J. Biochem. 121, 769-778 (1997)

Biochemical and Immunological Characterization of Deoxyhypusine Synthase Purified from the Yeast *Saccharomyces carlsbergensis*

Md. Ruhul Abid,* Kazuhiro Sasaki,* Koiti Titani,† and Masazumi Miyazaki*,1

*Department of Molecular Biology, School of Science, Nagoya University, Chikusa-ku, Nagoya 464-01; and †Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-11

Received for publication, December 13, 1996

Deoxyhypusine synthase catalyzes the NAD$^+$-dependent formation of deoxyhypusine in the eIF-5A precursor protein by transferring the 4-aminobutyl moiety of spermidine. This enzyme has recently been shown to be essential for cell viability and growth of yeast [Sasaki, K., Abid, M.R., and Miyazaki, M. (1996) *FEBS Lett.* 384, 151-154]. We have purified and characterized the enzyme from the yeast *Saccharomyces carlsbergensis*. The yeast and recombinant enzymes had a specific activity of 1.21 to 1.26 pmol per min per pmol of protein, and recognized both the eIF-5A precursor proteins almost equally as judged from their similar K_m and V_{max} values. Size exclusion chromatography and SDS-PAGE indicated that the active form of the enzyme is a homotetramer consisting of 43-kDa subunits. The enzyme showed a strict specificity for its substrates, NAD$^+$, spermidine and eIF-5A precursor protein. Among all the substrates tested, only NAD$^+$ showed a protective effect against heat inactivation of the enzyme suggesting that NAD$^+$ initiates some conformational change in the enzyme. NADH exhibited a strong non-competitive inhibition (product inhibition). Unexpectedly, FAD, FMN, and riboflavin showed a moderate competitive inhibition. The competitive inhibition by diamines was maximal with compounds resembling spermidine in carbon chain length. 1,3-Diaminopropane inhibited the enzyme strongly in a competitive manner (product inhibition). On the other hand, putrescine did not inhibit the enzyme or act as a substrate. A polyclonal antibody raised against the yeast recombinant enzyme specifically inhibited deoxyhypusine synthase activity. The cross-reactivity (by Western blotting) of this antibody with the crude extracts varied depending on the source, indicating species specificity.

Key words: deoxyhypusine synthase, eukaryotic initiation factor 5A, hypusine, post-translational modification, yeast.

Deoxyhypusine synthase catalyzes the initial step in the post-translational formation of a unique amino acid, hypusine, on eukaryotic translation initiation factor 5A (eIF-5A) (*2*). It catalyzes the oxidative cleavage and reductive transfer of the 4-aminobutyl moiety of spermidine to the ε-amino group of a specific lysine residue on the eIF-5A precursor protein (*3*). This reaction requires NAD$^+$ and results in the formation of deoxyhypusine [N^{ε}-(4-aminobutyl)lysine], which, in turn, is hydroxylated by deoxyhypusyl hydroxylase to produce hypusine (*3, 4*).

In spite of the pivotal role of hypusine for the *in vitro* activity of eIF-5A in methionyl-puromycin synthesis, which mimics the formation of the first peptide bond in protein synthesis (*5*), the precise *in vivo* function of this putative initiation factor is not known (*6*). However, the hypusine-containing protein, eIF-5A, which is ubiquitous and highly conserved in eukaryotes, appears to be vital for cell proliferation (*7*).

Deoxyhypusine synthase is a key enzyme catalyzing the first step of hypusine biosynthesis. Disruption of the deoxyhypusine synthase gene in yeast has recently been shown to be lethal (*1*). Taken together with the previous findings that inhibition of the enzyme by chemicals (*8*) or by limiting the level of the substrate spermidine (*9*) arrested the growth of eukaryotic cells, this indicates that the enzyme indeed plays an essential role in cell physiology. In addition, the enzyme is unique in its ability to modify a single lysine residue in one cellular protein through a multistep redox reaction involving the three substrates, NAD$^+$, spermidine, and eIF-5A precursor protein (*2*). A lower eukaryote, yeast, is an attractive organism for studies on these questions, owing to the availability of powerful genetic and biochemical tools, and molecular cloning techniques. Here, we report the characterization of deoxyhypusine synthase purified from yeast, and synthesis of the yeast recombinant enzyme in *Escherichia coli* to provide a basis for elucidation of the cellular function(s)

[1] To whom correspondence should be addressed. Tel: +81-052-789-2991, Fax: +81-052-789-3001, E-mail: j45933a@nucc.cc.nagoya-u.ac.jp

Abbreviations: DTT, dithiothreitol; DYS, deoxyhypusine synthase; eIF-5A, eukaryotic initiation factor 5A; ec-eIF-5A, the precursor of yeast eIF-5A expressed in *E. coli*; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; IPTG, isopropyl-1-thio-β-D-galactopyranoside; 2-ME, β-mercaptoethanol; NAD$^+$, nicotinamide adenine dinucleotide; NMN, nicotinamide mononucleotide; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

and reaction mechanism of the enzyme. We also compare some properties of the yeast enzyme with those of the enzymes recently purified from *Neurospora* (*10*) and mammals (*11-13*), revealing some species specificity.

MATERIALS AND METHODS

Materials—Aminobutyl-agarose and PMSF were purchased from Sigma. Superose 6 and hydroxyapatite were from Pharmacia and Koken (Tokyo), respectively. The facilities for FPLC (Pharmacia LKB) were kindly provided by Dr. Masahiro Sugiura, Center for Gene Research, Nagoya University. [^{14}C]Spermidine [*N*-(ϵ-aminopropyl)-[1,4-^{14}C]tetramethylene-1,4-diamine trihydrochloride] with a specific activity of 114 mCi/mmol was purchased from Amersham. Standard protein kits for SDS-PAGE and protein assay were from Bio-Rad. NAD^+, NADH, NADP, nicotinamide, ADP, FAD, and FMN were from Boehringer Mannheim. 1,2-Diaminoethane, 1,3-diaminopropane, putrescine, cadaverine, 1,6-diaminohexane, 1,7-diaminoheptane, 1,8-diaminooctane, 1,9-diaminononane, 1,10-diaminodecane, 1,12-diaminododecane, spermidine, and spermine were from Sigma.

Growth of Yeast—Saccharomyces carlsbergensis strain IFO 1265 was obtained from Fermentation Institute of Osaka. The yeast cells were grown at 30°C by aeration to a late log phase in 15 liters of rich medium containing (per 1 liter) 5 g of yeast extract, 10 g of peptone, 100 g of glucose supplemented with 4 g $(NH_4)_2SO_4$, 2 g of KH_2PO_4, 0.42 g of $MgSO_4 \cdot 7H_2O$, and 0.374 g of $CaCl_2 \cdot 2H_2O$, and the pH was adjusted to 5.0. Cells were harvested by continuous flow centrifugation at 10,000 rpm in a Kubota RC-1A rotor and washed once with buffer A containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM 2-ME, 30 mM KCl, 10% (v/v) glycerol, 0.2 mM EDTA, 1 mM PMSF, and 5 μg/ml antipain. The yield of packed cells was 200 g. The cells were frozen immediately in liquid nitrogen and stored at −80°C.

