J. Biochem. 2010;148(2):149–155 doi:10.1093/jb/mvq041

JB THE JOURNAL OF BIOCHEMISTRY

A Cys/Ser mutation of NDPK-A stabilizes its oligomerization state and enhances its activity

Received March 14, 2010; accepted April 8, 2010; published online April 18, 2010

Yunru Chen[1,*], Chuiwen Qian[1,*], Chaowan Guo[1,2], Feng Ge[1], Xiaowei Zhang[1,3], Xue Gao[1], Shan Shen[4], Bing Lian[4], Kaio Kitazato[2], Yifei Wang[1] and Sheng Xiong[1,†]

[1]Biomedical R&D Center, Guangdong Provincial Key Laboratory of Bioengineering Medicine, National Engineering Research Center of Genetic Medicine, Jinan University, Guangzhou, Guangdong, P. R. China 510632; [2]Division of Molecular Pharmacology of Infectious Agents, Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki City, 1-14 Bunkyo-machi, Nagasaki, 852-8521, Japan; [3]College of Translational Medicine, Nanchang University, Nanchang 330031, Jiangxi, P. R. China; and [4]The First Affliated Hospital, Jinan University, Guangzhou, Guangdong, P. R. China 510632

*These authors contributed equally to this work.

[†]Sheng Xiong, Biomedical R&D Center, Jinan University, 5/F, Building of Biology, Guangzhou, Guangdong, 510630, P. R. China, Tel: +86 20 85220504 311, Fax: +86 20 85220504 309, email: xiongsheng@jnu.edu.cn

Nucleoside diphosphate phosphate transferase A (NDPK-A) has been shown to play critical roles in the regulation of proliferation, differentiation, growth and apoptosis of cells. Our previous study suggested that the disulphide cross-linkage between cysteine 4 (C4) and cysteine 145 (C145) of NDPK-A might be a possible regulator of its activity. To confirm this hypothesis, the C145 residue of NDPK-A was mutated to serine, and the isomerization and biological activities of the mutant were investigated and compared with those of its wild-type counterpart. It was found the C145S mutation eliminated the intramolecular disulphide bond (DB) and prevented the formation of intermolecular DB, which was known to dissociate the hexameric NDPK-A into dimeric one. We also demonstrated that the C145S mutation didn't affect the autologous hexamerization of this protein, and the mutant had increased bioactivities including phosphate transferase and DNase. These findings support the hypothesis that the formation of DBs in NDPK-A is involved in the regulation of the oligomerization and bioactivity of this multiple function protein, and that C145 is a key residue in the regulation of NDPK-A. In addition, the C145S mutant that we have constructed might be an attractive candidate for use in applications that require NDPK-A.

Keywords: DNase/disulphide bond isomerization/NDPK-A/oligomerization/phosphate transferase.

Abbreviations: AC, affinity chromatography; CTL, cytotoxicity of T lympholeukocyte; DTT, dithiothreitol; GAAD, granuzyme A-activated DNase; IU, international unit; IEC, ion exchange chromatography; MW, molecular weight; NDP, nucleoside diphosphates; NTP nucleoside triphosphates; NS, normal saline; PMF, peptide mass fingerprinting; RP-HPLC, reverse phase high performance liquid chromatogram; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Nucleoside diphosphate phosphate transferase A (NDPK-A) is a multifunction protein encoded by the metastasis suppressor gene *nm23-H1* that can catalyse the transfer of a phosphoryl group from nucleoside triphosphates to nucleoside diphosphates to maintain the pool of nucleoside triphosphate required for biosynthesis (*1*, *2*). It is involved in cellular regulatory functions such as cell proliferation, differentiation, development and apoptosis. However, the regulation and mechanism of the multifunctions of NDPK are poorly understood, especially with respect to oxidative modification. It has been proposed that oxidative modification is a possible means of regulating the functions of NDPK in many cellular processes, based on *in vitro* experimental results (*3*). The activity of nucleoside diphosphate (NDP) transferase in human brain is decreased in Alzheimer's disease and Down syndrome, and this might result from the oxidative modification of NDPK (*4*). In a previous study, we found that the NDP transferase activity of NDPK increased 2-fold after treatment with *N*-acetylcysteine, and treatment with H_2O_2 resulted in inactivation of the enzyme for the formation of intermolecular disulphide bonds (DBs) and disruption of its oligomerization state (*5*). NDPK-A is catalytically active in its hexameric form and inactive in its dimeric form (*6–8*). It has been reported that the native hexameric structure is dissociated into a dimeric form by the intermolecular DBs of NDPK (*3*). There are three cysteine residues in NDPK-A, *i.e.* Cys4, Cys109 and Cys145. In previous peptide mapping study, we demonstrated that Cys4 and Cys145 form a DB in the oxidized state of NDPK-A (*5*). Here, we have mutated the cysteine 145 (C145) residue of NDPK-A to serine using site-directed mutagenesis, and the physicochemical characteristics and bioactivities of NDPK-A C145S were determined to investigate the possible role of

