Cell-free Synthesis of Deoxyhypusine

SEPARATION OF PROTEIN SUBSTRATE AND ENZYME AND IDENTIFICATION OF 1,3-DIAMINOPROPANE AS A PRODUCT OF SPERMIDINE CLEAVAGE*

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The post-translational formation of hypusine (N'-4-amino-2-hydroxybutyl)lysine) occurs in a precursor of eukaryotic initiation factor 4D by way of two major steps: 1) transfer of the 4-amino group from spermidine to the α-amino group of a specific lysine residue to form an intermediate, deoxyhypusine; 2) hydroxylation of the deoxyhypusine residue to form hypusine. The initial step of this modification, deoxyhypusine synthesis, was studied in fractionated lysates of Chinese hamster ovary cells, untreated, or treated with α-difluoromethylornithine (DFMO); the enzyme(s) and the protein substrate (eukaryotic initiation factor 4D precursor) were separated. The enzyme activity was found in the 0-45% ammonium sulfate fraction from both untreated and DFMO-treated cells. The protein substrate was detected in the 45-75% ammonium sulfate fraction from cells depleted of spermidine by treatment with DFMO, but not in any fraction from untreated cells. Upon further purification of the protein substrate by ion exchange chromatography, the requirement for a pyridine nucleotide, notably NAD+, became apparent. Free 1,3-diaminopropane was identified as a spermidine cleavage product formed concurrently with butylamine transfer, was not possible with competition with spermidine. 1,3-Diaminopropane exhibited a potent inhibition of deoxyhypusine formation, probably through a different mechanism.

The unusual amino acid hypusine (N'-(4-amino-2-hydroxybutyl)lysine) is known to occur in only one cellular protein, the eukaryotic initiation factor 4D (eIF-4D)1. This amino acid is formed by a post-translational event in which the 4-carbon moiety from the polyamine spermidine is conjugated to a specific lysine residue to form an intermediate, deoxyhypusine (N'-(4-amino-2-hydroxybutyl)lysine), which is subsequently hydroxylated (2, 3). The enzyme that catalyzes the final step, deoxyhypusine hydroxylase, has been partially purified and characterized (4).

The mechanism of deoxyhypusine synthesis was studied in mammalian cells by the use of specifically isotopically labeled precursors, i.e. spermidine and lysine (2, 3, 5). Based on the knowledge obtained from these studies as to the origin of the atoms of hypusine and evidence that one of the two protons on carbon 5 of spermidine is abstracted during transfer of the 4-amino group to the α-amino group of the lysine (6), we proposed a biosynthetic pathway that involves the oxidative cleavage of spermidine between carbon 5 and the secondary amino nitrogen (Scheme 1). The detection of free 1,3-diaminopropane, which was postulated to form in parallel with butylamine transfer, was not possible in cultured cells, however, due to the very low consumption of spermidine for hypusine synthesis (0.01-0.02%/h) (8-10) and the diverse metabolic pathways of polyamines.

Earlier attempts to develop a cell-free system for deoxyhypusine synthesis were unsuccessful, partly because the protein substrate is lacking in cells that contain a normal level of polyamines. Recent data suggest that a precursor (M, ~ 18,000, pl 5.1) of eIF-4D (M, ~ 18,000, pl 5.3) accumulates only upon depletion of spermidine (8). By treatment of cells with DFMO (11), an irreversible inhibitor of the initial enzyme of polyamine biosynthesis, ornithine decarboxylase, spermidine can be lowered to a level that depresses deoxyhypusine synthesis and causes accumulation of the protein substrate (8). Murphey and Gerner (12) recently accomplished cell-free synthesis of deoxyhypusine in lysates of DFMO-treated HTC cells and showed that the reaction is critically dependent on pH (optimum at 9.5). Under similar reaction conditions, we observed the synthesis of deoxyhypusine in lysates of DFMO-treated CHO cells. We report here a separation of the enzyme and the protein substrate by ammonium sulfate fractionation, a further purification of the protein substrate, and stimulation by NAD+ and certain other pyridine nucleotides. Using the reconstituted system, from which most of the cellular amines and other low molecular weight compounds had been removed, we were able to determine the fate of the propylamine moiety of spermidine during deoxyhypusine synthesis and have examined the effects of spermine analogues and other amines on this reaction.