Enzyme Assay—A rapid and simple assay method of deoxyhypusine synthase activity was developed, which is based on the incorporation of radioactivity from [^{14}C]-spermidine into the ec-eIF-5A precursor protein as a 3.8% PCA precipitate. Typical assay mixtures contained 1 mM NAD^+, 10 μM [^{14}C]spermidine, 1 μM ec-eIF-5A, 1 mM DTT, and various amounts of deoxyhypusine synthase in a total volume of 50 μl containing 0.25 M glycine-NaOH buffer, pH 9.5. Incubations were done at 30°C for 30 min. The reaction mixture was adsorbed onto a paper disk (Whatman 3 MM, 2.4 cm), which in turn was dipped in 3.8% (v/v) PCA and treated at 90°C for 10 min to precipitate deoxyhypusinated ec-eIF-5A in the paper disk. The disk was washed with cold 0.2 N HCl and ethanol, and dried. Radioactivity was counted.

The nature of inhibition by the compounds was determined by plotting reciprocal velocities of [^{14}C]deoxyhypusine production *versus* reciprocals of the concentrations of the substrate at constant fixed levels of the other components, ec-eIF-5A (1 μM), deoxyhypusine synthase (40 ng), and NAD^+ (1 mM) or [^{14}C]spermidine (10 μM), and at set inhibitor concentrations. Where the graphic patterns were indicative of competitive inhibition, K_I values were obtained by fitting the data to the following equation, where I = concentration of inhibitor, and $K_{m(app)}$ = apparent K_m value obtained at a fixed concentration of the inhibitor,

$$K_I = [I] \cdot K_m / (K_{m(app)} - K_m)$$

Where the inhibition was of a non-competitive type, the K_I value was obtained by fitting the data points to the following equation, where $V_{max(app)}$ = apparent maximum rate of the reaction at a fixed concentration of the inhibitor.

$$K_I = [I] \cdot V_{max(app)} / (V_{max} - V_{max(app)})$$

Purification of the Yeast eIF-5A Precursor Proteins—A DNA fragment from the cloned gene of yeast eIF-5A (*CSF1*, cycloheximide sensitivity factor gene, Ref. *14*; equivalent to *TIF51A*), containing the ORF from +1 to +550, was amplified by PCR. The PCR product was digested at the *Nde*I and *Bam*HI sites (built in the 5′- and 3′-end primers, respectively), inserted into the vector pET-3a and used to transform *E. coli* BL21 (DE3) cells. The transformants were grown in LB medium containing 50 μg/ml ampicillin and induced with 0.5 mM IPTG at an A_{600} of 0.6. The incubation was continued at 37°C for 2 h. The cells were harvested by centrifugation, washed with and suspended in 30 mM Tris-HCl, pH 7.5, 10 mM KCl, 10 mM 2-ME, 1 mM PMSF, 5 μg/ml antipain, and 10% glycerol. After sonication, crude extracts were subjected to conventional column chromatography using DEAE-Sephacel and CM-Sepharose. Fractions containing ec-eIF-5Aa expressed from *TIF51A* (*CSF1*) were identified by 12% SDS-PAGE, Western blotting, and measurement of deoxyhypusine synthesis activity. The third step of chromatography on a MonoQ (Pharmacia LKB) column provided a highly purified sample of eIF-5Aa, showing a single band on SDS-PAGE. The same procedures were applied to prepare an apparently homogeneous preparation of ec-eIF-5Ab expressed from the other one of the two genes for eIF-5A (*TIF51B*), which was also amplified by PCR on a template DNA from yeast (*15*).

Purification of Deoxyhypusine Synthase—All operations were carried out on ice or at 4°C. Frozen cells (freshly prepared, 200 g) of *Saccharomyces carlsbergensis* were ground in an electric mortar with quartz sand (500 g) for 13 min and extracted with 600 ml of buffer A. After successive centrifugations of the homogenate at 25,000 × *g* for 30 min and 100,000 × *g* for 4.5 h, the final supernatant (S-100) was subjected to the ammonium sulfate precipitation between 50 and 100% saturation. The precipitate was dissolved in buffer A for conventional chromatography using DEAE-Sephacel, DEAE-Toyopearl, and hydroxyapatite columns. Two activity peaks were observed on the first column of DEAE-Sephacel and purified separately. The final step of purification was performed on an aminobutyl-agarose affinity column equilibrated with buffer B [250 mM glycine-NaOH, pH 9.5, 10 mM KCl, 10 mM 2-ME, 0.1 mM EDTA, 0.1 mM PMSF, 0.5 mM NAD^+, and 25% (v/v) glycerol]. The activity peak fraction pooled from a hydroxyapatite column (step 5, Table I) was dialyzed against buffer B and loaded onto the aminobutyl-agarose column. The column was washed with buffer B and eluted with buffer A. Fraction Nos. from 25 to 95 (Fig. 1B) were pooled, and the remaining fractions from 16 to 24, and 96 to 110, were combined and rechromatographed. The rechromatographed fractions purified to homogeneity and the pooled fractions (25 to 95) were combined as purified peak I fraction, as shown in Table I. This purified peak I was used

in all the experiments of this study. The purified peak II fraction also gave similar results (data not shown).

The yeast recombinant enzyme overexpressed in *E. coli* was purified by only two steps of chromatography using DEAE-Toyopearl and aminobutyl-agarose columns. The experimental details are as described previously (*1*).

TABLE I. **Summary of the purification of deoxyhypusine synthase from yeast.** Purification was done from 400 g of yeast cells. The enzymatic assay and purification were carried out as described in "EXPERIMENTAL PROCEDURES." Two activity peaks obtained from the DEAE-Sephacel column were subsequently purified separately. One unit is defined here as the amount of enzyme required to catalyze the formation of 1 pmol of deoxyhypusine/h at 30°C.

Steps	Protein (mg)	Total activity (10^{-3} × units)	Specific activity (10^{-3} × units/mg)	Recovery (%)	Purification (fold)
1. Homogenate	27,000	3,430	0.13	100	1
2. Precipitate [50-100% $(NH_4)_2SO_4$]	8,800	2,610	0.29	76	2.4
3. DEAE-Sephacel					
Peak I	174	2,740	15.8	80	124
Peak II	172	2,580	15.0	75	118
4. DEAE-Toyopearl					
Peak I	149	2,060	13.9	60	109
Peak II	94	1,140	12.1	33	95
5. Hydroxyapatite					
Peak I	17	517	30.4	15	239
Peak II	13	488	37.5	14	295
6. Aminobutyl agarose					
Peak I	0.24	402	1,680	12	13,200
Peak II	0.16	280	1,750[a]	8	13,800

[a]Equivalent to 1.26 pmol·min^{-1}·pmol^{-1} of enzyme monomer (MW 43 kDa).

