Comparison of the Activities of Variant Forms of eIF-4D

THE REQUIREMENT FOR HYPUSEINE OR DEOXYHYPUSEINE*

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Eukaryotic protein synthesis initiation factor 4D (eIF-4D) (current nomenclature, eIF-5A) contains the unique amino acid hypusine (N\(^\gamma\)-(4-aminooxybutyl)lysine). The first step in hypusine biosynthesis, i.e. the formation of the intermediate, deoxyhypusine (N\(^\gamma\)- (4-aminooxybutyl)lysine), was carried out in vitro using spermidine, deoxyhypusine synthase, and ec-eIF-4D(Lys), an eIF-4D precursor prepared by overexpression of an eIF-4D cDNA in Escherichia coli. In a parallel reaction, using N-(3-aminopropyl)cadaverine in place of spermidine, a variant form of eIF-4D containing homodeoxyhypusine (N\(^\gamma\)-(5-aminopentyl)lysine) was prepared. Evidence that N-(3-aminopropyl)cadaverine can also act as the amine substrate for deoxyhypusine synthase in intact cells was obtained by incubating putrescine- and spermidine-depleted Chinese hamster ovary cells with [\(^3\)H]cadaverine. In these cells, in which [\(^3\)H]cadaverine is readily converted to N-(3-aminopropyl) [\(^3\)H]cadaverine, small amounts of [\(^3\)H]homodeoxyhypusine and another [\(^3\)H]-labeled compound, presumed to be N\(^\gamma\)-(5-aminooxy- \(^\gamma\)-hydroxy[\(^3\)H]pentyl)lysine, were found.

eIF-4D stimulates methionyl-puromycin synthesis, an in vitro model assay for translation initiation. Whereas the unmodified precursor ec-eIF-4D(Lys) appeared inactive, the deoxyhypusine-containing form provided a significant degree of stimulation. The variant form containing homodeoxyhypusine, on the other hand, showed little or no activity. These findings emphasize the importance of hypusine or deoxyhypusine for the biological activity of eIF-4D and demonstrate the influence of both the length and chemical nature of its amino alkyl side chain.

Eukaryotic initiation factor 4D (eIF-4D)\(^1\) is one of the protein factors that stimulate the initiation phase of eukaryotic translation by transient association with ribosomes (1). The activity of eIF-4D can be measured in an in vitro model assay for initiation of protein synthesis, the formation of methionyl-puromycin, in incubations containing AUG, Met-tRNA\(^\text{Met}\), puromycin, and the purified components necessary for the formation of 80 S initiation complexes (2, 3). Since eIF-4D also stimulates methionyl-puromycin synthesis with the preformed 80 S initiation complexes (4), it may be involved in formation of the first peptide bond. However, its exact in vivo role in eukaryotic protein synthesis is unknown.
eIF-4D is unique in that it is the only cellular protein known to contain the unusual amino acid hypusine (N\(^\gamma\)-(4-aminooxybutyl)lysine) (5). The biosynthesis of hypusine occurs post-translationally at one specific lysine residue of an eIF-4D precursor in two separate, enzymatically catalyzed steps: (i) transfer of the 4-aminobutyl moiety from the polypeptide spermidine to the \(\gamma\)-amino group of a specific lysine to form a deoxyhypusine residue and (ii) hydroxylation of the deoxyhypusine side chain to form a hypusine residue (6-8). Hypusine has been found in all eukaryotes examined (8, 9). The eIF-4D protein is highly conserved from yeast to human (9, 10). Especially noteworthy is the identity of the amino acid sequence around the hypusine residue (11-13), suggesting an important role for this residue of eIF-4D in protein synthesis and/or cellular metabolism. Recently, a human cDNA encoding the precursor of eIF-4D was cloned, and the cDNA sequence and the site of hypusine modification (lysine 50) determined (11).

Evidence suggesting an essential role of hypusine in eIF-4D was provided by a comparison of the biological activities of eIF-4D that contains hypusine and of precursors of eIF-4D that contain lysine in place of hypusine (14, 15). The two eIF-4D precursors, PI and PII, isolated from spermidine-depleted CHO cells (14), as well as the eIF-4D precursor, ec-eIF-4D(Lys)(formerly called ec-eIF-4D(Hpu-)), prepared by overexpression of a cloned human cDNA in Escherichia coli (15), failed to stimulate methionyl-puromycin synthesis. However, the possibility that the stimulatory activity of eIF-4D is the result of minor structural differences other than the presence of the hypusine residue per se or is due to differences in conformation could not be excluded, even though all these precursors were found to function as excellent substrates for enzymatic synthesis of the deoxyhypusine residue in vitro (14, 16).

