Oxidative Modification of Nucleoside Diphosphate Kinase and Its Identification by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry†

Eun Joo Song,‡ Yang Sun Kim,‡ Ji Young Chung,‡ Eunhee Kim,§ Suhn-Kee Chae,§ and Kong-Joo Lee*,‡

Center for Cell Signaling Research, Division of Molecular Life Sciences and College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea, and Research Center for Biomedicinal Resources and Division of Life Sciences, Paichai University, Taejon 302-735, Korea

Received February 3, 2000; Revised Manuscript Received April 26, 2000

ABSTRACT: Nucleoside diphosphate kinase (NDPK, Nm23) has been implicated as a multifunctional protein. However, the regulatory mechanism of NDPK is poorly understood. We have examined the modification of NDPK in oxidative stresses. We found that oxidative stresses including diamide and H_2O_2 treatment cause disulfide cross-linking of NDPK inside cells. This cross-linking was reversible in response to mild oxidative stress, and irreversible to strong stress. This suggests that disulfide cross-linked NDPK may be a possible mechanism in the modification of cellular regulation. To confirm this idea, oxidative modification of NDPK has been performed in vitro using purified human NDPK H_2O_2 inactivated the nucleoside diphosphate (NDP) kinase activity of NDPK by producing intermolecular disulfide bonds. Disulfide cross-linking of NDPK also dissociated the native hexameric structure into a dimeric form. The oxidation sites were identified by the analysis of tryptic peptides of oxidized NDPK, using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Intermolecular cross-linking between Cys109-Cys109, which is highly possible based on the X-ray crystal structure of NDPK-A, and oxidations of four methionine residues were identified in H_2O_2-treated NDPK. This cross-linkng was confirmed using mutant C109A (NDPK-A^{C109A}) which had similar enzymatic activity as a wild NDPK-A. Mutant NDPK-A^{C109A} was not cross-linked and was not easily denatured by the oxidant. Therefore, enzymatic activity and the quaternary structure of NDPK appear to be regulated by cross-linking with oxidant. These findings suggest one of the regulatory mechanisms of NDPK in various cellular processes.

Nucleoside diphosphate kinase (NDPK, Nm23)[1] has been considered a housekeeping enzyme catalyzing transfer of the terminal phosphate from nucleoside triphosphate (NTP) to nucleoside diphosphate (NDP) by forming a high energy phosphorylated enzyme intermediate. The primary role of NDPK in the cell is to maintain a pool of nucleoside triphosphate (NTP) required for biosynthesis. In humans, two isozymes have been identified as NDPK-A (Nm23-H1) (*1*) and NDPK-B (Nm23-H2) (*2*). NDPK-A acts as a suppressor of metastasis for some tumor types (*3*−*5*). NDPK-B was shown to be identical to PuF, a protein that binds to the nuclease-sensitive element in the promoter of c-*myc* oncogene, and activates its transcription (*6*−*9*). Very recently, new Nm23 genes, DR-nm23, nm23-H4, nm23-H5, and nm23-H6, have been identified (*10*−*12*). A protein encoded by DR-nm23 was shown to be involved in control of granulocyte differentiation and apoptosis of myeloid cells (*10*). Nm23-H4 possesses the region homologous to NDP kinase, with all residues crucial for nucleotide binding, and contains a NH_2-terminal region for targeting to mitochondria (*11*). Nm23-H5 has an additional 55 amino acid at the COOH-terminus and does not exhibit NDP kinase activity (*12*).

Interest in NDPK has been recently increasing because of its involvement in the cellular regulatory functions such as cell proliferation, differentiation, development, and apoptosis (*13*−*16*). For example, NDPK, product of the *awd* gene in *Drosophila*, is responsible for larvae growth and development (*17*−*20*). Human NDPK has been identified as a differentiation inhibitory factor for myeloid leukemia M1 cells (*21*, *22*). NDPK is essential for activating a response at the muscarinic K^+ channel by agonist (*23*, *24*). In arabidopsis, NDPK Ia specifically induces the transcription of HIS 4 in response to UV, suggesting that NDPK may be one of the components acting on a novel UV-responsive pathway (*25*).

However, it is not clear why NDPK has so many different functions and how each activity is regulated. In this study, we investigated the involvement of NDPK in the signaling pathway of oxidative stresses.

Redox signaling is mediated by reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), and lipid hydroperoxides. ROS has

† This work was supported by KRF (1998) and by KOSEF through the Center for Cell Signaling Research (CCSR) at Ewha Womans University. The students (E.J. Song and J.Y. Chung) were financially supported by the Brain Korea 21 Project

* To whom correspondence should be addressed. Phone: 82-2-3277-3038. Fax: 82-2-3277-3760. E-mail: kjl@mm.ewha.ac.kr.

‡ Ewha Womans University.

§ Paichai University, Taejon 302-735, Korea.

[1] Abbreviation: NDPK, nucleoside diphospahte kinase; MALDI-TOF MS, matrix assisted laser desorption and ionization time-of-flight mass spectrometry; TFA, trifluroacetic acid; HCCA, 4-hydroxy-α-cyano-cinnamic acid; H_2O_2, hydrogen peroxide; diamide, diazenedicarboxylic acid bis(*N*,*N*-dimethylamide) II; sinapinic acid, 3,5-dimethoxy-4-hydroxy cinnamic acid; DTT, dithiothreitol.

10.1021/bi000267a CCC: $19.00
Published on Web 07/29/2000

been implicated in the oxidative cell damage of physiological and pathological processes such as aging, apoptosis, neurodegenerative diseases, and cancer (*26*−*28*). Organisms possess repair systems for reversing the oxidative modifications and disposal systems for removing the damaged macromolecules. There are two kinds of repair systems, enzymatic and non-enzymatic systems. Non-enzymatic repair systems act, in general, as free radical scavengers (*29*, *30*), including glutathione, ascorbic acid, thioredoxin, and glutaredoxin. Enzymatic defense systems can remove oxygen radicals and their products and/or repair the damage caused by oxidative stress. Catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, and thioredoxin belong to the enzymatic defense systems (*31*, *32*). In addition to enzymatic and non-enzymatic systems, it is reported that some proteins function as a repair system through conformational change during oxidative stress (*33*, *34*). Sometimes, chaperon-like molecules and heat shock proteins are involved in these processes.

Here we have examined whether NDPK is involved in the signaling pathway of oxidative stress. We found that the alteration of NDPK occurred in oxidant-induced cell death, as well as the presence of oxidized high molecular weight (HMW) NDPK. To identify the characteristics of oxidized HMW NDPK, purified NDPK was oxidized in vitro with hydrogen peroxide and characterized. Disulfide cross-linking of NDPK occurred and the NDP kinase activity was lost by dissociating the native hexameric structure to dimer. Molecular modifications of oxidized NDPK were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Conventional procedures for the determination of molecular modifications of proteins involve multiple steps: cleavage of modified proteins with proteolytic enzymes, separation of peptides with chromatography, and sequencing of the peptides. However in this study, tryptic peptides were analyzed directly by MALDI-TOF MS without prior separation. Also these results were confirmed using mutant NDPK-A^{C109A}.