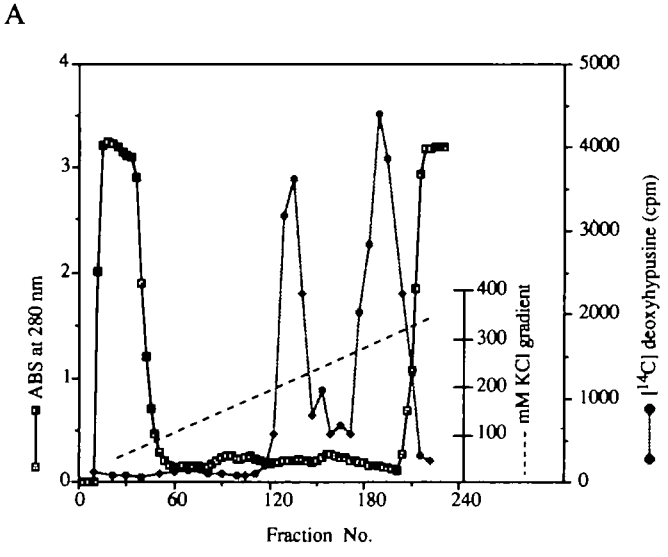
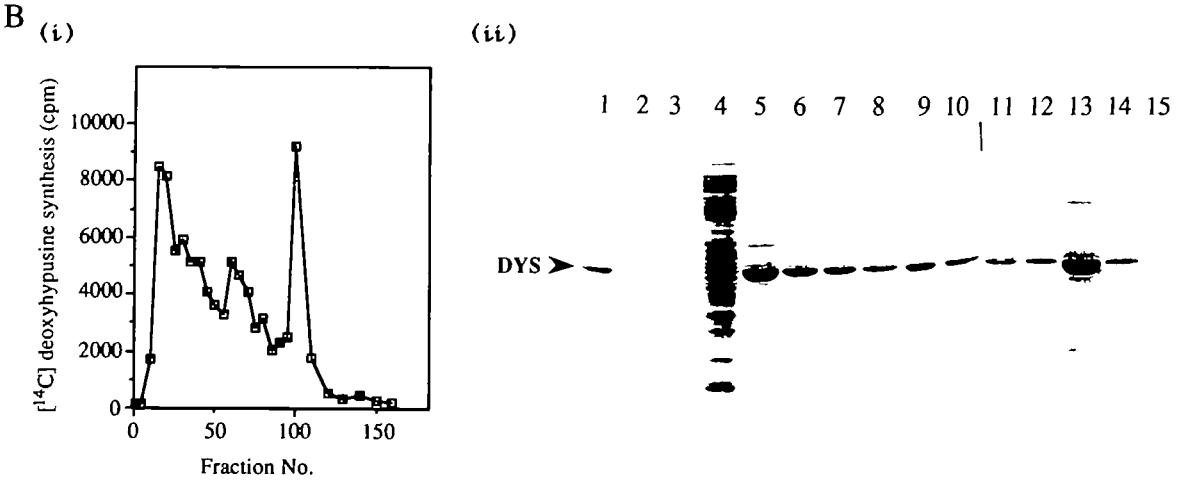


Fig. 1. **Purification of deoxyhypusine synthase by DEAE-Sephacel and aminobutyl agarose column chromatographies.** (A) Deoxyhypusine synthase fraction from step 2 (Table I) was chromatographed on a DEAE-Sephacel column. The first activity peak was eluted at 200-235 mM KCl and the second peak at 275-310 mM KCl. The two peak fractions I (tube Nos. 120 to 140) and II (175 to 205) were separately pooled, concentrated and subjected to the next step of chromatography on a DEAE-Toyopearl column. (Bi) The final step of chromatography on an aminobutyl-agarose affinity column. Peak I fraction from the hydroxyapatite column (step 5, Table I) was loaded onto an aminobutyl-agarose column, which was washed with buffer A containing 0.25 M glycine-NaOH, pH 9.5, 0.5 mM NAD^+, and 10 mM KCl, at tube No. 11, eluted with buffer B containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl (at tube No. 55), and finally purged with 0.1 M KCl in buffer B (at tube No. 97). Nearly the same elution profile was obtained for peak II fraction from step 5. (Bii) Analysis of the fractions from (Bi) on 10% SDS-PAGE. Lane 1, Purified deoxyhypusine synthase; lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15, show fraction Nos. 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120, respectively, from the aminobutyl-agarose column.



Production of a Polyclonal Antibody—A polyclonal antibody against deoxyhypusine synthase was raised in rabbit. The purified recombinant yeast enzyme was subjected to 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue at room temperature for 10 min. After destaining with acetic acid/methanol/water: 0.5 : 1.65 : 7.85 (v/v) at 4°C for 2.5 h, the band at 43 kDa was excised. The gel strip was washed and equilibrated with Milli-Q water for 1.5 h at 4°C. Protein was electroeluted from the minced gel. Electroelution was carried out at room temperature at a constant voltage (50 V) overnight with an elution buffer containing 0.25 M Tris, 1.92 M glycine (pH 8.6), and 0.1% SDS. The protein was dialyzed against phosphate-buffered saline (PBS) at 4°C and subjected to precipitation by addition of methanol/acetone (50 : 50 v/v). After microcentrifugation, the pellet was dissolved in PBS buffer and used to immunize rabbits (Medical and Biological Lab., Nagoya). The antibody was titered against the enzyme by dot blotting and Western blotting techniques.

Amino Acid Sequence Analysis—Limited digestion of the purified yeast deoxyhypusine synthase was carried out with 1% (w/w) trypsin-TPCK relative to the substrate in 36 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM 2-ME at 25°C. The intact protein and partial tryptic products were separated on 12.5% SDS-PAGE and electroblotted onto a PVDF membrane. After brief staining, portions of the membrane corresponding to major bands were excised and applied to an Applied Biosystems 477A Protein Sequencer equipped with an on-line 120A PTH analyzer (*16*).

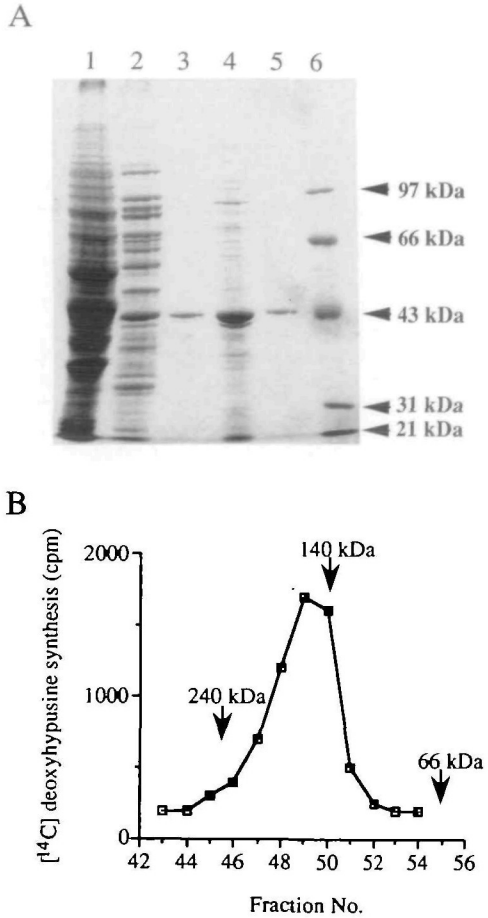


Fig. 2. **Estimation of molecular weight of deoxyhypusine synthase.** (A) 10% SDS-PAGE of the yeast and recombinant deoxyhypusine synthases. Lanes: 1, yeast cell extract (S-100); 2, a fraction from the first activity peak on DEAE-Sephacel; 3, purified yeast deoxyhypusine synthase from the first activity peak; 4, an activity peak fraction of the recombinant enzyme on the DEAE-Toyopearl column; 5, purified recombinant enzyme; 6, molecular weight markers. (B) Gel filtration analysis of the purified yeast enzyme on a Superose 6 column. Molecular weight markers used were human serum albumin (66 kDa), alcohol dehydrogenase from yeast (140 kDa), and pyruvate kinase from rabbit muscle (240 kDa), as indicated by arrows.

