Purification and characterization of human deoxyhypusine synthase from HeLa cells

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Received 16 March 1995

Abstract Post-translational modification of a specific lysine residue in eukaryotic initiation factor 5A is essential for cell viability. The amino acid hypusine, which is the product of this modification, is derived in two subsequent enzyme-catalyzed reactions. We have purified and characterized the enzyme responsible for the first step in hypusine modification, deoxyhypusine synthase, from HeLa cells. The human enzyme is multimeric with a native apparent molecular weight of 150,000 consisting of subunits of 41,000. The amino acid sequences of its peptide fragments share high sequence identity with a hypothetical protein (YHRO68w) on chromosome VIII of *Saccharomyces cerevisiae*. This protein appears to be the deoxyhypusine synthase of yeast.

Key words: Hypusine; Eukaryotic initiation factor 5A; Post-translational modification

1. Introduction

Eukaryotic initiation factor 5A is the only protein which contains the amino acid hypusine [1]. Hypusine formation is a spermidine-dependent post-translational event that appears to be ubiquitous in eukaryotic cells. The modification occurs in two enzyme-catalyzed reactions that involve (i) the transfer of the aminobutyl moiety of spermidine to the ε-amino group of lysine (at position 50 in human eIF-5A) and (ii) subsequent hydroxylation of this intermediate.

Eukaryotic initiation factor 5A is an essential protein which is abundantly expressed in eukaryotes and archaebacteria. Although its name was originally derived from its ability to stimulate the in vitro formation of the first peptide bond in protein synthesis [1,2] its precise function in vivo is not yet understood. Nevertheless, hypusine formation is central to eIF-5A function [3, 4] and efficient cell proliferation requires large amounts of the correctly modified protein [5–7].

Deoxyhypusine synthase catalyzes the first step in hypusine formation. The enzyme from *Neurospora crassa* has recently been purified [8]. Here we describe the purification and characterization of the human deoxyhypusine synthase. The enzyme appears to have similar properties as its *N. crassa* homologue, being most probably a homotetramer with an apparent subunit molecular weight of 41,000. Furthermore, the amino acid sequence information from the human enzyme has enabled us to identify the hypothetical protein YHRO68w, localized on chromosome VIII of *Saccharomyces cerevisiae*, as the deoxyhypusine synthase of yeast.

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Abbreviations: Deoxyhypusine synthase, spermidine dehydrogenase (NAD^+, 1-deoxyhypusyl-forming and aminobutyl-transferring); EDTA, (ethylenedinitrilo) tetraacetic acid; eIF-5A, eukaryotic initiation factor 5A; HPLC, high performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate.

2. Materials and methods

2.1 Materials

HeLa S3 cells were purchased from the Computer Cell Culture Centre, University of Mons, Belgium. [^{14}C]Spermidine trihydrochloride (*N*-(3-aminopropyl)-[1,4-^{14}C]tetramethylene-1,4-diamine trihydrochloride) with a specific activity of 114 mCi/mmol was from Amersham International plc, Little Chalfont, England. Chromatography materials and columns were obtained from Pharmacia, Freiburg, Germany, and NAD^+ from Boehringer Mannheim Corporation, Germany. All other reagents were of analytical grade.

2.2. Enzyme assay

The deoxyhypusine synthase activity assay is based on the incorporation of radioactivity from [^{14}C]spermidine into eIF-5A precursor protein [9]. The standard reaction mixture contained 6 μM [^{14}C]spermidine, 1 mM NAD^+, 2 μM recombinant eIF-5A precursor protein (purified according to João et al. [10]), and various amounts of deoxyhypusine synthase in 0.3 M glycine-NaOH buffer, pH 9.0, 1 mM DTT. 300 μl of the assay mixtures were incubated for 2 hours at 37°C. In order to separate the labeled eIF-5A protein from excess spermidine, the reaction mixtures were passed over Sephadex G-25 M gel filtration columns (PD-10 columns, Pharmacia) prior to measuring the incorporated radioactivity with a liquid scintillation counter.