EXPERIMENTAL PROCEDURES

Materials. Chemicals including 4-hydroxy-α-cyano-cinnamic acid (HCCA), 3,5-dimethoxy-4-hydroxy cinnamic acid (sinapinic acid), trifluroacetic acid (TFA), sodium bicarbonate, trizma base, HEPES, ATP, UDP, $MgCl_2$, and ATP-agarose were purchased from Sigma (St. Louis, MO). Radioisotope [γ-^{32}P]ATP was obtained from DuPont NEN (Boston, MA). PEI cellulose TLC plate were from Alltech (Deerfield, IL). *Escherichia coli* strain BL21 transformed with pET3c expression plasmids containing *nm23-H1* or *nm23-H2* were provided by Dr. P. S. Steeg at NCI. Sequencing grade trypsin (from bovine pancreas) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Superose 12 HR 30/10 column came from Pharmacia LKB (Uppsala, Sweden). H_2O_2 was obtained from Junsei Chemical Co. (Tokyo, Japan).

Cell Culture. Mouse radiation induced fibrosarcoma (RIF-1) cell line (*35*) (a gift from Dr. G. M. Hahn) cultured in RPMI 1640 containing 10% fetal bovine serum, 100 mg/mL of streptomycin, and 100 units/mL of penicillin G at 37 °C in an atmosphere of 5% CO_2/95% air (*35*). All experiments were performed on 50% more or less confluent cell cultures. For diamide and H_2O_2 treatments, monolayers of cells grown in tissue culture dishes were incubated with various concentrations of diamide or H_2O_2 in RPMI for 10∼30 min at 37 °C.

Profiling of Protein Synthesis by [^{35}S]Methionine Pulse Labeling. Patterns of cellular protein synthesis after oxidative stress were examined by pulse labeling with [^{35}S]methionine (1 μCi/mL) in methionine free RPMI media for 1 h during the recovery after treatment of 5 mM H_2O_2 for 30 min or 0.5 mM diamide for 1 h. The labeled proteins were separated on SDS-PAGE gels which were autoradiographed and quantified by BAS2500 (Fujiphotofilm Co. Ltd., Tokyo, Japan).

Mutagenesis. The coding region for NDPK-A^{C109A} mutant was PCR amplified by two rounds of PCR. First PCR was done in two separate reactions using nm23 in pET-3a (Novagen, madison, WI) as template DNA. Reaction I used 5′-AC *CAT ATG* CAT ATG GCC AAC TGT GAG CGT AC-3′ and 5′-AAC TTG TAT **GGC** GAA GTC TCC ACG GAT GGT-3′ primers, and reaction II used 5′-GGA GAC TTC **GCC** ATA CAA GTT GGC AGG AAC-3′ and 5′-TC *GGA TCC* TCA TTC ATA GAT CCA GTT C-3′ primers. PCR products of reaction I and II were combined and used as template DNA for second PCR. Primers for second PCR were 5′-AC *CAT ATG* CAT ATG GCC AAC TGT GAG CGT AC-3′ and 5′-TC *GGA TCC* TCA TTC ATA GAT CCA GTT C-3′. Mutated nucleotides are noted in boldface type and the linker sites, in italic type. The second PCR product was digested with *Nde*I and *Bam*HI enzymes and cloned into pET-3a cut with the same enzymes. The integrity of the mutant cDNA was verified by both strands sequencing.

Protein Purification. Native NDPKs from human erythrocytes consists of NDPK-A and NDPK-B. Native NDPK, recombinant NDPK-A, -B, and recombinant NDPK-A^{C109A} were purified with modified methods as described previously (*36*). Briefly, cytosolic fraction of human erythrocytes was prepared by lysis the 2 L packed erythrocytes with 8 vol lysis buffer (10 mM Tris-acetate, 10 mM NaCl, 0.1 mM EDTA, 2 mg/mL aprotinine, pH 7.4). Cytosolic fraction of *E. coli* strains BL21(DE3) transformed with expression pET-3c expression plasmids containing nm23-H1, nm23-H2 (gifts from Dr. P. S. Steeg at NCI) and nm23-H1^{C109A} coding region were obtained after inducing the expression of each protein with 0.2 mM IPTG with the method previously described (*37*). Each cytosolic fraction was applied to 2∼4 mL of ATP-sepharose column equilibrated with buffer A (20 mM Tris-acetate, 20 mM NaCl, 0.1 mM EDTA, 3 mM $MgCl_2$, 15 mM β-mercaptoethanol, pH 7.4) at a flow rate of 3 mL/min. The column was then washed with 200 mL buffer A and with 200 mL buffer A containing 0.25 M NaCl to remove nonspecifically binding proteins. Then NDPK was eluted with buffer A containing 1 mM ATP. The fractions containing NDP kinase activity were pooled.

NDP Kinase Activity Assay. Enzymatic activity of NDPK was measured with the modified method as previously described (*38*). Enzymes were incubated with 10 μL of total volume of reaction buffer B containing 20 mM HEPES (pH 7.4), 1 mM each of ATP and UDP as substrate, 0.5 μCi [γ-^{32}P]ATP and 3 mM $MgCl_2$ for 10 min at 37 °C. The process was stopped by adding nonreducing gel sample buffer containing 125 mM Tris base, 2.3% SDS, and 10%

glycerol. Aliquots were loaded onto PEI cellulose TLC plate and developed in a solution of 0.75 M KH_2PO_4 (pH 3.6). Dried TLC plates were exposed to X-ray film for autoradiogram and the formation of [γ-^{32}P]NTP was quantified with Fujiphotofilm BAS2000 (Tokyo, Japan). All measurements were made in duplicate or triplicate.

Size Exclusion Chromatography. Molecular size of NDPK was determined using Pharmacia FPLC Superose 12 HR 10/30 (Pharmacia) column (1 cm × 25 cm) equilibrated with buffer C (20 mM Tris-acetate, 20 mM NaCl, 0.1 mM EDTA, 3 mM $MgCl_2$, pH 7.4) and developed at a flow rate of 0.5 mL/min. Proteins used as molecular weight markers were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (132, 66 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa) and α-lactoalbumin (14 kDa) (*39*).

