS-NAD-GC7 (Form II) |
|----------------------|------------------------------|------------------|----------------------|
| Space group          | P42,2                        | P3,21            | P3,21                |
| Cell dimensions (Å)  |                              |                  |                      |
| a = b                | 108.6                        | 104.8            | 104.8                |
| c                    | 69.8                         | 159.7            | 159.3                |
| Resolution (Å)       | 2.1                          | 2.2              | 3.0                  |
| No. of observations  | 104309                       | 196239           | 68751                |
| No. of unique reflections | 22384                      | 50963            | 20171                |
| Completeness (%)     | 89.5 (67.4)                  | 99.9 (99.3)      | 96.3 (92.5)          |
| R_{free} (%)         | 5.8 (17.4)                   | 8.0 (30.3)       | 9.9 (25.4)           |
| Refinement statistics|                              |                  |                      |
| No. of reflections (I/σ(I) ≥ 0.0) | 22019                      | 48787            | 19545                |
| Working              | 20724                        | 46492            | 18603                |
| Test                 | 1295                         | 2985             | 942                  |
| R (R_{free})         | 0.199 (0.245)                | 0.177 (0.199)    | 0.196 (0.232)        |
| No. of protein atoms | 2678                         | 5144             | 5154                 |
| No. of NAD atoms     | 44                           | 88               | 88                   |
| No. of GC7 atoms     | 24                           |                  |                      |
| No. of waters        | 138                          | 423              |                      |
| (B-factor) (Å²)      |                              |                  |                      |
| Monomer A            | 16.9                         | 29.6             | 46.1                 |
| Monomer B            | 27.5                         | 42.3             |                      |
| NAD                  | 34.5                         | 40.2             | 19.0                 |
| GC7                  | 24.8                         | 38.7             |                      |
| Waters               |                              |                  |                      |
| Root mean square deviation from identity | 0.0090 | 0.0095 | 0.0104 |
| Bond lengths (Å)     | 1.8                          | 1.4              | 1.4                  |
| Bond angles (degrees)|                              |                  |                      |

Numbers in parenthesis refer to statistics for highest resolution shell.

pH, and high ionic strength. The inaccessibility of the DHS active site suggests that the Form I structure is unlikely to be the fully active form of the enzyme. This observation prompted continued screening for alternate crystallization conditions.

We report here a new crystal form (Form II) of the human DHS:NAD holoenzyme obtained under significantly different crystallization conditions, i.e. low ionic strength and pH 8.0. Importantly, in vitro assays have demonstrated that DHS is enzymatically active at this pH (16–18). In this new Form II crystal, the ball-and-clip motif has undergone a dramatic structural change allowing accessibility to the active sites. This structure is likely to be more representative of the active form of DHS. Furthermore, DHS Form II crystals soaked in the potent DHS competitive inhibitor, N2-guanyl-1,7-diaminoheptane (GC7) (19), yielded the crystal structure of the first DHS-NAD-inhibitor ternary complex. The observation of the spermidine analog GC7 bound in the DHS active site provides a new structural perspective to discuss mutational data, the proposed mode of spermidine binding, and the reaction mechanism.

MATERIALS AND METHODS

Protein Purification—Recombinant human DHS was overexpressed in Escherichia coli and purified as described previously (13, 15, 20). Crystallization—The new crystal form (Form II) of DHS was initially observed while using the Hampton Research 2-methyl-2,4-pentanediol (MPD) grid screen. Optimized crystallization conditions were 4.5–7.0 mg/ml DHS in 3 mM NAD, 50 mM Tris-HCl, pH 7.8, and 100 mM KCl combined in a 1:1 ratio with the reservoir solution (64–70% (v/v) MPD and 0.1 M Tris-HCl, pH 8.0). A typical initial droplet size was 3.5 μL. The crystallization droplets were equilibrated via vapor diffusion against 1 ml of the reservoir solution at room temperature. Well formed but small crystals (~0.1-mm maximum each dimension) grew within several days. Selected crystals were soaked in a stabilizing solution containing 64% (v/v) MPD, 2 mM GC7, 0.11 M Tris-HCl, pH 8.0, for several days to form the inhibitor–enzyme complex. GC7 was synthesized as described previously (19). Form II crystals were washed well with a stabilizing solution devoid of DHS, dissolved in water, and subjected to MALDI-TOF and SDS-PAGE analysis (data not shown). This analysis confirmed the presence of only full-length DHS in the Form II crystals. Fresh Form I crystals were grown following the published protocol (15) excluding 1,7-diaminoheptane. Crystals of both types were grown using DHS from the same sample aliquot. Form II crystals contain two DHS monomers/asymmetric unit, as opposed to the single monomer/asymmetric unit observed within the Form I crystal.

X-ray Diffraction Data Collection—Crystals were flash-cooled using liquid propane. No additional cryoprotectant was required because of the high concentration of MPD. Data collection was conducted at 95 K. Initial diffraction data sets were obtained using CuKα radiation produced from a Rigaku RU-200 rotating anode and a R-Axis IIC detector. Final data sets were collected at Beamline X-9B at the National Synchrotron Light Source using monochromatic x-rays with a wavelength of 0.98 Å and an ADSC Quantum 4 CCD detector. The data were processed with the HKL package (21). Please see Table I for data collection and refinement statistics.