For this reason and in an effort to define the role of the hydroxyl group of hypusine in the biological activity of eIF-4D, we decided to measure the stimulatory effect on methionyl-puromycin synthesis of a form of eIF-4D that contains deoxyhypusine. This intermediate, ec-eIF-4D(Dhp), was produced in vitro by the action of the enzyme deoxyhypusine...
synthesis on ec-eIF-4D(Lys). The finding reported here that this deoxyhypusine-containing form of eIF-4D produced a degree of stimulation in the methionyl-puromycin synthesis assay supplied the impetus for further investigation of the relationship of the structure of the hypusine residue to its function in eIF-4D. In this regard, we prepared and tested a variant form of eIF-4D in which homodeoxyhypusine (N°-(5-aminopropyl)lysine) replaced hypusine. This study was prompted by reports (17-19) that cadaverine can substitute for putrescine in supporting the growth of certain mammalian cells depleted of putrescine and spermidine and by our interest in whether the spermidine homolog N°-(3-aminopropyl)cadaverine, which is produced from cadaverine in cells, can replace spermidine as a substrate for deoxyhypusine synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**

DFMO was a gift from Merrell Dow Research Institute (Cincinnati, OH). [1,8-3H]Spermidine·3HCl ([terminal methylenes-3H]spermidine·3HCl) (>30 Ci/mmol) was purchased from Du Pont-New England Nuclear. N,N°-Diacetyl[1,5-3H]cadaverine was custom-synthesized (Amersham Corp.) by catalytic tritylation (platinum oxide catalyst) of glutaronitrile in a mixture of acetic acid and acetic anhydride. [1,5-3H]Cadaverine·2HCl (~18 Ci/mmol), prepared by acid hydrolysis of the diacetyl compound, was purified on silica gel as outlined (20).

N°-(3-Aminopropyl)[3H]cadaverine (~18 Ci/mmol) was isolated from the ether-extracted trichloroacetic acid supernatant of DFMO-treated CHO cells, after their incubation with [3H]cadaverine, by ion exchange chromatography on Bio-Rex 70 (NH4 form; 200–400 mesh, Bio-Rad) using elution with 10 mM NH4OH. Nonradioabeled N°-(3-aminopropyl)cadaverine·3HCl and N,N°-bis(3-aminopropyl)cadaverine·4HCl were prepared by a modification of a procedure used for preparation of radiolabeled spermidine and spermine (21). In the modification, cadaverine, instead of putrescine, was treated with acrylonitrile, and the products were isolated by ion exchange chromatography on Dowex 50 (H+, X2) using gradient elution with HCl (22).

Homodeoxyhypusine (N°-(5-aminopropyl)lysine) was synthesized according to a published procedure for the preparation of deoxyhypusine (23), except that 1-bromo-5-pthalimido-4-aminobutane. Homodeoxyhypusine was isolated by ion exchange chromatography on Bio-Rex 70 (NH4 form; 200–400 mesh) using a linear gradient of 0–0.3 M NH4OH and was recrystallized from water as the tri-p-hydroxyazobenzene-p′-sulfonate salt (m.p. 182–184 °C).

C6H12N6O4S: 3C3H6N6O8S·H2O (1084.2)

Calculated: C 52.06 H 5.30 N 11.63 S 8.87

Found: C 52.19 H 5.37 N 11.64 S 8.98

34CF plasma desorption mass spectroscopy, positive ion M + 1, 232.3 (calculated for homodeoxyhypusine, 232.33).

The eIF-4D precursor, ec-eIF-4D(Lys), was prepared by overexpression of a human eIF-4D cDNA in E. coli as described previously (15). Deoxyhypusine synthesis was partially purified from rat testis (16). Other materials and reagents were obtained as indicated in the text.

**Methods**

**Cell Culture and Labeling—CHO cells were cultured as outlined previously (7).** Addition of DFMO, the irreversible inhibitor of ornithine decarboxylase, was made at 4 mM to subconfluent, rapidly growing cells at a density of approximately 1–2 × 10⁶ cells/100-mm dish 24 h prior to addition of [3H]cadaverine or [3H]spermidine. After 24–42 h, the cells were washed and harvested as described (7).