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012

© The Authors 2010. Published by Oxford University Press on behalf of the Japanese Biochemical Society. All rights reserved

DB isomerization between C145 and the other cysteine residues.

Materials and Methods

Materials

DEAE-sepharose Fast Flow and Cibacron Blue 3GA Sepharose CL-4B were purchased from GE Healthcare and Millipore, respectively (USA). The plasmid extraction kit was from Shanghai Shenggong Biologic Engineering Company (China). The restriction enzymes EcoRI and BamHI were purchased from NEB Laboratory (USA); Pyrobest DNA Polymerase was from the Dalian Takara Company (China); The PCR purification kit, gel extraction kit and plasmid miniprep kit were obtained from Shanghai Biocolors Company (China); The expression plasmid pBV220-NDPK-A was constructed previously in our laboratory; *Escherichia coli* strain DH5α was purchased from Invitrogen (USA). The primers were synthesized by the Shanghai Sangon Company (China). All other chemicals were analytical reagent grade.

Construction of the expression plasmid

Taking pBV220-NDPK-A as template and the oligonucleotides (5′-AGTCgaattcATGGCCAACTGTGAGCGTAC-3′, 5′-GCGg gatccTCATTCATAGATCCAGTTCTGAGCGCTGCTCGTGTA ATCTACC-3′) as primer pairs, the coding sequence of WT NDPK-A was point mutated to NDPK-A C145S using polymerase chain reaction (30 cycles including denaturation at 95°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 2 min in each cycle). The amplified fragments were recovered and digested with BamHI and EcoRI before ligation with pBV220. *Escherichia coli* DH5a competent cells were transformed with the ligation mixture and the recombinant plasmid was subsequently screened and sequenced to confirm that the insert was correct.

Protein expression and purification

The plasmid pBV220-NDPK-A-C145S was subsequently transformed into the expression host, *E. coli* BL21 (DE3). Recombinant clones were screened and grown in fresh LB medium. After screening to discover the optimal conditions for protein expression, 1% glucose was added to the medium and the cells were cultivated at 37°C with shaking at 250 r.p.m. When the density of the culture reached $OD_{600} = 0.7$, the temperature was increased to 42°C, and the cells were allowed to grow for a further 6 h. After harvested by centrifugation at 4000*g* for 20 min, the cell pellets were freeze-thawed once and thoroughly resuspended in 10 ml of buffer A (20 mM Tris–HCl, 1 mM EDTA, 2 mM $MgCl_2$ and 1 mM DTT, pH 7.5) per gram of cell paste. Lysozyme (0.1 mg/ml) was then added to the cell suspension and the mixture was incubated at 30°C for 30 min. The suspension was disrupted by sonication at 600 W. After centrifugation at 20,000*g* for 30 min at 4°C, the pellets and the supernatant were analysed using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE).

To purify recombinant NDPK-A C145S, the supernatant was initially loaded onto an ion exchange chromatography column comprising 20 ml of DEAE Sepharose that had been equilibrated with buffer A. The non-specifically bound proteins were then eluted with buffer B (0.05 M NaCl added buffer A), and the crude proteins (including the NDPK-A C145S mutant) were then eluted with buffer C (0.1 M NaCl added buffer A). The crude proteins were then applied to a Cibacron Blue 3GA Sepharose CL-4B column (*9*), and the homogeneous NDPK-C145S was eluted with buffer D (1.5 mM ATP added buffer A). Finally, the purity of NDPK-A C145S was assessed by SDS–PAGE and density scanning, and the concentration of the protein was evaluated using the Bradford method (*10*) (BSA was used as the standard).

