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Characterization of yeast deoxyhypusine synthase: PKC-dependent phosphorylation *in vitro* and functional domain identification

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Abbreviations: PKC, protein kinase C; eIF-5A, eukaryotic initiation factor 5A; PMA, phorbol 12-myristate 13-acetate

Abstract

The biosynthesis of hypusine [N^{ε}-(4-amino-2-hydroxybutyl)-lysine] occurs in the eIF-5A precursor protein through two step posttranslational modification involving deoxyhypusine synthase which catalyzes transfer of the butylamine moiety of spermidine to the ε-amino group of a designated lysine residue and subsequent hydroxylation of this intermediate. This enzyme is exclusively required for cell viability and growth of yeast (Park, M.H. *et al.*, *J. Biol. Chem.* 273: 1677-1683, 1998). In an effort to understand structure-function relationship of deoxyhypusine synthase, posttranslational modification(s) of the enzyme by protein kinases were carried out for a possible cellular modulation of this enzyme. And also twelve deletion mutants were constructed, expressed in *E. coli* system, and enzyme activities were examined. The results showed that deoxyhypusine synthase was phosphorylated by PKC *in vitro* but not by $p56^{lck}$ and $p60^{c\text{-}src}$. Treatment with PMA specifically increased the relative phosphorylation of the enzyme supporting PKC was involved. Phosphoamino acid analysis of this enzyme revealed that deoxyhypusine synthase is mostly phosphorylated on serine residue and weakly on threonine. Removal of Met^{1}-Glu^{10} (ΔMet^{1}-Glu^{10}) residues from amino terminal showed no effect on the catalytic activity but further deletion (ΔMet^{1}-Ser^{20}) caused loss of enzyme activity. The enzyme with internal deletion, ΔGln^{197}-Asn^{212} (residues not present in the human enzyme) was found to be inactive. Removal of 5 residues from carboxyl terminal, ΔLys^{383}-Asn^{387}, retained only slight activity. These results suggested that deoxyhypusine synthase is substrate for PKC dependent phosphorylation and requires most of the polypeptide chains for enzyme activity except the first 15 residues of N-terminal despite of N- and C-terminal residues of the enzyme consist of variable regions.

Keywords: deoxyhypusine synthase, PKC, phosphorylation, deletion mutation

Introduction

The biosynthesis of hypusine [N^{ε}-(4-amino-2-hydroxybutyl)-lysine] occurs exclusively in one cellular protein, the precursor of eukaryotic translation initiation factor 5A (eIF-5A) through a unique two-step posttranslational modification (Park *et al.*, 1993a; Park *et al.*, 1993b). In the first step, deoxyhypusine synthase catalyzes NAD-dependent transfer of the butylamine moiety of the polyamine spermidine to the ε-amino group of a specific lysine residue of eIF-5A precursor (Lys^{51} in the yeast protein) to form a deoxyhypusine [N^{ε}-(4-aminobutyl) lysine] residue (Park *et al.*, 1982; Wolff *et al.*, 1995). Subsequent hydroxylation of this intermediate by deoxyhypusine hydroxylase completes hypusine conformation and eIF-5A maturation (Park *et al.*, 1993a; Park *et al.*, 1993b).

Several lines of evidence support the fact that eIF-5A and its hypusine modification play a pivotal role in eukaryotic cell proliferation (Park *et al.*, 1993a; Park *et al.*, 1993b). In the yeast *Saccharomyces cerevisiae,* inactivation of eIF-5A gene (Schnier *et al.*, 1991; Wöhl *et al.*, 1993) or of the deoxyhypusine synthase gene (Park *et al.*, 1998) results in the loss of cell viability. In mammalian cells, inhibitors of either deoxyhypusine synthase (Park *et al.*, 1994) or deoxyhypusine hydroxylase (Hanauske-Abel *et al.*, 1994) exert antiproliferative effects. The arrest in cell proliferation by inhibitors of polyamine biosynthetic enzymes has been attributed to depletion of eIF-5A following depletion of spermidine (Byers *et al.*, 1992).

