Biochimica et Biophysica Acta, 1036 (1990) 128–137
Elsevier

BBAGEN 23420

Characterization and reconstitution of a cell free system for NAD^+-dependent deoxyhypusine formation on the 18 kDa eIF-4D precursor

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(Received 15 January 1990)

Key words: Deoxyhypusine formation; NAD^+ dependence; Post-translational modification

Deoxyhypusine formation on the 18 kDa eIF-4D precursor is due to a covalent linkage between a lysine residue of the protein and the aminobutyl moiety derived from spermidine. The deoxyhypusine is then hydroxylated to form hypusine. This post-translational modification represents one of the most specific spermidine-dependent biochemical events in eukaryotic cells. Deoxyhypusine formation can be performed in vitro at pH 9.5 and is greatly stimulated by NAD^+. Using the labeling of the 18 kDa protein by [^{3}H]spermidine as an assay for deoxyhypusine formation, we found that (i) significant deoxyhypusine formation can be demonstrated in vitro at pH 7.2 only if NAD^+ is present, (ii) deoxyhypusine formation was sensitive to buffer composition; buffers made of basic amino acids and Tris were inhibitory, (iii) sulfhydryl reagents and metal ions such as Cu^{2+} and Fe^{3+} were potent inhibitors of deoxyhypusine formation and (iv) the 18 kDa protein substrate was heat-stable. The in vitro activity of deoxyhypusine formation, which depends on the presence of both enzyme and protein substrate, can be separated from the product, eIF-4D, by a one-step Cibacron blue dye affinity column. Taking advantage of this finding, we have developed a simple procedure, based on the use of Cibacron blue dye, for partially purifying both the deoxyhypusine-forming enzyme and the 18 kDa protein substrate. When the partially purified enzyme and protein substrate were mixed in the presence of 1 mM NAD^+ and [^{3}H]spermidine, the 18 kDa protein was radiolabeled, no labeling could be detected if any one component was absent. Using partially purified enzyme, we have also determined the half-life of the protein substrate in α-difluoromethyl ornithine (DFMO)-treated NB-15 cells and found it to be longer than 10 h.

Introduction

Hypusine formation on an 18 kDa cellular protein has been demonstrated in various mammalian cells by metabolic labeling with [^{3}H]putrescine or [^{3}H]spermidine [1–4]. This unique post-translational modification involves two steps: (i) the transfer of the butylamino moiety from spermidine to the ε-amino group of a lysine residue to form deoxyhypusine and (ii) the hydroxylation of the deoxyhypusine to form hypusine [1,5]. The first step is catalyzed by deoxyhypusine-forming enzyme, abbreviated as E_1 in this paper, and the second step is catalyzed by deoxyhypusine hydroxylase [6]. Metabolic labeling of the 18 kDa protein in cultured cells is generally increased by serum- or mitogen-treatment [3,5]. Both the differentiation of mouse neuroblastoma cells and the aging of human diploid fibroblasts have been shown to be accompanied by a decrease of metabolic labeling of the 18 kDa protein [2,7]. Hypusine formation also occurs in lower eukaryotes such as yeast [8] and *Neurospora crassa* [9,10]. The highly conserved nature, the unique dependence on spermidine and the responsiveness to growth stimulation of hypusine formation suggest that it may have important physiological function(s) in eukaryotic cells.

Murphey and Gerner [11] have shown deoxyhypusine formation in cell lysates of DFMO-treated HTC cells. Due to tight coupling of the synthesis and modification of 18 kDa protein [12,13], prior treatment with DFMO, a suicide inhibitor of polyamine biosynthetic enzyme ornithine decarboxylase, was found to be necessary for

Abbreviations: eIF-4D, eukaryotic initiation factor 4D; DFMO, α-difluoromethyl ornithine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; dansyl, 5-dimethylaminonaphthalenesulfonyl; PBS, phosphate-buffered saline.

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cells to accumulate the unmodified protein substrate for in vitro labeling [11]. They also found that deoxyhypusine formation in vitro is highly pH dependent and can not occur at pH below 8 [11]. Based on their work, we have found that NAD^+ significantly stimulated both the rate and magnitude of labeling of 18 kDa protein in cytosolic lysates derived from DFMO-treated mouse neuroblastoma cells [14]. In order to gain more insights on the biochemical properties of the protein components involved in deoxyhypusine formation, we have examined the effects of pH, buffer composition, putative effectors and temperature on deoxyhypusine formation. Since we have previously reported that metabolically labeled 18 kDa hypusine-containing protein binds very tightly to Cibacron blue dye [15], we also examined the binding affinity of the protein substrate to this dye in the present study. We found that Cibacron blue dye did not bind to either 18 kDa protein substrate or the enzyme E_1 when both were present in lysates of DFMO-treated cells. However, once these two proteins were separated from each other by gel filtration, both of them gained affinity to Cibacron blue dye. This finding enabled us to develop a procedure to isolate both the protein substrate and its modifying enzyme E_1 from DFMO-treated NB-15 mouse neuroblastoma cells. Using partially purified enzyme and protein substrate we showed that the labeling of the 18 kDa protein substrate by [^{3}H]spermidine in the reconstituted system exhibited an absolute requirement for NAD^+ at physiological pH. Since the 18 kDa protein was found to be heat stable, we have also determined the relative amount of 18 kDa protein in heated lysates by in vitro labeling assay and estimated the half-life of the protein substrate in DFMO-treated NB-15 cells to be longer than 10 h.

Deoxyhypusine formation represents, by far, one of the most specific polyamine-dependent biochemical reactions in eukaryotic cells. In view of the importance of polyamines in growth regulation [16], the present study should facilitate our further understanding of the biochemistry and function of proteins involved in deoxyhypusine formation.

Experimental procedures

Materials. Tissue culture media, sera and Earle's balanced salt solution were obtained from Gibco, Grand Island, NY. [2,3-^{3}H]Putrescine dihydrochloride (30 Ci/mmol), [terminal methylenes-^{3}H]spermidine trihydrochloride (40.1 Ci/mmol) and Enhancer were purchased from NEN, DuPont Research Products, Boston, MA. Reactive Blue −2 Sepharose CL-6B (Cibacron blue dye), Sephadex G-50, NAD^+, NADH and other nucleotides were from Sigma, St. Louis, MO. α-Difluoromethyl ornithine (DFMO) was a generous gift from Merrell Dow Research Center, Cincinnati, OH. Synthetic hypusine was kindly given by T. Shiba, Osaka University, Japan.