Peptide fingerprint map analysis

After resolved on a 12% SDS–PAGE gel with silver staining, the bands of WT NDPK-A and its C145S were manually excised and washed with solution A (25 mM NH_4HCO_3, 50% ACN in triple-distilled water). The gel pieces were then dried by 100% ACN after dye removal. The samples were then reduced for 30 min at 37 or 56°C in 55 mM dithiothreitol (DTT) and alkylated in iodoacetamide for 30 min in the dark at 37°C. After reduction and alkylation, the proteins were digested using 0.01 g/l trypsin (Promega) at 37°C overnight. The peptide fragments were extracted twice with 50% ACN. After concentration by vacuum centrifugation, the extracted peptides were re-suspended in 50% ACN and dispensed onto a MALDI target plate with an equal amount of cyano-4-hydroxycinnamic acid solution (2 mg/ml, 70% ACN in 0.1% TFA aqueous solution) in 1 µl. The plate was then left to dry under ambient conditions. The peptides were analysed by MALDI-MS mass chromatography using an ABI 4800 proteomics system (Applied Biosystems, USA) (*11*, *12*).

SDS–PAGE

The samples of WT NDPK-A and its C145S mutant were treated with loading buffer either containing or not containing DTT (a reducing agent) and then resolved on 12% SDS–PAGE as previous described (*13*) to determine its DB isomerization. After electrophoresed at 100 V, the gels were scanned in gray scale using a densitometer after staining with Coomassie Blue.

Multi-angle laser light scattering analysis

The MW of NDPK-A C145S in aqueous solution was determined by Multi-angle laser light scattering (MALLS) analysis (*14–16*). NDPK-A C145S was filtrated through a 0.2 µm filter and applied into a molecular exclusion column in Agilent 1100 HPLC system and then eluted at a flow rate of 1.0 ml/min. ASTRA program was used for light scattering data acquisition and processing, as well as the differential refractive index(DRI) and UV signals were recorded simultaneously. The intensity of the scattered light measured by multi-angle laser photometer at specific angles was directly relative to the MW and concentration of the sample in solution. Their relationship is the Zimm's formula as follow, $K^*C/R_\theta = 1/\mathrm{MW}\cdot P(\theta) + 2A_2CM^2P^2(\theta)$, whose parameters R_θ is the excess measured Rayleigh ratio, C is the sample concentration (g/ml) determined by DRI detector, A_2 is the second virial coefficient in (mol/g^2) and K* is a constant.

Western blot analysis

Proteins resolved in SDS–PAGE gel were transferred to polyvinylidene difluoride membrane in Tris–glycine buffer containing 20% methanol. Blocked in 0.5% TBS-Tween-20 containing 5% non-fat milk for 1 h followed by 4 × 15 min washes in TBS-Tween-20, the membrane was incubated with mouse anti-human NDPK-A McAb and corresponding secondary antibody HRP-IgG for 1 h in sequence. Finally, DAB reagent kit was used to visualize the antibody antigen complexes.

NDP transferase assay

The NDP transferase activity of NDPK-A was assayed by reverse phase high performance liquid chromatogram (RP–HPLC). Purified WT NDPK-A or its C145S mutant (22.5 ng) was incubated with 1 µM ATP and 1 µM UDP in buffer E (33 mM Na-Hepes and 5 mM $MgCl_2$, pH 7.4) in a total volume of 1000 µl at 37°C for 8 min. After quenched by 0.2 mol/l formic acid, the reaction mixture (10 µl) was applied to a C18 column (4.6 × 150 mm 5 m, Agilent) and eluted with 10% acetonitrile in the mobile phase (100 mM potassium dihydrogen phosphate, 20 mM acetic acid and 5 mM TBAB, pH 5.0) at a flow rate of 1 ml/min. The NDPK activity was determined from the difference in the peak area of the nucleotides before and after the catalytic reaction. One international unit (IU) of NDPK activity is defined as the amount of enzyme that forms 1 µmol of ATP/UTP per minute under the assay conditions. The specific activity was expressed in units of NDPK activity per milligram of protein (*17*).

