Deoxyhypusine synthase catalyzes the first step in hypusine (N²-(4-aminobutyl)lysine) synthesis in a single cellular protein, eIF5A precursor. The synthesis of deoxyhypusine catalyzed by this enzyme involves transfer of the 4-aminobutyl moiety of spermidine to a specific lysine residue in the eIF5A precursor protein to form a deoxyhypusine-containing eIF5A intermediate, eIF5A(Dhp). We recently discovered the efficient reversal of deoxyhypusine synthesis. When eIF5A(²H)Dhp, radiolabeled in the 4-aminobutyl portion of its deoxyhypusine residue, was incubated with human deoxyhypusine synthase, NAD, and 1,3-diaminopropane, [³H]permidine was formed by a rapid transfer of the radiolabeled 4-aminobutyl side chain of the [³H]deoxyhypusine residue to 1,3-diaminopropane. No reversal was observed with [¹⁴C]hypusine protein, suggesting that hydroxylation at the 4-aminobutyl side chain of the deoxyhypusine residue prevents deoxyhypusine synthase-mediated reversal of the modification. Purified human deoxyhypusine synthase also exhibited homospermidine synthesis activity when incubated with spermidine, NAD, and putrescine. Thus it was found that [¹⁴C]putrescine can replace eIF5A precursor protein as an acceptor of the 4-aminobutyl moiety of spermidine to form radiolabeled homospermidine. The Kₘ value for putrescine (1.12 mM) as a 4-aminobutyl acceptor, however, is much higher than that for eIF5A precursor (1.5 μM). Using [¹⁴C]putrescine as an acceptor, various spermidine analogs were evaluated as donor substrates for human deoxyhypusine synthase. Comparison of spermidine analogs as inhibitors of deoxyhypusine synthase, as donor substrates for synthesis of deoxyhypusine (or its analog), and for synthesis of homospermidine (or its analog) provides new insights into the intricate specificity of this enzyme and versatility of the deoxyhypusine synthase reaction.

The post-translational synthesis of an unusual amino acid, hypusine, converts the inactive eIF5A precursor to an active protein (see Refs. 1–3). The biosynthesis of hypusine occurs exclusively in this protein by way of two enzymatic steps. In the first step, deoxyhypusine synthase (EC 2.5.1.46) facilitates the transfer of the 4-aminobutyl moiety from spermidine, the donor substrate, to the eIF5A precursor (eIF5A(Lys)), the acceptor substrate, to form a deoxyhypusine-containing protein (eIF5A(Dhp)) (4–6). This intermediate is hydroxylated by deoxyhypusine hydroxylase (EC 1.14.99.29) (7) to complete hypusine synthesis and eIF5A maturation. The essential role of eIF5A and its hypusine modification is supported by gene disruption studies in yeast, Saccharomyces cerevisiae, in which inactivation of the two eIF5A genes (8), or of the single deoxyhypusine synthase gene (9, 10), causes a loss of cell viability.

Deoxyhypusine synthase is a tetrameric enzyme consisting of four identical subunits (11). The crystal structure of the human enzyme in a complex with its cofactor, NAD, revealed four active sites at the interfaces of subunit dimers (11). The enzyme catalyzes a complex reaction normally involving two substrates, the polyamine spermidine (the donor of the butylamine moiety) and the protein substrate, the eIF5A precursor (the acceptor of the butylamine moiety), and a cofactor, NAD. We have established that deoxyhypusine synthesis occurs by way of a covalent enzyme-imine intermediate (12) in four steps (Scheme 1, left-hand pathway): (i) NAD-dependent dehydrogenation of spermidine to form dehydrospermidine, (ii) transfer of the 4-aminobutyl moiety from dehydrospermidine to the e-amino group of an active site lysine residue (Lys-329 in the human enzyme) to form a covalent enzyme-imine intermediate, (iii) transfer of the 4-aminobutyl moiety from the enzyme-imine intermediate to the N²-amino group of a specific lysine residue of the eIF5A precursor (Lys-50 in the human protein (13)) to form an eIF5A-imine intermediate, and (iv) reduction of this intermediate by enzyme-bound NADH to form a deoxyhypusine residue (14). In the absence of eIF5A precursor, the enzyme has the ability to catalyze an alternate abortive reaction, cleavage of spermidine to 1,3-diaminopropane and 2-pyrroline (6). Deoxyhypusine synthase exhibits high specificity toward its substrates: it recognizes and modifies only one cellular protein, the eIF5A precursor. Previous studies with a large number of compounds structurally related to spermidine as inhibitors of deoxyhypusine synthesis (15–17), and the topology of the proposed spermidine-binding pocket of the enzyme (11) also revealed a narrow specificity toward the donor substrates, with spermidine being strongly favored.

It was not known whether deoxyhypusine synthase can catalyze other reactions in polyamine metabolism or perform...
other cellular functions. Recently, Ober and Hartman reported that deoxyhypusine synthases from tobacco (18) and Senecio vulgaris (19, 20) can accommodate putrescine as an alternate butylamine acceptor, thus resulting in the production of homospermidine from spermidine (donor) and putrescine (acceptor) (Scheme 1, right-hand pathway). Another plant enzyme, homospermidine synthase, which can also catalyze the synthesis of homospermidine, was identified (19, 21, 22) in the roots of certain plants, e.g., Asteraceae such as Senecio and Eupatorium, that produce pyrrolizidine alkaloids, defense chemicals against insects. Homospermidine is generated by this enzyme as the starting material for pyrrolizidine alkaloid biosynthesis (21).