Cell culture and lysate preparation. NB-15 mouse neuroblastoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum [2]. At 80–90% confluence, the cultures were replenished with fresh growth medium with or without 5 mM DFMO and incubated for another 48 h. Cells were then washed with phosphate-buffered saline (PBS, 50 mM sodium phosphate, 0.15 M NaCl, pH 7.2) and resuspended in an appropriate buffer as indicated in figure legends. The cell suspension was homogenized by a brief sonication at 4°C and separated into cytosolic lysates and pellets by a centrifugation at $27\,000 \times g$ for 30 min. The cytosolic lysates were either used directly for in vitro labeling assay or further processed by passing through a Sephadex G-50 spun column to remove small molecules. The eluent collected after the Sephadex G-50 spun-column was designated as G-50 lysates [14]. Previous study has shown that deoxyhypusine-forming activity resides completely in cytosolic lysates [14].

In vitro radiolabeling assay. Deoxyhypusine-forming activity was estimated by radiolabeling of the 18 kDa protein with [^{3}H]spermidine in vitro as we previously described [14]. Specific details of the amount of various components and additives and incubation time are given in the figure and table legends. The in vitro labeling reaction was terminated by the addition of sodium dodecyl sulfate-stop solution and the sample was analyzed by SDS-PAGE and fluorography [2,14]. Although the labeling of the 18 kDa protein was used as an index for the activity of deoxyhypusine formation, it should be noted that the labeled deoxyhypusine residue can be further hydroxylated in lysates to become hypusine. Thus the labeled 18 kDa protein may contain both deoxyhypusine and hypusine depending on the labeling condition.

Identification of radioactive species associated with labeled protein. The labeled 18 kDa protein bands were excised from gel and the proteins were recovered from gel slices by electroelution, dialysis and lyophilization. The dried proteins were acid hydrolyzed (110°C, 20 h) in 6 M HCl under N_2 gas. The acid hydrolysates were neutralized and dansylated as previously described [3]. The dansylated species were analyzed by thin layer chromatography. Authentic deoxyhypusine, prepared as described by Park et al. [5], and synthetic hypusine [17] were used as a standard.

Binding affinity of the enzyme E_1 and 18 kDa protein substrate in cell lysates to Cibacron blue dye. Cytosolic lysates prepared from DFMO-treated cells (approx. 2–4 mg proteins in 0.4 ml) were mixed with an equal volume of Cibacron blue dye resins that had been equilibrated with TE buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA). The mixture was incubated for 10 h at 4°C and then centrifuged at $13\,000 \times g$ for 5 min. The supernatant obtained was designated as non-bound fraction. Fraction eluted by 0.4 M KCl in TE buffer was desig-

nated as bound fraction. Both fractions were then tested for deoxyhypusine-forming activity by in vitro labeling assay.

Isolation of E_1 enzyme and 18 kDa protein substrate. For a typical purification, about 80 to 100 dishes (100 mm) of DFMO-treated cells were used for the preparation of cytosolic lysates. Approx. 20 ml of lysates containing a total of 120 mg proteins were loaded directly onto a Cibacron Blue column (2.5 × 50 cm) which had been equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2 mM β-mercaptoethanol and 5% glycerol). The flow-through fraction was loaded again onto the same column two more times. The non-bound fraction (50 ml) was collected and concentrated by Amicon ultrafiltration (PM 10 membrane) to a final volume of about 3 ml. The concentrated protein solution was mixed with 1 ml of 2 M NaCl and loaded onto a Sephadex G-50 column (2.5 × 110 cm) which had been equilibrated with 2.5 × PBS containing 5% glycerol and 2 mM β-mercaptoethanol. The chromatogram was developed by eluting the column with the same high ionic strength buffer. Two fractions were collected, one from the void volume was designated as high-molecular-weight fraction; the other after the void volume was designated as low-molecular-weight fraction. The high-molecular-weight fraction contained only enzyme activity and the low-molecular-weight fraction contained only protein substrate (see Results). Both fractions were then dialyzed against buffer A and rechromatographed onto a second Cibacron Blue column (1.5 × 10 cm) for further purification. Both of the second Cibacron Blue columns were eluted first with buffer A for non-bound fraction, followed by 0.4 M KCl in buffer A for bound fraction. Both bound and non-bound fractions from each Cibacron Blue column were dialyzed against buffer A and concentrated by PM-10 membranes. All fractions were frozen at −70°C in the presence of 15% glycerol. The presence of enzyme or protein substrate was established by in vitro labeling assay.

Reconstitution of deoxyhypusine formation system. For the reconstitution experiments, freshly thawed substrate fraction and enzyme fraction were used. Both fractions were added to Earle's balanced solution which contained [^{3}H]spermidine (50–100 μCi/ml) and various additives in a total volume of 50 μl. The labeling was carried out at 37°C for varying lengths of time as indicated in legends and the reaction products were analyzed by SDS-PAGE and fluorography.

Determination of the half-life of the 18 kDa protein substrate. NB-15 mouse neuroblastoma cells at 80% confluence were treated with 5 mM DFMO and 10% dialyzed fetal bovine serum for 48 h. Cycloheximide was then added to the cultures to a final concentration of 50 μg per ml. At indicated times, after the addition of cycloheximide, cells were harvested and cytosolic lysates prepared as described above. The lysates were heated at 85°C for 5 min to inactivate the endogenous E_1 activity. The relative amount of 18 kDa protein substrate in heated lysates was determined by in vitro labeling assay using exogenously added deoxyhypusine-forming enzyme.

Gel electrophoresis and other procedures. SDS-PAGE was performed according to the procedure of Laemmli [18]. The method of O'Farrell [19] was used for isoelectric focusing-SDS two-dimensional gel analysis. Fluorograms were prepared according to the method of Bonner and Laskey [20] except that Enhancer was used. Protein amount was determined by the procedure of Lowry et al. [21].