DNase assay

The DNase activity of NDPK-A was assayed by supercoiled plasmid digestion and agarose gel electrophoresis. Three groups of reaction mixtures (20 µl each) were prepared in reaction buffer (10 mM Tris–HCl, 1 mM EGTA, 5 mM $MgCl_2$ and 1 mg/ml BSA, pH 7.5) containing 0.5 µg supercoiled pcDNA 3.1(-) as the substrate, and then 0.5 mg NDPK-A C145S, WT NDPK-A or bovine pancreatic DNase I were added to each tube. After incubation for 24 h at 37°C, the reactions were stopped with 2 µl 10× loading buffer. Finally, the reactions were resolved using 1% agarose gel electrophoresis and the gel was scanned using a densitometer (*18*, *19*).

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012

Results

DNA sequencing and PMF analysis confirmed the NDPK-A C145S mutation

A primer pair was designed for the site-specific mutagenesis of NDPK-A C145S, which was inserted into the expression vector, resulting in the recombinant plasmid, pBV220-NDPK-A-C145S (Fig. 1A). The sequence of the C145S mutant was confirmed by DNA sequencing, using WT NDPK-A as a control. It was observed that the codon TGT (C145) in the WT NDPK-A was changed to AGT, thereby encoding a serine residue in NDPK-A C145S (Fig. 1B).

To further confirm the mutant from its amino acid sequence, the recombinant NDPK-A C145S mutant and WT NDPK-A were purified to homogeneity and subjected to peptide mass fingerprinting (PMF) analysis using MALDI-TOF mass spectroscopy. However, neither the peptide fragment containing C145 from WT NDPK-A (EIGLWFHPEELVDYTS CAQNWIYE) nor the corresponding fragment containing S145 from NDPK-A C145S mutant (EIGLWFHPEELVDYTSSAQNWIYE) could be detected due to the high molecular weight (MW) of this peptide fragment, which approached the upper bound of detection (4000 Da). In a parallel experiment, a NDPK-A C4S mutant was also prepared. The peptide fragment containing S4 (ANSER, 576.2742 Da) from NDPK-A C4S was determined as 576.2689 Da (Fig. 1D) and that of the corresponding fragment in WT NDPK-A (ANCER, 592.2508 Da) was determined as 592.2287 Da in the m/z profile (Fig. 1C). From this data and the DNA sequencing of C145S mutant, we concluded that the C145 in NDPK-A was correctly mutated into serine.

Soluble expression and purification of the NDPK-A C145S mutant

Escherichia coli BL21 (DE3) bearing the C145S expression plasmid was cultured to mid-logarithmic phase and induced at 42°C. It was found that the recombinant NDPK-A C145S mutant was located in the supernatant of the bacterial lysate, indicating that the protein is expressed in soluble form in the cytoplasm of *E. coli* (Fig. 2A).

For shake-flask cultures grown under optimal conditions, we were able to purify up to 19.44 mg of NDPK-A C145S from 4.98 g cell paste that was collected from 1 l of culture. During the purification process, the cell lysate was passed through a weak anion exchange column to remove most of the host proteins and the cellular DNA, and the NDPK-A C145S mutant was eluted using buffer C (Fig. 2B). The eluted fraction was then applied to a Cibacron Blue 3GA Sepharose CL-4B column, to which NDPK-A C145S bound specifically. In contrast, the host proteins were eluted in the flow-through fractions. SDS–PAGE analysis showed that the purity of the recombinant NDPK-A C145S obtained was 97% (Fig. 2B).