RESULTS

Purification and Molecular Weight Estimation of Deoxyhypusine Synthase—Table I summarizes a typical purification of deoxyhypusine synthase from *Saccharomyces carlsbergensis* including 4 steps of column chromatography. The first step of chromatography on DEAE-Sephacel gave occasionally two activity peaks (Fig. 1A). The cause of the two peaks is not known. The two peak fractions were separately subjected to the next steps of chromatography on DEAE-Toyopearl and on hydroxyapatite. The aminobutyl-agarose affinity column used in the final step of purification was found to be very efficient (Fig. 1Bi and ii). In this column we exploited the affinity of deoxyhypusine synthase for the aminobutyl moiety of spermidine in the presence of NAD^+. Specific retention of the enzyme on this column required the presence of 0.5 to 1 mM NAD^+ and a high pH of 9.5, a mimicry of the conditions optimal for deoxyhypusination (Fig. 1Bi). The enzyme preparation purified from each of the two peaks on DEAE-Sephacel gave a single band with a molecular weight of 43 kDa when analyzed on SDS-PAGE (see Fig. 1Bii and lane 3 in Fig. 2A). Finally, a purification of over 13,000-fold was achieved with a total recovery of 20% and a specific activity of 1,680 to $1,750 \times 10^3$ units per mg of protein, which is equivalent to 1.21 to 1.26 pmol per min per pmol of enzyme monomer (MW, 43 kDa) (Table I). From the data of Table I, the deoxyhypusine synthase was roughly estimated to represent 0.005 to 0.007% (w/w) of total soluble protein of yeast. The yeast recombinant enzyme overexpressed in *E. coli* was purified to homogeneity by only two steps of chromatography on DEAE-Toyopearl and on aminobutyl-agarose (Fig. 2A, lanes 4 and 5), as described previously (*1*). One liter of culture yielded *ca.* 5 mg of the purified recombinant enzyme.

TABLE II. **Requirements for the *in vitro* synthesis of deoxyhypusine.** A complete assay mixture contained 10 μM [^{14}C]spermidine, 1 mM NAD^+, 1 mM DTT, 1 μM ec-eIF5A, and 40 ng of enzyme in a total volume of 50 μl containing 0.25 M glycine-NaOH buffer, pH 9.5. Incubations were carried out at 30°C for 30 min.

Assay mixture	Production of [^{14}C]deoxyhypusine (cpm)
1. Complete	6,094
2. Without NAD^+	156
3. Without [^{14}C]spermidine	29
4. [^{14}C]Putrescine, without [^{14}C]spermidine	150
5. Without ec-eIF5A	120
6. Without DTT	1,798
7. Without enzyme	105

The molecular weight of deoxyhypusine synthase either purified from yeast or from *E. coli* (recombinant) was estimated to be ~170 kDa in its active form by size-exclusion chromatography on a Superose 6 column (see, Fig. 2B), suggestive of a homotetramer of a subunit of 43 kDa.

Partial Amino Acid Sequences of the Enzyme—The purified yeast deoxyhypusine synthase was not accessible to Edman degradation, indicating a blocked N-terminal amino acid. Six internal peptides were isolated by partial trypsin digestion and on SDS-PAGE. The sequences obtained for four of these are as follows: bands A (40 kDa) and E (8 kDa), ASVPIPDDFVQGIDYSKPEATNMRATDL; bands C (26 kDa) and D_2 (20 kDa), IGNLLVPNDNY(C)-KFEEXIVP. These peptide sequences were used to design oligonucleotide primers for subsequent cloning of the corresponding gene (*1*).

Requirements of the Purified Yeast Deoxyhypusine Synthase—As summarized in Table II, absolute requirements of NAD^+, spermidine and eIF-5A precursor protein, were observed for synthesis of deoxyhypusine by the enzyme. The yeast and recombinant enzymes exhibited a strict specificity for NAD^+ as a proton acceptor, which cannot be replaced by NADP+, FAD, or FMN of up to 20 mM. [^{14}C]Putrescine was not able to substitute for [^{14}C]-spermidine. The requirement for precursor protein is also specific. No [^{14}C]deoxyhypusine was formed by any cytosol protein extracted from *E. coli* transformed with a plasmid (pET-3a) alone (data not shown). When the system was deprived of DTT, the activity fell to *ca.* 30%. The residual activity may be partly due to the presence of 2-ME carried over from the enzyme and precursor protein preparations. The NAD^+-dependent deoxyhypusine synthase may be a dehydrogenase-like enzyme. Many dehydrogenases are known to be sensitive to sulfhydryl reagents and metal ions. At a concentration of 0.6 mM, Cu^{2+}, Fe^{3+}, and Fe^{2+} were the most effective inhibitors (85 to 100% inhibition) of deoxyhypusine synthase, Zn^{2+} and Mn^{2+} were weak inhibitors (around 20%), and Mg^{2+} and Ca^{2+} were not inhibitory (data not shown). Similar effects of these metal ions were observed on the formation of deoxyhypusine in mammalian cell lysates (*17*).

Optimal pH and Temperature—As shown in Fig. 3A, the purified enzyme exhibited a pH optimum at about 9.5 with a broad activity profile. Even at neutral and higher pH, the yeast enzyme displayed a substantial activity, in contrast to the mammalian enzyme (*11-13*). The optimal temperature for the yeast enzyme was observed at about 30°C, at which yeast can grow best. Lower temperature was better for the

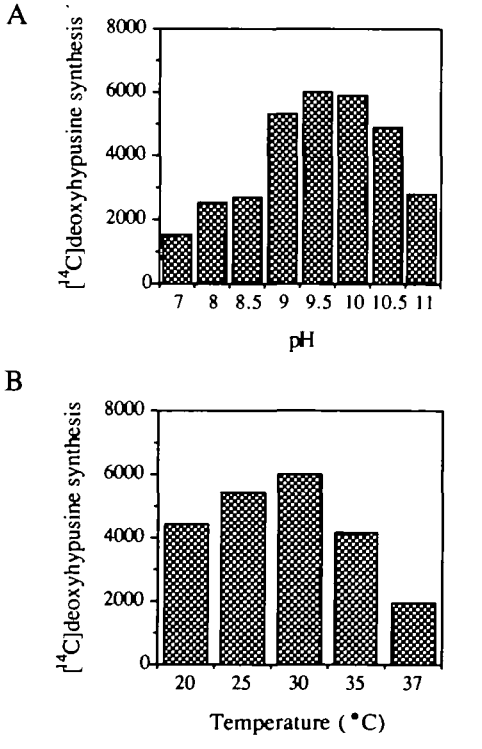


Fig. 3. **pH and temperature profiles for deoxyhypusine synthase activity.** (A) At a fixed concentration of all the substrates, as described in "MATERIALS AND METHODS," 40 ng of deoxyhypusine synthase was incubated for 30 min at 30°C at various pH values in 0.05 M Tris-HCl buffer (pH 7 to 9) and in 0.25 M glycine-NaOH buffer (pH 9 to 11). (B) The temperature was varied from 20 to 37°C under the same conditions as in (A) except for a fixed pH of 9.5.

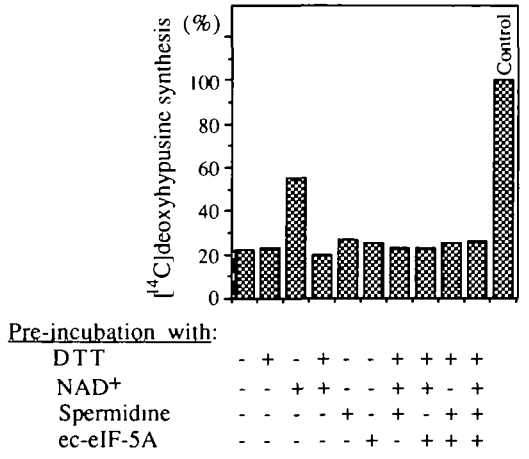


Fig. 4. **Protection of deoxyhypusine synthase from heat inactivation.** Purified deoxyhypusine synthase (40 ng) was preincubated at 42°C for 5 min in the presence (+) or absence (−) of DTT and/or the substrates as indicated. Deoxyhypusine synthesis was assayed as described in "MATERIALS AND METHODS." "Control" indicates deoxyhypusine synthesis without pre-incubation. Here, 100% activity means 6,094 cpm, *i.e.*, 1.26 pmol·min^{-1}·pmol^{-1} of enzyme.