**Analysis by Ion Exchange Chromatography—Proteins from cells or in vitro reaction mixtures were precipitated with 10% trichloroacetic acid.** The trichloroacetic acid precipitates were washed repeatedly with 5% trichloroacetic acid containing nonradioactive amines (1 mM spermidine, spermine, putrescine, and cadaverine and 0.5 mM N°-(3-aminopropyl)cadaverine) in order to remove noncovalently bound radioactive amines before hydrolysis in 6 N HCl (18 h at 100 °C). Ion exchange chromatographic analyses of protein hydrolysates and of trichloroacetic acid supernatant solutions were performed on a automated Dionex D-400 amino acid analyzer (16, 25) using a gradient buffer with two columns. The columns were packed with the 0.2 N sodium citrate/sodium chloride buffers (0.6 N Na+, pH 5.80; 1.5 N Na+, pH 5.55; and 3.0 N Na+, pH 5.55) described previously (25) for 5, 10, and 40 min, respectively, followed by reequilibration with the initial buffer for 17–19 min.

**Deoxyhypusine Synthase Reactions—Deoxyhypusine synthesis was measured by determination of the incorporation of radioactivity from [1,8-3H]spermidine into [3H]deoxyhypusine in the precursor protein, as described in an earlier publication (16), except that a purified precursor protein obtained by overexpression of eIF-4D cDNA in E. coli (ec-eIF-4D(Lys)) (15) was used in place of CHO cell precursor protein. The synthesis of the deoxyhypusine homolog, homodeoxyhypusine (N°-(5-aminopropyl)lysine), was carried out in mixtures containing N°-(3-aminopropyl)[3H]cadaverine in place of [3H]spermidine.

**Methionyl-puromycin Assay—**The activities of eIF-4D and its variant forms in stimulating methionyl-puromycin synthesis were measured as described previously (3, 15).

**RESULTS**

**ec-eIF-4D(Lys) as a Protein Substrate for Deoxyhypusine Synthase—**Hypusine appears to be ubiquitous in all eukaryotes. The prokaryote, E. coli, on the other hand, contains neither eIF-4D, eIF-4D precursors, nor the enzymes responsible for hypusine synthesis in these precursors. Thus, we were able to prepare an eIF-4D precursor in E. coli with the use of the human cDNA (15). This precursor protein, ec-eIF-4D(Lys), which contains lysine in place of hypusine, is efficiently converted to the deoxyhypusine-containing protein ec-eIF-4D(Dhp) by deoxyhypusine synthase from rat testis (Fig. 1A). Parenthetically, an altered form of eIF-4D precursor, ec-eIF-4D(Arg), isolated from E. coli transformed with a mutated cDNA coding for arginine at residue 50 (15), was not modified to a deoxyhypusine-containing form (data not shown). The apparent Kₘ value of 0.42 ± 0.045 μM for ec-eIF-4D(Lys), estimated from the data of Fig. 1A, is comparable to those for the cellular eIF-4D precursors isolated from spermidine-depleted CHO cells (16). Evidence that the lysine residue modified in vitro in ec-eIF-4D(Lys) is identical with that converted to hypusine in intact cells is provided by the two-dimensional peptide maps of Fig. 1B. As shown previously, this peptide-mapping system separates the hypusine-containing peptide from other tryptic peptides of eIF-4D (10, 26). In Fig. 1B, the peptide containing hypusine in the tryptic digest of eIF-4D from cultured CHO cells (left-hand panel) is compared to that containing deoxyhypusine from ec-eIF-4D(Dhp), produced from ec-eIF-4D(Lys) in vitro (right-hand panel). The hypusine-containing peptide from eIF-4D and the deoxyhypusine-containing peptide from the protein modified in vitro by deoxyhypusine synthase occupy identical positions on the peptide maps (Fig. 1B) and appear at a single position when applied as a mixture (data not shown).