2.3. Protein purification

35 g of HeLa cells were resuspended in 280 ml of 10 mM Tris-HCl, pH 7.0, 10 mM NaCl, 5 mM DTT and 1 mM PMSF, and disrupted in a Dounce homogenizer with 20 strokes (pistil B). After centrifugation at 40,000 × *g* for 10 min at 4°C the proteins contained in the supernatant were precipitated with ammonium sulfate in a stepwise manner: addition of ammonium sulfate to a concentration of 40% (w/v) was followed by a second precipitation step with ammonium sulfate at a final concentration of 80% (w/v). Following incubation for 30 min at 4°C and centrifugation at 27,000 × *g* for 10 min, the pellet of the second precipitation step was dissolved in 500 ml of 25 mM potassium phosphate buffer at pH 7.8, containing 1 mM DTT. The protein solution was loaded onto a 300 ml Q-Sepharose Fast Flow column which was equilibrated with 50 mM potassium phosphate, pH 7.8, 1 mM DTT. After washing with 0.35 M NaCl in the column buffer, deoxyhypusine synthase was eluted with 0.5 M NaCl in the same buffer. The eluted protein fraction was concentrated by ultrafiltration, dialyzed against 50 mM potassium phosphate buffer at pH 6.5, containing 1 mM DTT and 0.6 M ammonium sulfate (buffer A), and subjected to chromatography on Butyl-Sepharose. Proteins were eluted using a linear gradient of 0% to 100% buffer B (50 mM glycine-NaOH, pH 9.0, 10% glycerol, 1 mM DTT) in buffer A. Deoxyhypusine synthase containing fractions were pooled, dialyzed against 50 mM potassium phosphate, pH 7.8, 1 mM DTT, and loaded onto a Mono Q column equilibrated with the same buffer. For elution of the proteins a linear gradient of 0–1 M NaCl in this buffer was used. The deoxyhypusine synthase eluted at about

SSDI 0014-5793(95)00394-0

400 mM NaCl. After dialysis against 25 mM potassium phosphate buffer at pH 6.5, 1 mM DTT, the protein solution was chromatographed on hydroxylapatite using a linear gradient of 25–500 mM potassium phosphate in the same buffer. Fractions containing pure enzyme were pooled and either used directly for further studies or stored at −20°C after addition of 0.1% (v/v) Tween-20.

Protein purity was checked by electrophoresis on 11% polyacrylamide gels in the presence of SDS and β-mercaptoethanol [11]. The proteins were visualized by staining with silver nitrate [12].

2.4. Protein sequencing

Prior to the sequencing procedure, about 3 μg of purified deoxyhypusine synthase were cleaved with 10% (w/v) cyanogen bromide in 70% formic acid/water over night at room temperature. The resulting peptide mixture was resolved by reversed phase HPLC on a Supersher RP60 Select B (125 × 2 mm/4 μm; Merck, Darmstadt, Germany) using a linear gradient of 1% acetonitrile per min in 0.1% TFA. The subsequent amino acid sequence analysis of individual peptides was performed with an Applied Biosystems 477A gas phase sequencer equipped with a 120A PTH amino acid analyser according to the instructions of the manufacturer.

2.5. Analytical gel filtration

Gel filtration chromatography was performed on a Pharmacia FPLC system equipped with a Merck-Hitachi L-4200 variable wavelength detector using a column of Superose 12 (1 cm × 30 cm). Experiments were conducted at 22 ± 2°C in 50 mM potassium phosphate buffer, pH 6.5, containing 400 mM KCl and 1 mM DTT. The flow rate was 0.4 ml/min. After 200-μl samples of protein were injected, the elution profiles were monitored by absorption at 230 nm. The column was calibrated with proteins of known M_r: horse myoglobin (17,800); bovine erythrocyte carbonic anhydrase (29,000); chicken ovalbumin (44,000); bovine serum albumin (66,300); bovine γ globulin (158,000); bovine thyroglobulin (670,000); and gave a linear relationship between the logarithm of M_r and the elution volume.

3. Results and discussion

Deoxyhypusine synthase catalyzes the first two steps in hypusine synthesis: the initial spermidine dehydrogenation and the subsequent transfer of an aminobutyl group to the ε-amino group of lysine (at position 50 in human eIF-5A). The human enzyme was purified from HeLa cells using a 5-step procedure (Table 1). During the course of purification, deoxyhypusine synthase activity was measured according to a recently published method [9] except that the eIF-5A precursor protein did not carry a hexahistidine tag and the radiolabeled eIF-5A protein was separated from free [^{14}C]spermidine by small-scale gel filtration. The assay results were linear in respect to the enzyme concentration and the assay limited in its sensitivity only by the specific activity of [^{14}C]spermidine. After mechanical cell lysis, deoxyhypusine synthase was purified as a soluble protein from the cytoplasmic fraction. It appears to bind strongly to hydrophobic materials, and chromatography on Butyl-Sepharose as the third purification step is most effective in eliminating many of the other proteins (Fig. 1). A final chromatographic step on a hydroxylapatite column separates the enzyme from the major contaminating protein (apparent M_r of 60,000) after anion exchange chromatography on a Mono Q column. The purification procedure yielded a single protein with an apparent M_r of 41,000 as judged by gel electrophoresis in the presence of SDS (Fig. 1). The final protein solution possessed a specific activity of 3.2×10^{-3} U per mg of protein, representing a 1,400-fold purification from cell extracts (Table 1).