Structure Determination and Refinement—The structure of the new Form II crystal of the DHS-NAD binary complex was determined using the molecular replacement program AMORE (22). The search model was the DHS-NAD complex from the Form I crystal (PDB code 1DHS) (15). The unambiguous results from the rotation and translation functions were used to position two copies of the search model within the Form II asymmetric unit. CNS (23) and O (24) programs were used for refinement and model building, respectively. A test set of reflections (2295, 4.4% of total) was randomly selected prior to refinement to calculate R_{free}, to monitor the refinement progress. Bulk solvent correction and an overall anisotropic temperature factor correction were performed. Non-crystallographic symmetry 2-fold restraints were employed, with the weighting scheme guided by R_{free}. Initially the non-crystallographic symmetry restraints were strong, and as the model improved, the restraints were weakened to allow the model to reflect structural discrepancies observed in the electron density. Waters were included in the model at later stages of refinement, with positions located based on peaks ≥3σ in Fw − FF difference maps and possessing reasonable hydrogen bonding geometry. The final model included individual isotropic temperature factors.

Crystals of the DHS-NAD-GC7 ternary complex were isomorphous to the Form II crystals that did not undergo the inhibitor soak, and so additional phasing information was not required to calculate an initial electron density map. However, the soaked crystals exhibited weaker diffraction intensities. GC7 molecules were built into the strong (~3σ) Fw − FF difference present in each active site of the DHS tetramer. The refinement scheme was similar to that described above. The test data set used to calculate R_{free} was composed of reflections having the same indices as those in the test set used in the Form II DHS-NAD binary complex refinement, minimizing the bias in R_{free}. No
waters were included in the model of the inhibited holoenzyme because of the modest resolution of the data.

The Form I DHS crystal structure was refined using newly collected data from crystals grown in parallel with the Form II crystals. This experiment was done to ensure that structural differences between the DHS Form I and the Form II crystal structures were truly because of the dissimilar crystallization conditions (e.g., pH and ionic strength) and not because of the use of different DHS sample preparations. The original Form I structure was the starting model, and refinement commenced using simulated annealing protocols in CNS.

DHS in the Form II crystals exhibited several regions of structural disorder as judged by the absence of observed electron density following refinement. These regions include the N terminus (Met1-Glu27) and the C terminus (His364-Asp369) for both monomers within the asymmetric unit, and Ser78-Arg92 within monomer B only. The loop containing Ser78-Arg92 was also disordered in the Form I structure.

The geometry of all final models was analyzed with PROCHECK (25). Figures were created using RIBBONS (26) and GRASP (27).

Homospermidine Synthase (HSS) Homology Model—A model of the three-dimensional structure of HSS monomer from Senecio vulgaris was constructed by the Swiss-Model Server (28) using DHS as the homology template (58.1% identical, 73.8% similar).

RESULTS AND DISCUSSION

Overall Structure—DHS is present as a homotetramer in the new Form II crystal (Fig. 1A) as it was in the Form I crystal (Fig. 1B) (15). The only species observed corresponded to the expected molecular mass of a DHS tetramer (164 kDa) in size exclusion chromatography and dynamic light scattering solution studies (1, 12, 17). Thus the tetramer is highly likely to be biologically relevant and not a crystal-packing artifact. The tetramer in the Form II crystal exhibits pseudo-222 symmetry, governed by a single crystallographic 2-fold and two non-crystallographic 2-fold rotational symmetry elements; whereas, the Form I tetramer exhibits strict 222 symmetry. The two unique monomers within the Form II crystallographic asymmetric unit are denoted A and B, respectively. The tetramer may be considered as a dimer of dimers. This distinction is based upon the observation that each catalytic site is comprised of residues from two monomers. Each fundamental dimeric subunit of two closely associated monomers (Fig. 1A, A1 with B1 and A2 with B2) form two complete but antiparallel active sites at their interface, with 2830 Å²/monomer buried in the Form II crystal. These two crystallographically identical dimers form a looser association, creating interfaces between monomers A1 and B2, and between monomers B1 and A2. This second interface buries 2470 Å²/monomer in the Form II crystal. No additional active sites are created by the dimer-dimer interaction.

An essential question regarding DHS is why is it present as a tetramer when the fundamental catalytic unit of the enzyme appears to be a dimer? Data indicate the possible presence of negative cooperativity upon the successive binding of ligands, and the DHS tetramer appears to bind only a single eIF5A(Lys) monomer (14, 17). Fluorescence studies monitoring the conversion between NAD and NADH demonstrated that a certain population of the enzyme-bound NADH may not be readily available to reduce the eIF5A-imine intermediate in the final step of the proposed mechanism (14). These data have led to speculation that the DHS tetramer may undergo an asymmetric conformational change upon binding, either

Fig. 1. Human DHS crystal structures. A, stereoview of the Form II DHS-NAD tetramer, with each monomer indicated by a different color and label, and NAD in red. The black diamonds indicate the general location of the active sites, and the blue spheres denote Ser28. B, the DHS Form I structure (this study), emphasizing the ball-and-chain motif obstructing an active-site entrance. C, the Rossmann fold of the DHS monomer is green, the mobile ball-and-chain is magenta, and the secondary structure labeling scheme is from Ref. 15.

2 T. C. Umland and D. R. Davies, unpublished data.
singularly or in combination, the cofactor and one or both of the substrates. The relaxed symmetry requirements for the DHS tetramer in the new Form II crystal coupled with the ability of DHS to bind GC₇ within these crystals allowed for further analysis of the interplay between monomers within the tetramer.