TABLE III. **Kinetic properties of deoxyhypusine synthase.** The concentrations of eIF-5A precursor, spermidine, and NAD^+, as the non varied substrates, were 1 μM, 10 μM, and 1 mM, respectively, and 40 ng of enzyme was incubated in 50 μl of reaction mixture at 30°C for 30 min. Values were obtained by fitting the data to the Michaelis-Menten equation. The results are the averages obtained from more than three independent experiments.

Variable substrate	K_m (μM)	V_{max} (pmol/h)
Yeast deoxyhypusine synthase		
ec-eIF-5Aa (TIF51A protein)	0.15±0.01	43±4.5
ec-eIF-5Ab (TIF51B protein)	0.16±0.01	41±3.3
Spermidine	6.49±0.25	83±3.2
NAD^+	400±20	80±5.4
Recombinant deoxyhypusine synthase		
ec-eIF-5Aa (TIF51A protein)	0.18±0.01	38±3.6
ec-eIF-5Ab (TIF51B protein)	0.19±0.01	35±3.5
Spermidine	6.89±0.35	83±3.5
NAD^+	420±22	75±4.3

activity than higher temperature above 30°C. At 37°C, the activity was only *ca.* 30% of the optimal (Fig. 3B).

Protection against Heat Inactivation—The purified enzyme was sensitive to heat treatment. Pre-incubation of the enzyme alone in 30 mM Tris-HCl buffer (pH 7.5) decreased its activity to 65% of the original at 4°C for 1 h and to only a few percent at 65°C for 15 min. By utilizing this instability of the enzyme, we attempted to examine the protective effects of the substrates against heat inactivation through their interaction with the enzyme. Figure 4 depicts the protection by the substrates, NAD^+, spermidine and precursor protein, and their various combinations against heat inactivation at 42°C. Amongst a number of pre-incubation systems examined, only NAD^+ alone exerted a remarkable protective effect on deoxyhypusine synthase; further addition of DTT abolished this protection, suggesting that DTT might function as an antagonist to the oxidative agent, NAD^+, in the formation of deoxyhypusine. No significant protection was observed by the other substrates or any combination of the three substrates with or without (data not shown) DTT. These findings that only NAD^+ can protect the enzyme from heat inactivation probably suggest that the cofactor (one of the substrates) brings about some conformational change of the enzyme in an early step of the formation of deoxyhypusine. It should be noted that a parallel set of experiments was carried out with further addition of the compounds (except the enzyme) already included in the pre-incubation mixture without any observable increase in the activity, indicating that no inactivation of NAD^+, DTT, or precursor protein occurred during pre-incubation.

Kinetic Parameters—Table III gives a summary of the kinetic constants of the yeast deoxyhypusine synthase and recombinant enzyme expressed in *E. coli*. Plots of reciprocal velocities against reciprocals of variable substrate concentrations with each substrate showed good straight line relationships (see the lines drawn in the absence of inhibitors in Figs. 5 to 7). The data in Table III show that K_m and V_{max} values obtained for the same substrate are in good agreement between the yeast and recombinant enzymes and both enzymes can equally recognize the precursor proteins expressed from two different yeast genes.

Inhibitory Effects of Nucleotides and Related Compounds on the Yeast Enzyme—As mentioned above, the purified yeast enzyme strictly selected NAD^+ as a proton-acceptor for the formation of deoxyhypusine. Here, we examined the inhibitory effects of nucleotides and related compounds on the enzyme activity in order to elucidate the features of the NAD^+-binding site of the enzyme. Table IV gives the IC_{50} and K_I values of those compounds. Among the tested compounds related to NAD^+, only NADH exerted strong inhibition with an estimated IC_{50} value of 0.50 mM and K_I value of 0.85 mM, indicating an affinity to the enzyme comparable with that of the substrate NAD^+. Interestingly, this inhibition by the reduced product, NADH, was clearly of non-competitive type (Fig. 5). NADP, NMN, nicotinamide, and ADP, though structurally related to NAD^+, did not exhibit significant inhibition. Unexpectedly, FAD, FMN, and riboflavin, all of which contain flavin, exerted moderate inhibition (Table IV), and the type of inhibition appeared to be competitive, as shown for a typical inhibition pattern of FMN in Fig. 6.

Inhibition by Diamines and Polyamines—For characterization of the spermidine-binding site of deoxyhypusine synthase, diamines and polyamines structurally related to the substrate were tested as inhibitors of the purified yeast enzyme. Table V summarizes the IC_{50} and K_I values for a

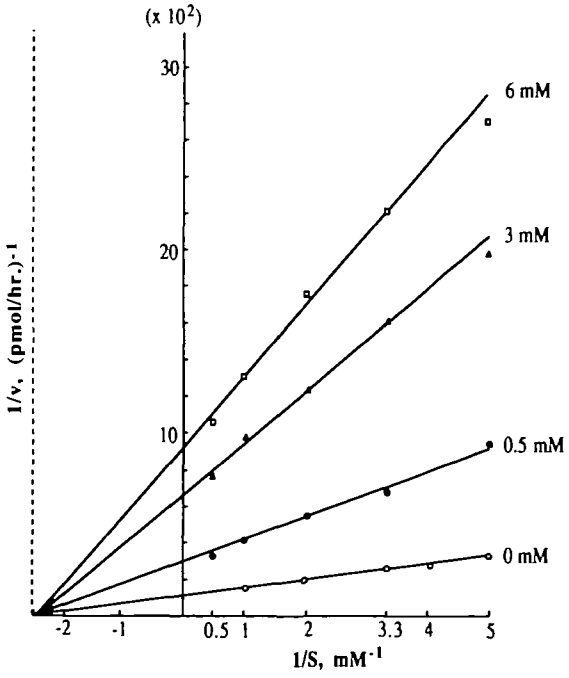


Fig. 5. **NADH inhibits deoxyhypusine synthase non-competitively.** The reaction was carried out with NADH as an inhibitor and various concentrations of the substrate NAD^+. All other substrates and conditions were the same as described in "MATERIALS AND METHODS." The NADH concentrations are given in mM at the ends of the lines. The initial velocities are given as the product, *i.e.* [^{14}C]-deoxyhypusine, formed per 40 ng of enzyme. Reciprocal values of the initial velocities of the product *versus* reciprocal values of the substrate (NAD^+) concentrations were plotted.