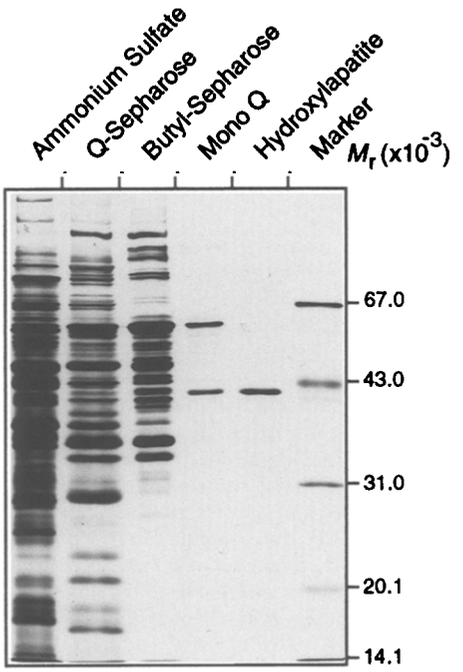

Fig. 1. Purification of human deoxyhypusine synthase. Polyacrylamide gel electrophoresis in the presence of SDS. Ammonium sulfate, protein fraction after ammonium sulfate precipitation; Q-Sepharose, protein fraction after anion exchange chromatography on Q-Sepharose; Butyl-Sepharose, protein fraction after chromatography on Butyl-Sepharose; Mono Q, protein fraction after anion exchange chromatography on a Mono Q column; hydroxylapatite, protein fraction after chromatography on hydroxylapatite; marker, molecular weight standard proteins with M_r values given on the right margin.

The purified enzyme eluted in a single symmetrical peak from a calibrated gel filtration column (Fig. 2). All of the deoxyhypusine synthase activity was found to be associated with this peak fraction indicating that the apparent native molecular weight of human deoxyhypusine synthase is about (150,000 ± 10,000). According to gel electrophoresis in the presence of SDS and

Table 1
Purification of human deoxyhypusine synthase from HeLa cells

Purification step[a]	Protein (mg)	Total activity[b] (×10^{-6} U)	Specific activity[b] (×10^{-6} U/mg)	Recovery (%)	Purification (x-fold)
Cell extract	620	1400	2.3	100	1
40–80% $(NH_4)_2SO_4$	550	1200	2.2	86	1
Q-Sepharose	34	130	3.8	9.3	1.7
Butyl-Sepharose	2.1	100	48	7.1	21
Mono Q	0.015	37	2500	2.6	1090
Hydroxylapatite	0.008	26	3250	1.9	1410

[a]1 international unit (U) of enzyme activity is defined as the formation of 1 pmole of product per min at 37°C.
[b]Protein fractions as described in Fig. 1.

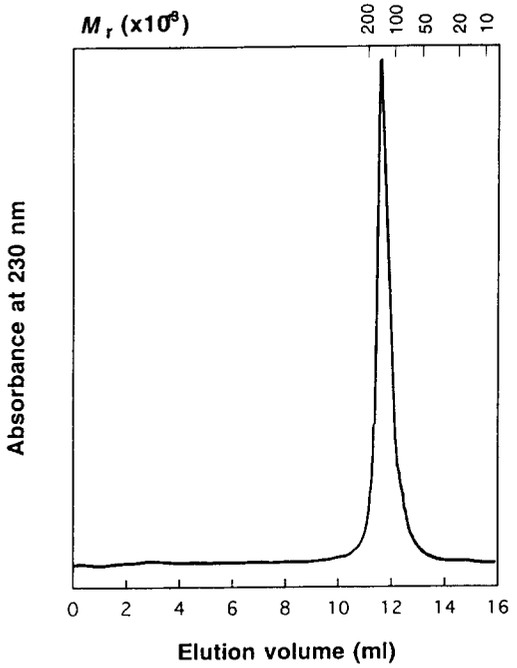

Fig. 2. Human deoxyhypusine synthase is a multimeric protein. Gel filtration chromatography on a Superose 12 column using a buffer of 50 mM potassium phosphate, pH 6.5, 400 mM KCl, 1 mM DTT with a flow rate of 0.4 ml/min. M_r values are given on upper ordinate from calibration with marker proteins.