Results

Effects of pH on the in vitro labeling of the 18 kDa protein. Deoxyhypusine formation on the 18 kDa protein can be demonstrated in vitro in a glycine-NaOH buffer at pH 9.5 but not at pH 7.0 [11]. Since pH 9.5 is not a physiological pH, it is desirable to search for an in vitro reaction condition at pH 7.0. Our finding that NAD^+ greatly stimulates the in vitro labeling of the 18 kDa protein in the glycine-NaOH buffer [14] prompted us to examine the possibility of labeling the 18 kDa protein by [^{3}H]spermidine at physiological pH in the presence of NAD^+. Fig. 1 shows that the 18 kDa protein could be labeled by [^{3}H]spermidine at pH 7.2 in Earle's balanced salt solution only if NAD^+ was present (Fig. 1, lane 2 vs. lane 1). FMN was only marginally effective (Fig. 1, lane 5 vs. lane 1). Other nucleo-

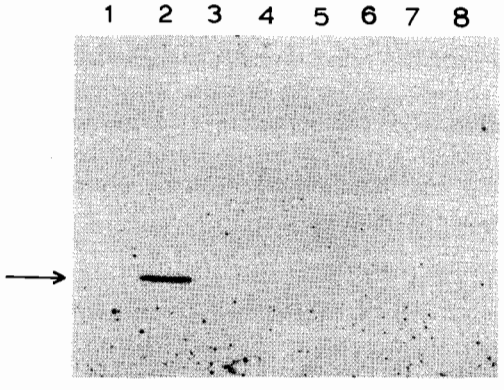

Fig. 1. Effect of various putative effectors on the labeling of the 18 kDa protein at pH 7.2 in cytosolic lysates derived from DFMO-treated NB-15 mouse neuroblastoma cells. Cytosolic lysates were prepared in Earle's balanced salt solution (pH 7.2). The in vitro labeling was carried out at 37°C in lysates (350 μg proteins) with 100 μCi/ml [^{3}H]spermidine (40.1 Ci/mmol) and various additives in a final vol. of 100 μl for 4 h. Additives added to the in vitro labeling system were: Lane 1, no addition; Lane 2, 1 mM NAD^+; Lane 3, 1 mM $NADP^+$; Lane 4, 1 mM FAD; Lane 5, 1 mM FMN; Lane 6, 1 mM ATP; Lane 7, 1 mM GTP; and Lane 8, 1 mM GDP. Each lane contained 300 μg proteins. Fluorogram of the gel was prepared as described in Experimental procedures.

tides tested including FAD were ineffective. In our previous study we have found that at pH 9.5, FAD and FMN can also stimulate the labeling of 18 kDa protein [14]. The rate of NAD^+-dependent deoxyhypusine formation at pH 7.2 was about 20–40% of that at pH 9.5 (data not shown).

Hypusine formation involves deoxyhypusine formation and subsequent hydroxylation of the deoxyhypusine residue [1,5]. Murphey and Gerner [11] reported that while deoxyhypusine formation occurs at pH 9.5, hydroxylation can only occur at pH around 7.0. We therefore examined the identity of the radioactive residue on the 18 kDa protein labeled at these two different pH values. The results summarized in Table I indicate that radiolabeled 18 kDa protein whether labeled at pH 7.2 or pH 9.5, contained both hypusine (25% for both samples) and deoxyhypusine (32% at pH 7.2 and 48% at pH 9.5). In addition, we found that 20–40% of radioactivity in the labeled 18 kDa protein was recovered as polyamines, mostly putrescine and spermidine (see Table I). Although the nature of linkages of these polyamines to the 18 kDa protein labeled in vitro is not known, the binding must be tight and highly specific because (i) it survived the SDS-PAGE, (ii) only the 18 kDa protein band was labeled on the fluorogram and (iii) the binding was NAD^+ dependent (Fig. 1, lane 2 vs. lane 1). Covalent binding of polyamines to cellular proteins have been reported, particularly on trans-

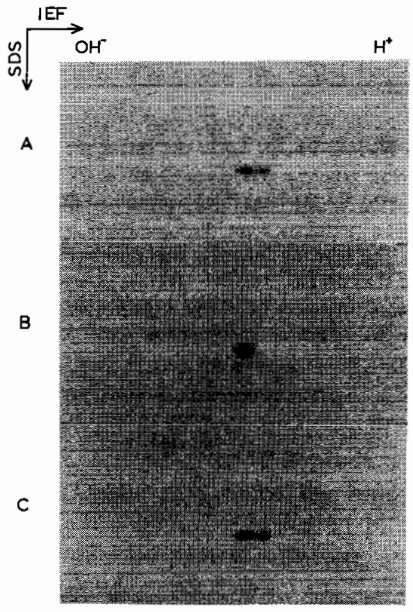

Fig. 2. Two dimensional gel analysis of radiolabeled 18 kDa protein obtained from the lysates at pH 7.2 and pH 9.5. The in vitro labeling was carried out in the presence of [^{3}H]spermidine (40.1 Ci/mmol) and 1 mM NAD^+ in either Earle's balanced salt solution (pH 7.2) or 0.1 M glycine-NaOH (pH 9.5) for 4 h at 37°C. (A) Lysates were labeled at pH 7.2 and 300 μg proteins were used for the isoelectric focusing gel; (B) Lysates were labeled at pH 9.5 and 300 μg proteins were used for isoelectric focusing gel; (C) Lysates were first labeled at pH 9.5 for 4 h, the pH was then adjusted to 7.2, the sample (250 μg proteins) was then incubated at 37°C for another 2 h before loading onto the tube gel for isoelectric focusing. Fluorograms were prepared as described in Experimental procedures.

TABLE I

Determination of deoxyhypusine and hypusine in labeled 18 kDa protein obtained from NB-15 cell lysates

	Sample 1 (pH 7.2) [a]			Sample 2 (pH 9.5)		
	R_f	cpm [b]	% [c]	R_f	cpm	%
Hypusine	0.17	870	26	0.17	2580	25
Deoxyhypusine	0.32	1060	32	0.32	5040	48
Putrescine	0.67	770	23	0.67	2200	21
Spermidine	0.82	440	13	0.82	370	4
Spermine	0.88	230	7	0.88	280	3
Total (cpm)		3300			10470	
Recovery (%) [d]		85			95	

[a] DFMO-treated NB-15 cells were harvested in Earle's balanced salt solution, pH 7.2 (Sample 1) and in 0.1 M glycine, pH 9.5 (Sample 2). Cell lysates were incubated with 50 μCi/ml of [^{3}H]putrescine and 1 mM NAD^+ for 4 h. Proteins (500 μg) from Sample 1 or 2 were loaded on a 12% SDS-polyacrylamide gel. After electrophoresis, the radiolabeled 18 kDa bands were excised from the gels and electroeluted. About 4000 cpm and 11000 cpm were collected from sample 1 and sample 2, respectively. The eluted proteins were acid hydrolyzed, dansylated and analyzed by Silica G TLC plates. Solvent system was chloroform/methanol/acetic acid (125/5/1, by volume).

[b] Radioactivity recovered from the TLC plates.

[c] Calculated as the percentage of the total radioactivity recovered from the TLC plates.

[d] Calculated as the percentage of the radioactivity associated with the 18 kDa protein after electroelution.

glutaminase-catalyzed formation of γ-glutamyl polyamine linkage in cells (e.g., Refs. 22 and 23). Since Ca^{2+} did not stimulate the in vitro labeling of the 18 kDa protein (Ref. 14, also Table III), it is doubtful that transglutaminase, a Ca^{2+}-dependent enzyme, was responsible for the binding of polyamines to the 18 kDa protein. Thus, the data shown in Table I differ from that reported by Murphey and Gerner [11]. Whether the discrepancy is due to difference in cell lines used or because of the presence of NAD^+ in our in vitro labeling system remains to be examined.