Analysis of Tryptic Peptides of NDPK by MALDI-TOF Mass Spectrometry. NDPK-A was incubated with or without H_2O_2. Approximately 10 μM purified NDPK-A was allowed to incubate with 5 mM H_2O_2 for 2 h at 37 °C. To find the location of modification, purified proteins were digested with trypsin in 100 mM ammonium bicarbonate (pH 8.5) at 10:1 ratio of NDPK-A to trypsin for 15 h at 37 °C. The digested sample was stored at −20 °C for further analysis. Mass analysis was performed with a PerSeptive Biosystems (Voyager-Elite Biospectrometry Workstation, PerSeptive Biosystems, Inc., Fremingham, MA) MALDI-TOF MS using a nitrogen laser (337 nm) with delayed extraction. The extraction voltage was 20 kV. Data were collected and analyzed and then compared with the Internet protein resource "MS-Digest" (http://prospector.ucsf.edu/, 2000). Samples were prepared for mass analysis in a matrix of either 3,5-dimethoxy-4-hydroxycinnamic acid (Sinnapinic acid) or 4-hydroxy-α-cyano-cinnamic acid (HCCA). Saturated matrix solutions were prepared in a 50% (v/v) solution of acetonitrile/0.1% TFA.

RESULTS

NDPK is a hexameric protein composed of 17 kDa monomer containing 152 amino acids. Cellular NDPK has heterohexameric form and consists of NDPK-A and NDPK-B (*36*). This hexamer is completely dissociated and changed into monomer under the reducing and nonreducing SDS−PAGE condition, which indicates that NDPK hexamers are associated with noncovalent weak interaction. However, it was found that NDPK in cells treated with oxidative stresses exists in the form of high molecular weight complexes. Identification of high molecular weight NDPK complexes has been demonstrated in this study.

Oxidative Modification of NDPK with Diamide and H_2O_2. Cellular modification of NDPK with oxidative stress was detected as disulfide cross-linking under a non-reducing SDS-PAGE gel (Figure 1). When RIF-1 cells were exposed to 0.5 or 0.75 mM of diamide and 5 mM of H_2O_2, the formation of high molecular weight NDPK was observed under the non-reducing condition, and these were dissociated into monomer under the reducing condition (Figure 1A). The extents of oxidative stress were monitored by metabolic labeling of cells with [^{35}S]methionine during recovery after stress treatment (Figure 1B). The protein synthesis was immediately inhibited by oxidative stresses and returned to normal during recovery. This indicates that high molecular weight NDPK can be formed in conditions in which the protein synthesis was inhibited. The formation of NDPK cross-linking was reversible in response to mild stress (0.5 mM diamide). High molecular weight NDPK appeared under non-reducing conditions and disappeared during recovery. In contrast, high molecular weight NDPK exposed to strong stress (0.75 mM diamide) remained unchanged. This high molecular weight NDPK produced by oxidative stress was dissociated and changed into monomers under reducing conditions, which suggests that high molecular weight NDPK is a product of oxidative disulfide bonding.

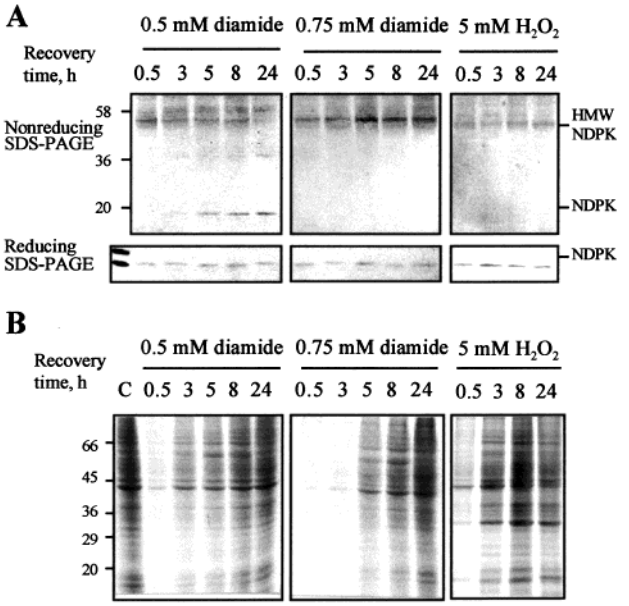

FIGURE 1: Cross-linking of NDPK in diamide and H_2O_2 treated cells. RIF-1 cells were incubated with 0.5 mM, 0.75 mM diamide for 1 h, and 5 mM H_2O_2 for 30 min and recovered at various times. (A) The same amount of cellular proteins was applied on 12% SDS−PAGE in non-reducing and reducing conditions. Western analysis of NDPK was performed using polyclonal anti-NDPK antibody. (B) The protein synthesis rate was monitored during various recovery time after oxidative stress by metabolic labeling the cells for 1 h with [^{35}S]methionine. Same amount of protein was applied on each lane of SDS-PAGE and detected with autoradiography.

To identify the oxidative modification of NDPK, purified proteins were oxidized with H_2O_2 in vitro. When purified NDPK proteins, including recombinant NDPK-A, recombinant NDPK-B, and human erythrocyte NDPK, were incubated with various concentrations of H_2O_2 at 37 °C, NDPK dimeric forms (36 kDa) were observed under nonreducing SDS-PAGE above 0.1 mM H_2O_2 (Figure 2A). As shown in Figure 2A, 5 mM H_2O_2 was adopted for producing the sufficient amounts of dimeric NDPK for the analysis of MALDI-TOF MS. These dimeric NDPKs were fully reduced to monomeric form (18 kDa) by adding β-mercaptoethanol. This indicates that dimeric NDPK formation can be formed by disulfide intermolecular cross-linking between Cys residues.

NDPK-A contains three cysteine residues, Cys-4, Cys-109, and Cys-145. NDPK-B contains two cysteine residues, Cys-109 and Cys-145. Cys-109 and Cys-145 conserved in both NDPK-A and -B may be involved in the formation of disulfide bonds because both NDPK-A and -B are capable of producing the same dimers.

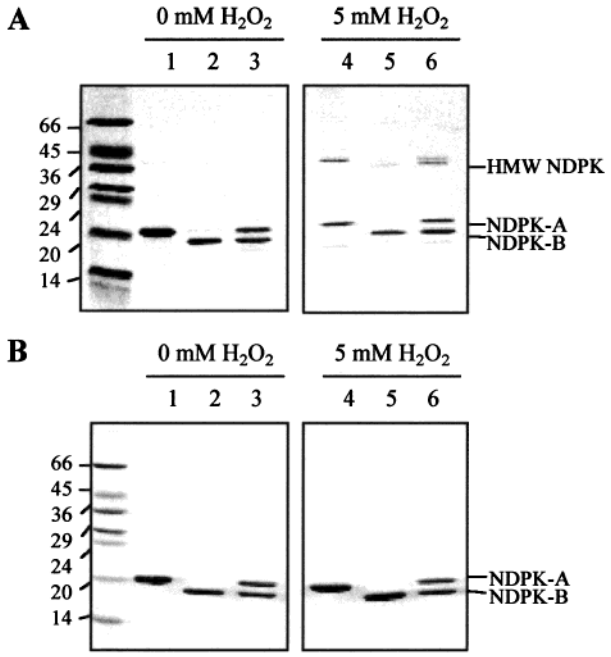

FIGURE 2: Disulfide cross-linking of various NDPK isoforms treated with H_2O_2. Various NDPKs (10 μM) were incubated with 5 mM H_2O_2 for 2 h at 37 °C and resolved on 13% SDS-PAGE under non-reducing (A) and reducing conditions (B). Lane 1 and 4, recombinant NDPK-A; lane 2 and 5, recombinant NDPK-B; lane 3 and 6, human erythrocyte NDPK.