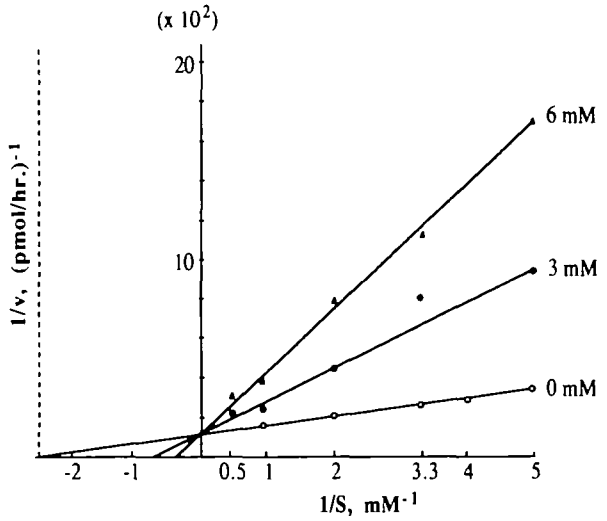


Fig. 6. **Competitive inhibition of deoxyhypusine synthase by FMN.** NAD^+ and FMN were used as a varied substrate and inhibitor, respectively. The FMN concentrations are given in mM at the ends of the lines. The initial velocities and double reciprocal plotting were as described in the legend to Fig. 5.

series of diamines and polyamines with various carbon chain lengths, including the apparent inhibition by unlabeled spermidine as a reference for comparison with other compounds. The efficiency of these compounds as inhibitors of the yeast enzyme varied depending on the number of methylene groups between their primary amino groups. Inhibition was maximal with 1,7-diaminoheptane and 1,8-diaminooctane, which resemble the substrate, spermidine, in carbon chain length, as was also observed with the partially purified rat testis enzyme (*18*). In an extended conformation, 1,7-diaminoheptane is slightly shorter and 1,8-diaminooctane a bit longer than spermidine in the distance between the primary amino groups. The yeast enzyme was also strongly inhibited by 1,9-diaminononane, carrying one more methylene group than 1,8-diaminooctane, in contrast to the weak inhibition of the rat enzyme by 1,9-diaminononane (*18*). However, 1,6-diaminohexane with a slightly shorter carbon chain than 1,7-diaminoheptane exerted similarly weak inhibition on both the yeast and rat enzymes. The strong inhibition by these compounds was shown to be of competitive type, as illustrated by a typical inhibition pattern in Fig. 7. A weak inhibition by 1,12-diaminododecane, but no inhibition by spermine, which resembles this diamine in carbon chain length, was observed. In spermine either one of the secondary amino groups might have some negative effect on its interaction with the enzyme. It is noteworthy that 1,3-diaminopropane exerted moderately strong competitive inhibition. It is plausible that 1,3-diaminopropane, an intermediary product of the spermidine cleavage reaction, exerts product inhibition on the enzyme. On the other hand, putrescine, which resembles the aminobutyl moiety of spermidine, did not show any inhibition. This is consistent with the finding that putrescine could not substitute for spermidine in the deoxyhypusine synthesis reaction *in vitro* (Table II).

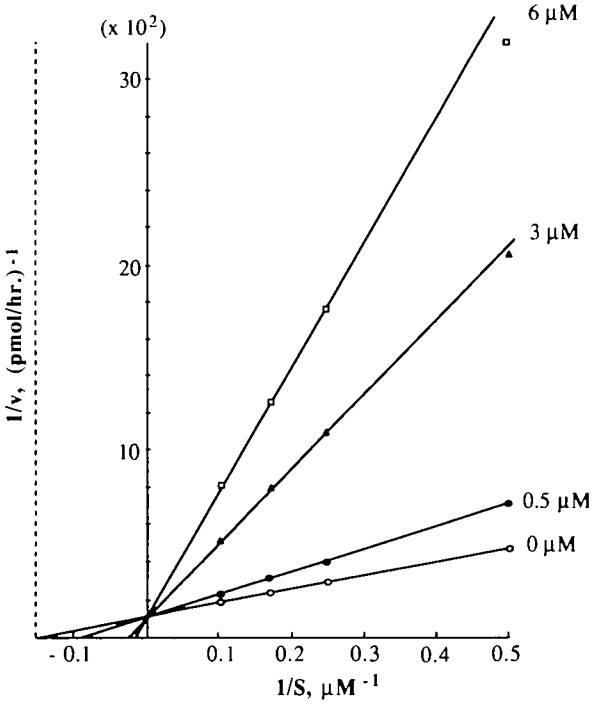


Fig. 7. **Competitive inhibition of deoxyhypusine synthase by 1,7-diaminoheptane.** The reaction was carried out with 1,7-diaminoheptane as an inhibitor and various concentrations of the substrate [^{14}C]spermidine. The initial velocity and double reciprocal plotting were as described in the legend to Fig. 5.

Recognition of Deoxyhypusine Synthase by a Polyclonal Antibody Raised against the Yeast Enzyme—A polyclonal anti-DYS antibody was raised against the purified yeast recombinant enzyme. The anti-DYS antibody was confirmed to recognize specifically deoxyhypusine synthase by inhibition of the enzyme activity and by Western blotting. As shown in Fig. 8A, the antibody inhibited the yeast enzyme activity by more than 90% while almost no inhibi-

TABLE IV. **Inhibition of deoxyhypusine synthase by nucleotides.** IC_{50} values, *i.e.* the concentrations inhibiting deoxyhypusine production by 50%, were calculated by fitting the data points to the equation, $IC_{50}=I(100-x)/x$; where x=% inhibition, I=concentration of inhibitor. The results are the average values of at least three independent experiments.

Compound	IC_{50} (mM)	K_i (mM)
NADH	0.50±0.03	0.85±0.15
NADP	<[a]	
Nicotinamide	15±0.54	
ADP	13.2±0.4	
NMN	<[a]	
Riboflavin	5±0.18	
FMN	3±0.15	1.28±0.06
FAD	7.19±0.27	2.27±0.23

[a]Less than 50% inhibition at 20 mM concentration of the inhibitor.

TABLE V. **Inhibition of deoxyhypusine synthase by diamines and polyamines.** The results are the average values obtained from at least three independent experiments.

Compound	Structure	IC_{50} (μM)	K_i (μM)
1,2-Diaminoethane	$H_2N\diagup\diagdown NH_2$	<[a]	
1,3-Diaminopropane	$H_2N\diagup\diagdown\diagup NH_2$	20	12.9±0.6
Putrescine	$H_2N\diagup\diagdown\diagup\diagdown NH_2$	<[a]	
Cadaverine	$H_2N\diagup\diagdown\diagup\diagdown\diagup NH_2$	<[a]	
1,6-Diaminohexane	$H_2N\diagup\diagdown\diagup\diagdown\diagup\diagdown NH_2$	225	
1,7-Diaminoheptane	$H_2N\diagup\diagdown\diagup\diagdown\diagup\diagdown\diagup NH_2$	1.3	0.90±0.08
1,8-Diaminooctane	$H_2N\diagup\diagdown\diagup\diagdown\diagup\diagdown\diagup\diagdown NH_2$	3	1.91±0.16
1,9-Diaminononane	$H_2N\diagup\diagdown\diagup\diagdown\diagup\diagdown\diagup\diagdown\diagup NH_2$	10	7.52±0.23
1,10-Diaminodecane	$H_2N\diagup\diagdown\diagup\diagdown\diagup\diagdown\diagup\diagdown\diagup\diagdown NH_2$	<[a]	
1,12-Diaminododecane	$H_2N\diagup\diagdown\diagup\diagdown\diagup\diagdown\diagup\diagdown\diagup\diagdown\diagup\diagdown NH_2$	236	
Spermidine	$H_2N\diagup\diagdown\diagup NH\diagup\diagdown\diagup\diagdown NH_2$	10[b]	6.49[c]±0.25
Spermine	$H_2N\diagup\diagdown\diagup NH\diagup\diagdown\diagup\diagdown NH\diagup\diagdown\diagup NH_2$	<[a]	

[a]Less than 50% inhibition at 1 mM concentration of the inhibitor. [b]Apparent inhibition by unlabeled spermidine. [c]K_m for spermidine.

