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Synthesis of deoxyhypusine in eukaryotic initiation factor 4D in rat hepatoma cells

Murphey, Roberta Jean, Ph.D.

The University of Arizona, 1989
SYNTHESIS OF DEOXYHYPUSINE IN EUKARYOTIC INITIATION FACTOR 4D IN RAT HEPATOMA CELLS

by

Roberta Jean Murphey

A Dissertation Submitted to the Faculty of the
DEPARTMENT ON MOLECULAR AND CELLULAR BIOLOGY
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THE UNIVERSITY OF ARIZONA

1989
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Roberta Jean Murphey
entitled "Synthesis of Deoxyhypusine in Eukaryotic Initiation Factor 40 in Rat Hepatoma Cells"

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ABSTRACT

The aim of this research was to study the mechanism involved in the synthesis of deoxyhypusine, the intermediate step in the synthesis of the amino acid hypusine. Deoxyhypusine is derived from the butylamine moiety of a spermidine molecule which is added to the ε-amino group of one lysine in the eukaryotic initiation factor 4D (eIF-4D). Initially, a hepatoma tissue cell (HTC) lysate with a pH of 9.5 in glycine buffer and with a depleted spermidine pool supported deoxyhypusine synthesis in protein. Since CHES buffer was as efficient as glycine buffer, the synthesis of deoxyhypusine was pH dependent (optimum ~9.2) and not buffer dependent. Next, several inhibitors were used in the cell-free system to block deoxyhypusine synthesis. Only guazatine, a plant amine oxidase inhibitor, completely inhibited deoxyhypusine synthesis. This suggested that an oxidase was involved in deoxyhypusine synthesis.

In addition factors were investigated as possible allosteric stimulators of deoxyhypusine formation. NAD⁺, NADH, FAD⁺, FMN⁺, and as nicotinamide were tested for effects on deoxyhypusine formation. NAD⁺ was the most efficient stimulator, but NADH and nicotinamide also stimulated deoxyhypusine formation. Although these factors increased the synthesis of deoxyhypusine, these assays were done in buffer with low concentrations of spermidine. When the spermidine pool was replenished, these effects were diminished. Thus, it appeared that
NAD$^+$ may lower the apparent $K_m$ for spermidine without affecting the $V_{max}$ of deoxyhypusine synthesis.

The inhibition of deoxyhypusine synthesis by guazatine implied the involvement of a polyamine oxidase. Therefore, the effect of oxygen depletion on deoxyhypusine formation was investigated. The depletion of oxygen reduced the level of deoxyhypusine synthesis to 12% of the control. This activity could be restored to 85% by reoxygenation of the lysate. Thus in support of the suggestion made by the guazatine data, a spermidine oxidase is involved in deoxyhypusine formation.

The most significant contribution of this work was the development of a cell-free system to study deoxyhypusine. This synthesis required an unusually high pH in vitro and required polyamine depletion (Chapter 2). In addition, synthesis requires a unique spermidine oxidase that is blocked by a guazatine and is conditionally stimulated by NAD$^+$ (Chapter 3).
CHAPTER 1

INTRODUCTION

In 1971, hypusine, an amino acid, was discovered in a free form in the bovine brain (Shiba, Mizote, Kaneko, Nakijima, Kakimoto and Sano, 1971). The structure of hypusine was established and confirmed as \( N^e-(4\text{-amino-2-hydroxybutyl})-2,6\text{-diaminohexanoic acid} \) (Shiba, Akijama, Umeda, Okada and Wakamiya, 1982; Tice and Ganem, 1983). Since its discovery, hypusine has been found as a free amino acid (Nakajima, Matsubayashi, Kakimoto and Sano, 1971) and bound to protein (Sano, Miyake and Kakimoto, 1984). Protein-bound hypusine primarily occurred in a protein, \( M_r \sim 18,000 \), which was originally observed in lectin-stimulated lymphocytes and Chinese hamster ovary (CHO) cells (Park, Cooper and Folk, 1981; Cooper, Park and Folk, 1982). The synthesis of protein-bound hypusine coincides with an increase in protein synthesis (Cooper et al., 1982) and this biochemical process was observed in several mammalian cell lines (Chen, 1983; Torrelio, Paz and Gallop, 1984). Because of the ubiquity of the modification in an \( M_r \sim 18,000 \) protein and its conservation among eukaryotes (Park Chung, Cooper and Folk, 1984; Gordon, Mora, Meredith, Lee and Lindquist, 1987), the biophysical characteristics of the protein were compared to those of known eukaryotic protein translation initiation factors. The \( M_r \sim 18,000 \) protein modified by hypusine was identified
as the putative eukaryotic protein synthesis initiation factor eIF-4D (Cooper, Park, Folk, Safer and Braverman, 1983).

Biosynthesis of hypusine in protein involves a multi-step reaction. Early investigations of the synthesis of hypusine led to speculation that a butylamine group was transferred to a protein-bound lysine (Imoaka and Nakajima, 1973). The use of radiolabel techniques allowed the demonstration that the lysine segment of the modification was contributed by a protein (Park, Cooper and Folk, 1982). Park and coworkers demonstrated that normal human peripheral lymphocytes supplemented with either \([^3H]\)putrescine or \([\text{terminal methylenes-}{^3}\text{H}]\)spermidine labelled a single protein. When the protein was analyzed, the label was found to be incorporated into hypusine. Thus, it appeared a polyamine was the substrate for hypusine synthesis. To further investigate the possibility of polyamine involvement, Park and coworkers (1981) used spermidine and the spermidine precursor, putrescine (see Polyamine Pathway, Figure 1) in addition to a potent inhibitor of S-adenosylmethionine decarboxylase, methylglyoxal bis(guanylylhydrazone) (Corti, Dave, Williams-Ashman, Mihich and Schenone, 1974) to study the substrate specificity of hypusine biosynthesis. The inhibitor, in the presence of labelled putrescine, blocked hypusine biosynthesis in lymphocytes. On the other hand, if the inhibitor was present, along with labelled spermidine, hypusine biosynthesis was not affected. By blocking the conversion of putrescine to spermidine, hypusine was not synthesized if exogenous spermidine was not supplied to the cells. Several amines have been
used to compete with spermidine as a substrate for hypusine. N\textsuperscript{1}-acetylspermidine, added to hepatoma tissue culture (HTC) cells, was unable to compete with the labelled spermidine in this regard (Gerner, Mamont, Bernhardt and Siat, 1986). Inhibition of polyamine oxidase when spermidine was added to polyamine depleted cells did not affect hypusine synthesis, which ruled out putrescine as the natural polyamine substrate. Spermine, the final polyamine in this biosynthetic pathway, was also a poor substrate for hypusine synthesis (Gerner et al., 1986). Thus, these data suggest that the butylamine portion of hypusine is donated by a spermidine molecule.
Figure 1. Polyamine biosynthesis pathway in mammalian cells. The enzymes: 1. ornithine decarboxylase (ODC) (E.C.4.1.1.17), 2. S-adenosylmethionine decarboxylase (SAM DC) (E.C.4.1.1.50), 3. spermidine synthase (E.C.2.5.1.16), 4. spermine synthase (E.C.1.5.1.22). Adapted from Pegg, 1986.
Protein-bound hypusine was identified in several eukaryotic cell lines. Studies in human lymphocytes and CHO cells revealed the hypusine-containing protein had a $M_r \sim 17,000-19,000$ and $pI \sim 5.1-5.5$ (Cooper et al., 1982). Because the protein was ubiquitous, and correlated with protein synthesis and cell growth (Cooper et al., 1982; Chen, 1983; Torrelio et al., 1984), it was compared to the protein translation initiation factors. The hypusine-containing protein was identical to the putative initiation factor eIF-4D (Cooper et al., 1983). Only 10% of this protein is found associated with ribosomes, thus the majority is found primarily in the post-ribosomal supernatant. It has also been shown to exist after the 80S initiation complex is formed (Benne and Hershey, 1977). This is unlike any other translation initiation factor, therefore, its classification as an initiation factor is questioned.

Because the only known function of eIF-4D was a 2-3 fold stimulation of the methionyl-puromycin assay (simulating the start of protein translation) and of poly(U)-dependent polyphenylalanine synthesis (Jagus, Anderson and Safer, 1981; Thomas, Benne and Voorma, 1981; Moldave, 1985), yet it had no effect on the level of translation, it was of interest to study a definitive role for the protein. EIF-4D is ten times more abundant than any other initiation factor. In rat hepatoma tissue culture cells, eIF-4D comprised 0.1% of whole cell protein (personnel communication). In order to learn more about the function(s) of eIF-4D-containing hypusine, attempts were made to isolate the unmodified protein from several cell lines.
Edman degradation of radiolabelled hypusine in eIF-4D showed that the radiolabelled hypusine was released as a single peak. For every mole of eIF-4D, there was a stoichiometric modification of the third amino acid (lysine) in the protein (Park et al., 1984; Park, Lui, Neece, Swiggard, 1986). All of the eIF-4D appeared to be modified in log phase CHO cells. When polyamine-depleted HTC cells were exposed to exogenous polyamines, the initial rate of hypusine synthesis was 5-10 times greater than in those cells that had normal levels of polyamines (Gerner et al., 1986). In this same study, the rate of hypusine synthesis was dependent on the final endogenous content of spermidine. Thus, these data suggest that a precursor substrate accumulated due to the lack of polyamine availability and this substrate could be modified by the addition of spermidine. This substrate may be an unmodified eIF-4D which does not exist in logarithmically growing cells.