After the purification of homospermidine synthase from S. vulgaris and cloning of its cDNA (19) (and a nearly identical one from Senecio vulgaris (23)), it became clear that this enzyme is closely related to deoxyhypusine synthase but not to a bacterial homospermidine synthase, which is known to catalyze synthesis of homospermidine from two molecules of putrescine (24). The S. vulgaris homospermidine synthase shows a high homology to S. vulgaris deoxyhypusine synthase (79%) and to those from human (61%) and tobacco (74%) (19). Based on the high amino acid sequence identity and similarity of the physical properties of the purified enzymes, homospermidine synthase of the plant homospermidine synthase was presumed to occur by a mechanism similar to, or identical with, that of deoxyhypusine synthase, using spermidine as the butylamine donor and putrescine as the acceptor. However, the plant homospermidine synthase is distinct from deoxyhypusine synthase in that it does not use eIF5A precursor as butylamine acceptor and does not catalyze synthesis of deoxyhypusine in plant eIF5A precursor (19, 20). Therefore, it is likely that this enzyme, which is specific for plant secondary metabolism, evolved from a ubiquitous critical enzyme, deoxyhypusine synthase (19, 20, 23).

Synthesis of hypusine in eIF5A precursors has been deemed irreversible, because there are no known reactions to convert the hypusine residue back to a lysine residue. The novel feature of the deoxyhypusine synthase reaction, namely, the facile reversibility of deoxyhypusine synthesis, caught our attention by a fortuitous observation made in the course of assays of deoxyhypusine hydroxylase, the enzyme that acts on eIF5A(Dhp) to form hypusine. We routinely prepared a radiolabeled substrate for deoxyhypusine hydroxylase, eIF5A([3H]Dhp), in an in vitro deoxyhypusine synthase reaction by incubation of eIF5A precursor, ec-eIF5A, with the enzyme, NAD, and [3H]spermidine, followed by removal of excess [3H]spermidine. During deoxyhypusine hydroxylase reactions, we often observed a loss of radioactivity from the labeled substrate protein that could not be attributed to proteolytic degradation, because protease inhibitors provided no protection. In fact the release of radioactivity from the substrate protein was greatly enhanced upon incubation of eIF5A([3H]Dhp) with a mixture of purified deoxyhypusine synthase, NAD, and 1,3-diaminopropane, suggesting a reversal of deoxyhypusine synthesis by the human enzyme (Scheme 2). This enhancement was also observed when 1,3-diaminopropane was replaced by putrescine. The flexibility of human deoxyhypusine synthase to adopt putrescine as a butylamine acceptor in this reverse reaction suggested that the human enzyme, like the plant enzymes, is also capable of homospermidine synthesis (Scheme 2, right-hand pathway).

The results presented here provide definitive evidence that generation of spermidine or homospermidine from eIF5A([3H]Dhp) in this reversal reaction occurs by way of the enzyme-imine intermediate through transfer of its 4-amino- butyl moiety to acceptors, 1,3-diaminopropylamine or putrescine, and reveal highly specific, yet versatile, activities of deoxyhypusine synthase.

**EXPERIMENTAL PROCEDURES**

**Materials**—[1,8-3H]Spermidine-3HCl (20–36 Ci/mol) and [1,4-3H]Putrescine-2HCl (107 mCi/mol) were purchased from PerkinElmer Life Sciences; diaminobutane, 1,3-diaminopropane, 1,4-diaminobutane (putrescine), 1,5-diaminopentane (cadaverine), 1,6-diaminohexane, 1,7-diaminooctane, 1,8-diaminononate, and 1,9-diaminononane, spermidine, and spermine were purchased from Sigma; coldine (sym-norspermidine) was from Eastman Kodak; AmplifyTM was from Amersham Biosciences. 6-Fluorospermidine-3HBr (MDL 72721), 6,6-difluorospermidine-3HBr (MDL 72766), 7,7-difluorospermidine-3HBr (MDL 72748) were generous gifts from Merrell Dow Research Institutes; 1-methylspermidine was from Dr. J. K. Coward, University of Michigan; and 5,5-dimethylspermidine was from Dr. Bruce Ganem, Cornell University. Sym-Homospermidine, N,N-monoguanylated-1,7-diamino- 

**Spermidine** (GC7) (15), N-(3-aminopropyl)-1,4-diamino-cis-but-2-ene, and N-(3-aminopropyl)-1,4-diamino-trans-but-2-ene (cis- and trans-unsaturated spermidines (25)), N,N-ethylspermidine, N6-ethylspermidine (15), N-(3-aminopropyl)cadaverine (26), and 8-ethylspermidine were synthesized in this laboratory. Recombinant human eIF5A precursor protein, ec-eIF5A, was purified from BL21(DE3) harboring pET11a encoding human eIF5A as described previously (27). Recombinant human deoxyhypusine synthase was purified as described previously (28). eIF5A([3H]Hpu) was prepared from Chinese hamster ovary cells cultured with [1,8-3H]spermidine (4).