N°-(3-Aminopropyl)cadaverine as an Amine Substrate for Deoxyhypusine Synthase in Cultured CHO Cells and in Vitro—When CHO cells were cultured with [3H]cadaverine, this labeled amine was transported into the cells and partly converted to N°-(3-aminopropyl)[3H]cadaverine and N,N°-bis(3-aminopropyl)[3H]cadaverine (Table 1). The uptake of cadaverine and an accumulation of N°-(3-aminopropyl)cadaverine and N,N°-bis(3-aminopropyl)cadaverine were previously reported in several different mammalian cells that were prevented from synthesizing putrescine by the use of DFMO (17–19). This in vitro production of polyamine derivatives of cadaverine is consistent with the observation that the isolated rat prostatic enzymes, spermidine synthase and spermine
syntehase, can use cadaverine in place of putrescine, and N-(3-aminopropyl)cadaverine instead of spermidine, respectively (18). In our CHO cells that were not treated with DFMO, a relatively low level of [3H]cadaverine accumulated and a limited conversion to polyamine derivatives occurred. Upon depletion of the cells of putrescine and spermidine by exposure to DFMO, however, pronounced increases in both [3H]cadaverine uptake and polyamine derivative formation occurred (Table I and Fig. 2A). The latter is presumably due to the high cellular level of the donor of the aminopropyl moiety, S-adenosylmethionine, which results both from its increased production and from depletion of its normal aminopropyl acceptors, putrescine and spermidine (27, 28).

In these DFMO-treated cells, a small portion of the total radioactivity was found to be associated with the trichloroacetic acid-insoluble fraction. When the hydrolysate of this fraction was examined by ion exchange chromatography, two distinct radiolabeled components, I and II, were found (Table I and Fig. 2B, arrows) along with some noncovalently bound [3H]cadaverine and N-(3-aminopropyl) [3H]cadaverine that was not removed even by repeated washings. The major component, I, eluted at 26 min, ~3 min after deoxyhypusine; the minor component, II, eluted at 21–22 min, ~1 min after hypusine. Evidence that these are produced through a common enzymatic step in which the [3H]-labeled aminopentyl moiety of N-(3-aminopropyl) [3H]cadaverine is transferred to a lysine residue in an eIF-4D precursor, in a manner parallel to that for the formation of deoxyhypusine, is as follows: (i) the radiolabeled protein from DFMO-treated cells that were incubated with [3H]cadaverine shows SDS-PAGE characteristics identical with those of the radiolabeled protein, eIF-4D, produced upon the growth of cells in the presence of [3H]putrescine (Fig. 3A, lanes 2 and 1, respectively); (ii) an amino acid residue (Component I) formed during incubation of cells with [3H]cadaverine was identified as a homolog of deoxyhypusine, homodeoxyhypusine [N'-(5-aminopentyl)lysine] (see Scheme 1), by comparison of its ion exchange chromatographic properties with those of a synthetic sample of this homolog (Table II); (iii) the [3H]-labeled protein produced in the enzymatic in vitro reaction between N-(3-aminopropyl) [3H]cadaverine and ec-eIF-4D(Lys) displays the same mobility on SDS-PAGE as that from the reaction with [3H] spermidine and ec-eIF-4D (Fig. 3A, lanes 2 and 3, respectively); (iv) the major [3H]-labeled component, [3H]homodeoxyhypusine, from protein hydrolysates of DFMO-treated cells grown with [3H]cadaverine (Figs. 2B and 3B, panel 2) corresponds in chromatographic properties to the labeled amino acid residue produced in vitro from ec-eIF-4D(Lys) and N-(3-aminopropyl) [3H]cadaverine (Figs. 2D and 3B, panel 4).

The fact that, when DFMO-treated cells were cultured with

![In vitro deoxyhypusine synthase-catalyzed conversion of lysine to deoxyhypusine in ec-eIF-4D(Lys). A, dependence of the rate of conversion on ec-eIF-4D(Lys) concentration. Reaction mixtures contained 2.7 μM [3H] spermidine (10 μCi), 0.5 mM NAD⁷, 1 mM DTT, the indicated concentration of ec-eIF-4D(Lys), 125 μg of bovine serum albumin, 7.4 units of enzyme, and 0.4 M glycine buffer, pH 9.5, in a total volume of 0.1 ml. After 1 h of incubation at 37 °C, reactions were stopped by addition of trichloroacetic acid. Analyses for [3H]deoxyhypusine were performed on the acid-insoluble fraction from CHO cells grown in the presence of [3H]putrescine as outlined previously (10). Peptide maps from these tryptic digests and the fluorograms of the maps were prepared as described (10). The fluorograms shown are of only a portion of the total map prepared from the CHO cell protein digest (left-hand panel) and of that from the CHO cell protein digest (right-hand panel). The origin is indicated in each panel by a small circle.