Deoxyhypusine synthase has been purified from rat testis (Wolff *et al.*, 1995), *Neurospora crassa* (Tao and Chen, 1995a), HeLa cells (Klier *et al.*, 1995), and yeast (Sasaki *et al.*, 1996). Human (Joe *et al.*, 1995; Tao and Chen, 1996) and *N. crassa* (Tao and Chen, 1995b) cDNAs for the enzyme have been cloned, and its gene has been identified (Kang *et al.*, 1995; Klier *et al.*, 1995; Tao and Chen, 1995b) and cloned (Sasaki *et al.*, 1996)

in yeast. The amino acid sequence of deoxyhypusine synthase is highly conserved and the native enzymes consist of tetramers of four identical subunits of 40 to 43 kDa, depending on the species.

Protein synthesis consumes a significant proportion of the available energy of eukaryotic cells. Rates of protein synthesis are regulated, thereby integrating the translation process with other metabolic pathways of the cell. In many cases, translation rates change within minutes of the inductive events and are readily reversed, suggesting that the regulatory mechanisms involve rapid changes in the specific activities of the protein synthesis machinery rather than changes in the cellular concentration of any of its components. Covalent modification of proteins by phosphorylation is well known to control many metabolic pathways and may likewise regulate translation rates (Hershey, 1989). Initiation stage, especially in translational machinery, is regarded as the most commonly observed target for physiological control. Several of the initiation factors are phosphoproteins but the clearest links between phosphorylation and the regulation of translation concern the factors eIF-2 and eIF-4E (Rhoads, 1993; Pain, 1996).

eIF-5A, the unique hypusine-containing initiation factor, is implicated in the final step of the initiation phase of protein synthesis (Benne *et al*., 1978). Besides hypusine modification, eIF-5A in yeast is phosphorylated on serine. Therefore eIF-5A undergoes two posttranslational modifications, hypusination and phosphorylation, where the activity of the factor is dependent on the first but is not influenced *in vitro* by the second. However, hypusine formation in eIF-5A is not reversed when rates or specificity of protein synthesis is altered (Gordon *et al*., 1987). Therefore another reversible modification for the regulation of eIF-5A activity may be implicated. Deoxyhypusine synthase, the first enzyme involved in hypusination, could be the primary target of the regulation of the factor.

We undertook a study to provide insight into the minimal structural requirements for the activity of yeast deoxyhypusine synthase through deletion mutation. Our data as well as those of Abid *et al*. (1997), reported while this work was in progress, indicate that even though the N- and C-terminal residues of the yeast deoxyhypusine synthase are outside the highly conserved regions, they play an important role in the activity of the yeast enzyme. In addition, study of internal deletion mutation, designed on the basis of a comparison of the amino acid sequences of the yeast and human deoxyhypusine synthases, provide insight into the structure-function relationship of the enzyme. Recently, we have identified that deoxyhypusine synthase essential for irreversible hypusine formation was phosphorylated by PKC *in vitro*. In this study, we report phosphorylation modification of yeast recombinant deoxyhypusine synthase and structural requirement of the enzyme through sequential deletion of non-conserved sequence segment of the deoxyhypusine synthase.

Materials and Methods

Materials

[1,8-^{3}H]Spermidine · HCl (15 Ci/mmol) and [γ-^{32}P]ATP (5,000 Ci/mmol) were purchased from New England Nuclear. Oligonucleotide primers were synthesized by Genotech, Korea. pET-11a expression vector and the host *Escherichia coli* B strain BL21(DE3) were from Novagen; *Taq* polymerase, T4 DNA ligase, restriction enzymes, casein kinase II (CKII), and PKC from Boehringer mannheim; p60$^{c\text{-}src}$ and p56^{lck} from Upstate Biotechnology; cellulose thin-layer chromatography (TLC) plates (without fluorescent indicator) from Merck. PMA was obtained from Sigma, and other chemicals were purchased as described in the text. Yeast eIF-5A precursor proteins expressed in *E. coli* were purified from *E. coli* lysates after overexpression of the yeast eIF-5A cDNAs, are described previously (Kang *et al*., 1995).