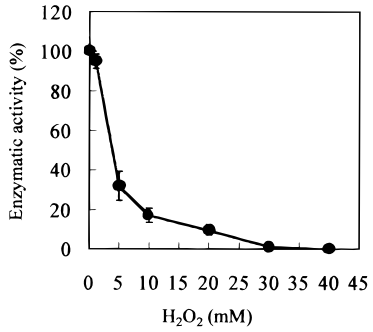

FIGURE 3: Effects of H_2O_2 on the NDP kinase activity of NDPK. NDPK-A was preincubated at the indicated concentration of H_2O_2 for 2 h at 37 °C. Enzymatic activity of NDPK-A was measured on TLC plate as described under Experimental Procedures.

Characterization of Cross-linked NDPK Protein. Incubation with H_2O_2 resulted in the inactivation of NDP kinase activity in the purified recombinant NDPK-A, in a manner dependent on time and H_2O_2 concentration (Figure 3). The formation of disulfide bonds on the surface of molecules also affects catalytic activity. To examine the mechanism of inactivating NDPK by H_2O_2, oligomeric structure changes were investigated.

To investigate whether the disulfide bond formation of NDPK with H_2O_2 affects hexameric structure of NDPK-A, the native molecular mass of NDPK was measured with size-exclusion chromatography using Superose 12. The average mass of NDPK-A without H_2O_2 was 100 kDa, corresponding to a hexamer (Figure 4A). On the other hand, NDPK-A treated with 5 mM H_2O_2, which had lost their enzymatic activity, showed heterogeneous populations of dimeric and intermediate species (Figure 4B). The disulfide bond formation with H_2O_2 could destabilize the hexameric state and induce the dimeric state. Intermediate species, being smaller

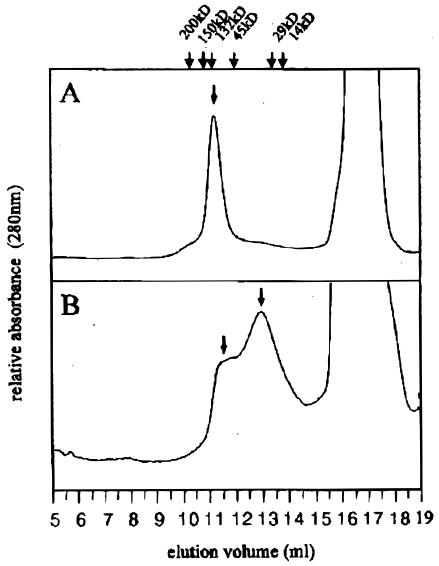

FIGURE 4: Effects of H_2O_2 on the quaternary structure of NDPK-A. Oligomeric structures of control (A) and oxidized NDPK-A incubated with 5 mM H_2O_2 (B) were determined by size-exclusion chromatography on a Superose 12 column (Pharmacia) calibrated with the marker proteins indicated. The *line* in each panel represents relative levels of absorption at 280 nm.

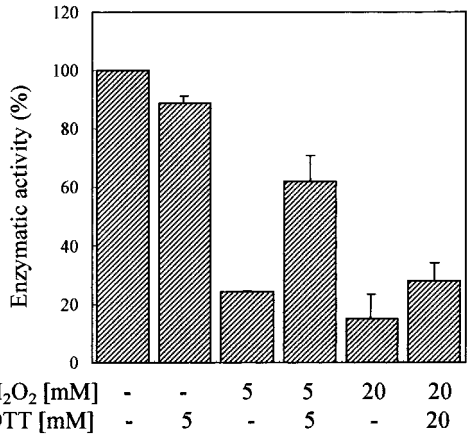

FIGURE 5: Reversibility of inactivated NDPK by H_2O_2. NDPK-A was preincubated at the indicated concentration of H_2O_2 for 2 h at 37 °C and reincubated with indicated concentration of DTT for 10 min. Then the enzymatic activity of NDPK was measured in TLC plate as described under Experimental Procedures.

than hexamer, could be produced during the process of dissociation from hexamer to dimer with H_2O_2. It is possible that the inactivation of catalytic activity of NDPK with H_2O_2 may be caused by the dissociation of hexameric to dimeric form. It has been previously reported that dimeric NDPK does not have catalytic activity in *Dictyostelium* (*39*).

To test if formation of dimeric NDPK is reversible in response to oxidative stress in vitro as well as in vivo (Figure 1A), reactivation studies using dimeric NDPK were performed in the presence of the reducing agent DTT in vitro. In the presence of a reducing agent, NDP kinase activity was monitored. NDPK with mild oxidative stress (5 mM H_2O_2) reactivated its enzymatic activity in the presence of DTT, but NDPK with a strong oxidative stress (20 mM H_2O_2) did not (Figure 5). This indicates that disulfide formation and simultaneous loss of enzymatic activity could be reversed by a reducing agent.

Table 1: Expected and Observed Peptide Fragments Obtained from Trypsin Cleavage of Control and 5 mM H_2O_2-Treated NDPK-A

tryptic peptide	No-treatment expected (*m*/*z*)	No-treatment observed (*m*/*z*)	H_2O_2-treatment observed (*m*/*z*)	modification
7−18	1344.76	1344.45	1344.61	none
19−27	984.62	984.39		
40−56	2066.03	2066.41	2082.88	Met-41 oxidized
57−66	1149.64	1149.36	1149.51	none
67−85	2116.07	2116.48	2148.04	Met-68 oxidized Met-76 oxidized
89−105	1785.92	1785.45	1801.63	Met-90 oxidized
106−114	994.48	994.25	994.37	none
115−128	1485.72	1485.82		
129−152	2942.33	2942.71		
106−114 plus 106−114	1986.96		1985.94	disulfide bond between 106 and 114

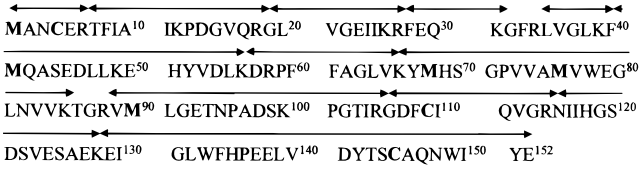

FIGURE 6: Sequence of NDPK-A showing the expected peptides in a tryptic digest of the unmodified protein. The peptides formed by cleavage C-terminal to Lys and Arg are given above the sequence.