There appear to be two reactions involved in hypusine biosynthesis. The enzyme(s) responsible for the synthesis of deoxyhypusine catalyzes the cleavage and transfer of the butylamine moiety of spermidine to the ε-amino group of lysine in eIF-4D. In order to learn more about the enzyme(s) involved in this synthesis, several inhibitors (including several polyamine oxidase inhibitors) were used in an attempt to block the reaction in whole cells (Gerner et al., 1986). Only diethyldithiocarbamate and iodoacetamide were partially effective. Therefore, there seemed to be a unique oxidase or dehydrogenase involved in deoxyhypusine synthesis. A cell-free system
was needed in order to closely analyze the type of enzyme(s) involved in the synthesis of deoxyhypusine. Attempts to devise an in vitro system to study the enzyme(s) involved were unsuccessful (Park et al., 1982). However, the enzyme involved in the second reaction in hypusine synthesis, the hydroxylation of deoxyhypusine, has been identified and partially purified. The enzyme is known as deoxyhypusine hydroxylase and was found primarily in the testes of rats although several other tissues were investigated (i.e. liver, heart) (Abbruzzese, Park and Folk, 1986). Initial studies using a cell lysate system suggested that the enzyme could be blocked by metal chelators such as \(\alpha,\alpha'-\)dipyridyl, desferoxamine and picolinic acid (Park et al., 1982) or the vasodialator, hydralazine (Paz, Torrello and Gallop, 1984). These results suggested that the enzyme required a metal ion for activation. Further investigations revealed that the block due to a metal chelator \((\alpha,\alpha'-\)dipyridyl) could be reversed by cobalt, not iron, as originally expected. The hydroxylase was found to be different from the hydroxylating enzyme(s) used to process propyl- and lysyl-procollagen, since it failed to stoichiometrically decarboxylate \(\alpha\)-ketoglutarate. Surprisingly, unlike the preliminary data, iron inhibited the enzyme activity after partial purification of the enzyme. It appeared that deoxyhypusine hydroxylase used a distinct catalytic mechanism which is specific for the synthesis of hypusine. Therefore, deoxyhypusine hydroxylase appeared to be unique for hypusine and required a metal ion for activity (possibly cobalt).
Complete purification will allow further characterization of this enzyme.

Focus of the Investigation

The major task of this investigation was to develop a cell lysate system capable of supporting the synthesis of deoxyhypusine in eIF-4D. Once the assay was developed, specific questions concerning the mechanisms of deoxyhypusine synthesis were addressed. In order to develop the assay, several parameters were considered such as buffer type, pH and lysate (cell) concentration. The types of enzymes capable of catalyzing such a reaction were considered and a suitable buffer was found (Holtta, 1977). Deoxyhypusine was quantitated based on the incorporation of radiolabelled spermidine into protein. The only protein known to contain hypusine is eIF-4D which has a Mr ~18,000 and a pI ~5.1-5.5 (Cooper et al., 1982). Deoxyhypusine synthesis was quantitated either by the incorporation of radiolabelled spermidine into eIF-4D which was separated on polyacrylamide gels (Laemmli, 1970) or by HPLC analysis of the hydrolyzed protein (Gerner et al., 1986). After the cell lysate system was developed, possible inhibitors of the enzyme(s), as well as allosteric effectors and conditional requirements were studied to derive information concerning the mechanism of deoxyhypusine formation.

This dissertation is divided into chapters which focus on the mechanism of protein-bound deoxyhypusine synthesis. Chapter 2 describes the development of an assay for monitoring deoxyhypusine formation. Then, Chapter 3 addresses the requirements for optimum
synthesis of protein-bound deoxyhypusine. Chapter 4 includes unpublished data on the partial characterization of the deoxyhypusine synthesizing enzyme(s). Included are data that reveal information about the type of enzyme(s) which catalyze deoxyhypusine synthesis (i.e. oxidase, dehydrogenase etc.). In addition, this chapter contains information about the protein modified by the enzyme(s) and a comparison of two related cell lines with respect to the protein and the enzyme(s). Finally, Chapter 5 contains a discussion of the findings of this dissertation and proposed future investigations.
CHAPTER 2

HYPUSINE FORMATION IN PROTEIN BY A 2-STEP PROCESS IN CELL LYSATES

Abstract

The putative protein synthesis initiation factor eIF-4D is post-translationally modified by the polyamine spermidine, forming the rare amino acid hypusine from a lysine residue. The hypusine precursor, deoxyhypusine, was formed in crude cell lysates at pH 9.5 and converted to hypusine at pH 7.1. The modification occurred in eIF-4D, since the isoelectric points and molecular weights of the proteins modified in intact cells and lysates were indistinguishable. Only lysates from cells treated with α-difluoromethylornithine, to deplete endogenous polyamine pools, supported the formation of deoxyhypusine, suggesting that unmodified eIF-4D accumulated in spermidine deficient cells. Guazatine, an inhibitor of enzymes which form Δ1-pyrroline from spermidine, blocked deoxyhypusine formation in lysates by nearly 70% at 100μM and completely at 1 mM. Other mammalian amine oxidase inhibitors had little or no effect on this reaction. Thus, deoxyhypusine formation in eIF-4D was catalyzed by a guazatine-sensitive enzyme with a basic pH optimum.

Introduction

Hypusine [Nε-(4-amino-2-hydroxybutyl)-2,6-diaminohexanoic acid] discovered in 1971 (Shiba et al., 1971), has been identified only in
the putative eukaryotic initiation factor 4D (eIF-4D) (Cooper et al., 1983). The modification of this protein occurs by the transfer of the butylamine moiety of spermidine to the ε-amino group of lysine in protein to form deoxyhypusine, which is then hydroxylated (Park et al., 1981; Abbruzzese et al., 1986).

The enzyme(s) responsible for the synthesis of deoxyhypusine is unknown. Park and Folk (1986), using a dual-label technique, suggested that the butylamine moiety is converted to an aldehyde by an oxidative cleavage and that an enzyme-bound amino intermediate is formed. Previous attempts to develop a cell free system for this synthesis were unsuccessful (Park et al., 1982). A cell lysate system was developed for synthesizing hypusine in protein and was used to investigate the influence of endogenous spermidine depletion and pH requirements of deoxyhypusine formation.

**Experimental Procedures**

**Materials**

[1,8-3H]spermidine•3HCl\(^1\) was purchased from New England Nuclear. The specific radioactivity was 24.3 Ci/mmol.

\(^1\)Tabor, Tabor and DeMeis (1971) were the first to order the number of atoms in spermidine. Convention requires the secondary amino-nitrogen be numbered as N-4 rather than N-5 because the lowest number for the secondary amino-nitrogen must be used. Therefore, the numbering is

\[ H_2N-\text{CH}_2-\text{CH}_2-\text{CH}_3 \quad \text{NH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_2 \]

and [terminal methylene-3H]spermidine is [1,8-3H]spermidine.
α-Difluoromethylornithine and MDL-72.521 (N¹-(Buta-2-3-dienyl)-N²-methylbutane-1,4-diamine) were kindly provided by the Merrell Dow Research Institute (Cincinnati, OH). Authentic hypusine was a generous gift from Dr. Tetsuo Shiba (Osaka University, Japan). Guazatine (38% solution, Kenopel) was a gift from Drexel Chemical Company (Memphis, TN).

Methods

Rat hepatoma tissue culture (HTC) cells were maintained as suspension cultures as described elsewhere (Mamont, Ohlenbusch, Nussli and Grove, 1981), except that McCoy's suspension medium minus sodium bicarbonate was used in place of Swim's medium (Gibco Laboratories, Grant Island, NY). Horse Serum (Gibco labs) was used in experiments employing exogenous polyamines (Blaschko, 1962; Tabor and Tabor, 1964).

Synthesis of Protein-bound Hypusine or Deoxyhypusine in a Cell Lysate. HTC cells were cultured for up to 48 hours in the presence or absence of 5 mM α-difluoromethylornithine. The cells were harvested by centrifugation (300 x g for 5 minutes) and then washed 2 times in sterile phosphate-buffered saline (PBS: 140 mM NaCl, 2.0 mM KCl, 8.1 mM Na₂HPO₄ and 0.9 mM K₂HPO₄). The cells were resuspended in 0.1 M glycine-NaOH buffer at a concentration of 10⁷ cells/ml and frozen in liquid nitrogen. Cells were lysed by thawing at room temperature and viewed by phase-contrast microscopy to confirm the lysing process. Protein was assessed by the method of Bradford (1976). [1,8-³H]spermidine (10 µCi/ml, unless otherwise stated) with or
without unlabeled spermidine and dithiothreitol was added to the lysate. The lysate was dispensed into sterile tubes which were sealed with parafilm (to avoid evaporation) and incubated, shaking, in a 37°C water bath for the indicated times. The assay was terminated by addition of HClO₄ (0.2N final concentration) or by freezing the reaction mixture at -80°C.

Analysis of Polyamines and Hypusine or Deoxyhypusine in Protein. Deoxyhypusine, hypusine and polyamines were quantitated using a high performance liquid chromatography (HPLC) method described elsewhere (Gerner et al., 1986). Deoxyhypusine identification was based on the retention time of radiolabel from spermidine in protein from cells treated with α,α'-dipyridyl or hydralazine, known inhibitors of deoxyhypusine hydroxylation (Park et al., 1982; Paz et al., 1984). In order to obtain adequate separation of deoxyhypusine and hypusine, 0.2 minute fractions were collected between 21-29 minutes during the total 40 minute gradient run.

Single dimension electrophoresis was performed as described elsewhere (Gerner et al., 1986). Isoelectric focusing followed the method of O'Farrell (1975), using 0.05% ampholytes of the total solution except a substitution of pH range 4-6: pH range 3.5-9.5 ampholytes (BioRad, Richmond, CA) at 1:1 were used in the tube gel preparation. The samples were loaded onto the gel and electrophoresed for 24 hours at 400 volts. The gels were removed from the tubes and cut into 2 mm segments, which were dissolved in 500 μl water and then counted in 10 mls of liquid scintillation fluor.
Results