In vitro phosphorylation

The phosphorylation reaction of yeast recombinant deoxyhypusine synthase was carried out at 37°C for 30 min with various kinases such as CKII, PKC, p56^{lck}, and p60$^{c\text{-}src}$, in a total reaction volume of 25 μl containing kinase buffer (20 mM Tris, pH 7.5, 10 mM magnesium acetate, 0.4 mM $CaCl_2$, and 1 mM dithiothreitol) in the presence of [γ-^{32}P]ATP. For time course phosphorylation, yeast deoxyhypusine synthase was reacted for 1, 10, 30, and 60 min. In the case of PMA treatment, up to 10 μM of PMA were used to stimulate the phosphorylation reaction. The reaction was terminated by mixing with SDS sample buffer, boiled, and run on a 10% SDS-polyacrylamide gel. The gel was stained in 0.125% Coomassie blue and destained in a solution of 40% methanol/10% glacial acetic acid. After drying, the gel was exposed to a film to visualize radiolabeled proteins.

Phosphoamino acid analysis

To analyze phosphoamino acid of yeast deoxyhypusine synthase, purified protein was incubated with PKC in the presence of [γ-^{32}P]ATP. Phosphorylated deoxyhypusine synthase was separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to Immobilon (Millipore) (Kamps and Sefton, 1989; Duclos *et al*., 1991). The Immobilon membrane was wet in methanol for 10 min and then incubated in transfer buffer (193 mM glycine, 25 mM Tris/base, 0.1% SDS, and 20% methanol) for 30 min. Transfer to the membrane was carried out using transfer buffer at 150 mA for 2 h. Proteins bound to Immobilon were visualized by staining with India ink for 1 h. The membrane was rinsed with several changes of

deionized water, and the rinsed membrane was then dried and subjected to autoradiography. ^{32}P-labeled deoxyhypusine synthase band was excised, transferred to screwcap microfuge tube, and hydrolyzed at 110°C for 2 h in 6 N HCl under nitrogen gas. After hydrolysis, the tubes were centrifuged for 5 min and the supernatant was transferred to a new tube. The aqueous hydrolysate was dried in a speedvac concentrator, dissolved in pH 1.9 buffer (acetic acid/formic acid/water, 78 : 25 : 897, v/v/v) containing 3 μg each of phosphoserine, phosphothreonine, and phosphotyrosine. Two-dimensional electrophoresis/chromatography was performed on cellulose TLC plate. Electrophoresis was done at pH 1.9 buffer at 2.5 kV for 25 min (first dimension) followed by ascending chromatography in isobutyric acid/0.5 M ammonium hydroxide (5 : 3, v/v) (second dimension). Autoradiography was performed at -70°C using intensifying screen. Standards were visualized by ninhydrin staining.

In vitro assay of deoxyhypusine synthesis activity

The enzyme activity was measured as described previously (Kang *et al.*, 1995). A typical reaction mixture contained, in a total volume of 20 μl, 0.2 M glycine-NaOH buffer, pH 9.5, 1 mM dithiothreitol, 5 μg of bovine serum albumin, 1 mM NAD, 7-9 μM (2-5 μCi) [1,8-^{3}H] spermidine, 10 μM human or yeast eIF-5A precursor protein, and wild type or mutant enzyme. Incubations were at 37°C for 60 min. After trichloroacetic acid precipitation, the precipitate was washed three times with 10% trichloroacetic acid containing putrescine, spermidine, and spermine (1 mM each), dissolved in 0.4 ml of 6 N HCl and hydrolyzed at 105°C for 16 h. The [^{3}H]deoxyhypusine formed was measured after its separation from other components of the acid hydrolyzed protein fraction by ion exchange chromatography as described previously (Wolff *et al.*, 1990). One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 pmol h^{-1} of deoxyhypusine.

Construction of deletion recombinant subclones of yeast deoxyhypusine synthase cDNA and their expression in *E. coli*

On the basis of the nucleotide sequence of the full length yeast deoxyhypusine synthase, the mutant subclones with a truncated N-terminus or a truncated C-terminus, were generated using synthetic oligonucleotide primers (Table 1). A mutant cDNA with an internal deletion was constructed by a two-step PCR procedure. In the first step, DHS M1 and DHS M2 cDNAs were generated in two separate reactions (Table 2). The PCR products of the expected size were isolated from an agarose gel, and the mixture of the two amplified fragments was used as template in the second PCR step to construct DHS M3. These mutants were cloned in the pET-11a vector and confirmed by sequencing.