Intimate contacts formed between each monomer with each of the remaining three partners within the DHS tetramer provide additional evidence that the tetramer is required for either a regulatory mechanism or enhanced stability. For example, consider monomer A1. It interacts with monomers B1 and B2, forming the two types of dimer interfaces discussed above. More interestingly, monomer B2 contributes an N-terminal ball-and-chain motif (Fig. 1) that is capable of blocking one of the active sites formed at the A1-B1 dimer interface, as observed in the Form I crystal (Fig. 1B). Monomer A1 lacks extensive contacts with the fourth monomer, monomer A2, but the limited interactions are distinctive. First, monomer A2 contributes a ball-and-chain motif capable of blocking the entrance to the second active site formed at the A1-B1 interface. Secondly, Phe⁵⁴ of monomer A2 makes extensive contacts to a loop in monomer A1 (Asp¹³¹ and Gly¹³⁴) and a loop in monomer B1 (Asn¹⁰⁶), both of which interact extensively with the NAD adenine moiety. Phe⁵⁴ is present in a hairpin turn connecting two helices, possessing the unusual strained main chain geometry of $\phi = -42^\circ$ and $\psi = -135^\circ$ in both crystal forms, and is well defined by electron density. These interactions allow for a potential transmission path for a regulatory conformation change.

The root mean squared deviation is 0.17 Å between the Ca atoms of the two independent monomers in the Form II crystal of the DHS:NAD holoenzyme, superimposable by a rotation of 180.0° coupled with a translation of 0.16 Å. Each NAD binding site is occupied to a comparable degree based upon a visual inspection of the electron density and the refined temperature factors. Similarly, the root mean squared deviation between the Ca atoms of the two independent monomers in the Form II crystal of the DHS:NAD:GC₇ ternary complex is also 0.17 Å, and each of the NAD and the GC₇ binding sites is equally occupied. Furthermore, the Form II crystals exhibited only two limited regions of the main chain possessing significant conformational differences between the two independent monomers. First, there is a lack of electron density to define the position of residues His³⁶⁴, Asp³⁶⁶, and presumably they are highly mobile. Met³⁶³ is the last residue of the chain defined by electron density, assuming different conformations in the independent monomers. Second, the loop composed of residues Ser³⁷⁸–Arg³⁹² is observed within monomer A but not within monomer B of the Form II crystals. It is also unobserved within the Form I crystal structure. No function has been identified with either one of these two regions, but both human and Saccharomyces cerevisiae DHS require the presence of the five C-terminal residues for full activity (20, 29). It should be emphasized that the weights of the non-crystallographic symmetry restraints employed during refinement were carefully selected so as not to artificially force the monomers to be similar, and $2F_o - F_r$ omit maps calculated following a short simulated annealing protocol were used to reduce model bias.

Thus, there is no structural indication from the Form II crystals that NAD alone or the combination of NAD and GC₇, when present in excess stimulates negative cooperativity within DHS because of an asymmetric conformational change lowering the affinity for the successive binding of ligands. However, any potential conformational changes to the DHS tetramer resulting from the binding of eIF5A(Lys) or from less than equivalent concentrations of NAD or GC₇, remains to be addressed.

**NAD Binding Site**—The NAD cofactor binds to a Rossmann dinucleotide-binding motif present within the core of the DHS monomer (Fig. 1C). A subdomain is inserted into this motif following strand β₂, partially forming the active site. The DHS polypeptide chain is extended on both the N- and C-terminal sides of the dinucleotide-binding motif. The NAD cofactor is bound in the canonical manner to the Rossmann fold of monomer A but is also subjected to an interaction with monomer B involving a loop (Thr³⁰⁸–Lys³¹⁵) following strand β₃. This loop also contributes crucial residues to the catalytic active site. The NAD buried surface area due to interactions with monomer A and B are ~476 and ~294 Å², respectively. The majority of the NAD-binding interactions are similar to those observed in the Form I crystal. However, in the Form I crystal Oε₂ of Glu¹³⁴ interacts with the amide nitrogen of the nicotinamide ring. In the Form II holoenzyme crystal, this residue assumes a different rotamer in each of the two independent monomers. In monomer A this side chain points away from the nicotinamide ring, hydrogen bonding to Oε₂ of Tyr²₇⁶ and several waters, whereas Glu¹³⁴ of monomer B resembles that seen in the Form I crystal. In the Form II holoenzyme/inhibitor complex, Glu¹³⁷ of monomer A interacts weakly with both the nicotinamide ring and the hydroxyl group of Tyr²₇⁶, and again this glutamate residue in monomer B resembles that observed in the Form I crystal. Only the nicotinamide ring of NAD has any appreciable solvent accessibility (~30 Å²) in the absence of a bound substrate or inhibitor and is completely solvent-inaccessible in the presence of GC₇.