**MATERIALS AND METHODS**

**Preparation of eIF5A([3H]Dhp)**—The deoxyhypusine-containing eIF5A intermediate, eIF5A([3H]Dhp), is the normal substrate for deoxyhypusine hydroxylase. It was prepared in radiolabeled form by deoxyhypusine synthase-catalyzed incorporation of the [3H]-labeled butylamine portion of [1,8-3H]spermidine into recombinant eIF5A precursor protein, ec-eIF5A. The reaction mixture contained, in 15 ml, 0.1 M glycine-NaOH buffer, pH 9.5, 1 mM DTT, 0.5 mM NAD, 5 μM (75 nmol, 1.25 mg) ec-eIF5A, 6.7 μM [100 nmol, 1.5 mCi] [1,8-3H]spermidine, and 40 μg of purified deoxyhypusine synthase. The reaction mixture was incubated at 37 °C for 2 h. After incubation, the radiolabeled eIF5A intermediate protein was separated from [1,8-3H]spermidine by ammonium sulfate precipitation (45–80% saturation). The precipitated pro-

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2 J.-H. Park, E. C. Wolff, J. E. Folk, and M. H. Park, to be published.
Reversal of Deoxyhypusine Synthesis

tein was redissolved in phosphate-buffered saline, and ammonium sulfate precipitation was repeated three to four times. Finally, the dissolved protein was dialyzed against phosphate-buffered saline containing 1 mM DTT at 4 °C for 4 h. Approximately 99% of the total radioactivity of this preparation was precipitable with 10% trichloroacetic acid, indicating a complete removal of free [3H]spermidine. This preparation was routinely used as substrate for deoxyhypusine hydroxylase assays and was used in preliminary experiments as substrate for the deoxyhypusine synthase-reversal reaction. However, it was found to contain unreacted ec-eIF5A protein and a small amount of the enzyme. Therefore, pure eIF5A[Dhp] (free of [1,8-3H]spermidine, ec-eIF5A, and deoxyhypusine-containing enzyme, hDHS[Dhp]) was used for determining the kinetic parameters and the results shown in Figs. 1–3. Virtually all of the radioactivity in acid hydrolysates of the trichloroacetic acid precipitates of both eIF5A[Dhp] preparations was found in the deoxyhypusine position upon ion exchange chromatographic separation (data not shown).

Reversal of Deoxyhypusine Synthesis—The radiolabeled deoxyhypusine-containing protein, eIF5A[Dhp], was incubated with purified human deoxyhypusine synthase, NAD, and a diamine or polyamine as indicated in the legends to the figures. The reaction was stopped after incubation at 37 °C for 5–60 min by addition of 250 µg of carrier BSA and 10% trichloroacetic acid. An aliquot of the trichloroacetic acid supernatant solution was counted for radioactivity, and another aliquot was used for ion exchange chromatography to identify radioactive products.

Homosperridmine Synthesis Activity—Unlabeled spermidine or various spermidine analogs were incubated with human deoxyhypusine synthase, NAD, and [1,4-14C]putrescine. After incubation for 5–90 min, carrier BSA was added and the reaction mixtures were treated with 10% trichloroacetic acid. Aliquots of the trichloroacetic acid supernatants were analyzed by ion exchange chromatography to identify the radiolabeled products and determine their amounts.

Synthesis of Non-radiolabeled Deoxyhypusine or Its Analogs—Reaction mixtures contained, in 0.4 ml, 0.1 M glycine-NaOH buffer, pH 9.5, 1 mM DTT, 0.5 mM NAD, 100 µg of BSA, 2.5 nmol (40 µg) of ec-eIF5A, 2 µg of the pure human enzyme, and 0.2 ml of spermidine or its analogs. After incubation at 37 °C for 2 h, the proteins in the reaction mixture were precipitated with 10% trichloroacetic acid, and the precipitates, after washing with trichloroacetic acid, were hydrolyzed in 6 N HCl at 108 °C for 18 h. After drying, the hydrolyzed samples were analyzed by ion exchange chromatography as reported previously (6) and basic amino acids and amines were detected fluorometrically after reaction with ortho-pthalaldehyde (29). The detection limit was ~0.05 nmol.

Kinetic Parameters—The conditions of reactions (enzyme amount and incubation time) were set to permit linearity of reaction with time and limited substrate consumption (~20%). K_m and V_max values were calculated with the "Enzyme Kinetics" program version 1.0c by D. G. Gilbert, dogStar software (Bloomington IN).

RESULTS

Reversal of Deoxyhypusine Synthesis Reaction and Identification of Products—When purified eIF5A[DHP] was incubated with deoxyhypusine synthase in the presence of NAD and 1,3-diaminopropane, a rapid loss of labeled protein was observed with a concomitant increase in trichloroacetic acid-soluble radioactivity (Fig. 1A, lane 5, top panel, radioactivity in protein after SDS-PAGE; lower panel, trichloroacetic acid-soluble radioactivity). The release of protein-bound radioactivity was dependent on deoxyhypusine synthase and NAD and was accelerated by addition of 1,3-diaminopropane (Fig. 1A, compare lanes 1–5) suggesting an enzyme-mediated reversal of deoxyhypusine synthase (Scheme 2). The disappearance of the radiolabeled protein was not due to a proteolytic degradation of eIF5A[Dhp], because it was not prevented by addition of protease inhibitors (not shown). No labeled deoxyhypusine was detected in the trichloroacetic acid supernatant solution before (as shown later in Fig. 2A) or after acid hydrolysis (not shown). Furthermore, this phenomenon was limited to eIF5A[Dhp]; no loss of the labeled protein was observed when the hypusine-containing protein, eIF5A[Hpu], was incubated with deoxyhypusine synthase, NAD, and 1,3-diaminopropane (Fig. 1C, lanes 13 and 14).