The isoelectric/electrophoretic behavior of the labeled 18 kDa protein obtained at two different pH values was compared in Fig. 2. The 18 kDa protein labeled at pH 7.2 gave two spots with p*I* values of 4.7 and 5.1, identical to the isoform structures obtained by metabolic labeling method [15]. In contrast, the 18 kDa protein labeled at pH 9.5 gave only one spot with the p*I* value of 5.1. If the sample labeled at pH 9.5 was reincubated at pH 7.2, the original one spot became two spots (Fig. 2C), suggesting the formation of isoforms. The cause of this pH-dependent shift of p*I* values is unclear. It is possible that an additional post-translational modification may have occurred on the 18 kDa protein at pH 7.2.

TABLE II

Effects of buffer composition on the labeling of the 18 kDa protein in NB-15 cell lysate

DFMO-treated cells were harvested, washed and resuspended in 0.1 M of various buffers. Except when indicated, the pH of all buffers was 9.5. In vitro labeling was carried out at 37°C for 4 h in the presence of 1 mM NAD^+ and [^{3}H]spermidine (20 μCi/ml). Labeled samples (350 μg proteins) were then analyzed by SDS-PAGE and fluorography. The labeling intensity was estimated by densitometric tracing of the fluorogram. Relative labeling intensity was calculated using the labeling intensity of the 18 kDa protein band obtained in glycine buffer as reference point. Each data point represents an average of two separate experiments.

Buffers	Relative intensity of the labeled 18 kDa protein
Glycine	100
Aspartate	125
Glutamate	130
Lysine	62
Histidine	63
Arginine	21
Ammonium phosphate	75
Sodium carbonate	3
Tris-base	5
Tris-HCl (pH 7.2)	0

Effect of buffer composition on the in vitro labeling of the protein substrate. We have compared the labeling intensity of the 18 kDa protein by [^{3}H]spermidine in lysates incubated in various buffers made from 0.1 M of either amino acids or bases. The data summarized in Table II indicate that deoxyhypusine formation in vitro was sensitive to buffer composition. Using the glycine-NaOH buffer (0.1 M, pH 9.5) as a reference point, buffers made of acidic amino acids appeared to be stimulatory, whereas buffers made of basic amino acids or bases were all inhibitory. For example, the labeling of 18 kDa protein in arginine buffer (pH 9.5) was only 20% of that in glycine buffer. More dramatically, the in vitro labeling of the 18 kDa protein was almost completely inhibited in 0.1 M Tris buffer.

Effect of inhibitors on the labeling of the 18 kDa protein. The dependence of deoxyhypusine/hypusine formation on NAD^+ suggest that the deoxyhypusine-forming enzyme may be a dehydrogenase-like enzyme. Since many dehydrogenases are known to be sensitive to sulfhydryl reagents and metal ions [24], we have examined the effects of these reagents on the in vitro labeling of the 18 kDa protein. As shown in Table III, both Cu^{2+} and Fe^{3+} were effective inhibitors. Oxidized glutathione at 1 mM inhibited the labeling by 44% and *p*-chloromercuribenzoate at 1 mM completely abolished the labeling reaction. These results are consistent with the notion that free sulfhydryl group(s) of the enzyme may be involved in deoxyhypusine formation.

Effect of temperature on deoxyhypusine formation. Fig. 3 shows that deoxyhypusine formation is sensitive to heat treatment. Since the labeling of the 18 kDa protein depends on activities of both deoxyhypusine-forming enzyme and the protein substrate, we then investigated whether heating inactivates the enzyme or the protein substrate or both. For this study, we have taken advantage of the finding that NB-15 cells grown in the absence of DFMO contained enzyme E_1 but not free protein substrate, similar to that reported for CHO cells [25]. We, therefore, prepared G-50 lysates from log-phase NB-15 cells and used them as a source of crude enzyme. The control experiment indicated that these lysates alone did not support the labeling of 18 kDa protein due to a lack of free protein substrate (data not shown). When the G-50 lysates from the log-phase NB-15 cells were added to heated lysates prepared from DFMO-treated cells, the labeling of the 18 kDa protein was fully restored in all heated samples including the one that had been treated at 100°C for 6 min (Fig. 3). These data suggested that while the deoxyhypusine-forming enzyme was sensitive to heat-treatment, the 18 kDa protein was extremely heat stable.

Binding affinity of the enzyme and protein substrate to Cibacron blue dye. Metabolically labeled 18 kDa protein (eIF-4D) has been shown to bind tightly to Cibacron Blue column [15]. Since the modified 18 kDa protein and its precursor may differ only by one amino acid residue (i.e., lysine vs. hypusine/deoxyhypusine), it is

TABLE III

Effects of metal ions and sulfhydryl reagents on the formation of deoxyhypusine in NB-15 cell lysates

Cytosolic lysates were incubated at 37°C for 4 h with 20 μCi/ml of [^{3}H]spermidine (40.1 Ci/mmol) and 1 mM NAD^+ in the presence of various agents. The inhibition was quantitated as following: % inhibition = (radioactivity measured in the absence of inhibitor − radioactivity measured in the presence of inhibitor)/(radioactivity measured in the absence of inhibitor). The radioactivity associated with the labeled 18 kDa protein bands were determined by dissolving the excised gel bands in H_2O_2 and counted by a liquid scintillation counter. Each data point represents an average of duplicate experiments.

Addition	Concentration (mM)	% Inhibition
None	0	0
GSH	0.5	0
	1.0	0
GSSG	0.5	29
	1.0	44
NEM [a]	1.0	50
PCMB [a]	0.5	75
	1.0	100
$CaCl_2$	1.0	0
$MgCl_2$	1.0	0
$MnCl_2$	1.0	39
$CuSO_4$	1.0	100
$FeSO_4$	1.0	84
$FeCl_3$	1.0	93

[a] NEM, *N*-ethyl maleimide; PCMB, *p*-chloromercuribenzoate.