**Active Site**—The observation of an enzyme-imine intermediate (Enz–Lys–N=CH(CHO)₂NH₂) identified Lys³¹⁵ of human DHS and Lys³⁵₀ of yeast DHS as the residue to which the butylamine moiety of sperridine is transferred in the second reaction step (13, 30). The Form I crystal structure (15) suggested further residues as having a role in the active site based on their proximity to Lys³¹⁵ (14, 30–32). The active site is contained within a deep narrow tunnel present at a dimer interface. The specificity is conferred by the large number of charged residues lining the tunnel walls and entrance coupled with the inaccessibility (see Figs. 3 and 4A). The tunnel is ~17 Å deep, as measured between the main chain carbonyl of Gly³¹⁴(A) to Oε₂ of Asp²⁴⁴(B). The entrance to the active-site tunnel is a broad funnel that is ~7 Å deep, resulting in the bottom of the tunnel being ~24 Å below the surface of the protein. The tunnel constricts to its narrowest at a ring-like arrangement of the side chains of His²⁸₈, Trp²⁷⁷, Lys³⁵₀, and the nicotinamide ring of the NAD cofactor. The distance between the Ne-2 of His²⁸₈ and Cε-3 of Trp²⁷⁷ is ~6.8 Å and between the Nε of Lys³⁵₀ and C-5 of the nicotinamide ring of NAD is ~6.4 Å in the Form II DHS:NAD structure. The groups forming this constriction have been proposed to participate in the reaction mechanism (15–17, 31). The tunnel then widens, leading to a side pocket at the bottom (see below). In the
absence of eIF5A(Lys), DHS catalyzes the NAD-dependent cleavage of spermidine to generate 1,3-diaminopropane and a reactive enzyme-imine intermediate (13). Spermidine binding to DHS is independent of the presence of eIF5A(Lys) but is enhanced by the presence of NAD (18, 31). These observations indicate that eIF5A(Lys) does not confer a significant conformational change to the active site that is crucial to the formation of dehydrospermidine and the DHS(Lys)-imine intermediate.

**Bound GC7 Inhibitor**—The structure of the Form II crystal soaked with GC7, a spermidine analog and a competitive inhibitor (Fig. 2) (19), provides further refinement of the proposed model of spermidine binding within the DHS active site (Fig. 3 and supplemental Fig. 1). Each of the two crystallographically independent active sites binds GC7 in a similar manner. The bound GC7 molecule is in an extended conformation. Electron density definitely indicates that its guanidinium moiety is positioned toward the bottom of the active-site tunnel, and its amino group is near the tunnel entrance. A notable feature of the active-site tunnel is that it is lined by a large number of charged residues that anchor GC7 in the active site. See Table II for a listing of close contacts with GC7. Specifically, Asp316(A) and Glu243(A) lie at the tunnel bottom and have close contacts with the GC7 guanidinium group. They presumably are also involved in contacts with one of the primary amino groups of spermidine, as mutation of either of these residues to alanine nearly eliminates spermidine binding (31). The main chain carbonyl group of Gly114(A) lies within hydrogen-binding distance of the GC7 guanidinium group. Near the entrance of the tunnel is Asp243(B), which forms a salt bridge to the GC7, terminal amino group. A second hydrogen bond is formed to this amino group by the side chain O\(^\text{6-1}\) of Asn292(A). The D243A mutation also nearly eliminates spermidine binding. The side chain carboxyl groups of Glu136(B), Glu137(B), and Asp135(A) further contribute to the overall negative charge of the binding site. In the tunnel, Glu136(B) lies on the level of the C-2 atom of GC7, and Glu137(B) lies on the level of the C-6 atom of GC7, and each is \(-5.4\) Å from GC7. The mutation of Glu137(B) to alanine reduces spermidine binding to \(<1\%\) of that of wild-type DHS. The Asp135(A) side chain interacts with the main chain amino group of Gly114(A), contributing to the overall negative charge of the guanidinium-binding pocket.