The reversal of deoxyhypusine synthesis was confirmed by the analysis of the reaction products by ion exchange chromatography, which showed the formation of spermidine in a complete reaction (Fig. 2A, filled circles). When the reaction mixture contained only eIF5A[HDP] and enzyme, and NAD, a small amount of 1-pyrroline was detected in the trichloroacetic acid supernatant (Fig. 1A, lane 4, lower panel, and Fig. 2A, filled diamonds). We have shown previously that 1-pyrroline is generated by cyclization of the 4-aminobutyl side chain of the enzyme-imine intermediate when deoxyhypusine synthase is incubated with [1,8-3H]spermidine and NAD in the absence of the acceptor, eIF5A precursor (6). Therefore 1-pyrroline was expected to be generated also in the reverse reaction in the absence of the acceptor, 1,3-diaminopropane. When 1,3-diaminopropane was included in the mixture, 1-pyrroline was no longer detectable and most of radioactivity from eIF5A[HDP] was found in spermidine (Fig. 1A, lane 5, Fig. 2A, filled circles), suggesting a rapid transfer of the 4-aminobutyl moiety to the acceptor, 1,3-diaminopropane, in this reverse reaction (Scheme 2).
Involvement of Enzyme-imine Intermediate in the Deoxyhypusine Synthase Reversal Reaction—The involvement of the enzyme-imine intermediate in the forward reaction of deoxyhypusine synthesis (Scheme 1) was demonstrated by its trapping into a stable adduct, Enz(Dhp), by NaBH₃CN reduction as reported previously (12). The evidence that conversion of eIF5A([³H]Dhp) back to eIF5A precursor and [³H]spermidine occurs by way of the same enzyme-imine intermediate (Scheme 2) was obtained by the trapping experiment shown in Fig. 1B. A radiolabeled enzyme band (41 kDa) was detected after NaBH₃CN reduction of an incubation mixture containing eIF5A([³H]Dhp), a large amount of enzyme (1 μg) and NAD (lane 9). The enzyme labeling was dependent on NAD and NaBH₃CN reduction. An identical reaction mixture to that in lane 9 but without NaBH₃CN treatment showed no labeling of the enzyme (lane 11). The trichloroacetic acid supernatant of the reduced reaction mixture contained, in 50 μl, 0.1 m Tris-HCl, pH 7.5, 100 μg of BSA, 1 mm DTT, 60,000 cpn (~5.3 pmol) of eIF5A([³H]Dhp) with or without 0.005 μg of human deoxyhypusine synthase, 1.0 mm NAD, and 0.2 mm 1,3-diaminopropane, as indicated by + and −. After incubation for 60 min at 37 °C, the reaction mixtures were treated with 10% trichloroacetic acid. The trichloroacetic acid-precipitated proteins were analyzed by SDS-PAGE and subsequent fluorography with Amplify™ (top panel), and the radioactivity in the trichloroacetic acid supernatant was measured (bottom panel). B, the reaction mixtures were the same as in A except that 1 μg of enzyme was used. Incubation was 2 min at 37 °C, and the mixtures were treated with NaBH₃CN as described (12) prior to trichloroacetic acid precipitation and analysis as in A. C, the reaction mixtures contained labeled hypusine protein, eIF5A([³H]Hpu) (lanes 12–14) in place of eIF5A([³H]Dhp) and were processed the same way as in B. Dap, 1,3-diaminopropane; hDHS, human deoxyhypusine synthase; Enz([³H]Dhp), labeled deoxyhypusine synthase containing [³H]deoxyhypusine residue.

pH Dependence of the Deoxyhypusine Synthase Reversal Reaction—The deoxyhypusine synthase-catalyzed production of deoxyhypusine is optimum at pH 9.2–9.5 (20, 30, 31). The proportion of the N⁴,N⁶-diprotonated species of spermidine, which is the active amine substrate for this enzyme, is highest at this pH (31, 32). The Kₘ values for spermidine at pH 7.5 and pH 9.5 are 150 and 7 μmol, respectively (28, 31). For the reverse reaction catalyzed by this enzyme, i.e. conversion of eIF5A([³H]Dhp) back to eIF5A(Lys) and spermidine, the Kₘ values at pH 7.5 and 9.5 for the acceptor substrate, 1,3-diaminopropane (95.4 and 105.8 μmol, respectively) are comparable, as are those for eIF5A([³H]Dhp) (0.66 and 0.86 μmol, respectively) (Table I). The finding of similar Kₘ values for eIF5A([³H]Dhp) at the two pH values is consistent with the effective binding of eIF5A([³H]Dhp) to deoxyhypusine synthase at neutral as well as at basic pH, as we reported previously (31). Thus, in contrast with the forward reaction, the first step of the reverse reaction, leading to the enzyme-imine intermediate, does not seem dependent on high pH. In fact, the formation of the enzyme-imine intermediate and its cleavage product, Δ₁-pyrroline, was readily detectable at pH 7.5 (Figs. 1 and 2) but not at pH 9.5.
Homospermidine synthesis
B) of homospermidine from spermidine and putrescine. We employed deoxyhypusine synthase is capable of catalyzing the synthesis of homospermidine from spermidine and putrescine. In A and B the incubations were 20 min; in C, 30 min. Other conditions were as described under "Experimental Procedures" and in the figure legends.