![Experimental Procedures.](https://example.com)

### Table I

**Distribution of radioactivity in untreated and DFMO-treated CHO cells after incubation with [3H]cadaverine**

Subconfluent CHO cells were incubated for 24 h with and without 4 mM DFMO. [3H]Cadaverine (50 μCi/dish) was added to both untreated and DFMO-treated cells, and incubation was continued for an additional 24 h. The cells were disrupted with trichloroacetic acid, the trichloroacetic acid-insoluble fractions were washed extensively and hydrolyzed with HCl, and the radioactivity of the individual components in both the acid-soluble and acid-insoluble fractions was measured after their separation by ion exchange chromatography, as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Treatment</th>
<th>In acid-soluble fraction</th>
<th>In acid-insoluble fraction (hydrolysate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cadaverine</td>
<td>N-(3-Aminopropyl)-cadaverine</td>
</tr>
<tr>
<td></td>
<td>N,N'-Bis(3-aminopropyl)cadaverine</td>
<td>Component I</td>
</tr>
<tr>
<td>None</td>
<td>280</td>
<td>43</td>
</tr>
<tr>
<td>DFMO</td>
<td>1300</td>
<td>9200</td>
</tr>
</tbody>
</table>

10⁶ x cpm/dish

<table>
<thead>
<tr>
<th>Component II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component I</td>
</tr>
<tr>
<td>ND*</td>
</tr>
<tr>
<td>ND*</td>
</tr>
</tbody>
</table>

* ND = none detected.
FIG. 2. Ion exchange chromatography of (A) the acid-soluble fraction of DFMO-treated CHO cells that were incubated with $[^{3}H]$cadaverine, (B) the hydrolysate of the acid-insoluble fraction from similarly treated cells, (C) the hydrolysate of the acid-insoluble fraction from DFMO-treated cells that were incubated with $[^{3}H]$cadaverine in the presence of $\alpha$,$\alpha$-dipyridyl, and (D) the hydrolysate of the precipitated proteins from an in vitro incubation of N-(3-aminopropyl)$[^{3}H]$cadaverine with ee-eIF-4D(Lys) and deoxyhypusine synthase. A, CHO cells were treated with DFMO and $[^{3}H]$cadaverine (60 $\mu$Ci/100-mm dish) as outlined in the legend to Table I, except that incubation with $[^{3}H]$cadaverine was for 42 h. A sample of the trichloroacetic acid supernatant solution of these cells was chromatographed as described under “Experimental Procedures.” B, the trichloroacetic acid-insoluble fraction from DFMO-treated CHO cells that had been incubated with $[^{3}H]$cadaverine as in A was hydrolyzed with 6 N HCl and chromatographed as described under “Experimental Procedures.” C, CHO cells were treated as in A, except that 0.1 mM $\alpha$,$\alpha$-dipyridyl was included together with $[^{3}H]$cadaverine. The protein fraction was hydrolyzed and chromatographed as in B, D, the in vitro reaction mixture contained 176 units of enzyme, 200 pmol of ee-eIF-4D(Lys), a portion of ether-extracted trichloroacetic acid supernatant from A containing 4 $\mu$Ci (~220 pmol) of N-(3-aminopropyl)$[^{3}H]$cadaverine, 0.5 mM NAD$,^+$, 1 mM DTT, and 0.2 M glycine buffer, pH 9.5, in a final volume of 0.025 ml. Incubation at 37 °C was for 2 h. A portion of the reaction mixture was treated with an equal volume of 10% trichloroacetic acid, and the precipitate was washed, hydrolyzed, and analyzed as in B.

$[^{3}H]$cadaverine in the presence of $\alpha$,$\alpha$-dipyridyl, an inhibitor of deoxyhypusine hydroxylase (7, 29), no Component II was formed (Fig. 2, compare C and B) suggests that this component is an hydroxylated form of homodeoxyhypusine. In order to test this possibility and to determine its structure, a sample of Component II was isolated (Table II). Assuming the location of the hydroxyl group shown in the postulated structure (Scheme I), as is the case in hypusine, one would expect two radiolabeled fragments from oxidative cleavage by periodic acid (6). Oxidation of Component II produced radiolabeled material (presumably HCHO) that appeared in the effluent from the cation exchange column and, under some conditions,
with both sets of cells. The pronounced difference in rates is protein/h in DFMO-treated cells supplemented with A'-pyrroline (Table 11). A'-Pyrroline could result from spontaneous cyclization of 4-aminobutyraldehyde, the aldehyde, and lysine (6); by analogy, oxidation products from Component II will be 4-aminobutyraldehyde, formaldehyde, and lysine.