The binding of GC7 results in the displacement by \(-1.3\) Å of the Lys228 ε-amino group. It shifts toward the imidazole ring of His288 yielding a close contact of \(-3.3\) Å (Lys228 N\(^{\text{ε-2}}\) to His288 Nε-2), as opposed to \(-3.9\) Å in the uninhibited holoenzyme. This is notable because Lys228 is the residue that is modified by the ε-amino group of spermidine during the initial spermidine dehydrogenation step. The spermidine binding of the H288A DHS mutant is less than 2%, and its NAD binding is less than 5% of the wild-type enzyme, respectively (31). The ε-amino group of Lys228 is abutted against GC7 atoms C-3, C-4, and C-5, forming contacts of 3.2, 3.5, and 3.6 Å, respectively. The location of the C-4 of GC7 is further constrained by close contacts to Ne-2 of His288 (3.4 Å) and to C-4 of the nicotinamide ring of NAD (3.3 Å), which is the atom that accepts the hydrogen generated from the dehydrogenation of spermidine (14). The secondary amine group of spermidine (Fig. 2, N-4) would lie in the position occupied by C-4 of GC7. Thus, by analogy this amine group is well positioned for the NAD-dependent dehydrogenation of spermidine to yield a N=C bond, which then is subjected to nucleophilic attack by the ε-amino group of Lys228 to form the enzyme-imine intermediate. A consideration of the likely protonation state of spermidine is important in light of the highly charged nature of the spermidine binding site. Each of the three amine groups of spermidine possess different pKa values (9.94, 8.40, and 10.81 for N-1, N-4, and N-8, respectively) (33). DHS displays maximum ac-
tivity in vitro at pH 9.0–9.5, and activity diminishes at lower pH until it is completely abolished below pH 7.0. At the optimal pH range for DHS activity, spermidine in solution is expected to exist primarily as the diprotonated species with its secondary amino group uncharged, and enzyme activity can be correlated with the concentration of this species (17). For the trip protonated spermidine to bind to DHS at a pH <9, in a similar orientation as GC7, the unfavorable charge interaction between the ε-amino group of Lys292 and the positively charged secondary amine of spermidine would have to be overcome. Furthermore, for the initial transamination reaction to occur at acidic pH, an additional proton would have to be removed from spermidine and ultimately from the DHS active site, and the His356 imidazole would become protonated, interfering with its proposed role as a general base for the dehydrogenation reaction. An interplay between NAD binding and spermidine binding exists (18, 31). The DHS-NAD-GC7 ternary structure allows this behavior to be more fully understood. There is essentially no binding of either spermidine or GC7, in the absence of NAD. The presence of spermidine or GC7 enhances NAD binding but is not required (18). Residues that line the NAD binding site had previously been subjected to mutational analysis. A general trend was observed in which mutants that reduced NAD binding also reduced spermidine and GC7 binding, with the most dramatic reductions in spermidine binding occurring for E137A, D235A, and D342A (31). Both Asp235 and Asp342 lie distant from the bound GC7, and thus the reduction in GC7 affinity upon mutation is very likely an indirect effect resulting from reduced NAD affinity. The nicotinamide moiety of NAD forms close contacts with 50% of the GC7 atoms, and this would be expected to be similar for bound spermidine. Thus, the absence of NAD would severely alter the geometry of the spermidine binding site. The E137A mutation only moderately reduces NAD binding but has a severe affect on spermidine and GC7 binding. Glu137 contributes to the overall negative charge of the spermidine binding site and has a close contact of 5.3 Å between its Oε-2 atom and C-4 of GC7. This GC7 atom likely corresponds to the position that the secondary amine group of spermidine, ω-4, would occupy when bound to DHS. Oε-2 of Glu137 also makes a close contact to the C-4 of the nicotinamide ring of NAD (3.5 Å). Because the carboxyl group of Glu137 lies near the donor and the acceptor of the spermidine dehydrogenation reaction, it is likely that Glu137 not only is important for spermidine binding but also plays a role in the DHS reaction mechanism. Glu137 is highly conserved in eukaryotic DHs and is either a glutamate or histidine in archaea. The observation of GC7 bound in the DHS active site confirms predictions (19, 34) regarding the binding mode of spermidine and its guananyl analogs through the extensive study of DHS inhibitors. The active site was predicted to be a narrow tunnel, with negatively charged sites near the entrance and the bottom of the tunnel optimized to anchor two positively charged groups (e.g. primary amino groups) of a linear molecule differing in length from spermidine by only plus or minus a methylene group. The monoguanylated analog of spermidine, GC7, was hypothesized and found to be a very potent DHS inhibitor with a K<sub>i</sub> value of 0.01 μM (versus K<sub>m</sub>app of 4.5 μM for spermidine) (19). GC7 is of the proper length, has a linear central moiety with positively charged groups at each end, and the additional characteristic of its guanidinium group to form a pair of zwitterionic hydrogen bonds. Such a pair of hydrogen bonds can potentially yield a stronger binding interaction than an amino group. The GC7 guanidinium group is positioned within the crystal structure to form a pair of hydrogen bonds to the carboxyl group of Glu323 and the carbonyl group of Gly314. The slightly more bulky group can be accommodated because of the side pocket at the end of the active site.

Active Site Side Pocket—The bottom of the DHS active-site tunnel expands to form an interior side pocket. Access to this side pocket is possible only by the long traverse of ~15 Å through the tunnel, passing the essential Lys327 catalytic residue midway (see Fig. 3 and supplemental Fig. 1). Interestingly, several charged and polar side chains (Lys327, Tyr355, Asp356, and Glu359) participate in defining the side pocket cavity despite its deeply buried nature. All four of these residues are highly conserved in DHS from different species, yeast to humans. The nature and location of these residues suggest that the side pocket has a functional importance that remains to be clarified, perhaps participating in the binding of a reaction intermediate.

Residues Asp316 and Glu323 are positioned at the junction of the main active-site tunnel and the side pocket (Fig. 3). They likely participate in the binding of spermidine in the active site, based on mutagenesis data (31) and the structure of the DHS-NAD-GC7 ternary complex. The Y305A mutation modestly reduces both spermidine and NAD binding, but the mutant DHS retains significant activity. Tyrosine at this position is strictly conserved in all eukaryotes. Tyr355 forms the back wall of the side pocket, and its solvent-exposed hydroxyl group is ~8 Å away from the active site, as measured from the ε-amino group of Lys327, and is ill positioned to directly interact with either spermidine or NAD. Because of the large difference in volumes occupied by tyrosine and alanine, the Y305A mutation may lower spermidine and NAD binding by disturbing the packing of the side chains of nearby residues. The conserved Lys327 forms a salt bridge with Asp316 and may serve to modulate the concentration of negative charges located in the active-site tunnel. The mutations K287R and K287A drastically reduce enzymatic activity to <1% of the wild type enzyme, as measured by several different assays (30, 32). The Lys327 side chain projects into the void of the side pocket, with sufficient space present to accommodate the bulkier arginine side chain of the K287R mutation without resulting in severe conformational changes of nearby residues (e.g. His356 and Asp356). This mutation preserves the negative charge while reducing the volume remaining within the pocket.