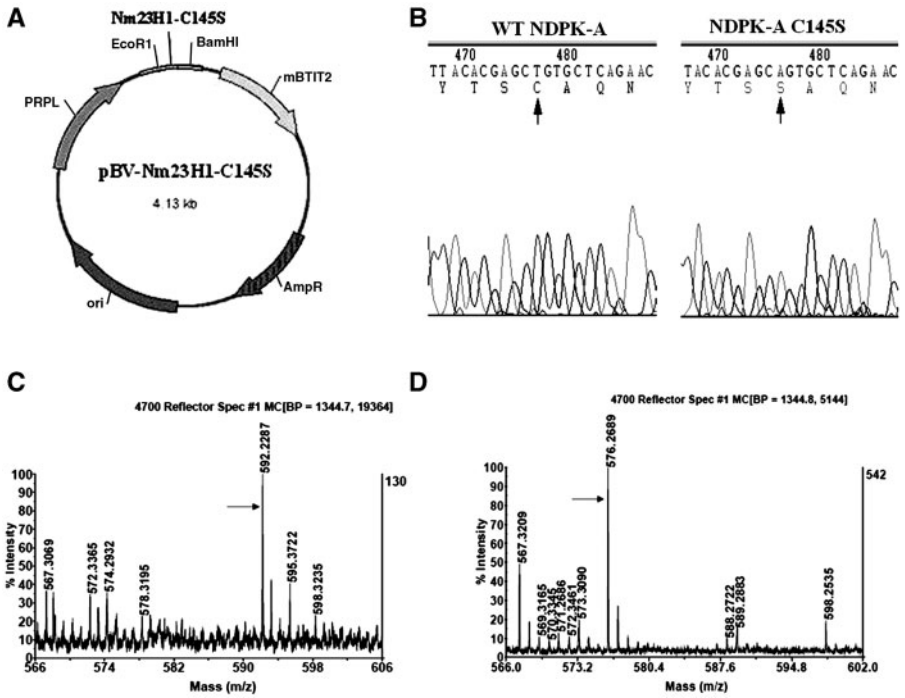

Fig. 1 Construction and identification of the NDPK-A C145S mutant. (A) A structural sketch of the recombinant plasmid pBV-ndpk-C145S. The coding sequence of ndpk-A-C145S was inserted into the plasmid pBV220 between the BamHI and EcoRI restriction sites. (B) The nucleotide sequence of the constructed NDPK-A C145S mutant. The arrows indicate codon for cysteine in WT NDPK-A (TGT) and that for serine in C145S mutant (AGT). (C, D): PFM analysis of NDPK-A C4S. After digestion with trypsin, the peptide fragment that contained residue S4 in NDPK-A C4S is ANSER, and its deduced MW is 576.2742 Da. The MW of the corresponding fragment in WT NDPK-A, ANCER, is 592.2508 Da. MALDI-TOF-MS revealed a m/z signal of 576.2689 (±0.01 Da) in the PMF profile of the C4S mutant. The m/z signal of 592.2287 is indicated with an arrow in the PMF profile of WT NDPK-A (±0.03 Da).

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012

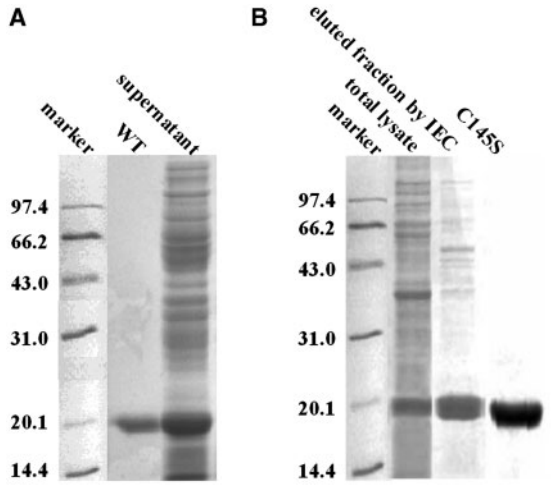

Fig. 2 SDS–PAGE analysis of the soluble expression and purification of NDPK-A C145S in *E. coli*. (**A**) Soluble expression of NDPK-A C145S. The total soluble proteins (supernatant) from the induced recombinant *E. coli* were resolved on reduced SDS–PAGE and stained with Coomassie Blue. The purified WT NDPK-A (WT) was used as a control. (**B**) Purification of NDPK-A C145S. The total soluble lysate was prepared and loaded onto an ion exchange column (IEC) and the eluted fraction containing NDPK-A C145S was then loaded onto an affinity column (AC). From this, NDPK-A C145S was obtained at a purity of 97%.

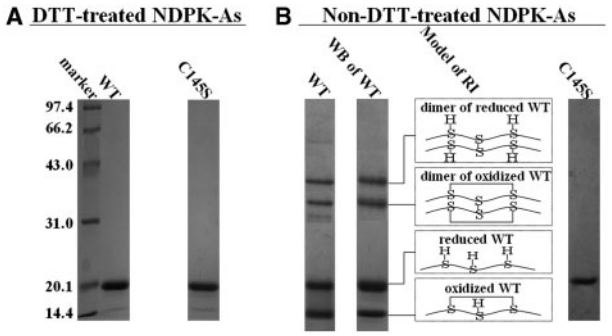

Fig. 3 DB isomerization of NDPK-A in different redox stress. (**A**) DTT treated WT NDPK-A and C145 mutant were resolved on 12% SDS–PAGE and stained with Coomassie Blue to observe its isomerization. Both proteins displayed to be one band (reductive isomers). (B) Isomerization of non-DTT treated WT NDPK-A and C145 mutant. Figures from left to right are the RI of WT NDPK-A, WB visualization of the four bands with DAB, model of the RI of NDPK-A, and the isomerization of NDPK-A C145S, respectively. Elimination of the RI in C145S mutant was observed from this analysis. These figures are from representative experiments carried out at least three times.