Analysis of Tryptic Peptides of NDPK-A by MALDI-TOF Mass Spectrometry. To identify the residues of NDPK modified by H_2O_2, the control and H_2O_2 treated NDPK-A were digested with trypsin, and resulting peptides were analyzed using a MALDI-TOF MS. The masses of these peptides were compared with masses of expected peptides (Table 1) calculated from the known sequence (Figure 6). It is reported that the quality of spectra is dependent on the matrix. Reduced forms of disulfide bond are observed in HCCA but this is not the case in sinapinic acid (*40*). However, survival of the disulfide bond in HCCA is also reported (*41*). Therefore, in our work, both HCCA and sinapinic acid were selected as matrix, HCCA for masses lower than 2000 and sinapinic acid for masses higher than 2000. Molecular weights of tryptic peptides from control NDPK-A, including residues 106−114 and 129−152, corresponded well with the expected peptides (Figure 7A and 7B). Mass spectra of H_2O_2-treated NDPK-A (Figure 7, panels C and D) showed the presence of several modifications. A 16 Da increase of predicted mass was detected in several peptides, which corresponds to the oxidation of Met in peptides containing Met-41, Met-68, Met-76, and Met-90. New mass at *m*/*z* 1985.94 was detected in oxidized NDPK-A. This was the expected mass of intermolecular dipeptide formed by a disulfide bond between two peptides 106−114 containing Cys-109. It is obvious that intermolecular disulfide cross-linking between two Cys-109 residues occurs in H_2O_2-treated NDPK-A. Disulfide formation of peptide 129−152 containing Cys-145 was not observed in H_2O_2-treated NDPK-A. However, it is not clear that Cys-145 is not involved in the formation of disulfide bonds, because the intensity of peptide 129−152 is weak in NDPK-A from control.

To determine the involvement of Cys residue in the formation of disulfide bonds, the control and the H_2O_2-treated NDPK-A were incubated with sulfhydryl-modifying reagent iodoacetamide in order to alkylate the sulfhydryl group. With this treatment, very complicated mass profiles were obtained, since alkylation of peptides with iodoacetamide at pH 8.5 was not specific to the Cys residue but rather labeled the Met, Lys, and His (data not shown). However, a clear 59 Da increase to the predicted mass in peptides 106−114, 129−152 containing Cys-109, and Cys-145 was detected by alkylating the sulfhydryl residues in control NDPK-A. In the case of H_2O_2-treated NDPK-A, a 59 Da increase by an iodoacetamide treatment was observed only in the peptide 129−152 (*m*/*z* = 3001.45), not in 106−114. The *m*/*z* 1985.94 corresponding to the disulfide bond of peptide 106−114 appeared in the H_2O_2-treated NDPK-A, regardless of iodoacetamide treatment. This suggests that only Cys-145 in H_2O_2-treated NDPK-A can be alkylated by iodoacetamide, and, on the other hand, that disulfude-formed Cys-109 cannot be alkylated. It is clear that only Cys-109 is involved in disulfide bond formation of NDPK following oxidative stress.

Oxidative Modification of NDPK-A^{C109A} with H_2O_2. To confirm that high molecular weight NDPK is formed by cross-linking between two Cys-109, NDPK-A^{C109A} mutant was prepared. The purified NDPK-A^{C109A} mutant had full enzymatic activity as wild NDPK-A. When purified NDPK-A^{C109A} was incubated with H_2O_2 for 2 h at 37 °C, cross-linked NDPK-A^{C109A} was not observed under the non-reducing SDS-PAGE (Figure 8A). In addition, the enzymatic activity of NDPK-A^{C109A} was not changed in the absence or presence of H_2O_2 (Figure 8B). This result confirms that only Cys-109 is involved in disulfide bond formation of NDPK and loss of enzymatic activity by oxidative stress.

DISCUSSION

Given recent observations that NDPK has multifunctional properties in various cellular processes, we newly found that high molecular weight oxidized NDPK was produced in response to oxidative stresses, but disappeared during the recovery. Simultaneously, the characteristics of oxidized NDPK were identified.

These findings suggest that NDPK may be involved in cell signaling by oxidative stress. Cross-linking of NDPK occurred in response to mild oxidative stress, and dissociation of cross-linking was observed during recovery. But with strong stress, cross-linked NDPK remained during cell death, indicating that NDPK could be one of the regulatory proteins in oxidative cell signaling.

Reactive oxygen species (ROS) have been implicated in a number of cellular processes, including proliferation, cell death, aging and cancer etc. (*26*). The modification of proteins occurs by ROS and oxidation-modified proteins