EXPERIMENTAL PROCEDURES

Materials

CHO cells WTB were kindly supplied by Dr. April R. Robbins (National Institutes of Health). DFMO was a generous gift from Merrell Dow Research Center; thermine and caldine from Dr. Alberto Abbruzzese (University of Naples, Naples, Italy); guazatine from Roberta J. Murphey and Dr. Eugene W. Gerner (University of Arizona Health Science Center); homospermidine from Dr. Thomas...
and 45-75% ammonium sulfate fractions were dissolved in 2-12 ml of phosphate-buffered saline (0.02 ml per 1×10^6 cells used for the original lysate preparation) and dialyzed against 2 liters of the same buffer for 3 h with two changes of buffer. The dialyzed fractions were frozen in aliquots and stored at -20°C. Further purification of the protein substrate was carried out by chromatography on a DEAE-Sephadex column as described in the legend to Fig. 2.

**Assay—**Deoxyhypusine synthesis was measured by direct determination of the incorporation of radioactivity from [1,8-3H]spermidine into the protein substrate as [3H]deoxyhypusine. Reaction mixtures contained ~0.5 M glycine-NaOH buffer, pH 9.5, 0.5 mM dithiothreitol, 3-10 µCi of [1,8-3H]spermidine (30-40 Ci/mmol, 1.3-1.8 µM), 10-25 µl of the 0.4-45% (NH₄)₂SO₄ fraction from untreated or DFMO-treated cells as enzyme, and 10-25 µl of the 45-75% (NH₄)₂SO₄ fraction from DFMO-treated cells or purified column fractions (Fig. 2) as protein substrate in a final volume of 70-150 µl.

Incubations were conducted at 37°C for 2 h. The reactions were terminated by the additions of an equal volume of 20% trichloroacetic acid. When the purified substrate was used, 500 µg of bovine serum albumin was added as carrier protein. The supernatants were separated, and the precipitates were washed three times with 5% trichloroacetic acid containing 1 mM each putrescine, spermidine, and spermine in order to remove noncovalently bound labeled polynucleotides. The washed precipitates were hydrolyzed in 6 N HCl at 110°C, and radioactivity in deoxyhypusine was measured after separation of this amino acid by ion exchange chromatography as described previously (13, 14). Specific details of each experiment are given in the figure and table legends.

**RESULTS**

Cell-free Synthesis of Deoxyhypusine: Separation of Protein Substrate and Enzyme Fractions—Rapid synthesis of [3H] deoxyhypusine was observed when crude lysate prepared from DFMO-treated cells, postmitochondrial supernatant from this lysate (25,000×g, 30 min), or a dialyzed 0-75% ammonium sulfate fraction of the lysate was incubated with [1,8-3H] spermidine in pH 9.5 buffer. Hypusine was not formed because the enzyme responsible for conversion of deoxyhypusine to hypusine is inactive at this high pH (4, 12). In each case deoxyhypusine synthesis occurred in an ~18,000-dalton protein (data not shown). Unlike the lysates from DFMO-treated cells, lysates from untreated cells did not support deoxyhypusine synthesis.

The data in Fig. 1 illustrate a clean, simple separation of the protein substrate and the enzyme(s) and further show that the failure of the lysate of untreated cells to synthesize deoxyhypusine is due to the lack of the protein substrate. Little, if any, deoxyhypusine synthesis was observed in a reaction mixture containing a single fraction from either untreated or DFMO-treated cells (Fig. 1, A, B, D, and E), in the same ammonium sulfate fractions from both cells after their combination (G and J), or in any combination of two fractions that did not include the 45-75% ammonium sulfate fraction from DFMO-treated cells (P and I). Only two combinations gave efficient deoxyhypusine synthesis: the 45-75% ammonium sulfate fraction from DFMO-treated cells with the 0-45% ammonium sulfate fraction from untreated cells (H) or from DFMO-treated cells (C). In view of previous evidence for the presence of an eIF-4D precursor in DFMO-treated cells but not in untreated cells (8), it follows that the 45-75% ammonium sulfate fraction from DFMO-treated cells contains the critical protein substrate. This is consistent with the fact that mature eIF-4D with approximately the same molecular weight as the precursor is precipitated between 45 and 75% ammonium sulfate. On the other hand, it seems that the 0-45% ammonium sulfate fractions of both untreated and DFMO-treated cells contain the enzymatic activity for deoxyhypusine synthesis.