DB isomerization in WT NDPK-A and the C145S mutant

The formation of DBs that are sensitive to DTT and β-mercaptoethanol (both reducing agents) can be detected by reduced and non-reduced SDS–PAGE electrophoresis (*20*). Under reduced conditions, the NDPK-A C145S mutant and WT NDPK-A appeared to be one band in the SDS–PAGE gel that were virtually indistinguishable (Fig. 3A). However, under non-reduced conditions, the intra- and intermolecular DBs occurred sequentially, and the intramolecular DBs could result in the compacter molecule that increased its migration rate in the gel while the intermolecular DBs doubled the molecular mass that decreased its migration rate. As shown in Fig. 3B, WT NDPK-A exhibited four bands all of which were proved to be NDPK-A by subsequent western blot analysis. The four bands are different DB isomers of WT NDPK-A, *i.e.* monomer of oxidized NDPK-A (14 kD), monomer of reduced NDPK-A (20 kD), dimer of oxidized NDPK-A (35 kD) and dimer of reduced NDPK-A (38 kD).

Whereas, the NDPK-A C145S mutant still exhibited to be one band, same as that in reduced condition (Fig. 3B). This data not only disclosed that a portion of WT NDPK-A was oxidized to form intra- and/or intermolecular DBs under non-DTT-treated condition, but also strongly suggested that C145 is a key residue in the formation of the DB isomers of NDPK-A. Further more, the intramolecular DB between cysteine 4 (C4) and C145 might be a precondition for the formation of intermolecular DB between C109s. Thus, point mutation of C145S not only disrupts the intramolecular in NDPK-A, but also prevents the intermolecular DB between two monomers (under weak oxidization condition, *e.g.* naturally dissolved oxygen in the solution). In summary of data disclosed here and reported previously, a model of the redox isomerization (RI) of NDPK-A was proposed (Fig. 3B). It is known that intermolecular cross-linkage will break the autologous hexamerization mediated by the non-covalent bonds of NDPK-A. From the isomerization character of NDPK-A, we deduced that the C145S mutation would facilitate and stabilize the hexamerization of NDPK-A, and therefore analysed its oligomerization state in aqueous solution.

When we first observe the four bands of WT NDPK-A, we thought the 14 kDa band might be a proteolitically cleaved segment of NDPK-A, or 20 kDa band might be a isomer of 14 kDa band via post-translational modification such as phosphorylation. But if the smallest two protein bands in Fig 3B are isomers of proteolitical cleavage or phosphorylation, DTT treated WT NDPK-A should also display to be two bands. *In situ* PFM analysis and other chemicophysical analysis such as N-terminal sequencing and RP-HPLC (data not shown) also supported that WT NDPK-A was an intact and homologous protein, and the migration difference of this protein in SDS–PAGE was only resulted by disulphide bond isomerization.

Autologous hexamerization of NDPK-A C145S in aqueous solution

NDPK-A is catalytically active in its hexameric form only. To analyse the oligomerization of NDPK-A C145S in aqueous solution, we determined the MW of the monomer (laser ionized) and oligomer (in aqueous solution) of this protein. The monomer of NDPK-A C145S was determined to be 17001 Dal by MALDI-TOF MS (data not shown), and the MW of this protein in aqueous solution was found to be 102 kD by MALS analysis. Figure 4A exhibited MALLS, DRI and UV signals during the elution of NDPK-A C145S, which was eluted from 9.078 to 9.786 min and peaked at 9.325 min. After data