**TABLE II**

**Effects of periodic acid on components of the radiolabeled protein produced in CHO cells grown with $^{14}N$-cadaverine**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Elution time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic homodeoxyhypusine</td>
<td>min</td>
</tr>
<tr>
<td>Component I</td>
<td>26</td>
</tr>
<tr>
<td>Component II</td>
<td>26</td>
</tr>
<tr>
<td>$\Delta'$-Pyrrylene</td>
<td>21</td>
</tr>
<tr>
<td>Radioactive compound from treatment of Component I with periodic acid</td>
<td>10</td>
</tr>
<tr>
<td>Radioactive compounds from treatment of Component II with periodic acid</td>
<td>26</td>
</tr>
<tr>
<td>of Component II with periodic acid</td>
<td>3</td>
</tr>
</tbody>
</table>

a basic second component that chromatographed at the position of $\Delta'$-pyrrylene (Table II). $\Delta'$-Pyrrylene could result from spontaneous cyclization of 4-aminobutyraldehyde, the aldehyde that would be produced from the postulated structure of Component II. Since the periodic acid oxidation of Component II proceeds as readily as does that of hypusine whereas no oxidation of deoxyhypusine or homodeoxyhypusine occurs under the same conditions, it seems reasonable to assume that the homodeoxyhypusine residue in the eIF-4D variant is hydroxylated at carbon 2 of its 5-aminopentyl side chain to form a $N'$-(5-amino-2-hydroxypropyl)lysine residue.

The rate of formation of homodeoxyhypusine plus Component II in DFMO-treated CHO cells grown with 0.3–0.6 $\mu$M cadaverine was estimated to be 0.01–0.1 pmol/mg of protein/h. This value is much lower than that calculated for the rate of deoxyhypusine plus hypusine formation (0.5–5 pmol/mg of protein/h) in DFMO-treated cells supplemented with 0.07–2.5 $\mu$M spermidine (31). Comparable growth was observed with both sets of cells. The pronounced difference in rates is understandable when one compares the kinetic constants determined for deoxyhypusine synthase with $N$-(3-aminopropyl)cadaverine ($K_{\text{m(app)}} = -6.6$ mM and $V_{\text{max}} = -12$ nM h$^{-1}$/unit of enzyme at 0.5 mM NAD and 10 $\mu$M ec-eIF-4D(Lys), data not shown) and those reported for the enzyme with spermidine ($K_{\text{m(app)}} = -0.83$ $\mu$M and $V_{\text{max}} = -12$ nM h$^{-1}$/unit of enzyme) (16). Thus it appears likely that the slow production of homodeoxyhypusine and Component II is a reflection of the slow affinity of deoxyhypusine synthase for $N$-(3-aminopropyl)cadaverine. That only small portions of total product are Component II indicates that the homodeoxyhypusine residue is an inefficient substrate for deoxyhypusine hydroxylase.

**Effects of ec-eIF-4D(Dhp) and ec-eIF-4D(Hdh) on Methionyl-puromycin Synthesis**—In order to determine the biological activities of the variants of eIF-4D that contain deoxyhypusine and homodeoxyhypusine, it was necessary to prepare these two proteins essentially free of contaminating materials. Fig. 4 shows the isolations of these proteins and defines their purities. Radiolabeled tracers were employed as a means of identifying the desired products. Satisfactory purifications were accomplished from in vitro enzyme-catalyzed reactions with the use of a single step chromatographic procedure (Fig. 4, A and B, insets). In each of the reactions, a significant portion of the substrate ec-eIF-4D(Lys) was converted to product, with somewhat more product formed in the deoxyhypusine synthase reaction. When samples of the purified ec-eIF-4D(Dhp) and ec-eIF-4D(Hdh) were hydrolyzed with 6 $N$ HCl and the hydrolysates analyzed by ion exchange chromatography, approximately 1 mol of deoxyhypusine and 1 mol of homodeoxyhypusine per mol of protein, respectively, were found (based upon the known arginine content of ec-eIF-4D(Lys)).