β-mercaptoethanol, this peak fraction contained only one polypeptide chain species with an apparent M_r of 41,000 (Fig. 1). Therefore, human deoxyhypusine synthase appears to be a multimeric enzyme, most likely a homotetramer. These results agree well with the recent finding that native deoxyhypusine from *Neurospora crassa* possesses a molecular mass of about 180 kDa while the protein migrated in a single band at 40 kDa in gel electrophoresis [8]. In addition, deoxyhypusine synthase, which was partially purified from rat testis, was also reported to have a native apparent M_r of 180–190 kDa [13].

Similarly to the enzymes from *N. crassa* and rat, human deoxyhypusine synthase exhibits a pH optimum at about 9.0 (Fig. 3). Since the enzyme appears to be almost inactive at neutral pH, deprotonation of primary or secondary amino groups may be important for efficient catalysis. Furthermore, addition of 5 mM EDTA to the enzyme assay did not cause any decrease in the catalytic efficiency indicating that no divalent metal ions are involved in the catalytic mechanism.

After cyanogen bromide cleavage of the purified human deoxyhypusine synthase, the following four internal peptide sequences were obtained: M-P-I-L-D-Q, M-E-Q-N-T-E-G-V-K-x-T-P-S, M-I-A-R-L-G-K-E-I-N-N-P-E-S-V-Y-Y-W-A-Q-K-N-K-I-P-V-F, and M-I-I-L-G-G-G-V-V-K-H-H-I-A-N-A-N-L-M (one additional peptide had a blocked N-terminus and was, therefore, not accessible to Edman degradation. Assuming a subunit molecular weight of 41,000 (about 375 amino acid residues), these sequences obtained represent about 17% of the total amino acid sequence. Comparison of these peptide sequences with the data base (EMBL Data Library) using the FASTA program within the GCG software package (Wisconsin Package Version 8, Genetics Computer Group, U.S.A. 1994) revealed that they have a high similarity to a recently reported hypothetical protein from *Saccharomyces cerevisiae*. The corresponding DNA locus for this protein was found on chromosome VIII during the course of the yeast genome sequencing project. This hypothetical protein contains 387 amino acid residues and possesses a theoretical molecular weight of 42,892 in its unmodified form [14].

An alignment of the four peptide sequences of human deoxyhypusine synthase with this hypothetical protein is shown in Fig. 4. 44 out of 65 amino acid residues are identical in the sequenced regions with no gaps in the aligned sequences. This represents a 68% sequence identity in these regions. Moreover, the subunit molecular weights of both the human and the hypothetical yeast protein are almost identical. Comparison of the amino acid sequence of the yeast protein with that of the recently published peptides from *Neurospora crassa* deoxyhypusine synthase reveals a sequence identity of 71% in the corresponding 75 amino acid residues so far sequenced (the *N. crassa* deoxyhypusine synthase peptides range from amino acid residues 127–144, 169–189, 246–265, and 319–334, respectively, when aligned to the yeast sequence). On the basis of the above data, we propose that the hypothetical protein YHR068w on chromosome VIII of *Saccharomyces cerevisiae* is the enzyme deoxyhypusine synthase, which is necessary for post-translational modification of eukaryotic initiation factor 5A in yeast. Our peptide sequence information for the human deoxyhypusine synthase will be the basis for the cloning of the corresponding cDNA.

Acknowledgements: We thank B. Bandilla and I. Mathes for their help in cleavage of the protein and amino acid sequencing, respectively. We are grateful to S. A. Ali for critical reading of the manuscript. T. W. was supported by SFB 369 of the Deutsche Forschungsgemeinschaft.