Effect of pH on the Synthesis of Peptide-bound Deoxyhypusine and Hypusine in Cell Lysates. Lysates from cells treated for 48 hours with \( \alpha \)-difluoromethylornithine were prepared as described, with initial solution pH values of 7.1, 8.0, 9.5 or 11.0. Acid hydrolysates of lysate protein, analyzed by HPLC, displayed a peak of radiolabel with a retention time equivalent to that of deoxyhypusine only when the lysate was incubated at pH 9.5 (Figure 2). Some radioactivity was apparent in all samples in a region corresponding to spermidine. No hypusine was observed in any of these lysates.
Figure 2. Effects of reaction pH on the synthesis of peptide-bound deoxyhypusine in cell-lysates. Lysates from cells treated for 48 hours with 5mM α-difluoromethylornithine were initially adjusted to different pH conditions: 7.1 (○), 8.0 (□), 9.5 (△) to 11.0 (□). All samples contained the same amount of protein and were incubated for 6 hours in the presence of [1,8-\(^3\)H]spermidine. Shown is the elution of radiolabel after separation of the hydrolyzed protein sample by HPLC. The early fractions (elution times less than 15 minutes) contained no radiolabel.
Folk and coworkers (Abbruzzese, et al., 1986; Park et al., 1982) had previously shown that the conversion of deoxyhypusine to hypusine occurred in lysates at pH 7.5. To determine whether deoxyhypusine and hypusine formation had different pH requirements, duplicate lysates were incubated at pH 9.5 for 6 hours to form deoxyhypusine. One sample was adjusted to pH 7.1 and then both were incubated for an additional 18 hours (Figure 3). All the deoxyhypusine in the sample shifted to pH 7.1 was converted to hypusine during the 18 hour incubation. The sample which remained at pH 9.5 produced more radiolabel in protein compared to that fraction shifted to pH 7.1. However, all the radioactivity in the former lysate had a retention time similar to deoxyhypusine indicating that deoxyhypusine, but not hypusine, was formed at pH 9.5 and hypusine, but not deoxyhypusine, was formed at pH 7.1.
Figure 3. Effect of pH shift on the synthesis of peptide-bound hypusine and deoxyhypusine. Duplicate lysates from α-difluoromethylornithine treated cells were initially adjusted to pH 9.5 and incubated in the presence of [1,8-3H]spermidine for 6 hours. At that time, one sample was shifted to pH 7.1 (○) while the other remained at pH 9.5 (□) for another 18 hours. This figure shows the elution of radiolabel in hydrolyzed protein after separation on HPLC. All other fractions did not have detectable radiolabel.
Comparison of the Protein Modification in Intact Cells and the Cell Lysate System. To confirm that the protein(s) modified in the lysate system was the same as those modified in the intact cell, protein bound hypusine labeled with [1,8-3H]spermidine in either intact cells or lysates were analyzed both by isoelectric focusing and SDS-polyacrylamide electrophoresis. The results, displayed in Figure 4 (Panel A and B), showed that the protein from both samples had a similar isoelectric point, pI \( \approx 5.5 \) and molecular weight, M, \( \approx 18,000 \), respectively, which are similar to authentic eIF-4D (Cooper et al., 1983). While the conditions used in Figure 3 result in the formation of deoxyhypusine and not hypusine in eIF-4D, others have shown that the hydroxylation of this moiety does not substantially change either the pI or M, of this protein (Park et al., 1982).
Figure 4. Analysis of proteins modified by radiolabeled spermidine in either intact cells or in the cell lysate system. HTC cells were grown in spinner culture in the presence of 5mM α-difluoromethylornithine for 48 hours. A portion of these cells (Θ) were incubated (10^7 cells/ml medium) in the presence of 10 μCi/ml [1,8-^3H]spermidine plus 1 μM unlabeled spermidine for 24 hours. A second aliquot, 2 x 10^7 cells (O), of the same spinner culture was lysed into 2 ml of buffer and incubated in the presence of [1,8-^3H] (100 μCi/ml) plus 41 μM (final) unlabeled spermidine for 24 hours. Panel A represents radiolabel in protein (100 μg total) in a 2 mm segment of each isoelectric focusing gel (O'Farrell, 1975). A control gel (A) was analyzed to assess the pH gradient. Panel B represents 100 μg of protein form intact cells or 200 μg of the lysate sample run on a NaDodSO₄/12.5% polyacrylamide gel. Molecular weight standards were from BRL, Bethesda MD. Percent of total counts applied are shown. Specific activity of the proteins were 266,870 cpm/mg protein for intact cell labeling and 40,370 cpm/mg when proteins were labeled in the lysates.
Effect of Spermidine Depletion on Formation of Deoxyhypusine in Cell Lysates. In order to determine if spermidine depletion was necessary for deoxyhypusine formation in the lysate system, HTC cells were treated with or without 5 mM α-difluoromethylornithine as described. Lysates from each culture were prepared and used in the cell free reaction for deoxyhypusine formation. Analysis of protein hydrolysates by HPLC showed a radioactive peak between fractions 27 and 30, corresponding to the elution time of deoxyhypusine, in the lysates of spermidine depleted, but not untreated, cells (Figure 5). Thus, only lysates from spermidine depleted cells were able to support the synthesis of deoxyhypusine in the cell free system.
Figure 5. Effect of α-difluoromethylornithine treatment on cells to be used as a source of protein in the cell lysate system. HTC cells were cultured in the presence (○) or absence (□) of 5 mM α-difluoromethylornithine for 48 hours. Lysates of both conditions were prepared into glycine-NaOH buffer at pH 9.5. [1,8-3H]spermidine (10 μCi/ml) was added to each sample and incubated for 24 hours. Sample proteins were analyzed by HPLC. The cpm per fraction are normalized to show values for equivalent protein contents (1 mg) of α-difluoromethylornithine treated and untreated cell lysates.
Effect of Inhibitors on Peptide-bound Deoxyhypusine Formation. Isotope studies in whole cells, reported by Park and Folk (1986), indicated that the cleavage of C-1 of the aminobutyl group of spermidine and subsequent transfer of this group to the ε-amino group of the peptide-bound lysine comprised the first step in hypusine formation in eIF-4D. Known inhibitors of amine oxidases were used in attempt to block this reaction in our lysate system. The results summarized in Table 1 indicated that inhibitors of the copper-dependent amine oxidases, such as diethyldithiocarbamate and iodoacetamide (Morgan, 1983) or the flavin-dependent amine oxidases such as MDL 72.521 (Bey, Bolkenius, Seiler and Casana, 1985) had little or no effect on deoxyhypusine formation. Diethyldithiocarbamate and iodoacetamide had 20% or less inhibitory activity, but this inhibition did not increase with inhibitor concentration. The diethyldithiocarbamate effect can be explained by its unusual biphasic responses reported by others (Smith, 1983). Guazatine, a plant amine oxidase inhibitor (Lin, Quamo, Ho and Baur, 1985) inhibited deoxyhypusine synthesis by nearly 70% at 100 μM and 100% at 1 mM final reaction mixture concentrations.
Table 1. Effect of Various Inhibitors of Amine Oxidases on Formation of Deoxyhypusine in HTC Cell Lysates

HTC cells were grown in the presence of α-difluoromethylornithine (5 mM) for 48 hours. Lysates were prepared as described and partitioned, such that each reaction tube contained approximately 3 mg protein (corresponding to about 10⁷ cells). [1,8-³H]spermidine (10 μCi/ml), and the designated amine oxidase inhibitor at the specified concentration were added to the various tubes and incubated for 24 hours. The samples were analyzed by HPLC as described. This table presents the percent inhibition of deoxyhypusine formation in protein relative to a control reaction containing no inhibitor.

<table>
<thead>
<tr>
<th>Inhibitor</th>
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<th>Concentration (μM)</th>
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<tr>
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<td>SH-group reagent</td>
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<td>100</td>
<td>6</td>
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<tr>
<td>MDL-72.521</td>
<td>Polyamine oxidase</td>
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</tr>
<tr>
<td></td>
<td>inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guazatine</td>
<td>Plant amine oxidase</td>
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<td>15</td>
</tr>
<tr>
<td></td>
<td>inhibitor</td>
<td>100</td>
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<td>100</td>
</tr>
</tbody>
</table>

This is representative of a single experiment which has been replicated.
Discussion

We report the formation of hypusine from a lysine residue in eIF-4D in a cell free system. The multi-step synthesis of hypusine proceeds by two reactions with distinct pH requirements. First, deoxyhypusine synthesis consists of the covalent linkage of the aminobutyl group of spermidine to protein bound lysine (Park, et al., 1981), and was optimal around pH ~ 9.5. No detectable deoxyhypusine was formed in lysates incubated at pH 7.1, 8.0 or 11.0. While these pH values vary from the pKₐs of the reaction buffer used, measurements indicated that the solution pH did not markedly vary from the initial pH (~ 0.3 units) during the incubation intervals (data not shown). Secondly, the formation of hypusine from deoxyhypusine was catalyzed by the enzyme deoxyhypusine hydroxylase (Abbruzzese, et al., 1986). This reaction occurred at pH 7.1, but not at pH 9.5.

In addition to specific pH requirements, our system for hypusine formation required that the source of lysates be from cells depleted of endogenous spermidine pools. Earlier studies from our group had suggested that unmodified eIF-4D accumulated in spermidine-depleted cells, since subsequent synthesis rates were 10 times higher, after spermidine restoration, than those in untreated cells (Gerner et al., 1986). Park, Cooper, and Folk (1986) have independently reported that a precursor protein accumulates in Chinese hamster cells treated with α-difluoromethylornithine, based on direct visualization of both the precursor and hypusine-containing protein on 2-dimensional gels. Therefore, lysates containing normal spermidine levels that did not
support deoxyhypusine formation were consistent with earlier suggestions that hypusine formation in protein occurs during or soon after translation (Park et al., 1981; Park, et al., 1982). Little or no unmodified eIF-4D would exist in these lysates.

The results of the inhibitor studies suggested that the enzyme(s) involved in the formation of deoxyhypusine is unique from the major class of amine oxidases found in mammalian cells (Seiler, Bolkenius, Bey, Mamont and Danzin, 1985), as inhibitors of the copper- and flavin- dependent amine oxidases had little or no effect on this reaction. Guazatine, which inhibited deoxyhypusine formation was chosen because it inhibits amine oxidases, enzymes which catabolize spermidine to 1,3-diaminopropane and \( \Delta^1 \)-pyrroline as in oat seedlings (Smith, 1983) and Serratia marcescens (Okada, Kawashima and Imahori, 1983). An amine oxidase may generate deoxyhypusine in eIF-4D since their action would be consistent with the known cleavage reaction which occurs in this process(es). Certain differences must exist between the mammalian and plant or bacterial enzymes, since the pH optima for spermidine varies between 6.3 and 8 for the plant amine oxidases (Smith, 1983) while the pH optimum for deoxyhypusine formation is near 9.5.
CHAPTER 3

DEOXYHYPUSINE SYNTHESIS IN RAT HEPATOMA CELLS

Abstract

The putative eukaryotic translation initiation factor 4D (eIF-4D) is modified by spermidine, by converting a specific lysine residue to the rare amino acid hypusine (Nε-(4-amino-2-hydroxybutyl)lysine) via a 2-step process. In rat hepatoma cell lysates, deoxyhypusine was synthesized (step 1) optimally at pH ~9.2. Specific factors, such as nicotinamide adenine dinucleotides (NAD⁺ and NADH) and to a lesser degree, nicotinamide, stimulated, while other mono- and dinucleotides (flavin mono- and adenine di-nucleotide) had little or no effect on the synthesis of deoxyhypusine. The stimulation displayed by all of the factors depended on the concentration of spermidine. When the spermidine concentration was 4 μM or less, the nicotinic acid ring-containing factors stimulated deoxyhypusine synthesis. This stimulatory activity was decreased at 15 μM, and lost at 40 μM, spermidine. The pH optimum for the reaction was unchanged by NAD⁺ when the spermidine concentration was saturating (40 μM).