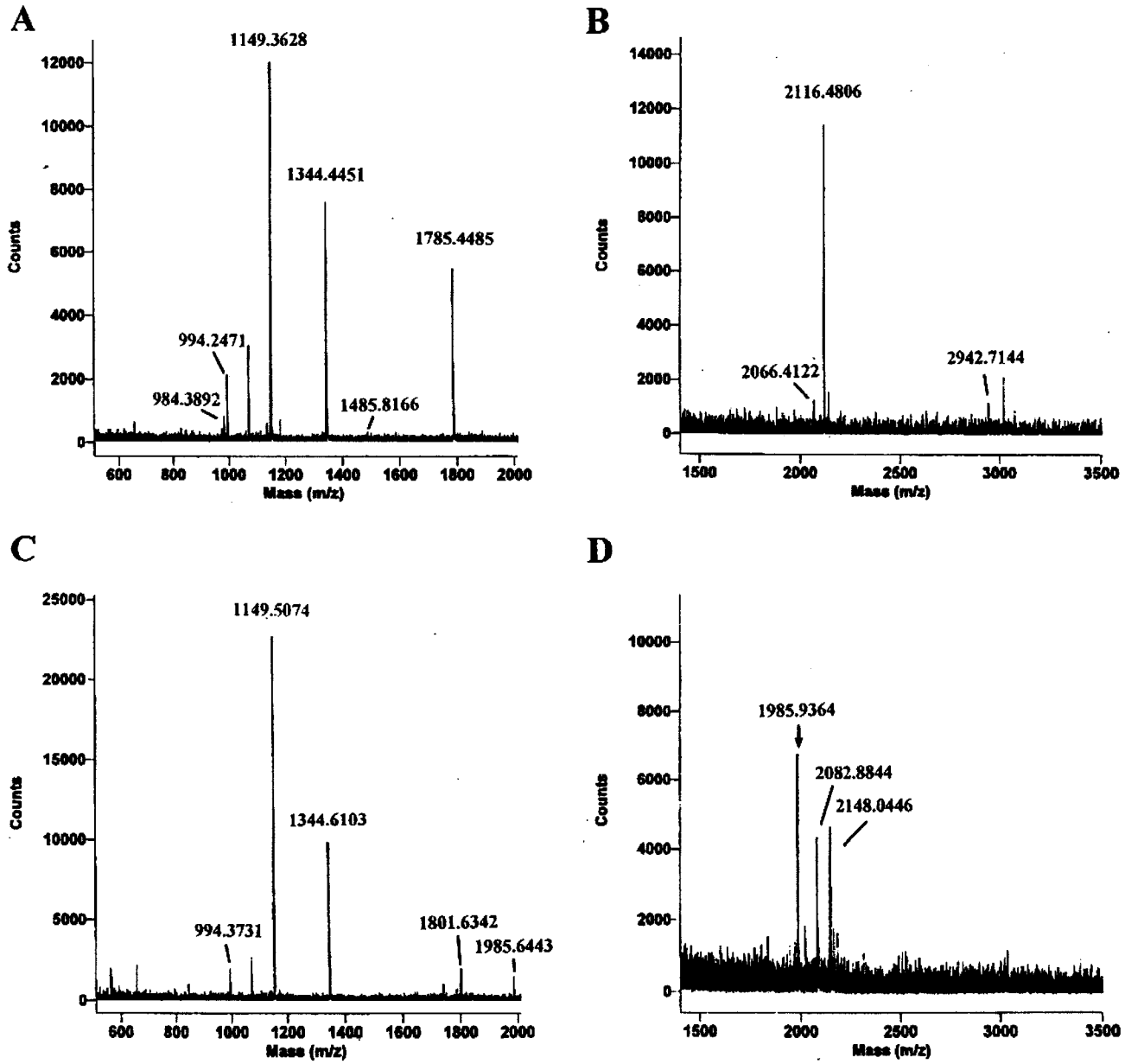

FIGURE 7: MALDI-TOF mass spectrum of tryptic peptides of control and H_2O_2-treated NDPK-A. Control (A, B) and 5 mM H_2O_2-treated NDPK-A (C, D) were digested with trypsin. Mass spectra A and C were obtained using matrix HCCA and spectra B and D using matrix sinapinic acid. Marked peaks in the spectrum were compared with calculated mass in Table 1.

accumulate during aging, oxidative stress and in some pathological conditions. ROS can induce oxidation of amino acid residue side chains, formation of protein−protein cross-linkages, and oxidation of the protein backbone. These oxidative damages to proteins affect their enzymatic activity and increase their susceptibility to proteolysis and aggregation in the cell (*42*, *43*). However, all of the oxidized proteins are not degraded and do not aggregate. Oxidized forms of some proteins are not only resistant to proteolysis but, in fact, can inhibit the ability of proteases to degrade the oxidized forms of other proteins (*44*−*47*). For example, the OxyR transcription factor is sensitive to oxidation and activates the expression of antioxidant genes, and OxyR is activated through the formation of a disulfide bond (*33*). Also, oxidized Hsp33 forms disulfide bond and activates its chaperone function, thus protecting cells from oxidants (*34*). These reports support the idea that the oxidative modification of proteins can be a regulatory cellular mechanism.

Oxidative modification of NDPK inactivated the catalytic NDP kinase activity in a manner dependent on the incubation time and concentration of H_2O_2, and dissociated the native hexameric NDPK into dimeric form. This suggests that oxidation of NDPK can be one way of NDPK functional regulation.

Two main NDPKs, NDPK-A and NDPK-B, have been identified for a long time. NDPK-A acts as a suppressor of metastasis and NDPK-B is identical to PuF, a protein that binds to nuclease-sensitive element in the promoter of c-*myc* oncogene, and activates transcription of the c-*myc* oncogene. But the DNA binding activity of human NDPK-B does not require catalytic activity (*9*). NDPK is essential for muscarinic K^+ channel gating by agonist through an unknown mechanism of action distinct from the phosphate transfer between nucleotides (*23*, *24*). NDPK has various functions, some of which are not related to its enzyme activity. However, the mechanism of NDPK in proliferation, dif-

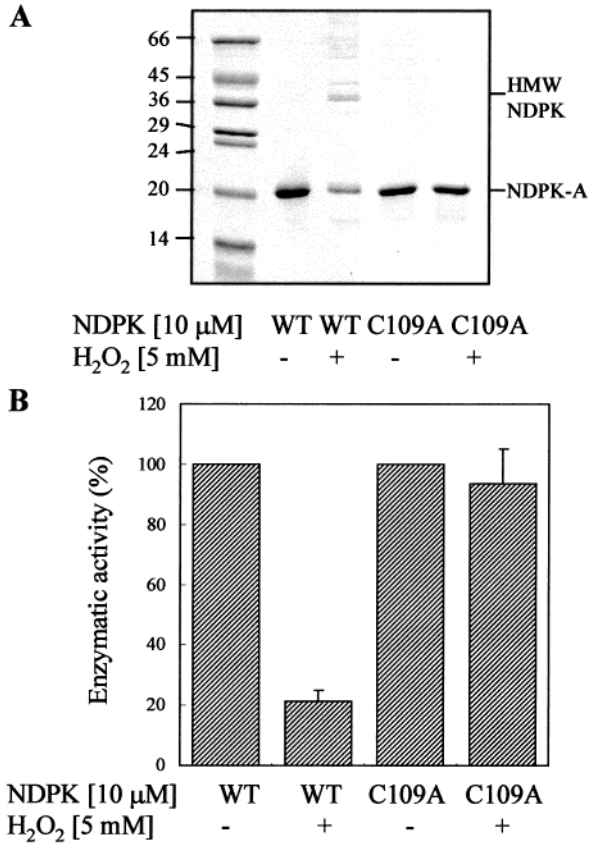

FIGURE 8: Effects of H_2O_2 on the NDPK-A^{C109A} mutant. Purified NDPK-A and NDPK-A^{C109A} (10 μM) were incubated with 5 mM H_2O_2 for 2 h at 37 °C and separated on 13% SDS-PAGE under nonreducing conditions (A). The enzymatic activity was monitored (B).

ferentiation and tumor promotion has not been identified clearly.