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012

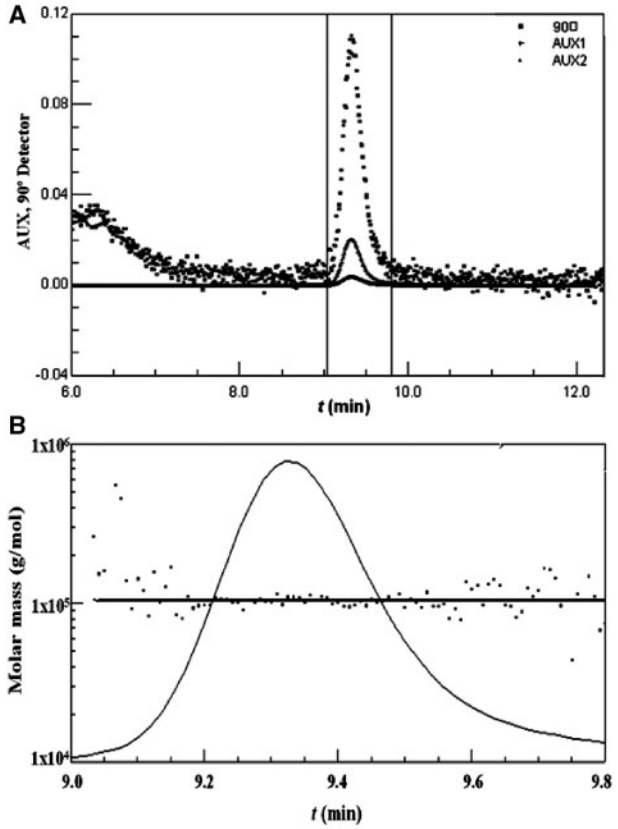

Fig. 4 Determination of the apparent MV of NDPK-A C145S in solution. NDPK-A C145S was loaded into a size exclusion HPLC system and the apparent MW of this protein was determined by MALLS and DRI analysis. (**A**) Signals detected by MALLS, UV and DRI detector. The line of scattered spots was the signal of MALLS, the lowest peak represented the DRI signal and the middle peak showed the UV signal. (**B**) Curve of apparent MW from MALLS/DRI analysis versus elution time of NDPK-A C145S. The scattered spots represented the distribution of the apparent MW of this protein. The line plotted from the spots is the average MW of NDPK-A C145S molecule in different time.

processed by ASTRA program, a curve of MALS signal versus MW versus elution time was plotted (Fig. 4B). From that the apparent MW of NDPK-A C145S in aqueous solution was confirmed to be 102 kD. Comparing the apparent MW of NDPK-A C145S in solution and the MW of its monomer, it is concluded that this protein has the same character with WT NDPK-A to form an autologous hexamer in solution.

The C145S mutation enhances the phosphate transferase activity of NDPK-A

NDPK-A is an essential enzyme that is required for the equilibration of the ratios of nucleoside triphosphates and diphosphates by catalyzing the reaction ATP + GDP $\longleftrightarrow$ ADP + GTP (*21*). Here, the phosphate transferase activities of WT and C145S mutant were assayed using ATP and UDP as substrates to evaluate the effect of C145S mutation on NDPK-A's activity. The results indicated that the C145S mutant had a higher phosphate transferase activity than that of WT NDPK-A (Fig. 5A), suggesting that the DB formed between cysteines might negatively regulate the enzyme activity of this protein.

The C145S mutation enhanced the DNase activity of NDPK-A

Another important activity of NDPK-A is its role as a granuzyme A-activated DNase (GAAD) in the process of cytotoxicity of T lympholeukocyte (CTL), which has recently been reported by Fan *et al.* (*22*). To assess the DNase activity of NDPK-A and its C145S mutant, we examined the cleavage activity of these two enzymes on supercoiled plasmid DNA. At a suitable temperature, NDPK-A will cut circular DNA into the linear form, which can be identified using agarose gel electrophoresis. Here, 1% agarose gel analysis showed that both NDPK-A C145S and WT NDPK-A indeed acted as a DNase after incubation with the substrate DNA at 37°C, although they were not as active as bovine pancreas DNase I. Furthermore, NDPK-A C145S exhibited significantly higher DNase activity than did WT NDPK-A (Fig. 5B). The percentage digestion of the supercoiled plasmid was calculated by scanning the agarose gel using a densitometer. An equal amount of substrate was used as a blank control. The results suggest that the NDPK-A C145S mutant could cut 90% of the supercoiled plasmid DNA, whereas WT NDPK-A was only able to cut ~68% (compared to specific bovine pancreas DNase I at 100%) (Fig. 5C). These results suggest that the C145S mutant can enhance the DNase activity of NDPK-A by eliminating the DB.