Deoxyhypusine formation was suppressed when oxygen was either displaced by nitrogen or removed by sodium hydrosulfite, an oxygen scavenger. Therefore, the synthesis of deoxyhypusine in eIF-4D on a protein-bound lysine is catalyzed by a pH-sensitive spermidine oxidase, where the activity is increased by NAD⁺ (NADH or
nicotinamide), possibly by lowering the apparent $K_m$ of the enzyme for spermidine, rather than by increasing the $V_{\text{max}}$.

**Introduction**

Hypusine (Nε-(4-amino-2-hydroxybutyl)lysine), an unusual amino acid that modifies the eukaryotic translation initiation factor, 4D (eIF-4D, $M_r=18,000$, pI=5.3), is derived from the butylamine moiety of spermidine. This moiety is added to the ε-amino group of lysine to form deoxyhypusine (Nε-(4-aminobutyl)lysine) bound to this protein (Cooper et al., 1983; Park et al., 1981). Hydroxylation of the second carbon of the spermidine moiety produces hypusine (Park et al., 1981; Park et al., 1982). The complete synthesis of hypusine requires at least two steps. The hypusine precursor, deoxyhypusine, has been synthesized in protein in cell lysates. This cell free synthesis requires depletion of the spermidine pool and is pH sensitive, requiring a pH ~9.5 (Murphey and Gerner, 1987, Chapter 2).

Presumably, the depletion of spermidine allows the accumulation of a precursor peptide, which is then available for subsequent modification in cell lysates (Murphey and Gerner, 1987, Chapter 2; Park and Wolff, 1988). The hydroxylation of deoxyhypusine to hypusine requires a pH ~7.2 and is catalyzed by deoxyhypusine hydroxylase. This hydroxylase has been partially purified (Abbruzzese et al., 1986). The enzyme(s) catalyzing deoxyhypusine synthesis is unknown.

The effects of specific factors on these enzyme(s) and various reaction conditions were investigated. Previous data suggested that the enzyme(s) involved in deoxyhypusine synthesis was NAD$^+$ dependent.
in mouse neuroblastoma cells (Chen and Dou, 1988) and Chinese hamster ovary cells (Park and Wolff, 1988) and that NAD⁺ removed the basic pH requirement for this reaction (Chen and Dou, 1988). However, these studies were conducted under suboptimal reaction conditions; specifically, the spermidine concentration was less than 2.5 μM, while endogenous spermidine concentrations of proliferating mammalian cells are often in the millimolar range (Pegg and McCann, 1982; Tabor and Tabor, 1984). We and others have postulated that the enzyme(s) involved in the cleavage of spermidine to form deoxyhypusine is a unique spermidine oxidase or a dehydrogenase. This idea was based on both inhibitor studies (Murphey and Gerner, 1987, Chapter 2) and identification of an expected product, 1,3-diaminopropane, which was synthesized in approximately the same amount as deoxyhypusine in protein when [1,8-$^3$H]spermidine was added to a spermidine-depleted, partially purified system (Park and Wolff, 1988). To distinguish between these two possibilities, we investigated the oxygen requirement for deoxyhypusine synthesis.

**Experimental Procedures**

**Materials**

$[1,8-{^3}$H]spermidine-3·HCl] was purchased from Du Pont-New England Nuclear. The specific radioactivity was 32.6 Ci/mmol. α-Difluoromethylornithine (DFMO) was kindly provided by the Merrell Dow Research Institute (Cincinnati, OH). The Clark electrode was from Yellow Springs Instrument Co. Yellow Springs, OH. NAD⁺, NADH,
nicotinamide, FAD+ and FMN+ were supplied by Sigma Chemical Company, St. Louis, MO.

Methods

Rat hepatoma tissue culture (HTC) cells were maintained as suspension cultures as described elsewhere (Mamont et al., 1981) except that McCoy's suspension medium minus sodium bicarbonate was used in place of Swin's medium (GIBCO). Horse serum (GIBCO) was used in experiments employing exogenous polyamines (Blaschko, 1962; Tabor and Tabor, 1964).

Synthesis of Protein-bound Hypusine or Deoxyhypusine.

Synthesis of hypusine or deoxyhypusine was as described elsewhere (Murphey and Gerner, 1987, Chapter 2), except the cells were resuspended in 0.1 M CHES (2-[N-cyclohexylamino]ethanesulfonic acid) (Sigma Chemical Company, St. Louis, MO.) pH ~9.2 (unless otherwise stated) at a concentration of 10^7 cells/ml and frozen in liquid nitrogen. The disrupted cells were thawed and the lysates were clarified by centrifugation at 600xg for 10 minutes.

[1,8-^3H]Spermidine (20 μCi/ml, unless otherwise stated) with or without unlabeled spermidine was added to the lysate. The assay mixture was incubated at 37°C for times up to 24 hours. The reaction was linear for up to 1 hour. The assay was terminated by adding HClO_4 (0.2 N final concentration).

Analysis of Polyamines and Hypusine or Deoxyhypusine in Protein. The analysis of polyamines or hypusine and deoxyhypusine in protein was performed using high performance liquid chromatography
(HPLC) methods described elsewhere (Murphey and Gerner, 1987, Chapter 2). Deoxyhypusine identification was based on the retention time of radiolabel from spermidine in protein (after hydrolysis) from cells treated with \(\alpha,\alpha'\)-dipyridyl, a known inhibitor of deoxyhypusine hydroxylation (Park et al., 1982). Hypusine identification was based on a synthetic standard. In order to obtain an adequate separation of the labelled amine, 0.2 min fractions were collected between 20-30 minutes during the 40-minute gradient run. The fractions were counted on a liquid scintillation counter. A unit was described as 1 pmol of deoxyhypusine formed per hour.

**Oxygen Depletion in the HTC Cell Lysate.** HTC cell lysates were prepared as described above. Oxygen levels were measured using a Clark electrode and recorded on a strip chart recorder. To saturate with oxygen, the buffer was aerated with 100% oxygen. An oxygen scavenger, sodium hydrosulfite, (Yellow Springs Instruments Co., Yellow Springs, OH.) was added to the buffer to deplete oxygen. The level of oxygen in the reaction resulting from sodium hydrosulfite addition was arbitrarily set to 0% on our scale. Oxygen was displaced in several samples by bubbling nitrogen into the tubes. Oxygen levels were monitored during the course of the experiment in the presence of \([1,8-^3\text{H}]\)spermidine. After completing each experiment, the protein samples were prepared for HPLC analysis of \([^3\text{H}]\)deoxyhypusine.

**Results**

**Buffer effects on the pH requirement for deoxyhypusine synthesis.** Lysates from HTC cells treated for 48 hours with
α-difluormethylornithine were prepared in glycine or CHES buffer at the indicated pH conditions (Fig. 6). The pH ranges for investigation were chosen with respect to the pKₐ of each buffer (glycine, 9.6; CHES, 9.3). The samples were frozen and thawed at the particular pH in each buffer. The pH dependence of deoxyhypusine formation was indistinguishable in these two buffers. Thus, the synthesis of protein-bound deoxyhypusine was pH-, but not buffer-, dependent with an optimum pH around 9.2.
Figure 6. Effect of reaction pH and buffer on deoxyhypusine synthesis. HTC cells were grown in the presence of the irreversible inhibitor of ODC, α-difluoromethylornithine (5 mM) for 48 hour. Lysates were prepared in either glycine (0) or CHES (O) buffer at varying pH conditions (as described in Methods). [1,8-3H]Spermidine (20 μCi/ml) was added to each sample and incubated for 30 minutes. Shown is the elution of total radioactivity in deoxyhypusine in protein which was separated by an HPLC. This is representative of a single experiment which has been replicated.
Effect of NAD$^+$ and other factors on the synthesis of protein-bound deoxyhypusine in HTC cell lysates. Lysates from cells treated for 48 hours with α-difluoromethylornithine were prepared in CHES buffer, pH ~9.2. The samples were aliquoted for treatment with NAD$^+$, NADH, FAD$^+$, FMN$^+$ and nicotinamide (Table 2). Acid hydrolysates of the protein samples analyzed by HPLC revealed that NAD$^+$ (300 µM) was the most effective factor, stimulating deoxyhypusine synthesis by approximately seven fold under conditions of low spermidine concentrations (0.6 µM). The lesser stimulation of deoxyhypusine synthesis by NADH and nicotinamide suggested that the nicotinic acid-ring was most likely the active moiety of the molecule, but that NAD$^+$ was the preferred effector. These data also suggested that the maximum stimulation of the reaction was near the physiological concentration of NAD$^+$ (300-400 µM) (Tischler, Friedrich, Coll and Williamson, 1977). Higher concentrations of NAD$^+$ (1000 µM) stimulated deoxyhypusine synthesis to a similar extent as did the 300 µM concentration.
Table 2. Effect of various factors on the formation of protein-bound deoxyhypusine in HTC cell lysates.