Recently, it is reported that different oligomeric states of NDPK have different biochemical functions. It is reported that a dimeric form of NDPK exists. NDPK-A dimer can interact with the dimer of glyceraldehyde-3-phosphate dehydrogenase, and this interaction activates the phosphotransferase function of NDPK (*48*). Also, *Dictyostelium* NDPK was dissociated into dimer by the double mutation P100S-N150stop (*39*) and the dimeric mutant is able to bind to DNA, whereas wild-type hexamer is not (*49*, *50*). It is thought, therefore, that NDPK may gain other functional characteristics through the process of dimer formation. The point mutation Ser^{120}→Gly in the human NDPK-A has been identified in several aggressive neuroblastomas (*51*). Recombinant NDPK-A containing Ser^{120}→Gly mutation exhibited reduced hexameric and increased dimeric oligomerization relative to the wild-type. Moreover, 28 kDa protein was coprecipitated preferentially with the Ser^{120}→Gly mutant of NDPK-A (*52*). By using immobilized antibodies against frog NDPK, 28 kDa protein was identified as a homologue of antioxidant proteins, while NDPK is associated with vimentin sensitive to oxidative damage (*53*). These investigations support the results of this report, that oxidative modification of NDPK inactivates the catalytic activity by dissociating the hexamer into dimeric form.

To identify the characteristics of oxidized NDPK, recombinant purified NDPK was oxidized with H_2O_2 in vitro and tryptic digests of peptides were analyzed by MALDI-TOF MS. Results demonstrate that cysteine and methionine residues of NDPK are particularly sensitive to oxidation. Methionine residues are converted to methionine sulfoxide (MeSOX) and cysteine residues are cross-linked into intermolecular disulfide bonds. Modification of methionine residues is distinct but may not be implicated in any functional regulation of NDPK, in accordance with reports that show that preferential oxidation of several exposed methionine residues in some proteins has little effect on their biological function (*54*).

Cross-linking of NDPK through formation of an intermolecular disulfide bond at Cys-109 affects the enzymatic activity and oligomeric structures. Eukaryotic NDPK has a hexameric quaternary structure, which consists of trimer of dimers (*55*, *56*). This oligomeric structure is coupled with enzymatic activity (*39*). We have demonstrated that H_2O_2 can dissociate native hexameric enzyme into dimeric form and inactivate the enzymatic activity. Intermolecular disulfide cross-linking occurred between Cys-109 and Cys-109. Cys-109 is located in the trimeric interface Kpn loops (residue 99−119) involving main contacts between subunits in hexameric structure. The X-ray structure of NDPK-A suggests that Cys-109 and Cys-109 are favorably positioned for easy formation of a disulfide bond (*57*). The formation of a disulfide bond can produce strain and disrupt the hexameric structure. It is reported that three so-called Kpn loops (residues 99−119) come together at the center of one trimer and induce productive conformation of the nucleotide binding site. Binding of a nucleotide results in the conformational change of the Kpn loop, so that strong contacts within the interface can occur (*39*). In NDPK oxidized by H_2O_2, the disruption of contacts in trimer interface results in conformational change of Kpn loops and inactivates enzymatic activity by damaging the binding of the nucleoside. Mutation of NDPK in dimeric interface can also inhibit catalytic activity (unpublished data). Hexameric structure is necessary for enzymatic activity.

Another functional possibility of oxidized NDPK is that oxidized NDPK in cell death dissociates hexamer into dimer, exposing a new surface. Then the dimer can interact with other proteins, such as an antioxidant protein and vimentin through a new surface not exposed in hexamer. High molecular weight NDPK formed in cells treated with H_2O_2 could be a complex of NDPK dimer and the proteins or DNA with which it has interacted. The function of this NDPK complex should be further explained. Modified NDPK may interact with other proteins and act in various cell signaling pathways as a savior or killer. Experiments are currently under way to further characterize the function of NDPK and to identify the target protein with which NDPK interacts in the signal transduction pathway of oxidative stress. Therefore, it is thought that cross-linking of cysteine residues sensitive to redox conditions of the environment may be involved in the functional regulation of NDPK.

In summary, we demonstrated that NDPK was modified by oxidative stress and identified the modification using MALDI-TOF MS. NDPK is oxidized with H_2O_2, and resulting dimers are composed of the intermolecular disulfide bond between Cys-109 and Cys-109. Oxidative modification is one possible way of regulating the functions of NDPK in many cellular processes.

ACKNOWLEDGMENT

We are grateful to Dr. P. S. Steeg for providing cDNA of nm23-H1 and nm23-H2. We thank Dr. H. J. Kim in Hyundai Pharm. Ind. Co. for letting us to use MALDI-TOF MS.