In the previously reported Form I crystal structure, continuous electron density was present within the side pocket but could not be attributed to DHS, NAD, nor any other chemical species known present in the mother liquor (15). It was suggested that this density was possibly a fortuitously captured reaction product or intermediate formed during purification. In contrast, the side pocket in the Form II crystal of the DHS-NAD binary complex contains several well defined spherical peaks in the F<sub{o}</sub> – F<sub>c</sub>, and the 2F<sub{o}</sub> – F<sub>c</sub> omit electron density maps. These peaks were assigned as waters, but they could also be monovalent potassium or chloride ions, as these were present in the mother liquor. Very similar arrangements of waters were observed in each of the two crystallographically independent side pockets, but no non-crystallographic symmetry restraints were applied to the waters during model building and refinement.

The active-site side pocket of the Form I crystal grown in parallel with the Form II crystals was examined. It failed to contain any continuous density in the solvent region. Rather, discrete spherical density was present in similar positions as observed in the Form II crystal of the two independent side pockets of the structure and interpreted as waters. Thus, it is likely that the extra but unassigned continuous density observed in the side pocket of the Liao et al. (15) structure is because of differences in crystal handling that may have resulted in a less well ordered water structure.
Ball-and-Chain Motif—The Form I DHS crystal structure exhibits an unusual ball-and-chain motif that tightly blocks the entrance to each active-site tunnel (15), as described above. The motif was defined as the N-terminal residues Ala9–Val17, forming a two-turn α-helix (the ball), and the extended structure (the chain) comprised of residues Leu18–Pro25 (Fig. 1, B and C). Five of the nine residues of the ball motif are alanines, and the majority of the interactions between the ball and the active-site entrance are hydrophobic.

In the Form II crystals, the ball-and-chain motif underwent a dramatic structural alteration. Electron density is absent for residues Met1–Glu27, a region including the ball-and-chain motif, for both crystallographically independent DHS monomers. The most likely explanation for the unobserved electron density is that the ball-and-chain motif is highly mobile (i.e. a swinging ball-and-chain) under the Form II crystallization conditions. Thus, in the Form II crystals the catalytic active-site entrances are unobstructed allowing access to the competitive inhibitor GC7.

The integrity of the crystallized DHS was confirmed using MALDI-TOF mass spectroscopy and SDS-PAGE (data not shown). The DHS integrity was further confirmed by growing Form I and II crystals in parallel using DHS from the same sample preparation. The electron density for the ball-and-chain motif was clearly observed in these Form I crystals but was absent in the Form II crystals (Fig. 1, B versus A). In both the Form I and II crystal structures, the residues immediately C-terminal to Glu27 are tightly associated to the globular body of the DHS monomer. Hence, residues Glu27 and Ser28 may be thought of as a hinge or elbow from which the ball-and-chain region extends.

The transition from the static to the swinging ball-and-chain is likely because of the significantly different crystallization conditions utilized to grow the two crystal forms. The Form I crystallization used 1.7 M sodium/potassium phosphate as the precipitating agent, and the pH of the mother liquor was ~4.5. This high salt condition would tend to enhance the hydrophobic interactions that stabilize the ball-and-chain binding at the active-site entrance. The crystallization pH is also near that of the calculated pI (5.2) of the DHS monomer. Moreover, in vitro the enzyme is inactive at this pH (16–18). The Form II crystallization employs low salt conditions, using the polyalcohol MPD as the precipitating agent, and the mother liquor pH is ~8.0 at which DHS remains active.

The two crystallographically independent monomers in the Form II crystal permit consideration of any effect that the crystal-packing environment may have upon the conformation of the motif. Crystal contacts are present near the entrance to one of the two active sites in the crystallographic asymmetric unit. These contacts have the potential to sterically interfere with the association of the ball-and-chain motif to the entryway in a manner identical to that observed in the Form I crystal. However, the second active-site entrance borders a large solvent channel, lacking any nearby crystal-packing contacts. In both cases the ball-and-chain motif is disordered and presumably highly mobile. Thus, the crystal-packing environment appears not to have a strong influence upon the ball-and-chain motif under the Form II crystallization conditions. The presence of the inhibitor GC7 in the Form II crystals does not visibly stabilize the structure of the ball-and-chain motif, as it is also disordered in the ternary complex crystals.

A study to identify the core catalytic fragment of S. cerevisiae DHS found that the Δ1–5 and Δ1–10 deletion mutants yielded fully active DHS. The Δ1–15 deletion mutant exhibited very reduced activity, and the Δ1–20 deletion mutant was inactive (29). These data indicate that although the chain portion of the motif (Leu18–Pro25) is crucial for enzymatic activity, the ball portion also plays a role. This appears to be true even though the ball-and-chain region of DHS is not as highly conserved across species as much of the remainder of the DHS sequence.

The Form II crystals demonstrate that the structure of the ball-and-chain motif can be dramatically transformed solely by altering the solvent conditions, while leaving the structure of the globular portion of the protein relatively unchanged. This does not rule out the ball-and-chain as serving a regulatory role. However, it does indicate that a specific interaction between DHS and another protein or small molecule ligand is not necessary to switch the ball-and-chain motif between the bound state, where it obstructs access to an active site, to a disordered mobile state.

eIF5A(Lys) Binding—The minimal fragment of eIF5A(Lys) required for modification by DHS is almost its entire N-terminal domain (residues 20–80) (35). The lysine residue to be modified, Lys50 of human eIF5A(Lys), is present at the tip of a flexible finger-like loop in the structures of archaea eIF5A (36–38). The presentation of this lysine residue is as might be expected given the buried nature of the DHS active site.