HTC cells were grown in the presence of α-difluoromethylornithine (5mM) for 48 hours. The lysates were prepared and then [1,8-^3H]spermidine (20 µCi/ml) and the factor indicated was added for a 30 minute incubation (as described). The samples were analyzed by HPLC elution of precipitated protein containing [^3H]deoxyhypusine.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc. (µM)</th>
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<th>%</th>
</tr>
</thead>
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<tr>
<td></td>
<td>10^7 cells</td>
<td>control</td>
<td></td>
</tr>
<tr>
<td>NAD⁺</td>
<td>50</td>
<td>1545</td>
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</tr>
<tr>
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<tr>
<td>FMN⁺</td>
<td>1000</td>
<td>1466</td>
<td>80</td>
</tr>
</tbody>
</table>

This is representative of a single experiment which has been replicated.
A previous finding that NAD\textsuperscript{+} stimulated deoxyhypusine synthesis in mouse neuroblastoma cells (Chen and Dou, 1988) was conducted only at low spermidine concentrations (less than 1 \( \mu \text{M} \)). Since it had been previously shown that the rate of hypusine formation in intact cells was dependent on the endogenous spermidine concentration (Gerner et al., 1986), the effect of NAD\textsuperscript{+} was tested to see if it was physiologically significant, or simply a reflection of low spermidine concentration. Lysates were incubated with varying concentrations (0.6, 4, 15, and 40 \( \mu \text{M} \)) of spermidine, in the presence or absence of 300 \( \mu \text{M} \) NAD\textsuperscript{+}, and then analyzed for deoxyhypusine formation. While the stimulatory effect of NAD\textsuperscript{+} was apparent at 0.6 and 4 \( \mu \text{M} \) spermidine, the effect was decreased at 15 \( \mu \text{M} \) and lost at saturating concentrations of spermidine, 40 \( \mu \text{M} \) (Fig. 7). These data show that NAD\textsuperscript{+} does not affect the reaction \( V_{\text{max}} \) under these conditions, but instead might lower the \( K_m \) for spermidine.
Figure 7. Effect of spermidine concentration and NAD$^+$ on the formation of protein-bound deoxyhypusine. HTC cells grown in the presence of $\alpha$-difluoromethylornithine for 48 hours. Lysates were prepared with [1,8-$^3$H]spermidine (20 $\mu$Ci/ml) in the presence of increasing spermidine concentrations, as well as in the presence (0) or absence (0) of 300 $\mu$M NAD$^+$. The samples were incubated for 30 minutes. Shown is the total deoxyhypusine synthesized per mg protein of HPLC analyzed protein. This is representative of a single experiment which has been replicated.
Effect of NAD\(^+\) on the pH requirement for deoxyhypusine formation in protein in HTC cell lysates. NAD\(^+\) was reported to stimulate the synthesis of deoxyhypusine and eliminate the requirement for a basic pH (9.5) in mouse neuroblastoma cell lysates (Chen and Dou, 1988). Since NAD\(^+\) stimulates deoxyhypusine formation only when spermidine concentrations are not saturating (Fig. 7), we considered whether deoxyhypusine synthesis was pH-dependent when saturating (40 \(\mu\)M) concentrations of spermidine were included in the reaction mixture. We also determined whether the NAD\(^+\) effect was evident at pH values other than ~9.2 when a saturating spermidine concentration was used. Lysates from a-difluoromethylornithine treated cells were prepared as before in CHES buffer at the specified pH, and were supplemented with 40 \(\mu\)M spermidine, with or without 300 \(\mu\)M NAD\(^+\). Analysis of deoxyhypusine formation showed that the pH-dependence of this reaction was similar at ~0.6 \(\mu\)M (Fig. 6) and 40 \(\mu\)M (Fig. 8) spermidine. In addition, 300 \(\mu\)M NAD\(^+\) had no effect on this dependence in the HTC cell lysates when the spermidine concentration of the reaction mixture was 40 \(\mu\)M (Fig. 8).
Figure 8. The pH dependency of deoxyhypusine formed in lysate protein in 40 μM spermidine in the presence or absence of 300 μM NAD⁺. HTC cells were grown in the presence of α-difluoromethylornithine for 48 hours. The cells were harvested and cell lysates were prepared (as described) in CHES buffer ranging from pH 7.2-10.2. The lysates were then supplemented with 40 μM spermidine (labelled concentration=20 μCi/ml and remaining was unlabelled) in the presence (○) or absence (●) of 300 μM NAD⁺. These samples were incubated for 30 minutes. The graph represents the HPLC analysis of total deoxyhypusine synthesized per mg protein of each sample. This is representative of a single experiment which has been replicated.
Oxygen dependence of deoxyhypusine synthesis in protein. In order to determine whether the enzyme(s) responsible for deoxyhypusine synthesis might be an oxidase, oxygen was depleted from the reaction by aerating with nitrogen or by addition of sodium hydrosulfite, an oxygen scavenger, to the reaction solution. The inset in Figure 9 shows the level of oxygen in samples which were either left untreated (control), gassed with nitrogen or gassed with nitrogen and subsequently aerated with oxygen (reoxygenated) for the times shown. The 100% level was buffer aerated with oxygen and the 0% level was buffer treated with sodium hydrosulfite. The untreated control oxygen level was approximately 62% that of the maximal level and the nitrogen treated sample contained approximately 5% of this level. The oxygen levels were monitored throughout the experiment. The control sample which contained cell lysate and labelled spermidine was considered to be maximal (100%) deoxyhypusine synthesis. Nitrogen treatment of the lysate decreased deoxyhypusine synthesis to 12% of this value. The reoxygenation of the sample resulted in nearly complete (85% of control) restoration of deoxyhypusine synthesis. An additional sample in this experiment was treated with sodium hydrosulfite. This treatment resulted in a total inability of the lysate to support deoxyhypusine synthesis. After this treatment, the enzyme could not be reactivated by oxygen (data not shown). This was probably due to other effects of sodium hydrosulfite on the lysate, perhaps by reduction of thiol groups. These data demonstrated that the enzyme(s) responsible for deoxyhypusine synthesis required oxygen.
Figure 9. Effect of oxygen depletion on deoxyhypusine synthesis in HTC cell lysates. HTC cells were grown in the presence of α-difluoromethylornithine for 48 hours. Lysates were prepared and then conditions were as follows: 1. Control (CON); [1,8-3H]spermidine (20 μCi/ml) was added and the oxygen level of the sample was measured throughout the experiment. 2. Nitrogen treated (N₂); this sample was depleted of oxygen by bubbling with nitrogen then labelled spermidine (20 μCi/ml) was added. Nitrogen was continually bubbled into the sample throughout the experiment. 3. Reoxygenated sample (N₂/O₂); this sample was first treated with nitrogen and then oxygen was added in the presence of [1,8-3H]spermidine (20 μCi/ml). The protein was precipitated from each sample and analyzed for [3H]deoxyhypusine content by the HPLC. The insert represents the relative level of oxygen (as percent) of each sample throughout the incubation period. The bar graph represents percent of control deoxyhypusine in protein. This data is representative of a single experiment which has been replicated.
Discussion

The synthesis of the amino acid hypusine in lysates requires two pH-sensitive steps; deoxyhypusine synthesis at basic pH and hypusine synthesis at neutral pH (Murphey and Gerner, 1987, Chapter 2). In this chapter, the pH sensitivity of deoxyhypusine synthesis was verified by comparing two buffers CHES (pKₐ=9.3) and glycine (pKₐ=9.6). Deoxyhypusine synthesis had a pH optimum of ~9.2, independent of the buffer used, spermidine concentration and presence or absence of the allosteric effector NAD⁺. In intact, a-difluoromethylornithine-treated HTC cells, approximately 10 pmols of hypusine was synthesized during the first hour after readdition of spermidine (Gerner et al., 1986). Initial attempts at reconstituting this synthesis in cell lysates produced a rate of only about 10% of this level. This finding suggested that certain factors might be diluted by the assay conditions. NAD⁺ was subsequently shown to stimulate deoxyhypusine synthesis in several cell lines (Chen and Dou, 1988; Park and Wolff, 1988). However, both of these studies only investigated deoxyhypusine synthesis under conditions of spermidine concentrations much lower (less than 1 µM) than those which exist in proliferating cells and those which are known to affect hypusine formation in intact cells (Gerner et al., 1986). Therefore, the effects of several factors were tested on deoxyhypusine formation in the cell free system under conditions of varying spermidine concentrations.
NAD$^+$, and to a lesser degree NADH and nicotinamide, stimulated deoxyhypusine formation but only at low spermidine concentrations. In agreement with others (Chen and Dou, 1988), flavin-containing factors had little or no effect on deoxyhypusine synthesis in our system. The stimulatory effect of NAD$^+$ was diminished when the spermidine concentration was increased to 40 μM, which is near saturation for this reaction (see Fig. 7). Thus, these data suggest that NAD$^+$ might lower the apparent $K_m$ of the reaction for spermidine, without affecting the $V_{max}$. This occurs at physiologically relevant concentrations of NAD$^+$ (150-300 μM) and spermidine (0.6-40 μM). The rate of deoxyhypusine synthesis in these cell free reactions, when spermidine was included at 40 μM, was approximately 50% (5 pmols per hr per mg protein) of the rate reported in α-difluoromethylornithine treated whole cells (Gerner et al., 1986). These data presented here and elsewhere (Gerner et al., 1986) suggested that the rate of deoxyhypusine synthesis was sensitive to physiological amounts of spermidine, and that NAD$^+$ might diminish the $K_m$ for spermidine under conditions of spermidine depletion. Although Chen and Dou (1988) suggested that NAD$^+$ alleviated the pH requirement for deoxyhypusine synthesis, no effect of NAD$^+$ (300 μM) was found on this requirement in HTC lysates when the concentration of spermidine was replenished to 40 μM. The reason for this discrepancy is unclear at this time, although it may reflect a cell-line dependent effect.