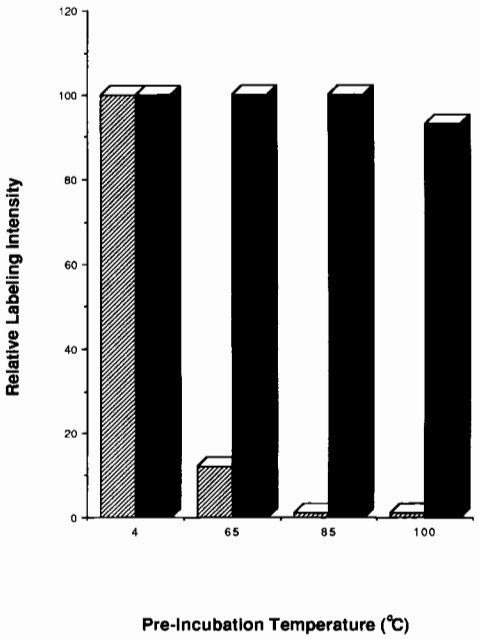

Fig. 3. Effect of heating on the in vitro labeling of 18 kDa protein. The G-50 lysates were prepared as previously described [14] in Earle's balanced salt solution (pH 7.2). The G-50 lysates were divided into four aliquotes and incubated at different temperatures as indicated for 6 min. Each sample was then divided into two portions (about 300 μg each) for in vitro labeling experiments. The labeling was carried out using pre-incubated G-50 lysates in the presence (solid block) or absence (shaded block) of exogenously added crude E_1 enzyme (200 μg lysates prepared from log-phase NB-15 cells). The reaction mixtures were incubated with 20 μCi/ml of [^{3}H]spermidine and 1 mM NAD^+ at 37°C for 6 h. The samples were then analyzed by SDS-PAGE and fluorography. The relative labeling intensity of the 18 kDa protein band on the gel was determined by densitometric tracing.

possible that the procedure we developed for the purification of hypusine-containing 18 kDa protein [15] may be adopted for isolating the precursor protein. As a preliminary test, the freshly prepared cytosolic lysates were divided into bound and non-bound fractions based on their affinity to the Cibacron Blue column. Each fraction was then tested for deoxyhypusine-forming activity by the in vitro labeling assay. Fig. 4 shows that the deoxyhypusine-forming activity was found only in the non-bound fraction but not in the bound fraction (Fig. 4, lane 2 vs. lane 3), indicating that both the enzyme and the protein substrate existed in the non-bound fraction. We also noticed that this one step Cibacron Blue column offered a 10-fold increase of in vitro deoxyhypusine-forming activity (Fig. 4, lane 2 vs. lane 1). Since there is a possibility that residual enzyme or the protein substrate may still exist in the bound fraction, we also examined deoxyhypusine-forming activity in the mixture of bound and non-bound fraction (Fig. 4, lane 4). The labeling intensity of the 18 kDa protein shown in lane 4 was comparable to that shown in lane 2, suggesting that almost all the enzyme and substrate were in the non-bound fraction. Fig. 4 also shows that once the 18 kDa protein was modified in vitro by [^{3}H]spermidine, the labeled protein gained complete affinity to Cibacron blue dye (Fig. 4, lane 6 vs. lane 5), consistent with our previous report that metabolically labeled 18 kDa protein shows strong affinity to Cibacron blue dye [15].

Isolation of enzyme E_1 and 18 kDa protein substrate. Once the 18 kDa protein was modified via deoxyhypusine formation, we found that the enzyme E_1 in cell lysates gained binding affinity to Cibacron blue dye (data not shown). One possible explanation is that, before modification, E_1 enzyme and the 18 kDa protein might form a complex preventing either of them from binding to Cibacron blue dye. After modification, the enzyme was dissociated from the product and then gained affinity to the dye. Since the binding of the enzyme to Cibacron blue dye appeared to depend on the presence of 18 kDa protein substrate, we reasoned that it may be possible to employ the Cibacron Blue column first to remove all bound proteins from both E_1 and the protein substrate in cytosolic lysates. Once the enzyme and the protein substrate are separated, a second Cibacron blue columns can then be used to remove all contaminant non-bound proteins. Based on this hy-

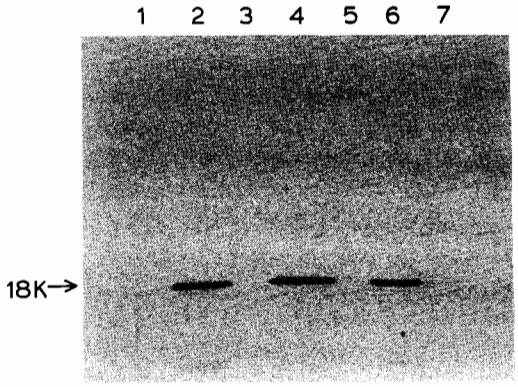

Fig. 4. The binding affinity of deoxyhypusine-forming activity and the labeled 18 kDa protein to Cibacron blue dye. Cytosolic lysates were prepared in Earle's balanced salt solution (pH 7.2) and separated into bound and non-bound fractions by Cibacron Blue column chromatography. In the first set of experiments (lanes 1–4), the in vitro labeling was carried out in control lysates and in each fraction in the presence of 1 mM NAD^+ and 10 μCi/ml of [^{3}H]spermidine for 6 h at 37°C. The samples were then analyzed by SDS-PAGE and fluorography. Lane 1, control cytosolic lysates (150 μg proteins); lane 2, non-bound fraction (130 μg proteins), lane 3, bound fraction (20 μg proteins), and lane 4, mixture of bound (20 μg proteins) and non-bound (130 μg proteins) fraction. In a second set of experiments (lanes 5–7), the cytosolic lysates were divided into non-bound and bound fraction by Cibacron Blue column chromatography. In vitro labeling was carried out only in the non-bound fraction (300 μg protein) in the presence of 1 mM NAD^+ and 20 μCi/ml of [^{3}H]spermidine for 4 h at 37°C. The radiolabeled sample was then rechromatographed on a second Cibacron Blue column, the non-bound fraction (130 μg protein, lane 5) and the bound fraction (20 μg protein, lane 6) were analyzed by SDS-PAGE and fluorography. Lane 7, control lysates identical to the sample used in lane 1.

pothesis, we have developed a scheme for isolating both the enzyme and the protein substrate simultaneously from DFMO-treated NB-15 mouse neuroblastoma cells. The 3-step procedure, summarized in Fig. 5, included a Sephadex G-50 column 'sandwiched' between two Cibacron Blue columns. The first Cibacron Blue column was used (i) to enrich both E_1 enzyme and protein substrate; (ii) to ensure that any modified 18 kDa protein (i.e., eIF-4D) present will be removed from the cytosolic extracts; and (iii) to remove other proteins that bind to Cibacron blue dye. Gel filtration under a high ionic strength condition was used to separate the enzyme from the protein substrate. As shown in Fig. 6A, the high-molecular-weight fraction from the G-50 column could stimulate the labeling of 18 kDa protein in both control and heated lysates, whereas the low-molecular-weight fraction stimulated the labeling only in control but not in heated lysates. No labeling of the 18 kDa protein could be detected in either fraction when tested alone. These data suggested that the high-molecular-weight fraction contained the enzyme activity, whereas the low-molecular-weight fraction contained the protein substrate. These two fractions were then rechromatographed on a second Cibacron Blue column and separated into bound and non-bound fractions. Fig. 6B shows that both the enzyme and protein substrate were recovered separately in the bound fraction. The fact that the binding affinity of the enzyme and protein substrate to Cibacron blue dye depends on the presence of each other, allows us to use this dye column twice in a complementary fashion, first to remove the proteins bound to the dye and subsequently to remove the proteins that did not bind to the dye.