DHS binds eIF5A(Lys) with a very high affinity (KD < 0.5 nM in the absence of NAD or spermidine) (17). Formation of a DHS-eIF5A(Lys) complex, and thus the conformation of the eIF5A(Lys) binding site, appears to be independent of NAD or spermidine binding (17). A full mutational analysis of DHS...
residues predicted to be present at the DHS:eiF5A(Lys) binding interface has not been performed, but the distribution of charge within or around the active-site tunnel is likely important, as significant ionic contributions to the binding were predicted from thermodynamic calculations (17). A mutational analysis of residues forming the NAD and spermidine binding sites revealed a single mutation (D313A) that significantly reduced eiF5A(Lys) binding (31). Paradoxically, Asp313 lies near the bottom of the active-site tunnel, and its side chain carboxyl group interacts with the adenine moiety of NAD. The D313A mutation also severely reduces NAD binding affinity. Two additional DHS mutations (D243A and K329A) enhanced eiF5A(Lys) binding. Asp329 lies near the tunnel entrance. Although Asp243 and Lys329 appear not to be optimal for eiF5A(Lys) binding to DHS, these two residues are catalytically important. Asp329 forms a salt bridge to the terminal amine group of GC7, and presumably interacts similarly with bound spermidine. Lys329 participates in forming the enzyme-imine reaction intermediate, which then participates in the imine transfer to eiF5A(Lys).

Comparison with Homospermidine Synthase—It is instructive to compare DHS with a closely related plant enzyme, HSS when considering (eiF5A(Lys)) binding. HSS likely evolved from DHS via gene duplication in a progenitor of certain plants that now produce pyrrolizidine alkaloids as defense compounds against herbivore insects (39–41). A major difference between these two highly related enzymes is that HSS is incapable of binding eiF5A(Lys) and thus is incapable of using it as a substrate (41).

HSS catalyzes the NAD-dependent transfer of the butyramine moiety from spermidine to putrescine, yielding the higher homolog, homospermidine [(N-4-aminobuty)-1,4-butanediamine]. DHS is also capable of producing homospermidine from spermidine as a side reaction in the absence of eiF5A(Lys) and presence of putrescine and may be responsible for the presence of homospermidine within cells lacking HSS (39, 41–43). DHS and HSS employ the same enzymatic mechanism, and the residues defining the active-site tunnel and the NAD binding site are highly conserved. HSS retains an aspartate residue (Asp315) at a position equivalent to Asp313 in DHS, and so loss of eiF5A(Lys) binding by HSS is not because of the loss of this residue as occurs in the DHS D313A mutant. A S. vulgaris HHS structural model was constructed by homology modeling using the Form I DHS crystal structure. A sequence alignment exhibited 58.1% identical and 73.8% similar residues. This homology-modeled HSS structure was then compared with the DHS crystal structures with the aim of locating regions about the active-site tunnel and entrance that differ significantly between the two enzymes, and may account for the difference in eiF5A(Lys) binding capability. With the exception of several conservative substitutions, the active-site tunnel and side pocket of both enzymes were structurally very similar. However, the funnel-like entrance to the active-site tunnel contains a number of non-conserved substitutions that are likely to affect eiF5A(Lys) binding. Perhaps the most important substitutions are those that likely lead to different electrostatic potential distributions about the active-site tunnel entrance (Fig. 4). In both DHS and HSS, the electrostatic potential of the entrance is negative overall, but qualitatively the HSS is significantly more negative. Moreover, the small patches of positive electrostatic potential near the entryway are non-overlapping. eiF5A(Lys) must interact with this region to insert its reactive lysine residue into the active site, and surface charges are often extremely important for providing binding specificity.

The amino acid sequence within the human DHS ball-and-chain motif (Ala2-Pro25) was compared with the corresponding region within S. vulgaris HSS (Ala2-Glu24), and they exhibit 35.3% identity and 47.1% similarity. This degree of sequence similarity is less than that observed for the two proteins as a whole but is still at a significant level. This may indicate a conserved functional role for the ball-and-chain motif but not as a conserved eiF5A(Lys)-binding motif.

Concluding Remarks—The crystallization of human DHS in a second crystal form has allowed a deeper investigation of the unique features of this vital enzyme. The DHS Form II crystal demonstrates that the structure of the ball-and-chain motif can be dramatically altered by changes in ionic strength and pH and does not require the binding of a specific molecule (e.g., eiF5A(Lys) or spermidine). N-terminal deletion mutants demonstrate that a portion of the ball-and-chain motif is required for catalysis. This would not be expected if the only function of the motif is to regulate access to the active site. In fact, if its only function was access control, then it would be expected that the deletion of this motif would increase activity. The ball-and-chain motif is not vital for structural stability of the DHS tetramer. It is highly mobile within the Form II crystal structure with no significant effect on oligomerization. It remains an open question whether this motif aids in the binding of eiF5A(Lys).

The first structure of the inhibited DHS:NAD holoenzyme is reported here. The GC7 inhibitor is a spermidine analog that competes very effectively with spermidine and thus is expected to bind to the holoenzyme in a manner very similar to the actual substrate. The bound inhibitor confirms the catalytic importance of several residues and the NAD cofactor identified originally by biochemical techniques. It also provides further evidence that His288 serves as a proton acceptor/donor during catalysis. This would not be expected if the only function of this residue as occurs in the DHS D313A mutant.