Table I

| Donor | $K_m$ $\mu\text{M}$ | $V_{\text{max}}/\text{mg}$ | $V_{\text{max}}/K_m$ | Receiver $\mu\text{M}$ | $V_{\text{max}}/\text{mg}$ | $V_{\text{max}}/K_m$ |
|-------|---------------------|------------------------|---------------------|---------------------|------------------------|---------------------|
| A) Reversal of deoxyhypusine synthesis | | | | | | |
| eIF5A(Dhp) | | | | | | |
| pH 9.5 | 0.86 ± 0.075 | 1239 ± 25.8 | 1447 | Diaminopropane | pH 9.5 | 105.8 ± 12.6 | 1137 ± 62.4 | 10.7 |
| pH 7.5 | 0.66 ± 0.083 | 178.2 ± 7.08 | 269 | Putrescine | pH 9.5 | 95.4 ± 11.6 | 164.7 ± 6.0 | 1.73 |
| B) Homospermidine synthesis | | | | | | |
| Spermidine | 28.0 ± 2.8 | 90.9 ± 5.25 | 3.25 | Putrescine | 203 ± 61 | 90.0 ± 7.17 | 0.44 |
| Homospermidine | 19.3 ± 2.7 | 34.5 ± 1.92 | 1.79 | | | | |
| C) Deoxyhypusine synthesis | | | | | | |
| Spermidine | 7.26 ± 1.06 | 67.5 ± 2.68 | 9.30 | eIF5A(Lys) | 1.51 ± 0.17 | 65.7 ± 3.36 | 43.5 |

(data not shown). This result could be explained by a less efficient subsequent step, namely the transfer of the butylamine moiety from the enzyme-imine intermediate to 1,3-diaminopropane, at pH 7.5, as reflected in the 6-fold lower overall $V_{\text{max}}/K_m$ (1,3-diaminopropane) value at pH 7.5 than at pH 9.5 (1.73 versus 10.7, Table I).

1,3-Diaminopropane and Putrescine as Acceptors in the Deoxyhypusine Synthase Reverse Reaction—We tested a variety of diamines and polyamines for their ability to accept the 4-amino-1pyrroline. When 1,3-diaminopropane was tested as an acceptor as evidenced by their enhancement of the release of the radioactive 4-amino-1-pyrroline moiety from eIF5A(13H)Dhp (Fig. 3A). Both 1,3-diaminopropane and putrescine served as acceptors as evidenced by their enhancement of the release of the radioactive 4-amino-1-pyrroline moiety from eIF5A(13H)Dhp as trichloroacetic acid-soluble products (Fig. 3A and Table I). Other amines, namely 1,2-diaminoethane, 1,5-diamonopentane, 1,6-diamino-hexane, 1,7-diaminoheptane, 1,8-diaminooctane, 1,9-diaminononane, N7-monoguanyl-1,7-diaminoheptane, and spermidine, showed little increase in the radioactivity released from the protein, suggesting that they do not bind to the 1,3-diaminopropane binding pocket to act as an alternate acceptor. Rather, 1,7-diaminoheptane, N7-monoguanyl-1,7-diaminoheptane, and spermidine appeared to inhibit the reverse reaction when compared with the level of the control reaction (no acceptor amine addition) in which 5–10% of total radioactivity was converted to $\Delta^1$-pyrroline. When N7-monoguanyl-1,7-diaminoheptane was added together with 1,3-diaminopropane, it suppressed 1,3-diaminopropane-mediated release of radioactivity from eIF5A(13H)Dhp. The inhibition by N7-monoguanyl-1,7-diaminoheptane appears to be due to interference with the binding of 1,3-diaminopropane to the enzyme active sites, because GC7 did not interfere with the binding of eIF5A(Lys) to the enzyme (31). 1,3-Diaminopropane was by far the best acceptor ($K_m$ values, 105.8 $\mu$M at pH 9.5, and 95.4 $\mu$M at pH 7.5, Table I). Putrescine also acted as an acceptor at pH 9.5, but its affinity was 10-fold lower than that of 1,3-diaminopropane ($K_m$ values, 1117.5 $\mu$M at pH 9.5, Table I). The products formed from the two acceptors, 1,3-diaminopropane and putrescine, were identified by ion exchange chromatographic separation as spermidine and homospermidine, respectively (Fig. 3B).