The initial step of hypusine synthesis involves the cleavage of the butylamine moiety of spermidine, and Park suggested that this
cleavage occurred oxidatively (Park and Wolff, 1988). Previous experiments in this dissertation showed a polyamine oxidase inhibitor in plants, guazatine, substantially inhibited deoxyhypusine synthesis (Murphey and Gerner, 1987, Chapter 2). Here is shown directly that deoxyhypusine synthesis is diminished by oxygen depletion and can be restored with oxygen repletion. Therefore, a spermidine oxidase appears to be involved in deoxyhypusine synthesis. The reaction shown below summarizes our current knowledge of deoxyhypusine and hypusine synthesis in eukaryotic cells.

\[
\text{spermidine oxidase/}
\]
\[
\text{deoxyhypusine synthetase}
\]
\[
\text{pH } \sim 9.2
\]

\[(1) \text{lysine in eIF-4D } + \text{spermidine } \rightarrow \text{deoxyhypusine in eIF-4D}
\]

\[
\text{effectors:}
\]
\[
\text{spermidine, NAD}^+
\]

\[
\text{deoxyhypusine hydroxylase}
\]

\[(2) \text{deoxyhypusine in eIF-4D } \rightarrow \text{hypusine in eIF-4D}
\]

\[
\text{pH } \sim 7.2
\]

As shown here and elsewhere (Chapter 2), the synthesis of deoxyhypusine in eIF-4D is catalyzed by a guazatine-sensitive spermidine oxidase/deoxyhypusine synthetase, which is conditionally
influenced by NAD$^+$ (NADH and nicotinamide) at low spermidine concentrations and which has a pH optimum ~9.2. The conversion of deoxyhypusine to hypusine is catalyzed by a second enzyme, deoxyhypusine hydroxylase (Abbruzzese et al., 1986), which has been partially purified. These new findings should facilitate efforts to purify the spermidine oxidase involved in deoxyhypusine synthesis.
CHAPTER 4

PARTIAL CHARACTERIZATION OF DEOXYHYPUSINE AND HYPUSINE SYNTHESIS
IN RAT HEPATOMA CELLS

Abstract

Eukaryotic translation initiation factor 4D (eIF-4D) is a putative initiation factor and the only known protein containing hypusine. The synthesis of this rare amino acid is comprised of at least two steps. First, the butylamine moiety of spermidine is donated to the ε-amino group of a lysine in eIF-4D to form deoxyhypusine. Then, this moiety is hydroxylated on the second carbon. Although several studies have proposed a mechanism for the synthesis hypusine, very little attention has been focused on the physiological parameters of the protein. Cell fractionation studies were used to locate the modified protein in a mammalian cell. EIF-4D was not associated with lipids, DNA, RNA or structures soluble in less than 0.1 M NaCl. The protein was completely soluble in 0.25 M NaCl, which suggests that the protein was associated with a stable structure. Next, the stability of the modified protein was analyzed prior to a partial purification. Since the second step in hypusine synthesis could be blocked by α,α'−dipyridyl [3H]spermidine was used to trace eIF-4D that contained [3H]deoxyhypusine or [3H]hypusine in pulse chase experiments. Hydroxylation of deoxyhypusine to hypusine did not affect the stability of the protein, as the half-life of the
protein was greater than 24 hours regardless of the hydroxylation step. In addition, ts85 cells which are temperature sensitive for the ubiquitin degradative pathway were used to analyze the degradation of modified eIF-4D. The modification of eIF-4D and its stability were not affected by the ubiquitin degradation system. EIF-4D is not rapidly turned over. Therefore an attempt to study the enzyme(s) involved in deoxyhypusine synthesis was made. A partial purification was executed. Preliminary data indicated that there was more than one enzyme involved in this synthesis. The enzyme activities were compared in two cell lines, HTC and HMOa, using a partial purification technique. The enzyme(s) appear to be similar in these cell lines.

Introduction

The overall focus of this project has been to elucidate the mechanism of deoxyhypusine synthesis in eIF-4D. In this chapter, studies of the possible function of the modification in the protein were done. Since previous data (Gerner et al., 1986) suggested that eIF-4D was a long-lived (>24 hr) protein, experiments using known inhibitors of deoxyhypusine hydroxylase were used to investigate the possibility that the hydroxylation of deoxyhypusine might protect the protein from proteolytic digestion. If eIF-4D was targeted for degradation without the hydroxyl group, then the half-life of the protein would be less than 24 hr. Thus, deoxyhypusine and hypusine-containing eIF-4D turnover rates were compared. Ubiquitin is a highly conserved protein that targets certain proteins for degradation (Hershko, 1988). Temperature sensitive mutants for the ubiquitin
conjugation system were used to evaluate whether this process was involved in the degradation of eIF-4D. These studies on turnover suggested that eIF-4D was a long-lived protein. Therefore, a partial purification of the protein was carried out. Cell fractionation studies were used to localize eIF-4D in HTC cells as a means of disclosing information on the possible function of this protein. Finally, the rate of the enzyme(s) responsible for deoxyhypusine synthesis in eIF-4D were compared in two related cell lines, HTC and HMOa cells. The data in this chapter suggest that not only is eIF-4D highly conserved but so are its modifying enzymes.

Experimental Procedures

Materials and Methods

Cell fractionation. HTC cells were treated for 24 hours with DFMO (5 mM). Then the cells were labelled for 24 hr with [3H]putrescine (2 μCi/ml). After labelling, cells were treated as previously described (Staufenbiel and Deppert, 1984). The extracts were collected and the cell components remaining on the plates were processed. A 0.1-2.0 M NaCl gradient was used, instead of a one step NaCl wash, to more closely define the concentration of NaCl needed to dislodge the protein. Each plate was scraped and the remaining matrix was solubilized in denaturation buffer (62.5 mM tris-base, 8% sodium dodecylsulfate, 20% β-mercaptoethanol, 12% sucrose and .004% bromophenol blue). The protein were separated by 12.5% polyacrylamide electrophoresis (100 μg per lane). The radiolabelled protein was either detected by autoradiography or by slicing the gel and
dissolving the slices in liquid scintillant counted in a scintillation counter.

**Partial purification of the enzyme(s) involved in deoxyhypusine formation.** HTC cells grown in spinner culture and treated for 48 hr with α-difluoromethylornithine were harvested and counted. The cells (10^7/ml) were washed and lysed in 0.1 M CHES buffer (pH=9.2) by freezing and then thawing. The cell membranes, as well as other components, were precipitated at 600 x g for 20 min. Then, the protein was precipitated by the addition of ammonium sulfate to 45%. The sample was centrifuged at 20,000 x g for 30 min to clarify the supernatant. This supernatant was treated with ammonium sulfate to 70% saturation. The 0-45% and 45-70% ammonium sulfate fractions were rehydrated in CHES buffer (pH=9.2). An aliquot of the 0-45% fraction was assayed for activity and then the remaining protein was dialyzed for 18 hr with frequent buffer changes. The protein concentration was assayed by the Bradford technique (1976). The 0-45% fraction protein was subsequently equilibrated with CHES buffer and applied to a CNBr-activated Sepharose; 1,8 diaminooctane column. The column was eluted in two steps after washing with several void volumes with 0.1 M CHES buffer (pH=9.2). The column was eluted first with 250 mM NaCl and then with 1 M NaCl, both in CHES buffer. One ml fractions were collected and dialyzed. These fractions were assayed for activity.

The components of the Deoxyhypusine assay were; 250 μl sample, 250 μl of 45-70% ammonium sulfate fraction in CHES buffer, 10 μCi/ml [1,8-^3H]spermidine incubated at 37°C for 30 min. After the incubation
period, the protein in the sample was precipitated and hydrolyzed. The hydrolyzed protein was then analyzed by HPLC. The \[^3\text{H}\]deoxyhypusine-containing fractions were counted on a liquid scintillation counter.

**Results**

**Association of eIF-4D(hypusine) with subcellular structures.**

HTC cells were fractionated to locate \[^3\text{H}\]hypusine-containing eIF-4D. After each wash, the soluble components were collected and analyzed by SDS-PAGE gel analysis. Table 3 shows \[^3\text{H}\]spermidine incorporation into eIF-4D and distribution of the protein into certain fractions. The results show that the CPM in the Mr \(\sim 18,000\) protein decreased by only 30% with RNase treatment and little more with DNase treatment. Since only 30% of the protein is disrupted by RNase this implies that the majority of the protein is associated with the remaining matrix. The sodium chloride treatment dislodged the remaining protein in the matrix. There was an abrupt decrease in the CPM in 0.25 M NaCl and there was little or no protein left bound to the matrix. Closer analysis of the concentration of NaCl necessary to completely disrupt the association of the protein with a cellular structure revealed that greater than 0.1 M and less than 0.25 M NaCl was required. Thus, eIF-4D appeared to be bound to at least one as yet unknown structure which is sensitive to 0.25 M NaCl and is partially disrupted by RNase.
Table 3. Cell localization of radiolabelled eIF-4D.

HTC cells were treated with DFMO for 24 hrs with 
α-difluoromethylornithine (5 mM) and then labelled in vivo with
[^3H]putrescine (2 μCi/ml). After the labelling period, cells were
-treated as indicated in Methods and processed for protein analysis.
The samples (100 μg each) were loaded onto a 12.5% polyacrylamide gel.
The gels were sliced and radiolabelled protein corresponding to a Mr ~
18,000 were counted using a scintillation counter. This table shows
the total remaining CPM of radiolabel in eIF-4D per sample after the
indicated treatment.

<table>
<thead>
<tr>
<th>Treatment (in sequential order)</th>
<th>CPM in Mr ~18,000 protein (Percent control)</th>
<th>percent of protein remaining on the plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cell</td>
<td>5,800 (100%)</td>
<td>100%</td>
</tr>
<tr>
<td>NP40</td>
<td>5,340 (92%)</td>
<td>100%</td>
</tr>
<tr>
<td>RNase</td>
<td>4,023 (69%)</td>
<td>71%</td>
</tr>
<tr>
<td>DNase</td>
<td>3,935 (68%)</td>
<td>31%</td>
</tr>
<tr>
<td>0.10 N NaCl</td>
<td>3,867 (67%)</td>
<td>15%</td>
</tr>
<tr>
<td>0.25 N NaCl</td>
<td>0 (0%)</td>
<td>14%</td>
</tr>
</tbody>
</table>

This data is representative of a single experiment which has been
replicated.
Effects of blocking the hydroxylation of deoxyhypusine on the half-life of eIF-4D. Previous work had shown that eIF-4D containing hypusine had a half-life on the order of the population doubling time of HTC cells (doubling time ~24 hours) (Gerner et al., 1986). In order to determine if the hydroxylation step affected the stability of the modification in protein, HTC cells were subcultured in the presence of α-difluoromethylornithine for 42 hours to deplete endogenous spermidine pools. Replicate cultures were then incubated in the presence or absence of α,α'-dipyridyl for 2 hours to inhibit the hydroxylation of deoxyhypusine (Park et al., 1982). These cells were then pulse-labeled. The data in Table 4 show the radioactivity incorporated in eIF-4D at 0, 6, and 24 hours after the pulse. The results show that the percent of radioactivity in eIF-4D after the pulse of [³H]spermidine is very similar in deoxyhypusine or hypusine-containing eIF-4D. Approximately, 45% of the radiolabeled protein remains after 24 hours. Therefore, the half-life in either species is around 24 hours. These data suggest that the hydroxylation of deoxyhypusine in eIF-4D does not affect the stability of the protein.
Table 4. Effect of hypusine hydroxylation on stability of hypusine or deoxyhypusine in protein.