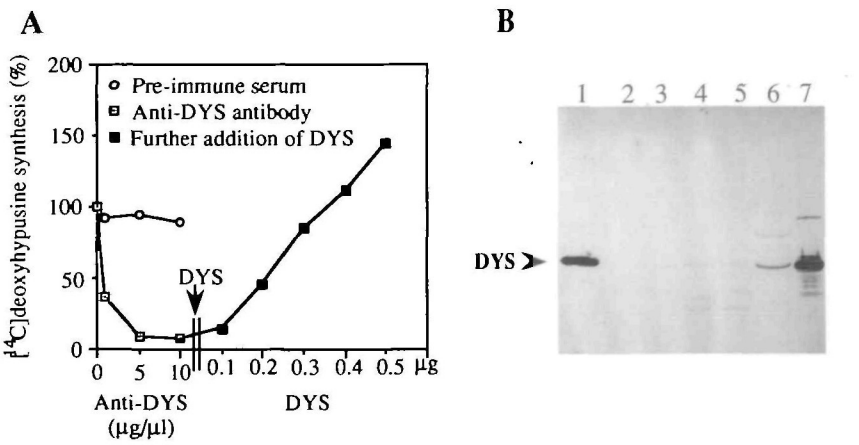


Fig. 8. **Specific inhibition of deoxyhypusine synthase activity by a polyclonal anti-DYS antibody (A), and cross-reactivity of anti-yeast DYS antibody with crude cell extracts (S-100) from different organisms (B).** (A) The reaction conditions were as described in "MATERIALS AND METHODS" with the exception of the presence of an increased level of either anti-EF-3 antibody as a control (pre-immune) serum or anti-DYS antibody as indicated. In order to follow the recovery of the inhibited activity, increasing amounts of the purified enzyme were added to reaction mixtures containing 10 $\mu g/\mu l$ anti-DYS antibody as indicated. (B) Equal amounts (200 μg) of total protein (S-100) from different organisms were loaded onto each lane (lanes 2 to 6). After electrophoresis on 10% SDS-PAGE, the proteins were transferred onto a PVDF membrane. The membrane was blotted with anti-DYS antibody (50×dilution). Lanes: 1, purified yeast DYS (15 ng); 2, 3, 4, 5, and 6, S-100 from *Tetrahymena pyriformis, Neurospora crassa, Aspergillus nidulans, Artemia salina*, and *Saccharomyces cerevisiae*, respectively; 7, *E. coli* cell extract (total protein, 15 μg) overexpressing recombinant yeast DYS.

tion was observed with the same amount of anti-EF-3 antibody used as a control (pre-immune) serum. Thereafter, the inhibited activity was restored proportionally to the increasing amount of the yeast enzyme added. The cross-reactivity of this antibody with crude extracts (S-100) from different eukaryotes was evaluated by Western blotting, as shown in Fig. 8B. The reactivity of the antibody varied depending on the protein source. The anti-DYS antibody reacted intensely with the yeast S-100 (lane 6), weakly with the *Aspergillus nidulans* (lane 4) and *Artemia salina* (lane 5) S-100, and faintly with the *Neurospora crassa* S-100 (lane 3), but not at all with the *Tetrahymena* S-100 (lane 2) or the purified recombinant human enzyme expressed in *E. coli* (data not shown), probably due to species specificity.

DISCUSSION

At the start of this study, we developed a simple and rapid assay method for deoxyhypusine synthase as described in "MATERIALS AND METHODS." This method is especially useful to large numbers of samples, and greatly facilitated the purification, characterization, and functional studies of the enzyme. The validity of this method was supported by a number of features of the yeast deoxyhypusine synthase characterized in this study, as well as in the previous paper (*1*).

We have described here the characterization of deoxyhypusine synthase purified from yeast and of the yeast recombinant enzyme expressed in and purified from *E. coli*. As judged from SDS-PAGE and size-exclusion chromatography, both the purified yeast and recombinant enzymes were homotetramers in their native form, consisting of 43-kDa subunits. Deoxyhypusine synthases from *Neurospora crassa* (*10*), rat testis (*11*), and human Hela cells (*12-14*) were also reported to be homotetramers. However, the "active" form of this enzyme in reaction systems synthesizing deoxyhypusine has not yet been confirmed to be the tetramer, since the associated form observed on gel filtration was at pH 6.5 to 7.5, which is different from the optimal pH required for deoxyhypusination. The yeast enzyme shows a high optimal pH of 9.5 and, even at pH 11, it retains a half of its activity (Fig. 3). It would be interesting to know how the first step of hypusination at such a high pH is smoothly linked to the second step of the reaction (deoxyhypusine hydroxylation) at a lower pH of 7.0 (*4*) to form hypusine within eukaryotic cells. The optimal reaction temperature of the yeast enzyme was 30°C, which is also optimal for yeast cell growth. The temperatures optimum for the enzymes from *Neurospora* (*19*) and mammalian cells (*11-13*) were reported to be 30 and 37°C, respectively, reflecting a preference for the same temperature for deoxyhypusination and optimal growth of the respective organism.

The yeast enzyme as well as the yeast recombinant one (see also Ref. *20*) has similar properties to those of deoxyhypusine synthases from *Neurospora* (*10*), rat (*11*), and human (*12, 13*). These enzymes recognize NAD^+, spermidine and the highly conserved eIF-5A precursor with strict specificity as the three substrates for deoxyhypusination. Interestingly, only NAD^+ alone was able to show a protective effect against heat inactivation of the yeast enzyme; almost no protection was observed with the other two substrates or with any combination of the three. The findings that the enzyme binds to a spermidine analogue (Fig. 1Bi) and the eIF-5A precursor protein (*21*) only in the presence of NAD^+ suggest that the initial binding with the cofactor NAD^+ plays an important role in causing some conformational change of the enzyme triggering deoxyhypusine synthesis. Since deoxyhypusine synthase is a dehydrogenase-like enzyme and many dehydrogenases are sensitive to sulfhydryl reagents and metal ions, we tested the effects of these compounds on the enzyme. The observations that the protective effect of NAD^+ was abolished by the addition of DTT and that the metal ions Cu^{2+} and Fe^{3+} strongly inhibited the enzyme activity suggest the presence of cysteine residues around the NAD^+-binding site. This assumption is consistent with the finding that NAD^+ protected the *Neurospora* enzyme against the inhibitory effects of the SH-reagents NEM and iodoacetamide (*10*). Whereas the yeast deoxyhypusine synthase specifically recognizes NAD^+ as a proton-acceptor, this enzyme activity was strongly inhibited by the reduced form NADH in a non-competitive manner. This suggests that the enzyme provides another binding site for NADH different from that for NAD^+. On the other hand, FAD, FMN, and

riboflavin unexpectedly exerted moderate competitive inhibition on the enzyme. The inhibition by FAD and FMN was also observed with the rat enzyme, except for the inhibition type (*11*). Although the reaction mechanism of the enzyme with these flavin compounds structurally unrelated to the substrate, NAD^+, must await further studies, it is interesting to note that FAD is a cofactor for diamine oxidase, which functions as a regulating enzyme in polyamine metabolism. The interaction of FAD with both deoxyhypusine synthase and diamine oxidase might have a role in controlling cell growth and proliferation.

The inhibitory effect of a series of diamines of various carbon chain lengths on the yeast enzyme was maximal with the compounds resembling spermidine in chain length, though an exceptionally high level of inhibition was exerted by 1,3-diaminopropane (product inhibition). All the diamines, including 1,3-diaminopropane, that inhibited the enzyme strongly showed a competitive inhibition pattern. The observations were consistent with those on the rat enzyme (*18*), indicating that the spermidine-binding site of the yeast enzyme is essentially the same as that of the mammalian enzyme. The binding of the yeast enzyme with the substrate, spermidine, in the presence of NAD^+ is plausibly initiated by both ionic and hydrophobic interactions. The ionic interaction may occur between negatively charged acidic amino acid residues on the enzyme and positively charged primary amino groups separated from each other by a distance equivalent to the chain length of spermidine; the hydrophobic interaction may occur between some hydrophobic region in the substrate-binding cavity of the enzyme and the hydrophobic carbon chain of spermidine. It seems noteworthy that some differences in the spermidine-binding pocket appear to exist between the yeast and mammalian enzymes, as indicated by the inhibition levels seen with putrescine and 1,9-diaminononane.