The effects of ec-eIF-4D(Dhp) and ec-eIF-4D(Hdh) on methionyl-puromycin synthesis are shown in Fig. 5. Whereas it was reported earlier (15), and is verified here, that ec-eIF-4D(Lys) provides little or no stimulation of methionyl-puromycin synthesis, ec-eIF-4D(Dhp), in which the lysine at position 50 of ec-eIF-4D(Lys) is changed to deoxyhypusine, is active in this respect. This stimulation, although clearly notable, is not as pronounced as that seen with eIF-4D, which contains hypusine. The stringent requirement for a deoxyhypusine or a hypusine residue in place of a specific lysine in imparting significant biological activity is emphasized by the finding that ec-eIF-4D(Hdh), in which the residue at position 50 differs from deoxyhypusine only by one methylene in chain length, shows little or no methionyl-puromycin synthesis-stimulating activity. Consistent with this observed specificity for deoxyhypusine and hypusine is the finding that ec-eIF-4D(Arg), in which an arginine residue replaces the lysine residue at position 50, is also without stimulatory activity (data not shown).

**DISCUSSION**

In earlier papers, we reported that hypusine plays an essential role in the in vitro activity of eIF-4D (14, 15). This conclusion was based on a comparison of the methionyl-puromycin synthesis-stimulating activity of eIF-4D with that of its precursors that contain lysine in place of hypusine. Because the posttranslational formation of the deoxyhypusine residue from a precursor lysine residue and hydroxylation of this deoxyhypusine residue to produce hypusine are separate events catalyzed by two different enzymes, and because the hydroxylation step proceeds in an efficient manner in vitro and in cultured cells, it seemed logical to question the contribution to the biological activity of eIF-4D of each event in...
Fig. 4. Preparation of the variant forms of eIF-4D, cc-eIF-4D(Dhp) (A), and cc-eIF-4D(Hdh) (B). Reaction mixtures contained 1.0 mg of cc-eIF-4D(Lys), 0.5 mM NAD\(^+\), 1 mM DTT, 61,000 units of deoxyhypusine synthase, 30 \(\mu\)M nonradioactive spermidine (in A) or 5.26 \(\mu\)M nonradioactive N-(3-aminopropyl)cadaverine (in B), and 0.25 M glycine NaOH buffer, pH 9.5, in a total volume of 4 ml. Incubations were for 4 h at 37 °C. The reaction mixtures were concentrated to \(-1\) ml using a YM-5 membrane (Amicon), diluted to 4D(Dhp) (A), and ec-eIF-4D(Hdh) proteins were prepared in 0.2-ml reaction mixtures of the compositions given above, except using 13.5 \(\mu\)M (100 \(\mu\)Ci) [\(^{3}H\)]Met-tRNA\(^{Met}\) (specific activity, 4.45 \(\times\) \(10^{4}\) cpm/pmol of Met), 0.8 mM GTP, 1 mM puromycin, 35 \(\mu\)M AUG, 0.06 \(A_{260}\) units of 40 S ribosomal subunits, 0.15 \(A_{260}\) units of 60 S ribosomal subunits, 0.5 \(\mu\)g of eIF-2, 3.4 \(\mu\)g of eIF-3, 0.2 \(\mu\)g of eIF-4C. For experimental details, see Ref. 3. The indicated amounts (\(\mu\)g) of ec-eIF-4D(Lys), eIF-4D from HeLa cells, ec-eIF-4D(Dhp), and cc-eIF-4D(Hdh) were added. Hpu, hypusine.