Table 1. Nucleotide sequences of primers used in PCRs

Forward primers	
Primer N0 (1-8) (for WT)	cttccagtatgctcatATG TCC GAT ATC AAC GAA AAA CTC
Primer N1 (6-12)	cttccagtatgctcatATG GAA AAA CTC CCA GAG TTA CTA
Primer N2 (11-17)	cttccagtatgctcatATG TTA CTA CAA GAC GCT GTC TTG
Primer N3 (16-22)	cttccagtatgctcatATG GTC TTG AAA GCA TCT GTT CCT
Primer N4 (21-27)	cttccagtatgctcatATG GTT CCT ATT CCA GAT GAC TTC
Primer N5 (26-32)	cttccagtatgctcatATG GAC TTC GTT AAG GTT CAA GGT
Primer M1 (190-196, 213-221)	TTG GAT AAG ATG TTG GAA GAA CAA GAC GTG GAT TCA CCA ATC TGG ACC
Reverse primers	
Primer C0 (387-381) (for WT)	cttccagtatggatccTCA ATT CTT AAC TTT TTT GAT TGG
Primer C1 (382-376)	cttccagtatggatccTCA GAT TGG TTT ACC ACT GGC AAA
Primer C2 (377-371)	cttccagtatggatccTCA GGC AAA GGT AGC AGC AAC AAT
Primer C3 (372-366)	cttccagtatggatccTCA AAC AAT CAA TGG AAG AAC AGT
Primer C4 (367-361)	cttccagtatggatccTCA AAC AGT GGT GAC ATC AGC AAA
Primer M2 (219-213, 196-188)	GAT TGG TGG ATC CAC GTC TTG TTC TTC CAA CAT CTT ATC CAA AAT TGG

The numbers in parentheses indicate the amino acid residue numbers of the coding or complementary sequences of yeast deoxyhypusine synthase; these sequences are denoted in upper-case letters. The *Nde* I sites for the forward primers, and the *Bam* HI sites for the reverse primers are underlind.

Table 2. Construction of full-length and deleted deoxyhypusine synthase cDNAs by PCR and the activities of the recombinant proteins

Recombinant subclones	Recombinant protein	Coding region	Activity
Full-length clone	WT	1–387	++++
5′ deletion clones	N1	6–387	++++
	N2	11–387	++++
	N3	16–387	+
	N4	21–387	-
	N5	26–387	-
3′ deletion clones	C1	1–382	+
	C2	1–377	-
	C3	1–372	-
	C4	1–367	-
Internal deletion clone	M1	1–197, 212–387	n.d.
	M2	1–197, 212	n.d.
	M3	1–197, 212–387	-

In the schematic representation, yeast coding sequences are black boxes, and the deleted regions are white boxes. In the case 5'-terminal deletions, methionine was artificially introduced as the NH_2-terminal amino acid. The numbers indicate the amino acid residue number in the yeast coding sequence. n.d., not determined; M, mutant; WT, wild type; N, NH_2-terminus; C, COOH-terminus.

Expression of the deletion mutations of yeast deoxy-

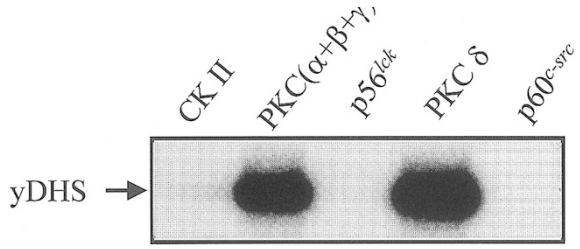

Figure 1. Phosphorylation of yeast recombinant deoxyhypusine synthase (DHS) by various kinases. Purified yeast recombinant deoxyhypusine synthase was incubated with different kinases; casein kinase II (lane 1), protein kinase C α+β+γ mixture (lane 2), $p56^{lck}$(lane 3), protein kinase C δ (lane 4), and $p60^{c\text{-}src}$(lane 5), respectively, in the presence of [γ-^{32}P]ATP. After drying of the SDS-polyacrylamide gel, autoradiography was performed with intensifying screen at -70°C. Phosphorylated DHS with molecular mass of 44 kD was indicated by arrow. yDHS denotes yeast deoxyhypusine synthase.