HTC cells were subcultured in the presence of 5 mM a-difluoromethylornithine for 42 hours to deplete endogenous spermidine pools. Replicate cultures were then incubated in the presence or absence of a,a'-dipyridyl for 2 hours to inhibit the hydroxylation of deoxyhypusine. These cells were then pulse-labeled for 2 hours with 20 µCi/ml [1,8-3H]spermidine. Culture were then washed to remove the label and medium containing 5 µM unlabeled spermidine and a,a'-dipyridyl was added. At various times, culture were harvested and radioactivity in both deoxyhypusine and hypusine was determined.

<table>
<thead>
<tr>
<th>Chase Interval (hrs)</th>
<th>Inhibitor present prior to or during label</th>
<th>Species Labeled</th>
<th>Radioactivity in Species in protein (% of zero value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+</td>
<td>deoxyhypusine</td>
<td>2496 (100)</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>deoxyhypusine</td>
<td>2426 (97)</td>
</tr>
<tr>
<td>24</td>
<td>+</td>
<td>deoxyhypusine</td>
<td>1071 (43)</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>hypusine</td>
<td>1820 (100)</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>hypusine</td>
<td>1856 (102)</td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>hypusine</td>
<td>821 (45)</td>
</tr>
</tbody>
</table>

This data is representative of a single experiment which has been replicated.
Effect of the ubiquitin conjugation system on eIF-4D degradation.

Polyamines can act as substrates for the enzymes involved in the ubiquitin conjugation system. To test whether this enzyme pathway was involved in hypusine-containing eIF-4D degradation, the cell line (ts85) temperature sensitive for this conjugation system was used (Finley, Ciechanover and Varshavsky, 1984; Ciechanover, Finley and Varshavsky, 1984). The cells were prepared (as described) and one group was incubated at the permissive temperature (32°C), while the other was incubated at the nonpermissive temperature (39°C) (Table 5). The results show the HPLC analyzed total amount of [3H]spermidine taken up by the cells and the [3H]hypusine found in the hydrolyzed protein. These results were used to calculate the pmols of protein-bound hypusine synthesized. The pmols of hypusine at 32°C and 39°C were approximately the same (0.34 and 0.33, respectively). Hypusine-containing eIF-4D was stable at either temperature. Therefore, these data, as well as the pulse-chase data (Table 4), demonstrated that eIF-4D was not a rapidly turning over protein and the stability of the modified protein was unaffected by the hydroxylation step converting deoxyhypusine to hypusine.
Table 5. Comparison of protein-bound hypusine levels in ts85 cells at permissive and non-permissive temperatures for ubiquitin conjugation.

Ts85 cells (temperature sensitive for the ubiquitin conjugation system) were treated with DFMO for 48 hours at 32°C. After that time one culture was transferred to the nonpermissive temperature, 39°C for 1.5 hours to inactivate the conjugation system. Then fresh media containing 20 μCi/ml (24.3 Ci/mmol) [1,8-3H]spermidine was added to both groups for two hours. At that time cells were harvested and acid-soluble and insoluble fractions were analyzed by HPLC.

<table>
<thead>
<tr>
<th>TEMPERATURE: CONDITION FOR UBIQUITIN CONJUGATION</th>
<th>HYPUSINE CPM/ALIQUOT</th>
<th>SPERMIDINE SRA* CPM/PMOL</th>
<th>HYPUSINE PMOL/ALIQUOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 (permissive)</td>
<td>1512</td>
<td>8860</td>
<td>0.34</td>
</tr>
<tr>
<td>39 (nonpermissive)</td>
<td>2496</td>
<td>15358</td>
<td>0.33</td>
</tr>
</tbody>
</table>

*specific radioactivity

This data is representative of a single experiment which has been replicated.
Partial purification of the deoxyhypusine synthesizing enzymes.
The enzyme(s) involved in deoxyhypusine synthesis was partially purified from HTC cells treated with α-difluoromethylornithine for 48 hours. The purification was based on the ability to separate the enzyme(s) from the substrate using ammonium sulfate precipitation (see Methods). The majority of the enzyme activity precipitated between 0 and 45% ammonium sulfate saturation (Park and Wolff, 1988). The substrate precipitates between 45 and 70% ammonium sulfate (Thomas, Goumans, Amesz, Benne and Voorma, 1979; Park and Wolff, 1988). 1,8-diaminooctane sepharose was used to prepare an affinity column to separate the enzyme(s) from other proteins because previous data indicated 1,8-diaminooctane was a weak inhibitor of the enzyme activity (Park and Wolff, 1988). Figure 10 shows the results of the two step NaCl wash of the column. The results show the elution of the protein and the CPM of [3H]deoxyhypusine in eIF-4D as a measure of enzyme activity. At least two peaks of activity were seen in the elution. The data suggested that there was more than one enzyme involved in deoxyhypusine synthesis. When the enzyme activity (CPM in deoxyhypusine) was compared to the total protein in these fractions, the majority eluted at the 1 M NaCl step. Approximately 35% of the activity to protein concentration was eluted with the 250 mM NaCl buffer, while another 65% was eluted in the 1 M NaCl buffer. These data suggest that the synthesis of deoxyhypusine involves at least two enzymatic steps.
Figure 10. Partial purification of the deoxyhypusine synthesizing enzymes. HTC cells treated with α-difluoromethylornithine for 48 hours were used to partially purify the enzyme(s) involved in deoxyhypusine synthesis. The enzyme(s) (0-45%) were separated from the substrate (45-70%) using ammonium sulfate precipitation. Then, the enzyme fraction was bound to a 1,8-diaminooctane affinity column and eluted with a two step elution (250 mM and 1 M NaCl). The fractions were assayed for enzyme activity by dialyzing the fraction and then combining an aliquot with a substrate fraction in the presence of 10 μCi/ml [1,8-3H]spermidine. These mixtures were incubated for 30 minutes at 37°C. The hydrolyzed proteins were analyzed by HPLC. This data represents the protein absorbance measured by Bradford technique (−○−) and the counts per minute (CPM) (−−) found after assaying the fraction for deoxyhypusine formation.
Comparison of protein-bound deoxyhypusine synthesis in HTC versus HMO\textsubscript{a} cells. The enzyme(s) involved in the synthesis of the intermediate, deoxyhypusine, was compared in two cell lines to see if they had similar activities. HMO\textsubscript{a} are cells which are selected for growth in \textit{\textalpha}-methylornithine and as a result have a defective spermidine-dependent pathway (Mamont, Duchesne, Grove and Tardif, 1978; Glass and Gerner, 1987). The alteration in these cells compared to the parental cell line, HTC, is that the half-life of ornithine decarboxylase (the enzyme which catalyzes the synthesis of ornithine to putrescine) is increased from \~30 min to several hours (Pritchard, Pegg and Jefferson, 1982). Thus, these cells were compared for difference(s) that might exist in another spermidine-dependent pathway, deoxyhypusine synthesis. The enzyme(s) were separated from the substrate using ammonium sulfate precipitation as described in the preceding section. Then each substrate was combined with its enzyme(s) as a control. Additionally, each substrate was mixed with the enzyme of the other cell line. These later samples were compared to the control samples under increasing spermidine concentrations (15-40 \textmu M). The data presented in Figure 11 show the HPLC separated \textsuperscript{3}H]deoxyhypusine in hydrolyzed protein and the actual endogenous spermidine measured in the lysate. The results show that under conditions of low spermidine (0.6 \textmu M), the deoxyhypusine synthesized was low in all combinations. As the spermidine concentration was increased, the deoxyhypusine synthesized was increased. The rate of this increase was similar in all the HTC and HMO\textsubscript{a} combinations. The
rate appeared to be slowing down at the highest concentration used whereby approximately 0.9 units per mg. protein were synthesized in all combinations. These data suggest there was no difference in the enzyme(s) of deoxyhypusine synthesis in these two cell lines, even at the lowest spermidine concentration tested.
Figure 11. Comparison of protein-bound deoxyhypusine synthesis in HTC versus HMO cells. HTC and HMO cells were incubated with α-difluoromethylornithine for 48 hours. The enzyme(s) for deoxyhypusine formation (0-45%) were separated from the substrate (eIF-4D; 45-70%) using ammonium sulfate precipitation. Then all possible combinations were mixed in the presence of 10 μCi/ml of [1,8-3H]spermidine and increasing concentrations of spermidine for a 30 minute incubation period. Then the hydrolyzed protein was separated by reverse phase HPLC. The data shown represents the deoxyhypusine synthesized relative to the HPLC analyzed soluble spermidine in the assay. The combinations were as follows: HTC enzyme/HTC substrate ( ), HTC enzyme/HMO a-substrate ( ), HMO a-enzyme/HTC substrate ( ) and HMO a-enzyme/HMO a-substrate ( ). A unit is equal to 1 pmol of deoxyhypusine synthesized per hour. This data is representative of a single experiment which has been replicated.
Discussion

The data in this report show that modified eIF-4D is quite stable. The half-life of eIF-4D containing deoxyhypusine was greater than 24 hours which was similar to the turnover rate seen in the hypusine-containing protein. These data were similar to the turnover rate reported for the hypusine-containing eIF-4D of HTC whole cells (Gerner et al., 1986). This rate of turnover was not influenced by the ubiquitin degradative pathway. Thus, it is concluded from these reports that eIF-4D is a long-lived protein, if at least the intermediate deoxyhypusine was present. It is unknown at this time whether the butylamine addition stabilizes the unmodified protein.

In addition to the rate of turnover of eIF-4D containing hypusine, cellular compartmentalization and component association were investigated. Previous reports indicated that although eIF-4D is classified as an initiation factor, only ~10% was associated with ribosomes. The other 90% of the protein was found in the supernatant of a 100,000xg centrifugation used to pellet ribosomes (Thomas et al., 1979). Preliminary data (personal communication) indicated that eIF-4D was associated with the nuclear fraction, not the membrane fraction, of dounced homogenized cells. In support of these data, a cell fractionation technique revealed that eIF-4D was not a membrane component or associated with lipids. The data in Table 3 suggested that the protein was associated with a structural component at less than 0.25 M NaCl but was not associated with DNA but may associate with RNA. This latter association was uncoupled at 0.25 M NaCl.
These experiments were conducted in cell culture dishes and after each
treatment the remaining matrix was removed and electrophoresed for
protein separation. It is possible that the matrix remaining could
collapse and trap the proteins. Therefore, further characterization
is needed to clarify with what, if any, structure eIF-4D was
associated. A sucrose gradient of unlabelled HTC cells and use of an
antibody (when it becomes available) to probe the fractions might help
answer the questions surrounding the fractionation presented here.