REFERENCES

1. Rosengard, A. M., Krutzsch, H. C., Shearn, A., Biggs, J. R., Barker E., Margulies, I. M., King, C. R., Liotta, L. A., and Steeg, P. S. (1989) *Nature 342*, 177−180.
2. Stahl, J. A., Leone, A., Rosengard, A. M., Porter, L., King, C. R., and Steeg, P. S. (1991) *Cancer Res. 52*, 445−449.
3. Steeg, P. S., Bevilacqua, G., Kopper, L., Thorgeirsson, U. P., Talmadge, J. E., Liotta, L. A., and Sobel, M. E. (1988) *J. Natl. Cancer Inst. 80*, 200−204.
4. Nakayama, T., Ohtsuru, A., Nakao, A., Shima, M., Nakata, K., Watanabe, K., Ishii, N., Kimura, N., and Nagataki, S. (1992) *J. Natl. Cancer Inst. 84*, 1349−1354.
5. De la Rosa, A., Williams, R. L., and Steeg, P. S. (1995) *Bioessays 17*, 53−62.
6. Postel, E. H., Berberich, S. J., Flint, S. J., and Ferrone, C. A. (1993) *Science 261*, 478−480.
7. Berberich, S. J., and Postel, E. H. (1995) *Oncogene 10*, 2343−2347.
8. Ji, L., Arcinas, M., and Boxer, L. M. (1995) *J. Biol. Chem. 270*, 13392−13398.
9. Postel, E. H., Weiss, V. H., Beneken, J., and Kirtane, A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 6892−6897.
10. Venturelli, D., Martinez, R., Melotti, P., Casella, I., Peschle, C., Cucco, C., Spampinato, G., Darzynkiewicz, Z., and Calabretta, B. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7435−7439.
11. Milon, L., Rousseau-Merck, M.-F., Munier, A., Erent, M., Lascu, I., Capeau, J., and Lacombe, M. L. (1997) *Hum. Genet. 99*, 550−557.
12. Munier, A., Feral, C., Milon, L., Pinon, V. P., Gyapay, G., Capeau, J., Guellaen, G., and Lacombe, M. L. (1998) *FEBS Lett. 434*, 289−294.
13. Sorscher, S. M., Steeg, P., Feramisco, J. R., Buckmaster, C., Boss, G. R., and Meinkoth, J. (1993) *Biochem. Biophys. Res. Commun. 195*, 336−345.
14. Keim, D., Hailat, N., Melhem, R., Zhu, X. X., Lascu, I., Veron, M., Strahler, J., and Hanash, S. M. (1992) *J. Clin. Invest. 89*, 919−924.
15. Moisyadi, S., and Harrington, H. M. (1989) *Plant. Physiol. 90*, 1156−1162
16. Ohneda, K., Fukuda, M., Shimada, N., Ishikawa, N., Ichou, T., Kaji, K., Toyota, T., and Kimura, N. (1994) *FEBS Lett. 348*, 273−277
17. Dearolf, C. R., Hersperger, E., and Shearn, A. (1988) *Dev. Biol. 129*, 159−168.
18. Biggs, J., Hersperger, E., Steeg, P. S., Liotta, L. A., and Shearn, A. (1990) *Cell 63*, 933−940.
19. Timmons, L., Xu, J., Hersperger, G., Deng, X.-F., and Shearn, A. (1995) *J. Biol. Chem. 270*, 23021−23030.
20. Xu, J., Liu, L. Z., Deng, X. F., Timmons, L., Hersperger, E., Steeg, P. S., Veron, M., and Shearn, A. (1996) *Dev. Biol. 177*, 544−557.
21. Okabe-Kado, J., Kasukabe, T., Honma, Y., Hayashi, M., Henzel, W. J., and Hozumi, M. (1992) *Biochem. Biophys. Res. Commun. 182*, 987−999.
22. Okabe-Kado, J., Kasukabe, T., Baba, H., Urano, T., Shiku, H., and Honma, Y. (1995) *Biochim. Biophys. Acta 1267*, 101−106.
23. Xu, L., Murphy, J., and Otero, A. S. (1996) *J. Biol. Chem. 271*, 21120−21125.
24. Otero, A. S., Doyle, M. B., Hartsough, M. T., and Steeg, P. S. (1999) *Biochim. Biophys. Acta 1449*, 157−168.
25. Zimmermann, S., Baumann, A., Jaekel, K., Marbach, I., Engelberg, D., and Frohnmeyer, H. (1999) *J. Biol. Chem. 274*, 17017−17024.
26. Berlett, B. S., and Stadtman, E. R. (1997) *J. Biol. Chem. 272*, 20313−20316.
27. Stadtman, E. R. (1992) *Science 257*, 1220−1224.
28. Jacobson, M. D. (1996) *Trends Biochem. Sci. 21*, 83−86.
29. Stephen, D. W., and Jamieson, D. J. (1996) *FEMS Micobiol. Lett. 141*, 207−212.
30. Prinz, W. M., Aslund, F., Holmgren, A., and Beckwith, J. (1997) *J. Biol. Chem. 272*, 15661−15667.
31. Jamieson, D. J. (1998) *Yeast 14*, 1511−1527.
32. Grant, C. M., Collinson, L. P., Roe, J.-H., and Dawes, I. W. (1996) *Mol. Microbiol. 21*, 171−179.
33. Zheng, M., Aslund, F., and Storz, G. (1998) *Science 279*, 1718−1721.
34. Jakob, U., Muse, W., Eser, M., and Bardwell, J. C. (1999) *Cell 96*, 341−352.
35. Sorger, P. K., Lewis, M. J., and Pelham, H. R. (1987) *Nature 329*, 81−84.
36. Kim, S. Y., Chang, K. H., Doh, H.-J., Jung, J. A., Kim, E., Sim, C. J., and Lee, K.-J. (1997) *Mol. Cells 7*, 630−634.
37. MacDonald, N. J., De la Rosa, A., Benedict, M. A., Freije, J. M., Krutsch, H., and Steeg, P. S. (1993) *J. Biol. Chem. 268*, 25780−25789.
38. Ulloa, R. M., Muschietti, J. P., Veron, M. Torres, H. N., and Tellez-Inon, M. T. (1995) *Mol. Biochem. Parasitol. 70*, 119−129.
39. Mesnildrey, S., Agou, F., Karlsson, A., Bonne, D. D., and Veron, M. (1998) *J. Biol. Chem. 274*, 4436−4442.
40. Patterson, S. D., and Katta, V. (1994) *Anal. Chem. 66*, 3727−3732.
41. Gorman, J. J., Ferguson, B. L., Speelman, D., and Mills, J. (1997) *Protein Sci. 6*, 1308−1315.
42. Gilbert, H. F. (1990) *Adv. Enzymol. Relat. Areas Mol. Biol. 63*, 69−172.
43. McDuffee, A. T., Senisterra, G., Huntley, S., Lepock, J. R., Sekhar, K. R., Meredith, M. J., Borrelli, M. J., Morrow, J. D., and Freeman, M. L. (1997) *J. Cell. Physiol. 171*, 143−151.
44. Friguet, B., Szweda, L. I., and Stadtman, E. R. (1994) *Arch. Biochem. Biophys. 311*, 168−173.
45. Grune, T., Reinheckel, T., Joshi, M., and Davies, K. J. (1995) *J. Biol. Chem. 270*, 2344−2351.
46. Grant, A. J., Jessup, W., and Dean, R. J. (1993) *Free Radical Res. Commun. 18*, 259−267.
47. Suarez, G., Etlinger, J. D., Maturana, J., and Weitman, D. (1995) *Arch. Biochem. Biophys. 321*, 209−213.
48. Engel, M., Seifert, M., Theisinger, B., Seyfert, U., and Welter, C. (1998) *J. Biol. Chem. 273*, 20058−20065.
49. Karlsson, A., Mesnildrey, S., Xu, Y., Morera, S., Janin, J., and Veron, M. (1996) *J. Biol. Chem. 271*, 19928−19934.
50. Mesnildrey, S., Agou, F., and Veron, M. (1997) *FEBS Lett.* 418, 53−57.
51. Lascu, I., Schaertl, S., Wang, C., Sarger, C., Giartosio, A., Briand, G., Lacombe, M. L., and Konrad, M. (1997) *J. Biol. Chem. 272*, 15599−15602.
52. Chang, C. L., Strahler, J. R., Thoraval, D. H., Qian, M. G., Hinderer, R., and Hanash, S. M. (1996) *Oncogene 12*, 659−667.
53. Otero, A. S. (1997) *J. Biol. Chem. 272*, 14690−14694.
54. Levine, R. L., Mosoni, L., Berlett, B. S., and Stadtman, E. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 15036−15040.
55. Giartosio, A., Erent, M., Cervoni, L., Morera, S., Janin, J., Konrad, M., and Lascu, I. (1996) *J. Biol. Chem. 271*, 17845−17851.
56. Lascu, I., Deville-Bonne, D., Glaser, P., and Veron, M. (1993) *J. Biol. Chem. 268*, 20268−20275.
57. Min, K., Kim, S. Y., Song, H. K., Chang, C., Cho, S.-J., Moon, J., Yang, J. K., Lee, J. Y., Lee, K.-J., and Suh, S. W. (2000) *Acta Crystallogr. Sect. D 56*, 503−504.

BI000267A