Homospermidine Synthetic Activity of Human Deoxyhypusine Synthase—The results shown in Fig. 4 demonstrate that deoxyhypusine synthase is capable of catalyzing the synthesis of homospermidine from spermidine and putrescine. We employed unlabeled spermidine as a donor and [14C]putrescine as an acceptor in deoxyhypusine synthase reaction mixture and measured the amount of radioactive homospermidine generated (Scheme 1, right-hand pathway). [14C]Putrescine was chosen as the radioactive acceptor substrate instead of [3H]spermidine as a radiolabeled donor substrate, because the difference in elution times between homospermidine and putrescine is much greater (8 min) than that between spermidine and homospermidine (1 min). It is evident from Fig. 4A that a portion of [1,4-14C]putrescine was converted to [14C]homospermidine and that the reaction is dependent upon the donor and acceptor substrates, spermidine and putrescine, respectively, and the cofactor NAD (Fig. 4B). Furthermore, the pH optimum appears to be the same as for deoxyhypusine synthase.

Spermidine Analogs as Donor Substrates for Deoxyhypusine Synthase—Spermidine and aminopropylcadaverine were previously shown to be substrates for deoxyhypusine synthase in vitro as well as in cells, by the use of the radiolabeled compounds, [1,8-3H]spermidine and aminopropyl[1,5-3H]cadaverine (1, 26). Use of the radioactive amines enabled us to measure the incorporation of radioactivity into eIF5A precursor protein by SDS-PAGE and allowed identification of the labeled component. Because those analogs that are not available in radioactive forms cannot be tested in this manner, their substrate activities were determined based on the fluorometric detection of expected products by reaction with ortho-phthalaldehyde (Table II) (6, 29). Of the compounds tested by this method, deoxyhypusine, or its analog product that chromatographs at or near the position of deoxyhypusine, was formed from donor amines, including spermidine, homospermidine, aminopropylcadaverine, cis- and trans-un saturated spermidines, 1-methylspermidine, 8-methylspermidine, N7-ethyl spermidine, and N7-ethyl spermidine. Little or no product was detected from caldine, 6-fluorospermidine, 6,6'-difluorospermidine, 7,7'-difluorospermidine, and 5,5'-dimethylspermidine (Table II). Although this procedure provided a direct measure of deoxyhypusine or its analogs produced, it is not a convenient method for screening a large number of compounds, because the sensitivity of the fluorometric detection is relatively low (detection limit being 0.05 nmol) and a large-scale reaction (>2 nmol of protein substrate per reaction) is required.

Spermidine Analogs as Donor Substrates for Synthesis of Homospermidine or Its Analogs—The utilization of [1,4-14C]putrescine as an acceptor of the 4-amino-1-pyrroline moiety from the enzyme-imine intermediate provides a new means to determine the capacity of various spermidine analogs to act as donor substrates and permitted detection of picomole amounts of...
products in a more sensitive and highly reproducible manner. When the reaction mixture contained only [1,4-14C]putrescine, but no donor amines (spermidine or its analogs), no homospermidine was formed (Fig. 5, no donor). We tested a number of spermidine analogs at two concentrations, 30 and 200 μM, as donors (Fig. 5 and Table II). It is clear that spermidine, homospermidine, caldine, cis- and trans-unsaturated spermidines, 1-methylspermidine, and N₁-ethylspermidine can act as donors to putrescine to form homospermidine or its analogs. Other analogs, including aminopropylcadaverine, 6-fluorospermidine, 6,6'-difluorospermidine, 7,7'-difluorospermidine, and 5,5'-dimethylspermidine, did not work as donor substrates. N₁-Monoguanyl-1,7-diaminoheptane, a potent inhibitor of deoxyhypusine synthesis, also inhibited synthesis of homospermidine, presumably by competitive binding at the spermidine binding site. The products from reaction with spermidine, homospermidine, 1-methylspermidine, and N₁-ethylspermidine were identified as homospermidine, as expected, by ion exchange chromatography. The elution positions of the products from cis- and trans-unsaturated spermidine, cis- or trans-unsaturated homospermidine, were indistinguishable from that of homospermidine. The product originating from caldine was identified as spermidine by ion exchange chromatography (as seen for the separation of spermidine and homospermidine in Fig. 3).

Whereas most spermidine analogs act as donor substrates in both deoxyhypusine synthesis and in homospermidine synthesis, discrepancies were noted for some compounds, e.g. caldine and aminopropylcadaverine (compare Fig. 5 and Table II). No deoxyhypusine analog was detectable from a reaction using caldine as donor and ec-eIF5A as an acceptor. Yet it definitely served as a donor of the 3-aminopropyl moiety to form spermidine when putrescine was the acceptor substrate (Fig. 5). Conversely, aminopropylcadaverine, which is a donor substrate for homodeoxyhypusine synthesis in the eIF5A precursor, did not.

FIG. 3. Aminobutyl acceptors and pH dependence of the deoxyhypusine synthesis reversal reaction. The reaction mixtures contained all the components and were processed in the same way, as described in the legend to Fig. 1, panel A, except that 1,3-diaminopropane was replaced by various diamines and polyamines, at 0.2 mM, and two different buffers, 0.1 M Tris-HCl, pH 7.5, or 0.1 M glycine-NaOH, pH 9.5, were used. A, an aliquot of the trichloroacetic acid supernatant from each sample was counted to determine the total amount of radioactivity released from eIF5A[1H]Dhp. B, an aliquot of the pH 9.5 reaction mixture with 1,3-diaminopropane or with putrescine as the acceptor was analyzed by ion exchange chromatography, and the radioactive product in each case was identified as spermidine or homospermidine, respectively. Dae, 1,2-diaminoethane; Dap, 1,3-diaminopropane; Ptc, putrescine; Cad, cadaverine; Dha, 1,6-diaminohexane; Dah, 1,7-diaminoheptane; Doo, 1,8-diaminooctane; Dan, 1,9-diaminononane; Spd, spermidine; Hspd, homospermidine; ApCad, aminopropylcadaverine; Gct, N₁-monoguanyl-1,7-diaminoheptane; in each case the various numbers following the abbreviation indicate the number of methylene groups between amino groups.