Several studies were conducted on the possible enzyme(s)
involved in deoxyhypusine synthesis in eIF-4D (Murphey and Gerner,
1987, Chapter 2; Chapter 3). The findings from these reports provided
the information needed to partially purify the enzyme(s). Reported in
this chapter is a simple method using ammonium sulfate precipitation
to separate the unmodified eIF-4D from the deoxyhypusine synthesizing
enzyme(s) (Park and Wolff, 1988). Once these were separated, the
enzyme fraction was bound to an affinity column using a weak inhibitor
(1,8-diaminooctane) of the deoxyhypusine synthesis assay described in
Chapter 2 and 3. The data presented in Figure 10 suggested that at
least two enzymes catalyzed the butylamine cleavage and transfer to
the ε-amino group of protein-bound lysine to synthesize deoxyhypusine.
Previous data suggested that at least one of these enzyme(s) was an
oxidase, probably responsible for the cleavage of spermidine.

HMO_{a} cells were reported to have an increased ornithine
decarboxylase half-life (Pritchard et al., 1982). Since ornithine
decarboxylase degradation was affected by polyamines (Glass and
Gerner, 1986), another polyamine dependent pathway might also vary from the HMOa parental cell line, HTC. Thus, deoxyhypusine synthesis was investigated. Preliminary reports on the labelled protein compared in whole cell HTC or HMOa cells suggested that the modified protein content was approximately 5 to 10 times less in cells provided with low levels of labelled spermidine. Therefore, the ammonium sulfate precipitation technique was used in the partial purification to separate the enzyme(s) (0-45%) from the substrate (45-70%) in HTC versus HMOa cells to study this phenomenon in the cell lysate system. The data presented in Figure 11 suggest that there was no difference in the synthesis of deoxyhypusine in HTC or HMOa cells at any concentration of spermidine used. Neither the substrate nor the enzyme(s) varied in deoxyhypusine synthesizing capabilities under these conditions. Thus, in order to evaluate whether a difference exists in any aspect of eIF-4D synthesis in these two cell lines, a complete purification of the components and development of a reconstitution assay is required.
CHAPTER 5

DISCUSSION

Development of a cell lysate system for the formation of protein-bound deoxyhypusine was the major task undertaken in this investigation. This provided the tool necessary for a closer analysis of the mechanism of the butylamine cleavage and transfer from the donor spermidine molecule to the ε-amino group of protein-bound lysine. Perhaps, the most interesting information gained from the assay development was the basic pH requirement; not a neutral pH requirement. Notably, this pH ~9.2 was required for optimum deoxyhypusine synthesis in the cell lysate system. Since the pH of a cell is neutral, this unusual pH requirement might be explained by the existence a subcompartment due to the folding of the protein around the site of the modification or other cellular components which create this environment. It is also possible that some as yet unknown factor changes the pH in proximity of the active site of the enzyme(s). Most importantly, the pH requirement was found in vitro not under intact cell conditions. The in vitro requirement for an optimum pH of ~9.2 might not reflect the in vivo situation. In addition to a basic pH requirement, a precursor substrate (unmodified eIF-4D, see Fig. 5) must be available for sufficient synthesis to occur, thus, spermidine pools were depleted in extracts prior to synthesis assays. The depletion of spermidine was accomplished using the ODC irreversible
inhibitor, α-difluoromethylornithine (DFMO). The protein modified by spermidine in this assay system was confirmed as eIF-4D by analysis of Mr and pI; ~18,000 and ~5.1-5.5 respectively (Fig. 4). Thus, the cell lysate system contained the necessary components for the synthesis of deoxyhypusine at pH ~9.2. A shift in the pH to ~7.2 allowed conversion of pre-existing deoxyhypusine to hypusine (Fig. 3). Deoxyhypusine hydroxylase was also present in the lysate.

Further analysis of the assay system revealed that certain components might be limiting. Investigations by others (Chen and Dou, 1988; Park and Wolff, 1988) revealed that certain factors in particular NAD+, might be required for optimal synthesis of deoxyhypusine. It was also suggested that NAD+ replaced the pH requirement in a mouse neuroblastoma cell line system (Chen and Dou, 1988). In order to address this possibility, several allosteric effectors were tested on the HTC cell lysate system (Chapter 3). NAD+ was the most effective factor under limiting spermidine concentrations (0.6-4 μM). At a spermidine concentration of 40 μM, which is near saturation, the stimulation by NAD+ of deoxyhypusine formation was abolished. Protein-bound deoxyhypusine formation is increased by NAD+ (300 μM) if the spermidine pool is limiting. One possible explanation for the conditional NAD+ dependence is the km of the enzyme(s) for spermidine but it may also be a pH effect as suggested by Chen and Dou (1988). If NAD+ affects the pH, this is also conditional (Fig. 8), since at concentrations of spermidine near saturation (40 μM) this NAD+ effect is abolished.
Several inhibitors were used in an attempt to block deoxyhypusine synthesis in whole cells (Gerner et al., 1986) and in the cell lysate system (Table 1). The most effective inhibitor in blocking deoxyhypusine was the plant amine oxidase inhibitor, guazatine. Guazatine blocked polyamine oxidase of oat seedlings (Smith, 1983) and inhibited spermidine conversion to Δ¹-pyrroline, 1,3-diaminopropane and H₂O₂. Spermidine dehydrogenase of Serratia marcescens (Bachrach, 1962; Campello, Tabor and Tabor, 1965) also catalyzed the spermidine conversion to Δ¹-pyrroline and other products. The Δ¹-pyrroline product could be an intermediate in deoxyhypusine synthesis. Recently, Park and Wolff (1988) presented results using 1,3-diaminopropane as an inhibitor of deoxyhypusine synthesis in a cell-free system. Diaminopropane markedly inhibited the synthesis of deoxyhypusine. Since guazatine effectively inhibits deoxyhypusine synthesis and diaminopropane also inhibits this synthesis, these data suggest that Δ¹-pyrroline might be a transient intermediate between the butylamine moiety of spermidine and deoxyhypusine. In order to distinguish between an oxidase and dehydrogenase, the requirement for oxygen was investigated (Fig. 9). Once the oxygen was depleted from the cell-free reaction mixture, deoxyhypusine synthesis was diminished. The repletion of the oxygen in the reaction mixture restored the enzyme activity such that deoxyhypusine synthesis was 85% of the control level. This finding further supports the hypothesis that deoxyhypusine is synthesized at least in part by a spermidine oxidase which may be similar to a plant amine oxidase.
Using the information gained from the cell free assay system, the enzyme(s) involved in deoxyhypusine synthesis were partially purified. The enzyme(s) could be separated from the substrate using ammonium sulfate. Park and Wolff (1988) showed the enzyme precipitated between 0-45% and eIF-4D precipitated between 45-70% ammonium sulfate (Thomas et. al., 1979; Park and Wolff, 1988). This separation technique was used and then the enzyme fraction was applied to a 1,8-diaminoctane-bound affinity column. Preliminary results suggested there were at least two enzyme(s) involved in deoxyhypusine synthesis (Fig. 11). The majority (65%) of the activity (unit per mg protein) was in the 1 M NaCl fraction. Thus, these data suggested more than one enzyme was involved in deoxyhypusine synthesis and that these enzyme(s) had different affinities for the substrate 1,8-diaminoctane. If there are two enzyme(s) involved in deoxyhypusine synthesis, at least one is a spermidine oxidase while the other is sensitive to the factor NAD\textsuperscript{+} (NADH and nicotinamide).

HTC cells were used in all of the experiments executed so far, therefore, we decided to compare what was known about deoxyhypusine synthesis in HTC cells to an HTC variant, HMO\textsubscript{a} (Chapter 4). Since the enzyme(s) and substrate (eIF-4D) were easily separated by ammonium sulfate precipitation, HTC and HMO\textsubscript{a} cells were treated with ammonium sulfate to separate these proteins. The enzyme(s) and substrates were analyzed by all possible combinations. Since the cell lines might differ in the enzyme \( k_m \) for spermidine, increasing concentrations of spermidine (15-40 \( \mu M \)) were used in the deoxyhypusine synthesizing
assay. The data in figure 11 show that the proteins, both the enzyme(s) and eIF-4D were similar in the two cell lines compared. The total deoxyhypusine synthesized was the same for both cell lines regardless of the eIF-4D-enzyme combination or the concentration of the reactants. However, further investigation is needed at low concentrations (<4 μM) of spermidine, which may exist in certain compartments of the intact cell, to determine whether a difference exists in these cell lines.

A summary of the information gained from this investigation is presented in Figure 12. Sufficient information about the deoxyhypusine synthesizing enzyme(s) has been acquired to permit complete purification. The enzyme or enzyme complex was demonstrated as a spermidine oxidase-deoxyhypusine synthetase which requires a basic pH (9.2) and is conditionally sensitive to NAD⁺ (NADH and nicotinamide). Once the substrate (eIF-4D) is modified by the butylamine portion of spermidine to synthesize deoxyhypusine, the protein is stable. The synthesis of hypusine-containing eIF-4D has been further characterized since deoxyhypusine hydroxylase has been isolated and partially purified (Abbruzzese et al., 1986). Therefore, to completely characterize the mechanism of hypusine synthesis, the emphasis should be to purify and characterize the enzyme(s) of deoxyhypusine synthesis and attempt to isolate the potential intermediate (A¹-pyrroline). In addition to the mechanism of hypusine synthesis, the function of this modification could be studied by generating the unmodified cDNA clone versus a modified cDNA clone and
transfecting each into HTC cells under controlled levels of polyamines. By limiting the precursor (spermidine) to the modification, information about the unmodified protein versus the modified protein could reveal a function of the modification. Since the function of eIF-4D is as yet unknown, these clones could also be used to study the function of the protein under varied conditions. For example: the level of polyamines could be limited and replenished in several different cell lines and the effect of eIF-4D in its proposed role as an initiation factor could be studied by using a reconstitution assay for protein translation.
Spermidine oxidase
Deoxyhypusine synthetase

Deoxyhypusine Hydroxylase

Figure 12. A Summary of the Synthesis of Hypusine in eIF-4D.
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