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REFERENCES

1. Park, M. H., Lee, Y. B., and Joe, Y. A. (1997) J. Biol. Chem. 272, 115–123
2. Good, E. D., Mora, R., Meredith, S. C., Lee, C., and Lindquist, S. L. (1987) J. Biol. Chem. 262, 16585–16589
3. Bartig, D., Lemkeneiker, K., Frank, J., Lottspeich, F., and Klinik, F. (1992) Eur. J. Biochem. 204, 753–760
4. Chen, K. Y., and Liu, A. Y. (1997) J. Biol. Chem. 272, 105–109
5. Hanuske-Abel, H. M., Park, M. H., Hanuske, A. R., Popowicz, A. M., Lamode, M., and Folk, J. E. (1994) Biochim. Biophys. Acta 1221, 115–124
6. Park, M. H., Wolff, E. C., Lee, Y. B., and Folk, J. E. (1994) J. Biol. Chem. 269, 27827–27832
7. Chen, Z. F., Yan, Y. P., Ding, Q. J., Knapp, S., Potenza, J. A., Schugar, H. J., and Chen, K. Y. (1996) Cancer Lett. 105, 235–239
8. Jansson, B. P., Malandrin, L., and Johansson, H. E. (2000) J. Bacteriol. 182, 1158–1161
9. Nishimura, K., Ohki, Y., Fukuchi-Shimogori, T., Sakata, K., Saiga, K., Bepgu, T., Shirahata, A., Kashigawa, K., and Igarashi, K. (2002) Biochem. J. 363, 761–768
10. Xu, A., and Chen, K. Y. (2001) J. Biol. Chem. 276, 2555–2561
11. Lipowsky, G., Bischoff, F. R., Schwarzermaier, K., Kraft, R., Kostka, S., Hartmann, E., Kutay, U., and Gorlich, D. (2000) EMBO J. 19, 4362–4371
12. Wolff, E. C., Park, M. H., and Folk, J. E. (1999) Biochim. Biophys. Acta 1251, 479–499
13. Wolff, E. C., Folk, J. E., and Park, M. H. (1997) J. Biol. Chem. 272, 15865–15871
14. Wolff, E. C., Wolff, J., and Park, M. H. (2000) J. Biol. Chem. 275, 9170–9177
15. Liao, D. I., Wolff, E. C., Park, M. H., and Davies, D. R. (2000) J. Biol. Chem. 275, 11581–11585
16. Wolff, E. C., Wolff, J., and Park, M. H. (1999) Biochem. J. 340, 273–281
17. Lee, Y. B., Joe, Y. A., Wolff, E. C., Dimitriadis, E. K., and Park, M. H. (1999) Biochem. J. 352 Pt 3, 851–857
18. Jansson, B. P., Wolff, E. C., Park, M. H., and Folk, J. E. (1995) J. Biol. Chem. 270, 13151–13159
19. Joe, Y. A., Wolff, E. C., and Park, M. H. (1995) J. Biol. Chem. 270, 22986–22992
20. Owszczak, L., and Minn, W. (1997) in Methods in Enzymology (Carter, C. W., Jr., and Sweet, R. M., eds) Vol. 276, pp. 307–326, Academic Press, Orlando
22. Navaza, J. (2001) Acta Crystallogr. Sect. D Biol. Crystallogr. 57, 1367–1372
23. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D 54, 805–921
24. Jones, T. A., Cowan, S., Zou, J.-Y., and Kjeldgaard, M. (1991) Acta Cryst. Sect. A 47, 110–119
25. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Cryst. 26, 283–291
26. Carson, M. (1991) J. Appl. Cryst. 24, 958–961
27. Nicholls, A., Sharp, K. A., and Honig, B. (1991) Proteins 11, 281–296
28. Peitsch, M. C. (1996) Biochem. Soc. Trans. 24, 274–279
29. Kang, K. R., and Chung, S. I. (1999) Exp. Mol. Med. 31, 210–216
30. Wolff, E. C., and Park, M. H. (1999) Yeast 15, 43–50
31. Lee, C. H., Um, P. Y., and Park, M. H. (2001) Biochem. J. 355, 841–849
32. Joe, Y. A., Wolff, E. C., Lee, Y. B., and Park, M. H. (1997) J. Biol. Chem. 272, 32679–32685
33. Baillon, J. G., Mamont, P. S., Wagner, J., Gerhart, F., and Lux, P. (1988) Eur. J. Biochem. 176, 237–242
34. Lee, Y. B., and Folk, J. E. (1998) Bioorg. Med. Chem. 6, 253–270
35. Joe, Y. A., and Park, M. H. (1994) J. Biol. Chem. 269, 25916–25921
36. Kim, K. K., Hung, I. W., Yokota, H., Kim, R., and Kim, S. H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10419–10424
37. Peat, T. S., Newman, J., Waldo, G. S., Berendzen, J., and Terwilliger, T. C. (1998) Structure 6, 1207–1214
38. Yao, M., Ohsawa, A., Kikukawa, S., Tanaka, I., and Kimura, M. (2003) J. Biochem. (Tokyo) 133, 75–81
39. Ober, D., and Hartmann, T. (1999) J. Biol. Chem. 274, 32040–32047
40. Ober, D., Gilbas, L., Witte, L., and Hartmann, T. (2003) Phytochemistry 62, 339–344
41. Ober, D., Harms, R., Witte, L., and Hartmann, T. (2003) J. Biol. Chem. 278, 12805–12812
42. Park, J. H., Wolff, E. C., Folk, J. E., and Park, M. H. (2003) J. Biol. Chem. 278, 32683–32691
43. Ober, D., and Hartmann, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14777–14782