Reconstitution of deoxyhypusine formation system in vitro. To examine whether the combination of the enzyme and the protein substrate was sufficient for deoxyhypusine formation we have performed the reconstitution experiments as described in Fig. 7. Neither enzyme nor protein substrate alone was capable of supporting deoxyhypusine formation whether in the absence or presence of NAD^+ (Fig. 7A, lanes 1–4). When mixed and incubated in the absence of NAD^+, no labeling of the 18 kDa could be observed either (Fig. 7A, lane 5). Labeling of the 18 kDa protein was found in the reconstituted system only in the presence of NAD^+ (Fig. 7A, lane 6). NADH at 1 mM showed a slight stimulatory effect on the labeling of the 18 kDa protein. The effectiveness is likely due to the oxidation of NADH to NAD^+ during incubation. The dependence of deoxyhypusine formation on NAD^+ is further

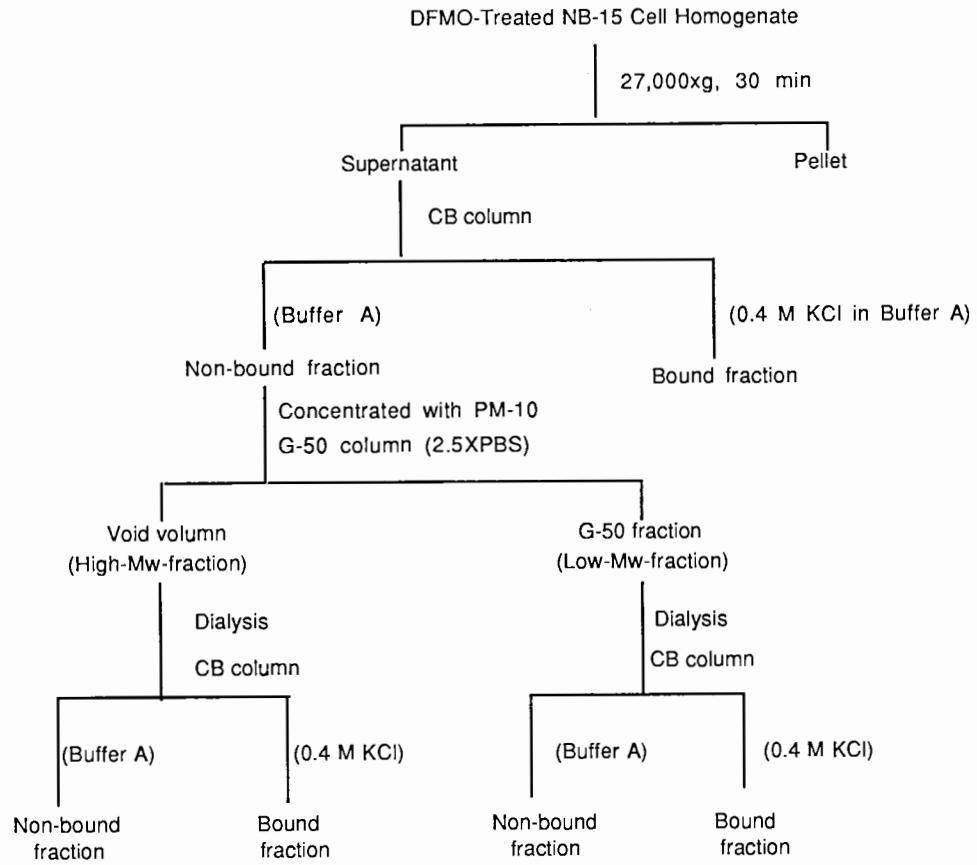

Fig. 5. Purification scheme for both deoxyhypusine-forming enzyme E_1 and the 18 kDa protein substrate from DFMO-treated NB-15 mouse neuroblastoma cells. CB, Cibacron Blue column; G-50, Sephadex G-50 column. Details are explained in Experimental procedures.

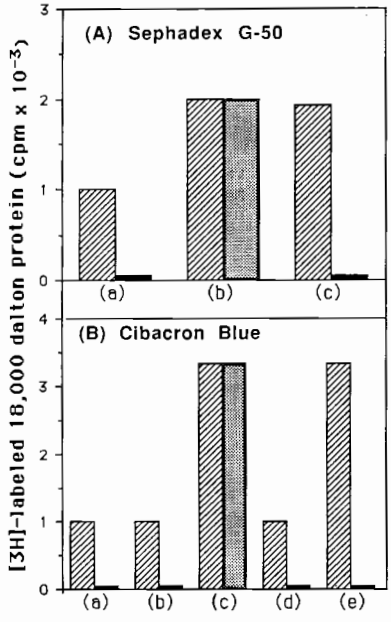

Fig. 6. Effect of various column chromatographic fractions on the in vitro labeling of 18 kDa protein in control and heated cytosolic lysates. Cytosolic lysates were prepared from DFMO-treated cells and used for the separation of E_1 enzyme and the protein substrate as described in Experimental procedures. Two fractions obtained after Sephadex G-50 column and four fractions from the second Cibacron Blue column were tested for the presence of E_1 and the protein substrate. Each fraction (10 μl) was added to either control lysates (300 μg proteins, shaded block) or heated lysates (300 μg proteins, solid block) and the in vitro labeling assay was carried out in the presence of 1 mM NAD^+ and [^{3}H]spermidine (20 μCi/ml) at 37°C for 2 h. Cytosolic lysates prepared from DFMO-treated NB-15 cells were used as control lysates. Heated lysates were made by heating the control lysates at 85°C for 5 min. The reaction products were analyzed by SDS-PAGE and deoxyhypusine formation was estimated by radioactivity associated with the 18 kDa protein band excised from the gel as determined by liquid scintillation counting. (A) Sephadex G-50 column chromatography: (a) no addition; (b) high-molecular-weght fraction added; (c) low-molecular-weight fraction added. (B) Second Cibacron Blue column chromatography: (a) no addition; (b) high-molecular-weight/non-bound fraction added; (c) high-molecular-weight/bound fraction added; (d) low-molecular-weight/non-bound fraction added; and (e) low-molecular-weight/bound fraction added. All fractions tested did not support in vitro labeling of the 18 kDa protein by themselves. All protein samples were prepared in Earle's balanced salt solution (pH 7.2).

studied in Fig. 7B. The dose-response curve of the effect of NAD^+ on the labeling of the 18 kDa protein at pH 7.2 in the reconstituted system gave a K_d value of NAD^+ about 0.2 mM.