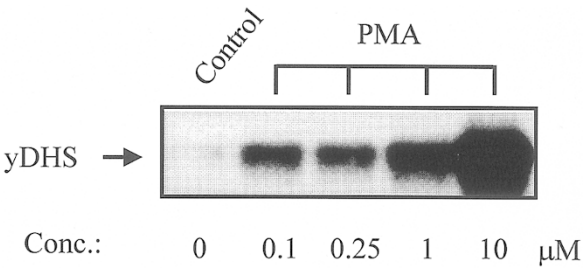

Figure 2. PMA (phorbol 12-myristate 13-acetate) stimulates the phophorylation of yeast recombinant deoxyhypusine synthase by PKC *in vitro*. Deoxyhypusine synthase was incubated with [γ-^{32}P]ATP and PKC in the absence (lane 1), or presence of 0.1 μM (lane 2), 0.25 μM (lane 3), 1 μM (lane 4), and 10 μM (lane 5) PMA, respectively. Phosphorylated DHS was visualized by SDS-PAGE followed by autoradiography. Conc., concentration.

hypusine synthase in *E. coli* and purification of the altered recombinant proteins were carried out as described earlier (Kang *et al*., 1995).

Results

Yeast deoxyhypusine synthase contains multiple potential phosphorylation sequence motifs for CKII, PKC, and tyrosine kinases. This suggests that yeast deoxyhypusine synthase could be phosphorylated on tyrosine as well as serine and threonine. To examine whether these kinases are involved in deoxyhypusine synthase phosphorylation, purified yeast recombinant deoxyhypusine synthase (Kang, *et al*., 1995) was incubated with different kinases. Deoxyhypusine synthase was phosphorylated by PKC isoforms, α + β + γ mixure or δ, *in vitro* (Figure 1, lane 2 and 4). Although CKII, a typical Ser/Thr kinase, was able to phosphorylate yeast enzyme protein, but the level of phosphorylation was significantly lower than that of PKC (Figure 1, lane 1). Other tyrosine kinases such as $p56^{lck}$ and $p60^{c\text{-}src}$, however, were not involved in the phosphorylation reaction (Figure 1, lane 3 and 5).

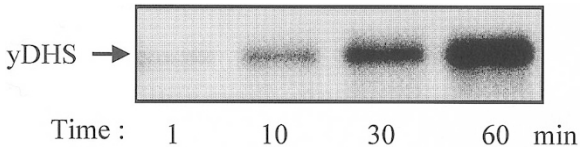

Figure 3. Time-dependent phosphorylation of yeast recombinant deoxyhypusine synthase by PKC. The kinase reaction of yeast enzyme was carried out at 37°C for various times in a total reaction volume of 25 μl containing kinase buffer. The reaction was terminated by mixing with SDS sample buffer, boiled, and run on a 10% SDS-PAGE. After staining with Coomassie blue, the gel was dried and exposed to a X-ray film.

PMA, tumor promoter, stimulates the phosphorylation of yeast deoxyhypusine synthase by PKC *in vitro*. As shown in Figure 2, the amount of ^{32}P-incorporation into deoxyhypusine synthase started to increase at 0.1 μM PMA (5-folds), and 10 μM treatment of this tumor promoter increased deoxyhypusine synthase 15-folds. This result reveals that deoxyhypusine synthase was stimulated by 0.1 μM PMA and increased up to 10 μM treatment. Additionally, the maximal stimulatory effect with PMA was within 1 h (data not shown). Figure 3 shows that yeast deoxyhypusine synthase was phosphorylated in a time-dependent manner by PKC *in vitro*. Phosphoamino acid analysis of yeast deoxyhypusine synthase phosphorylated by PKC revealed that this enzyme was phosphorylated mainly on serine, and trace amount of ^{32}P-incorporation into threonine was visible (Figure 4). But no detectable spot on tyrosine in