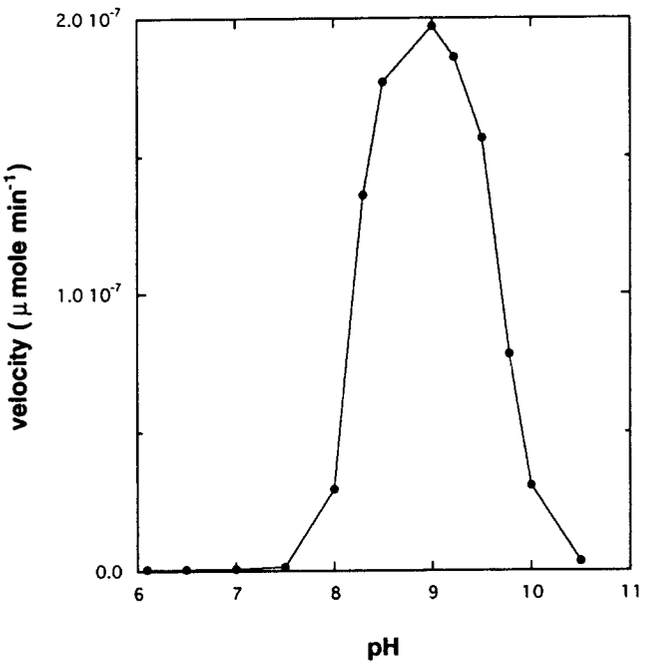

Fig. 3. Determination of optimal pH for human deoxyhypusine synthase activity. The reaction mixtures contained 6 μM [^{14}C]spermidine, 1 mM NAD^+, 2 μM recombinant eIF-5A precursor protein, and 0.13×10^{-6} U deoxyhypusine synthase. The assay buffer was 0.3 M glycine-NaOH, 1 mM DTT. The reaction mixtures were incubated for 2 h at 37°C.

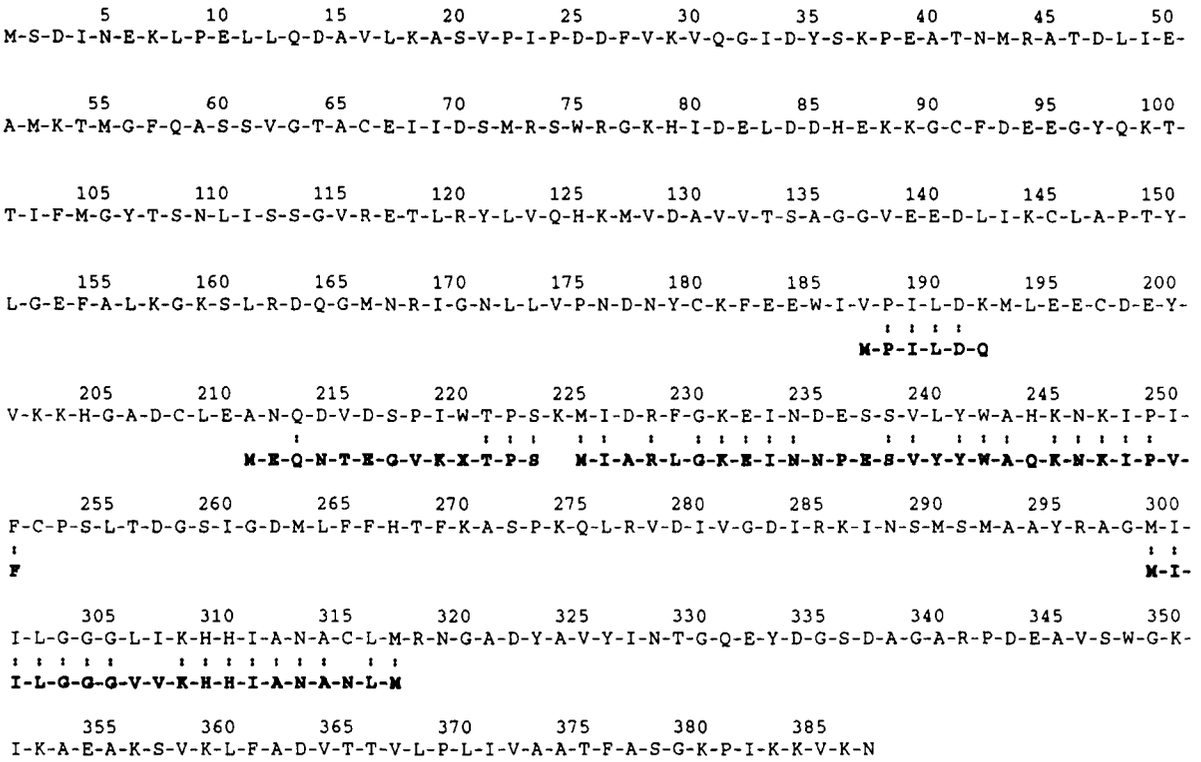

Fig. 4. Alignment of the amino acid sequences of the hypothetical protein YHRO68w from *Saccharomyces cerevisiae* (residues 1–387) and peptides derived from human deoxyhypusine synthase. The peptides of the human deoxyhypusine synthase are shown in bold. Amino acids are given in single letter code; X represents an amino acid residue which was not identified by peptide sequencing. Residues that are identical in the sequences of the YHRO68w protein and the peptides are connected by dots. Residue numbering is according to the hypothetical yeast protein.

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