**Partial Purification of the Protein Substrate and Effect of NAD⁺**—The protein substrate was further purified from the
45–75% ammonium sulfate fraction from DFMO-treated cell extract by chromatography on DEAE-Sephacel under conditions analogous to those used in the purification of eIF-4D (15). Similar behavior of the two in ion exchange chromatography could be anticipated since the two proteins differ little in pl (5.1 versus 5.3). The results presented in Fig. 2 demonstrate that the substrate (●) was eluted from this column in the same region as tracer eIF-4D (○) biosynthetically labeled in the hypusine residue and was well separated from the majority of 280-nm absorbing material.

A requirement for NAD$, or other pyridine nucleotides, became apparent after this purification step. In earlier studies we observed variable degrees of deoxyhypusine synthesis in different preparations and considerable loss of this activity after extensive dialysis. Chen and Dou (16) have recently reported a stimulation of labeling of an 18-kilodalton protein from $[^3H]$spermidine by NAD in lysates from a neuroblastoma (16) and by NAD and NADP in lysates from a mutant of Neurospora (17). Although we had observed a 3–4-fold stimulation of deoxyhypusine synthesis by 1 mM NAD in the combined ammonium sulfate fractions, partial purification of the substrate protein from the 45–75% ammonium sulfate fraction led to an almost absolute requirement for NAD$ in this reaction. The effects of increasing concentrations of NAD$ and the comparison of various cofactors added at 1 mM are shown in Fig. 3.

1,3-Diaminopropane as a Product of the Deoxyhypusine-synthesizing Enzymes—When the partially purified protein substrate was used for deoxyhypusine synthesis, no interconversion of polyamines was observed. Labeled putrescine was not utilized for deoxyhypusine formation using this substrate preparation (data not shown), confirming that spermidine is the direct polyamine precursor of deoxyhypusine (2, 5, 6).
Identification of 1,3-diaminopropane by chromatography of its dansyl and benzoyl derivatives

Cell-free synthesis of deoxyhypusine and chromatography of the trichloroacetic acid supernatant were carried out as described in the legend to Fig. 4. The fraction of labeled material that eluted from the column at the position of 1,3-diaminopropane (fraction 15, Fig. 4B, ○) was mixed with 50 nmol of unlabeled 1,3-diaminopropane. A portion of this mixture was treated with dansyl chloride, and the derivative was chromatographed on silica gel 60 (18). The dansyl derivatives were localized under UV light. The location of radioactivity was determined by removing 2-3-mm segments of the layer along the development track and examining each for radioactivity in liquid scintillation fluid. The benzoyl derivative was prepared using another portion of the mixture, and separation was conducted by high performance liquid chromatography according to Redmond and Tseng (19) using a Bondapak C18 column (Waters) and isocratic conditions (methanol:water, 58:42) and a flow rate of 1.5 ml/min. Absorbance was monitored at 254 nm, and 0.5-min fractions were collected for measurement of radioactivity. The abbreviations used are: DAP, 1,3-diaminopropane; PTC, putrescine; DAH, 1,6-diaminohexane; SPD, spermidine; TCA, trichloroacetic acid.

A. Dansyl derivatives

| Chloroform: triethylamine (10:2, v/v) | Cyclohexane: diethyl ether (1:9, v/v) |
|--------------------------------------|-------------------------------------|
| Dansyl DAP                           | 0.49                                |
| Dansyl PTC                           | 0.58                                |
| Dansyl derivative of the labeled component in TCA supernatant from cell-free deoxyhypusine synthesis | 0.49 |

B. Benzoyl derivatives

| Elution time |
|--------------|
| Chloroform: triethylamine (10:2, v/v) | Cyclohexane: diethyl ether (1:9, v/v) |
| Benzyol DAP | 6.45 |
| Benzyol PTC | 6.15 |
| Benzyol DAH | 9.31 |
| Benzyol SPD | 11.64 |
| Benzyol derivative of the labeled component in TCA supernatant from cell-free deoxyhypusine synthesis | 6.5-7.0 |
| Benzyol derivative of [3H]PTC | 6.0-6.5 |