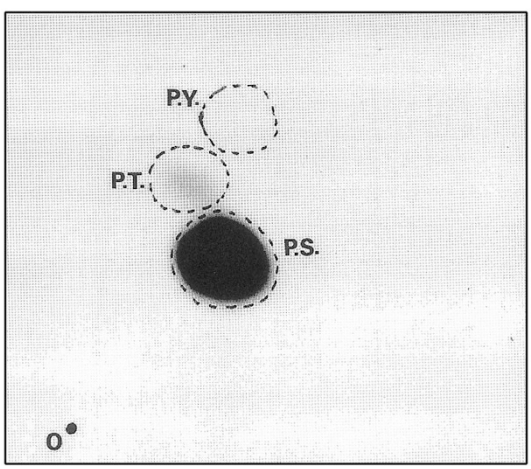

Figure 4. Phosphoamino acid analysis of yeast DHS phosphorylated by PKC. ^{32}P-labeled DHS by PKC was separated by SDS-PAGE, and tranferred to Immobilon membrane. The membrane piece corresponding to DHS was hydrolyzed under vacuum in 6 N HCl at 110°C for 2 h, and dried in speedvac concentrator. Dried sample was subjected to electrophoresis at pH 1.9 in acetic acid/formic acid/water (78 : 25 : 897, v/v/v) at 2.5 kV for 25 min (first dimension) followed by ascending chromatography in isobutyric acid/0.5 M ammonium hydroxide (5 : 3) (second dimension). Autoradiography was performed for 5 h at -70°C. Standard phosphoamino acids were visualized by ninhydrin staining. P.S., P.T., and P.Y. denote phosphoserine, phosphothreonine, and phosphotyrosine, respectively, and O denotes origin for sample.

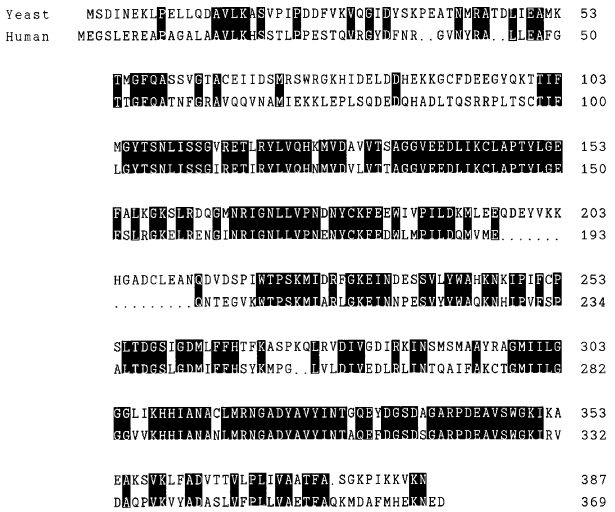

Figure 5. Comparison of amino acid sequences of yeast and human deoxyhypusine synthase. The amino acid sequences deduced from yeast cDNA are compared with that of human. To allow for maximal alignment of the yeast and human sequences, gaps have been introduced (·····). The amino acid residue numbers for the yeast and human sequences are indicated on the right. Identical amino acid residues are boxed (Joe *et al.*, 1995).

TLC plate was identified. When the enzyme was tested for the possible alteration of deoxy hypusine synthase activity during the phosporylation processes by PKC, there was no observable changes in enzyme activity.

In order to assess the minimal structural requirements for the activity of the yeast deoxyhypusine synthase, we designed mutant clones with serial deletions from the NH_2-terminus or from the COOH-terminus. As shown in Table 2, lysates of *E.coli* cells overexpressing recombinant proteins with truncation of either 5 or 10 amino acids from the N-terminus was found to display same enzyme activity with that of wild type enzyme. Deletion of 15 amino acids from the N-terminus drastically reduced enzyme activity, and 20 amino acids or more deletion showed no enzyme activity. Only 10 amino acid deletion from the C-terminus of the enzyme, however, exhibited no enzyme activity.

In an attempt to examine the basis of significant difference in the K_m value for NAD for the yeast and human enzyme, a mutant clone was generated with deletion of internal 16 amino acids of the yeast sequence (Gln^{197}-Asn^{212}, not present in the human enzyme) (Figure 5 and Table 2). *E. coli* lysate of the resultant mutant clone was isolated by Mono Q ion-exchange column chromatography (data not shown), displayed no detectable enzyme activity (Table 2).

Discussion

Deoxyhypusine synthase is a bifunctional enzyme that catalyzes the NAD-dependent oxidative cleavage of spermidine and the subsequent transfer of an aminobutyl moiety to a specific lysine residue of the eIF-5A precursor (Park *et al.*, 1993a: Park *et al.*, 1993b). We have identified and functionally expressed yeast deoxyhypusine synthase cDNA (Kang *et al.*, 1995). The yeast recombinant deoxyhypusine synthase enzyme is a 387-amino acid protein. This enzyme is a homotetramer consisting of 43 kDa subunits in its native form. Deoxyhypusine synthase exhibits a remarkable substrate specificity in its recognition of a single lysine residue of the eIF-5A precursor. eIF-5A is highly conserved in a wide range of eukaryotic species, and the amino acid sequence identity is especially high in the region surrounding the lysine residue that undergoes modification to hypusine (Park *et al.*, 1993a). Furthermore, a large portion of the substrate protein is required for recognition and modification by deoxyhypusine (Joe and Park, 1994).