hypusine. It became apparent from our present studies that a degree of in vitro activity is associated with the unhydroxylated intermediate, cc-eIF-4D(Dhp) (Fig. 5). However, the stimulation exerted by this deoxyhypusine-containing protein was less than that given by eIF-4D, which contains hypusine. On the basis of this difference, it is tempting to speculate that specific hydroxylation, i.e., conversion of deoxyhypusine to hypusine, is essential for full activity of eIF-4D. However, the role of hydroxylation cannot be determined at this time because the in vitro production of a hypusine-containing protein from cc-eIF-4D(Dhp), in the quantity and purity necessary for activity testing, is prohibited by the insufficient purity and the instability of the hydroxylation enzyme, deoxyhypusine hydroxylase. With the exception of the hypusine-for-lysine change, eIF-4D and cc-eIF-4D(Lys) possess almost identical amino acid sequences (11, 15). One difference, however, is known. The amino-terminal residue of cc-eIF-4D(Dhp) is alanine, whereas the amino terminus of eIF-4D appears to be blocked. If the amino-terminal region of eIF-4D plays a role in its activity, it is possible that the hypusine-containing protein prepared from cc-eIF-4D(Dhp) could display a significantly different degree of biological activity than eIF-4D. We find that neither free hypusine, deoxyhypusine, nor a peptide, Lys-Thr-Gly-deoxyhypusine-His-Gly-His-Ala-Lys, the sequence of which is identical with that around hypusine in eIF-4D, exert methionyl-puromycin synthesis-stimulating activity, even at high levels (data not given). Clearly, the biological activities displayed by eIF-4D and cc-eIF-4D(Dhp) depend upon macromolecular structural features in addition to the residues of hypusine and deoxyhypusine, respectively.

The polyamine requirement for the growth of mammalian cells is well documented (32, 33). These polyamines are known to bind to nucleic acids and to specific sites on tRNA (34, 35) order to conserve enzyme. The fractions of deoxyhypusine synthase that eluted from the column in the first 10 min in A were combined, reduced in volume, and employed as the major portion of the enzyme for the reaction described in B. For this reason, there is a significant reduction in the 280 nm absorbance between 10 and 35 min in B, as a result of the removal of impurities from the enzyme preparation used in A.
and to affect protein synthesis in vitro (36, 37) and in vivo (38). Yet the specific molecular mechanisms by which they modulate cellular processes are not known. Cadaverine was reported to support growth in various mammalian cells that were prevented from synthesizing putrescine and spermidine by treatment with DFMO (17–19). Growth stimulation by cadaverine appears to be linked to the ability of this diamine to function as a substrate for spermidine synthase (18). We present evidence here that N-(3-aminopropyl)cadaverine, the product of this reaction, can serve as an amine substrate for deoxyhypusine synthase. However, in consideration of the poor efficiency of the reaction and the inactivity of the product ec-eIF-4D(Dhd) in the methionyl-puromycin assay (Fig. 5), it is unlikely that restoration of growth of polyamine-depleted cells by cadaverine is due to eIF-4D variants containing structural elements of this diamine. It is possible, however, that cadaverine and N-(3-aminopropyl)cadaverine contribute indirectly to growth in polyamine-depleted cells by replacing putrescine and spermidine, respectively, in charge interactions, thus freeing the very small amounts of spermidine produced in these cells for use in hypusine synthesis.

Two radioactive components, I and II, were found in hydrolysates of the protein fraction from DFMO-treated CHO cells that were cultured with [3H]cadaverine (Fig. 4). One of these, Component I, was identified as homodeoxyhypusine. The other, Component II, displayed properties consistent with those of an hydroxylated form of homodeoxyhypusine, a form in which the hydroxyl group is vicinal to an amino group (Table II and Scheme 1). Although we were unable to formulate the optimum oxidation conditions needed to prove the structure of this amino acid due to its limited availability, it seems safe to conclude that it is a product of the enzyme deoxyhypusine hydroxylase and, thus, that the specificity of this enzyme extends to the homolog of deoxyhypusine.

Our present finding that substitution of deoxyhypusine, but not homodeoxyhypusine for lysine, at position 50 in ec-eIF-4D(Lys) causes stimulation of methionyl-puromycin synthesis (Fig. 5) provides further definition of the structural requirements for this in vitro activity. The true physiological function of eIF-4D in protein synthesis and the contribution by hypusine are not as yet known, however. Recently, a molecular and genetic approach was made by one of our laboratories to derive the in vivo function of eIF-4D and hypusine in yeast. Schnier et al. (13) report evidence that inactivation of both yeast genes for eIF-4D (TIF51A and TIF51B) is lethal and that the lysine codon at the site of hypusine synthesis is also a critical element for yeast growth. However, it is still an open question whether the required functions of hypusine and eIF-4D in yeast cells involve the translation pathway. Further studies are in progress to evaluate the role of the hypusine residue and of eIF-4D in protein synthesis in vivo.

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REFERENCES