FIG. 4. Synthesis of [14C]homospermidine from [14C]putrescine by human deoxyhypusine synthase. A, ion exchange chromatogram of a complete reaction mixture; B, [14C]homospermidine formed, expressed as percent of the total radioactivity. A complete reaction mixture contained, in 50 μl, 0.1 M glycine-NaOH buffer, pH 9.5, 50 μg of BSA, 1 mM DTT, 1 μCi [14C]putrescine (200 μM), 0.5 mM NAD, 0.2 mM spermidine, and 1.0 μg human enzyme and was incubated at 37°C for 1.5 h. At the end of incubation, 250 μg of carrier BSA was added, and the reaction mixture was precipitated with 10% trichloroacetic acid. Spd, spermidine; Ptc, putrescine; Hspd, homospermidine.
work as a donor when putrescine was the acceptor. Apparently both compounds are capable of forming an enzyme-imine intermediate (Table II), judging from the finding that they can function as a donor in either one of the two reactions. However, there seem to be differences at the step of transfer of the 3-aminopropyl or 5-aminopentyl moieties from the enzyme-imine intermediate depending on the acceptor, eIF5A precursor or putrescine.

**DISCUSSION**

The results presented in this report demonstrate the reversal of an established post-translational modification and provide new insights into the versatile activities of deoxyhypusine synthase. Studies prior to this work suggested a unidirectional pathway of reactions catalyzed by deoxyhypusine synthase, namely 1) the synthesis of deoxyhypusine from spermidine (donor) and eIF5A precursor (acceptor) (1–3) and 2) the side reaction, synthesis of homospermidine from spermidine (donor) and putrescine (acceptor) (18, 20) (Schemes 1 and 3A). With respect to the latter, our data with the human enzyme are largely consistent with those of Ober and Hartmann (18–20) who showed that deoxyhypusine synthase from plants (tobacco and *S. vernalis*) can synthesize homospermidine as well as deoxyhypusine. The present demonstration of the reversal of deoxyhypusine synthesis by way of the common enzyme-imine intermediate (Enz-Imine) offers a dynamic new view of the deoxyhypusine synthase reaction. Scheme 3B depicts the reversible nature of the inter-conversions between spermidine, homospermidine, and eIF5A precursor or putrescine.

**TABLE II**

Comparison of the inhibitory activity (IC₅₀) of spermidine and its analogs toward deoxyhypusine synthesis (5, 15), with the structure of the expected enzyme intermediate and the product formed with the two alternate acceptors; growth support was determined in the cited references. Deoxyhypusine synthesis activity was assayed with eIF5A(Lys) as acceptor, and the products were determined fluorometrically, as described under "Experimental Procedures." Assays of homospermidine synthesis were done with [¹⁴C]putrescine as acceptor, as described in the legend to Fig 5. Abbreviations used are as defined in the Fig. 5 legend.

| Donor Amine   | IC₅₀ μM | Enzyme Intermediate | Product       | Growth Support |
|---------------|--------|---------------------|---------------|---------------|
| Spermidine (3, 4) | 7.8    | Enz-Imine(NH₂)      | deoxyhypusine homospermidine | + (33)        |
| Homospermidine (4, 4) | ~20    | Enz-Imine(NH₂)      | deoxyhypusine homospermidine | n.d. c        |
| Calmodine (3, 3) | 41.2   | Enz-Imine(NH₂)      | deoxyhypusine homospermidine | n.d. c        |
| ApCad (3, 5)   | 118    | Enz-Imine(NH₂)      | homodeoxyhypusine cis-deoxyhypusine cis-homospermidine | + (34)        |
| cis-Spd <10    |        | Enz-Imine(NH₂)      | deoxyhypusine homospermidine |               |
| trans-Spd <3   |        | Enz-Imine(NH₂)      | trans-deoxyhypusine trans-homospermidine |               |
| 1-MeSpd 184    |        | Enz-Imine(NH₂)      | deoxyhypusine homospermidine |               |
| 8-MeSpd >1000  |        | Enz-Imine(NH₂)      | methyldeoxyhypusine methylhomospermidine | n.d. c        |
| N⁺-EtSpd >1000 |        | Enz-Imine(NH₂)      | deoxyhypusine homospermidine | n.d. c        |
| N⁺⁺-EtSpd >1000|        | Enz-Imine(NH₂)      | N-ethyldoxyhypusine N-ethylhomospermidine | n.d. c        |
| 5,5'-McpSpd >1000 |    |                     |               | - (33)        |
| 6-F-Spd 48     |        |                     |               | n.d. c        |
| 6,6'-F-Spd 14  |        |                     |               | n.d. c        |
| 7,7'-F-Spd >1000|        |                     |               | n.d. c        |

a Numbers in parentheses indicate number of methylene groups between amino groups.
b Detectable, but ≤3% of the amount of product from spermidine.c n.d., not determined.