The half-life of the 18 kDa protein substrate. Since the protein substrate was heat stable whereas the enzyme was not, it is possible to use exogenous E_1 to estimate the relative amount of free 18 kDa protein by in vitro labeling assay in heated cell extracts. Using this approach we found that the protein substrate in DFMO-treated NB-15 cells was very stable. A loss of only 20% of protein substrate could be observed 10 h after cycloheximide treatment as shown in Fig. 8. Based on the extrapolation of the data in Fig. 8 we estimated that the half-life of the 18 kDa protein to be as long as 24 h.

Discussion

We showed in this study that deoxyhypusine/hypusine can be formed on the 18 kDa protein, the eIF-4D precursor, in cell lysates at a physiological pH provided that NAD^+ is present (Fig. 1). The 18 kDa protein labeled in vitro at pH 7.2 shows two isoelectric structures with pI values of 4.7 and 5.1, similar to that obtained by metabolic labeling [15]. In contrast, only the basic form of the 18 kDa protein (pI = 5.1) was radiolabeled in lysates incubated at pH 9.5 (Fig. 2, panel B vs. panel A). The pH dependence of the labeling of these two forms could be due to (i) an existence of isoforms of the 18 kDa protein substrate or (ii) an additional post-translational modification of the 18 kDa protein, or (iii) both. Although the cause is not clear, we noticed that by incubating the pH 9.5 sample in a pH 7.2 buffer for another 2 h, the original one spot (pI = 5.1) could be converted to two spot with pI values similar to that of the pH 7.2 sample (Fig. 2, panel C).

The effect of buffer composition on the in vitro labeling of the 18 kDa protein (Table II) suggests that the buffering molecules may participate in deoxyhypusine formation, possibly in the stabilization of the reaction intermediate or transition-state complex. Since buffers made of basic amino acids or Tris-base were inhibitory in supporting in vitro labeling as compared to the glycine buffer, we speculated that the key reaction intermediate (or transition-state complex) may be positively charged. This notion is consistent with the observation that the labeling reaction was more favored at high pH [11,14].

Murphey and Gerner [11] reported that guazatine, an inhibitor of plant polyamine oxidases, can block the in vitro deoxyhypusine formation at 1 mM. Park and Wolff [25] have shown recently that a number of spermidine analogs can inhibit deoxyhypusine via competition mechanism. Our study on the effect of metal ions and sulfhydryl reagents (Table III) strongly suggests that the deoxyhypusine-forming enzyme may require free sulfhydryl group(s) for its action. If this is the case, the functional sulfhydryl groups can also become a target site for designing potent inhibitors for this enzyme.

The labeling intensity of the 18 kDa protein depends on both enzyme activity and the amount of protein substrate. Our finding that the 18 kDa protein is extremely heat-stable allows us to heat-inactivate the endogenous enzyme without affecting the substrate protein in cell extracts. Thus, it becomes possible to use exogenous enzyme to quantitate the amount of protein substrate in heated cell extracts by in vitro labeling assay. Using this approach, we have determined the

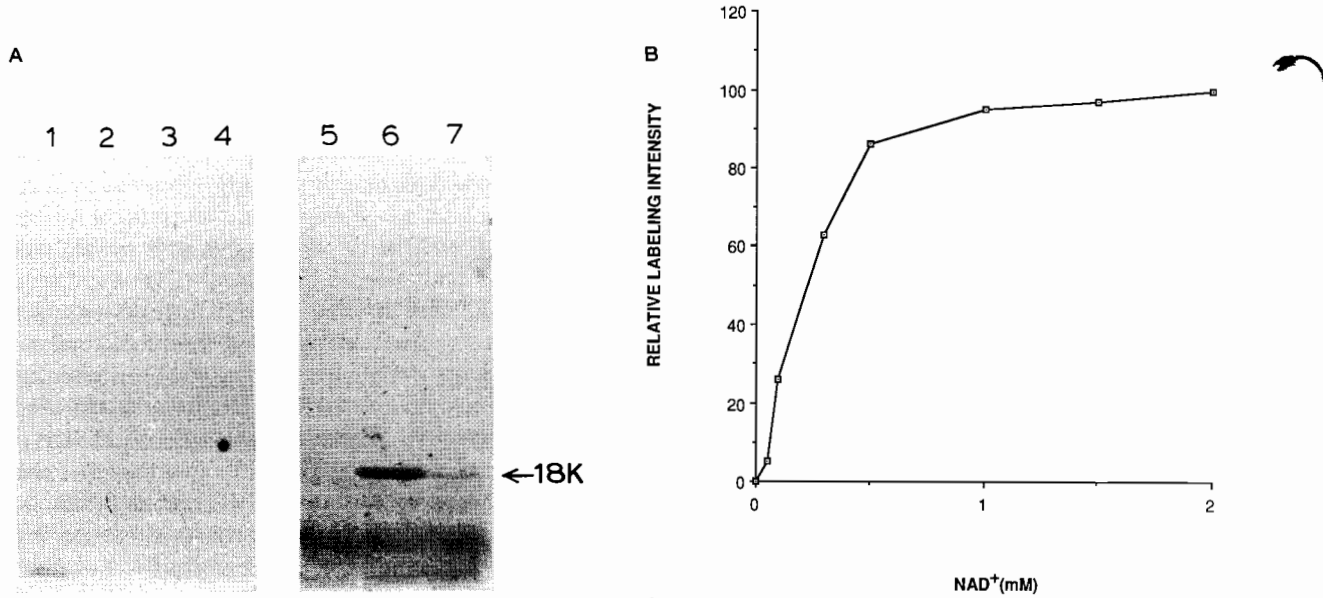

Fig. 7. (A) Reconstitution of the in vitro labeling system. The E_1 enzyme and the 18 kDa protein substrate were prepared as described in Experimental procedures. The in vitro labeling assay was carried out in Earle's balanced salt solution (pH 7.2) at 37°C for 4 h. Each assay contained 50 μCi/ml [^{3}H]spermidine and various components as indicated: Lane 1, 15 μl E_1 fraction; lane 2, 15 μl E_1 and 1 mM NAD^+; lane 3, 3 μl of 18 kDa protein fraction; lane 4, 3 μl 18 kDa protein fraction and 1 mM NAD^+; lane 5, 15 μl of E_1 and 3 μl of 18 kDa protein; lane 6, 15 μl E_1 and 3 μl 18 kDa protein and 1 mM NAD^+; and lane 7, 15 μl E_1 and 3 μl 18 kDa protein and 1 mM NADH. (B) Dose-response curve of the effect of NAD^+ on the labeling of the 18 kDa protein in the reconstituted system. Reaction mixtures contained 15 μl of E_1 fraction and 3 μl of the 18 kDa protein fraction in Earle's balanced salt solution (pH 7.2) in the presence of 50 μCi/ml [^{3}H]spermidine and various amount of NAD^+. The reaction was carried out at 37°C for 15 h. The relative intensity of the radiolabeled 18 kDa protein band was measured by densitometric tracing of the fluorogram.