Two eIF-5A precursor proteins from yeast (ec-eIF-5Aa and ec-eIF-5Ab) were equally recognized by the purified yeast and recombinant enzymes as shown in Table III. The two yeast precursor proteins are derived from two distinct genes TIF51A and TIF51B (*7*), which are reciprocally regulated by oxygen (*15*). However, the two proteins seem to be functionally indistinguishable *in vitro* and *in vivo* (*7, 15*). We have reported the existence of a single-copy gene encoding and expressing deoxyhypusine synthase in yeast (*1*). In the course of purification, two activity peaks appeared but the finally purified enzyme preparations were uniform in respect of molecular mass, kinetic parameters, and other physical and functional properties. There is no evidence for the occurrence of post-translational modification, such as phosphorylation, glycosylation and so on. It seems that a single enzyme interacts equally with the two precursor proteins in yeast.

Despite certain similarities in physical, chemical, and enzymatic properties among the *Neurospora*, rat, human, and yeast enzymes, these enzymes exhibit obvious differences, indicating species specificity. Some differences in primary structures, the failure of a polyclonal antibody to the yeast enzyme to recognize the *Tetrahymena* and human enzymes, the differences in the levels of inhibition by the substrate analogues depending on the enzyme source, and the different kinetic parameters are clear. The K_m value for NAD^+ of the yeast enzyme (400 μM) is very high compared with that of the rat (40 μM) and human (4.8 μM) enzymes. It would be interesting to know how the different K_m values for NAD^+ effectively function in the deoxyhypusine synthesis mechanism and its control in eukaryotes.

In addition to the various characteristic features of the yeast deoxyhypusine synthase revealed in this study, the availability of the recombinant enzyme and the ability to manipulate the deoxyhypusine synthase gene using proven techniques (*1*) should provide a powerful basis for future studies on the molecular mechanisms of the biosynthesis of deoxyhypusine (also hypusine) and control of the expression of this essential gene.

We wish to thank Dr. Masahiro Sugiura of the Center for Gene Research of this University for allowing us to use FPLC apparatus and Dr. Masami Suzuki of the Institute for Comprehensive Medical Science of Fujita Health University for his skillful amino acid sequencing.

REFERENCES

1. Sasaki, K., Abid, M.R., and Miyazaki, M. (1996) Deoxyhypusine synthase gene is essential for cell viability in the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **384**, 151-154
2. Park, M.H., Wolff, E.C., and Folk, J.E. (1993) Hypusine: its post-translational formation in eukaryotic initiation factor 5A and its potential role in cellular regulation. *BioFactors* **4**, 95-104
3. Park, M.H. and Wolff, E.C. (1988) Cell-free synthesis of deoxyhypusine. *J. Biol. Chem.* **263**, 15264-15269
4. Murphey, R.J. and Gerner, E.W. (1987) Hypusine formation in protein by a two-step process in cell lysates. *J. Biol. Chem.* **262**, 15033-15036
5. Smit-McBride, Z., Schnier, J., Kaufman, R.J., and Hershey, J.W.B. (1989) Protein synthesis initiation factor eIF-4D. *J. Biol. Biochem.* **264**, 18527-18530
6. Kang, H.A. and Hershey, J.W.B. (1994) Effect of initiation factor on protein synthesis and proliferation of *S. cerevisiae*. *J. Biol. Chem.* **269**, 3934-3940
7. Schnier, J., Schwelberger, H.G., Smit-McBride, Z., Kang, H.A., and Hershey, J.W.B. (1991) Translation initiation factor 5A and its hypusine modification are essential for cell viability in the yeast *S. cervisiae*. *Mol. Cell. Biol.* **11**, 3105-3114
8. Park, M.H., Wolff, E.C., Lee, Y.B., and Folk, J.E. (1994) Antiproliferative effects of inhibitors of deoxyhypusine synthase. *J. Biol. Chem.* **269**, 27827-27832
9. Byers, T.L., Ganem, B., and Pegg, A.E. (1992) Cytostasis induced in L1210 murine leukaemia cells by the *S*-adenosyl-L-methionine decarboxylase inhibitor 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine may be due to hypusine depletion. *Biochem. J.* **287**, 717-724
10. Tao, Y. and Chen, K.Y. (1995) Purification of deoxyhypusine synthase from *Neurospora crassa* to homogeneity by substrate elution chromatography. *J. Biol. Chem.* **270**, 383-386
11. Wolff, E.C., Lee, Y.B., Chung, S.I., Folk, J.E., and Park, M.H. (1995) Deoxyhypusine synthase from rat testis: purification and characterization. *J. Biol. Chem.* **270**, 8660-8666
12. Klier, H., Csonga, R., Steinkasserer, A., Wöhl, T., Lottspeich, F., and Eder, J. (1995) Purification and characterization of human deoxyhypusine synthase from HeLa cells. *FEBS Lett.* **364**, 207-210
13. Joe, Y.A., Wolff, E.C., and Park, M.H. (1995) Cloning and expression of human deoxyhypusine synthase cDNA. *J. Biol. Chem.* **270**, 22386-22392
14. Kitaoka, Y. and Miyazaki, M. (1990) Sequence determination of cDNA encoding yeast cycloheximide sensitivity factor and its plausible role at a first peptide bond formation step in the initiation process of protein synthesis. *Nucleic Acids Res.* (Symposium series) No. 22, 69-70
15. Schwelberger, H.G., Kang, H.A., and Hershey, J.W.B. (1993) Translation initiation factor eIF-5A expressed from either of two yeast genes or from human cDNA. *J. Biol. Chem.* **268**, 14018-

14025
16. Lee, I.S.M., Takio, K., Kido, R., and Titani, K. (1994) Purification and amino- and carboxyl-terminal amino acid sequences of alanine-glyoxylate transaminase from human liver. *J. Biochem.* **116**, 12-17
17. Dou, Q.-P. and Chen, K.Y. (1990) Characterization and reconstitution of a cell free system for NAD^+-dependent deoxyhypusine formation on the 18 kDa eIF-4D precursor. *Biochim. Biophys. Acta* **1036**, 128-137
18. Jakus, J., Wolff, E.C., Park, M.H., and Folk, J.E. (1993) Features of the spermidine-binding site of deoxyhypusine synthase as derived from inhibition studies. *J. Biol. Chem.* **268**, 13151-13159
19. Tao, Y., Skrenta, H.M., and Chen, K.Y. (1994) Deoxyhypusine synthase assay based on the use of polyhistidine-tagged substrate and metal chelate-affinity chromatography. *Anal. Biochem.* **221**, 103-108
20. Kang, K.R., Wolff, E.C., Park, M.H., Folk, J.E., and Chung, S.I. (1995) Identification of YHR068w in *S. cerevisiae* chromosome VIII as a gene for deoxyhypusine synthase. *J. Biol. Chem.* **270**, 18408-18412
21. Tao, Y. and Chen, K.Y. (1994) PCR-based cloning of the full-length *Neurospora* eukaryotic initiation factor 5A cDNA: polyhistidine-tagging and overexpression for protein affinity binding. *Biochem. J.* **302**, 517-525