Fig. 5. Spermidine analogs as donor substrates for synthesis of homospermidine or its analogs by deoxyhypusine synthase. The reaction mixture and the conditions were the same as in Fig. 4, except that spermidine or various spermidine analogs were added at two concentrations 0.03 (dashed bar) and 0.2 mM (solid bar). The amount of radioactivity in the product, homoserpermidine, or its analog, is indicated as percent of the total radioactivity in putrescine and the product. Spd, spermidine; GC7, N⁺-monoguanyl-1,7-diaminohexane; Hspd, homospermidine; ApCad, aminopropylcadaverine; cis-Spd, cis-ununsaturated spermidine (N-(3-aminopropyl)-1,4-diamino-cis-but-2-ene); trans-Spd, trans-ununsaturated spermidine (N-(3-aminopropyl)-1,4-diamino-trans-but-2-ene); 1-MeSpd, N⁺-methylspermidine; 8-MeSpd, 8-methylspermidine; 5,5'-Me₂Spd, 5,5'-dimethylspermidine; 6-F-Spd, 6-fluorospermidine; 6,6'-F₂Spd, 6,6'-difluorospermidine; 7,7'-F₂Spd, 7,7'-difluorospermidine; 5⁺-EtSpd, 5⁺-ethylspermidine; in each case the various numbers following the abbreviation indicates the number of methylene groups between amino groups.
Hence diamines with methylene chains longer than C₄ are not effective acceptors, as shown in Fig. 3. Similar considerations apply to the lysine residue on the eIF5A precursor, which also has a C₄ chain length, but in this case the exact positioning of the acceptor amino group is greatly facilitated by the binding of the eIF5A precursor protein over the surface of the enzyme around the active site, as reflected in its very low $K_{ma}$.

Use of eIF5A([³H]Dhp) as a butylamine donor provided a convenient method to screen many diamines and polyamines as potential butylamine acceptors (Fig. 3) for production of spermidine, homospermidine, or their analogs. The radioactivity released from the labeled protein, eIF5A([³H]Dhp), as a trichloroacetic acid-soluble component was easily measured and identified by ion-exchange chromatography (Fig. 3). Conversely, use of [¹⁴C]putrescine as an acceptor offered a simple and unambiguous method to determine donor activity of a number of spermidine analogs and their ability to form an enzyme-imine intermediate (Fig. 4 and Table II). Comparison of activities of these amines as donors for the synthesis of homospermidine (or its analogs) or deoxyhypusine (or its analogs) provides insight into the precise selectivity of the transfer reaction at the active site of deoxyhypusine synthase. Spermidine, homospermidine, 1-methylspermidine, and cis- and trans-unsaturated spermidines form enzyme-imine intermediates and function as donors to both eIF5A(Lys) and putrescine. Interestingly, inconsistencies are noted for aminopropylcadaverine and cadine (sym-norspermidine). Aminopropylcadaverine functions as a donor substrate for eIF5A(Lys) but not for putrescine. It is vice versa for cadine. That these compounds can function as donors to one means that they are both capable of forming enzyme intermediates. However, the structural constraints at the narrow active site pocket or different conformational changes induced in the active site by the two enzyme imine intermediates may permit transfer to one acceptor and not the other.

It is of interest to consider how the versatile activities of deoxyhypusine synthase may be reflected in living cells or animal tissues. Homospermidine has been found in small amounts even in plants that do not produce pyrrolizidine alkaloids (38) and, along with canavalamine (C₄C₃C₄), has been detected in certain mammalian tissues (39, 40). In these plants, and in mammals, where homospermidine synthase is not present, the production of homospermidine can be attributed to deoxyhypusine synthase. The three substrates/products of deoxyhypusine synthase, spermidine, eIF5A(Dhp), and homospermidine are in dynamic equilibrium by way of the enzyme-imine intermediate (Scheme 3B). The balance among these three will depend on the kinetic parameters of each step and concentration of each donor and/or acceptor substrate in the cellular environment.

Deoxyhypusine synthase, as well as eIF5A, is ubiquitous in all eukaryotes, and both are essential for eukaryotic cell proliferation and survival. The vital role of this enzyme and the hypusine modification has been demonstrated from gene disruption studies in yeast, whereas the role of the second step enzyme, deoxyhypusine hydroxylase, remains to be fully elucidated. That the reversal of modification is not observed with mature eIF5A-containing hypusine (Fig. 1) suggests that hydroxylation of deoxyhypusine side chain prevents this reversal and locks the protein in a bioactive form. It may represent an important role for hydroxylation, although the hydroxyl group on the hypusine residue most likely performs additional vital functions in the interaction of eIF5A with other macromolecules. Besides its established function in hypusine modification, and a proposed role in the production of homospermidine...
and unusual polyamines in a few mammalian tissues, deoxyhypusine synthase may have other independent cellular functions. It has been reported to be increased upon induction of senescence in certain plants (41) and has thus been implicated in plant senescence or cell cycle regulation. Recently, the deoxyhypusine synthase gene has been reported as one of the eight metastasis signature genes selected in human cancer (42). Further studies on the physiological function of this enzyme and eIF5A are warranted.

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