half-life of the 18 kDa protein substrate and found it to be extremely stable in DFMO-treated cells (Fig. 8). It has been speculated that deoxyhypusine/hypusine formation on the 18 kDa protein may protect the protein from the attack of lysine-specific intracellular proteinases, and thus enhance the stability of the protein [26]. The long half-life of the protein substrate seems to preclude this possibility in NB-15 cells.

The availability of an assay method for deoxyhypusine formation and the finding that the protein substrate is heat-stable are essential for developing the isolation scheme shown in Fig. 5. The key feature of this scheme is the use of Cibacron blue dye, a group-affinity resin [27,28]. Cibacron Blue column was initially chosen for two reasons: (i) We have previously found that hypusine-containing 18 kDa protein binds tightly to Cibacron blue dye [15] and (ii) Cibacron blue dye column is known to have high affinity with proteins which possess a dinucleotide fold (e.g., NAD^+) or adenine-containing nucleotide [27,28] and E_1 enzyme appears to be an NAD^+-requiring enzyme. In this study, we found that both the E_1 enzyme and the 18 kDa protein substrate, once separated, could bind to Cibacron blue dye (Fig. 6B). However, when both were present together in lysates, neither could bind to the Cibacron blue dye (Fig. 4). It is possible that E_1 enzyme and the protein substrate may form a complex with unknown stoichiometry which prevents either one from binding to Cibacron blue dye. To support this notion, we found that when partially purified E_1 and the 18 kDa protein were mixed in the absence of spermidine and NAD^+, the deoxyhypusine formation activity was recovered in the non-bound fraction, but not in the bound fraction after the Cibacron Blue column chromatography (Dou, Q.P. and Chen, K.Y., unpublished data). Such a unique binding property of E_1 enzyme and 18 kDa protein substrate allowed us to employ two Cibacron Blue columns, one before and one after the separation of these two proteins, to partially purify them simultaneously. Park and Wolff [25] have recently succeeded in separating the enzyme from the protein substrate in DFMO-treated CHO cell lysates by ammonium sulfate fractionation. In the present study, the enzyme and substrate were separated by gel filtration at high ionic strength. The use of the Cibacron Blue column as an initial step also ensures that the modified protein, if present, will not contaminate the preparation of protein substrate during subsequent isolation procedure. This is important because the modified protein may differ from the substrate protein by only one amino acid residue and thus, may be difficult to separate by conventional means. Since Cibacron blue dye is a group affinity resin, further purification should be achievable by fine-tuning the ligands chosen for elution.

In summary, we have shown that (i) deoxyhypusine formation on the 18 kDa protein can be carried out at

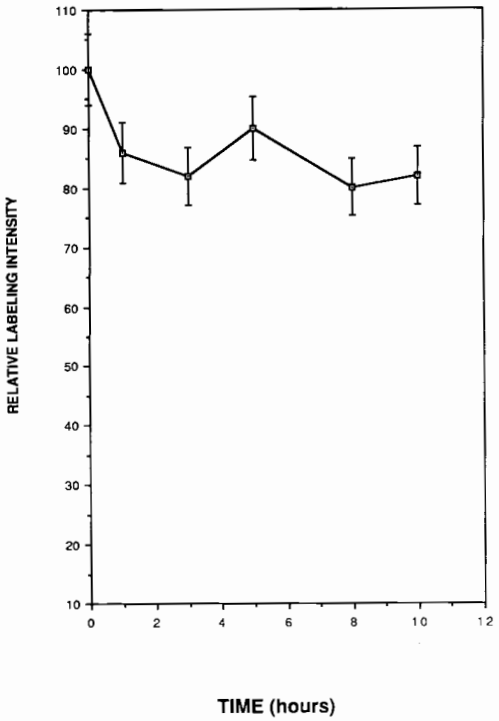

Fig. 8. Effect of cycloheximide on the stability of the 18 kDa protein substrate in DFMO-treated NB-15 cells. NB-15 cells at 80% confluence were treated with 5 mM DFMO in fresh Dulbecco's medium containing 10% of fetal bovine serum for 48 h. Cycloheximide was then added to the culture to a final concentration of 50 μg/ml. At designated times, cells were harvested for the preparation of cytosolic lysates. The lysates prepared were heat-treated at 85°C for 6 min and used as a source of protein substrate. In vitro labeling assay was initiated by adding 15 μl partially purified enzyme to the heated lysates in the presence of 1 mM NAD^+ and [^{3}H]spermidine (20 μCi/ml). The reaction was carried out at 37°C for 6 h to ensure complete reaction. The reaction mixture was analyzed by SDS-PAGE and fluorography. The intensity of the labeled 18 kDa band on the fluorogram was used as an estimate of the relative amount of protein substrate in the lysates.

pH 7.2 provided that NAD^+ is present, (ii) the labeled 18 kDa protein gives pH-dependent isoform structures, (iii) the buffer composition affects the activity of deoxyhypusine formation, (iv) the deoxyhypusine-forming enzyme may require free sulfhydryl group(s) for activity and (v) the 18 kDa protein is heat stable. In addition, we have developed a simple procedure for a simultaneous isolation of the E_1 enzyme and the 18 kDa protein from DFMO-treated mouse neuroblastoma cells. Using partially purified enzyme and protein substrate, we have demonstrated the NAD^+-dependency of deoxyhypusine formation in the reconstituted system. We have also determined the half-life of the protein substrate in DFMO-treated NB-15 cells and found it to be longer than 10 h.

Acknowledgements

This work was supported by a research grant from Charles and Johanna Busch Memorial Funds. We wish to thank Ms. Te-hua Chu for performing some of the experiments described in this paper and Mr. Peter Halajdida for graphic work. We also thank Dr. P. McCann for DFMO and Dr. T. Shiba for synthetic hypusine. A preliminary account of this work has been presented (Dou, Q.P. and Chen, K.Y. (1989) J. Cell Biol. 107, 3633a).

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