Inhibition of Deoxyhypusine Synthesis by Amines—In Fig. 5 are shown the effects of various amines on deoxyhypusine synthesis. Compounds closely related, in structure, to spermidine, e.g. cadine, homospermidine, and N⁴-benzylspermidine, strongly inhibited deoxyhypusine synthesis. Diamines with the two primary amino groups spaced at a similar distance to that in spermidine, e.g. 1,7-diaminoheptane and 1,8-diaminooctane, also exerted a strong inhibition. A spermine analogue, thermine, and a longer chain diamine, 1,9-diaminononanone, were slightly less inhibitory. The diamines with greater than 10 methylene groups or those with less than 6 methylenes, 1,10-diaminodecane, 1,12-diaminododecane, diaminoethane, 1,5-diaminopentane, and 1,6-diaminohexane, were poor inhibitors (<40% inhibition at 10⁻⁸ M). A notable exception was 1,3-diaminopropane which displayed a stronger inhibition than any other amine tested. Monoamines, e.g. n-propylamine, n-heptylamine, n-octylamine, and n-nonylamine, were ineffective.

Several inhibitors of amine oxidases were found to be less effective inhibitors than 1,3-diaminopropane or the spermidine analogues tested. Guazatine, an inhibitor of a plant amine oxidase (21) that catalyzes a similar spermidine cleavage, was reported by Murphey and Gerner (12) to inhibit deoxyhypusine synthesis in the crude lysate of DFMO-treated...
in DFMO-treated CHO cells (8), and further support the notion that accumulation of the eIF-4D precursor that serves as an acceptor of the 4-aminobutyl moiety from spermidine occurs upon spermidine depletion (20).

Because [1,8-3H]spermidine, in which the tritium is distributed equally between the two terminal methyl groups, was used for deoxyhypusine synthesis, equal amounts of radioactivity would be expected in the two products. That approximately the same amount of radioactivity was found in 1,3-diaminopropane and deoxyhypusine is in accordance with the proposed cleavage between C5 and the secondary amino nitrogen of spermidine as the initial step in hypusine biosynthesis (Scheme 1). A cleavage of spermidine at the same position to produce 1,3-diaminopropane and 4-aminobutyraldehyde, which spontaneously cyclizes to A'pyrroline, has been described in oat seedlings (21) and Serratia marcescens (22), but has not yet been detected in animals. Since little radioactivity is found in the early fractions (Fig. 4B) where A'pyrroline is expected to elute, it is likely that 1,3-diaminopropane is produced in a coupled reaction with deoxyhypusine formation in our cell-free system. In the mechanism in Scheme 1 the formation of a proposed Schiff base between the 4-aminobutyraldehyde, or other intermediate, and the ε-amino group of a specific lysine residue of the protein substrate may occur without dissociation of the intermediate from the enzyme and with provision for the prevention of nonenzymic cyclization to A'pyrroline. The formation of the initial intermediate, whether an aldehyde or not, probably involves removal of a proton from carbon 5 adjacent to the cleavage site in spermidine. This is evident from labeling studies in intact CHO cells that showed the loss of one of the hydrogens from carbon 5 of spermidine during its conversion of deoxyhypusine (6). In view of the stimulation by NAD it is tempting to postulate a role for a pyridine nucleotide at this step. The reduction of the double bond of the postulated Schiff base may be accomplished at a later stage by a different pyridine nucleotide or by another coupled cofactor.

The inhibition studies with various amines also shed light on this unique enzymatic reaction. In view of the evidence for the coupled formation of 1,3-diaminopropane and deoxyhypusine, the potent inhibition by 1,3-diaminopropane may be postulated as product inhibition. On the other hand, the inhibition by compounds that structurally resemble spermidine may be caused by competition at the substrate amine binding site. The strong inhibition by 1,8-diaminooctane, 1,7-diaminoheptane, homospermidine, N'-benzylspermidine, and caldine suggests a structural requirement for the two amino groups spaced at a distance close to that between the two primary amino groups of spermidine and also suggests that the secondary amino group is not necessary for binding to the active site of the enzyme.

Although the efficiency of deoxyhypusine synthesis in the combined ammonium sulfate fractions (~4 pmol/mg protein in 2 h) is comparable to the maximum rate observed in DFMO-treated intact CHO cells (~8 pmol/mg protein in 2 h) (8), some variations were observed in different preparations. Loss or dissociation of NAD+ or other cofactors is probably the cause of this variability. Whether NAD+ is the physiological cofactor for this reaction and, if so, in which step of the reaction it participates, remains to be determined after further purification of the enzyme.

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