Hypusine modification is essential for eIF-5A activity; the modification is actually irreversible. However, eIF-5A must be regulated reversibly because this protein is one of important translation initiation factors needed for synthesizing other cellular proteins. The most promising target for reversible regulation of eIF-5A is deoxyhypusine synthase, the first enzyme involved in its hypusination. Recently, we have searched for the primary structure of yeast deoxyhypusine synthase, and found to have motifs for multiple potential phosphorylation modification. On the basis of this finding, yeast recombinant deoxyhypusine synthase was used for *in vitro* phosphorylation with various kinases such as CKII, PKC, $p56^{lck}$, and $p60^{c\text{-}src}$. As results indicated that PKC is a powerful candidate for *in vivo* phosphorylation.

Protein kinase C denotes a family of isozymes that play a central role in signal transduction in eukaryotes. PKC is the major cellular receptor for the tumor-promoting phorbol esters, which activate PKC in a manner very similar to diacylglycerol (Zhang *et al.*, 1995). Treatment with PMA specifically increased the relative phosphorylation of deoxyhypusine synthase in yeast and also in human (data not shown), supporting PKC was implicated in the control of this system. The data that yeast recombinant deoxyhypusine synthase was phosphorylated in a time-dependent manner by PKC may suggest that several PKC phosphorylation motifs in the deoxyhypusine synthase protein could exist and participate in an allosteric enhancement of phosporylation (Jung, *et al.*, 1997). Although there were no previous report on the regulation of deoxyhypusine synthase by PKC-mediated phosphorylation in the cell, our findings of PKC catalyzed phosporylation of yeast deoxyhypusine synthase *in vitro* raises such possibility *in vivo.* However, we observed no direct evidence of close correlation between the phosphorylation state and the activity of deoxyhypusine synthase.

Our results of structural requirements for the activity of yeast recombinant deoxyhypusine synthase were con-

sistent with those of Abid *et al.* (1997). Truncations of 20 NH_2-terminal amino acids (Met^1-Ser^{20}) or 10 COOH-terminal amino acids (Ser^{378}-Asn^{387}) were devoid of enzymatic activity. These findings suggest that even though the NH_2- and COOH-terminal residues of deoxyhypusine synthase are not highly conserved, these regions also contain amino acid sequences important for the proper conformation of the enzyme in monomeric unit stability and/or for tetramer formation necessary for binding substrates and for catalysis. The most striking difference between the primary structures of the yeast and human enzymes is no existence of a human counterpart matching Gln^{197}-Asn^{212} in the yeast enzyme (Figure 5) (generated by the program GCG "BestFit" [Genetics Computer Group, Madison, WI]). Unexpectedly, a mutant protein with this internal 16-amino acid deleted yeast sequence displayed no enzyme activity. Since deletion of yeast sequence (Gln^{197}-Asn^{212}) not present in the human enzyme did not result in a dramatic decrease in its K_m value for NAD, one cannot attribute the large differences in affinities toward NAD specifically to this region of the molecule. On the other hand, it is intriguing that this deletion have a significantly detrimental effect on the yeast enzyme activity. However, it could be possible that there is another alignment of the yeast deoxyhypusine synthase amino acid sequence from plant tobacco, human, yeast, *N. crassa*, and the archaebacterium *M. Jannaschii* (Ober and Hartmann, 1999) using different software ESPript analysis (Gouet *et al*., 1999). According to this result, Gln at 193 of the tobacco deoxyhypusine synthase is well conserved from archaebacterium to human in all species studied. Especially, Gln residue of the yeast deoxyhypusine synthase corresponding to Gln^{193} of the tobacco enzyme in sequence alignment is identical to Gln^{197} deleted in M3 mutant protein (Figure 5 and Table 2). Therefore, it remain to be elucidated whether the loss of activity in internal deletion clone of the yeast enzyme is due to absence of Gln^{197}, highly conserved amino acid located in the mid portion of the enzyme.

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