Discussion

NDPK-A has been implicated in the regulation of proliferation, differentiation, growth and apoptosis in various cellular processes, especially as a suppressor of metastasis in tumor cells, as well as in its role in the process of CTL (*22*). It is reported that NDPK-A may function in cell signaling by oxidative stress (*3*). In previous study, we determined the NDP activity of this protein after treated with oxidizing and reducing agents, respectively. The reduced form of NDPK-A displayed much higher enzymic activity than the oxidized form. PFM analysis showed that cross-linking of NDPK-A occurred in response to mild oxidative stress, involving Cys4, Cys109 and Cys145, and subsequent bioactivity analysis suggested that the formation of DBs might play a role in the functional regulation of NDPK-A.

To confirm this hypothesis and get bioactivity enhanced NDPK-A derivative, we constructed the recombinant expression plasmid pBV220-NDPK-A-C145S and over expressed this mutant in genetically engineered microorganisms. In previous purification, which we optimized, we isolated WT NDPK-A by anion exchange chromatography and affinity chromatography involving the substrate (ATP). This protocol also worked well for NDPK-A C145S, suggesting that the DB might not affect the binding of NDPK-A to its substrate, or possibly even, its oligomeric structure. Further more, the NDPK-A C145S mutant exerted increased bioactivity, suggesting that this mutant

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012

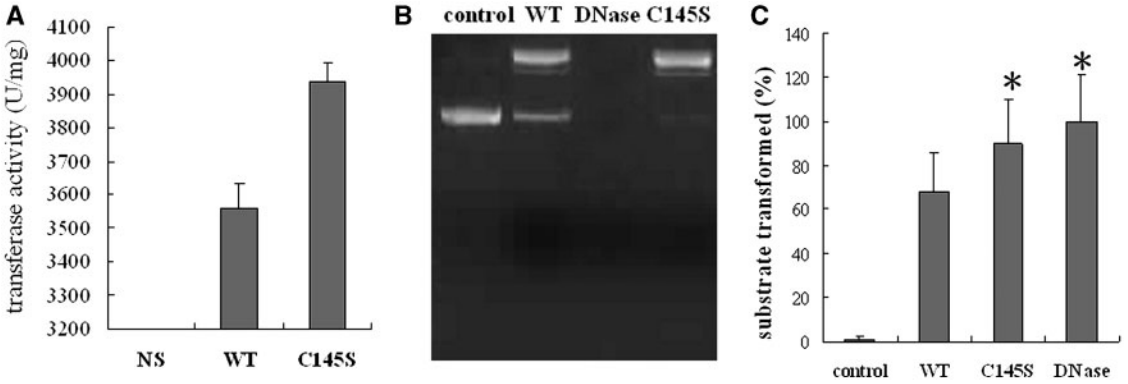

Fig. 5 Phosphotransferase and DNase activitives of the NDPK-A C145 mutant. (**A**) Phosphotransferase activity of NDPK-A C145 mutant. The phosphotransferase activity of NDPK-A C145S (C145S) was quantified with ADP and UTP as the substances and WT NDPK-A (WT) and normal saline (NS) were taken as the control groups. Mean ± SD, $n=3$. (**B, C**) DNase activity of the NDPK-A C145S mutant. Supercoiled DNA was incubated with WT, C145S and bovine pancreatic DNase 1 (DNase), respectively and the digestion was analysed using an agarose gel. The digestion of the supercoiled DNA (B) were scanned (gray scale) and the decrease in their percentages were calculated and compared (**C**). Mean ± SD, $n=3$, $^*P<0.05$ versus WT.

might be an important candidate for practical application of NDPK-A in future.

NDPK-A is only fully functional in its hexameric state mediated by non-covalent bonds and almost inactive in dimeric form (*23*, *24*). For example, Dictyostelium NDPK with P100S-N150stop double mutation will be dissociated into the dimeric form and lost its activity (*23*). The point mutation S120G in human NDPK-A has been found in several aggressive neuroblastomas (*25*) and this mutant exhibited reduced hexameric and increased dimeric oligomerization. We found that oxygen from air can partially dissociate native NDPK-A into a mixture of hexameric and dimeric form, decreasing its NDP transferase activity following the formation of DBs. Here, we found that the C145S mutation eliminated the intramolecular DB and prevented the formation of intermolecular DB which was reported to break the hexameric NDPK-A into dimeric one, but the mutation didn't affect the autologous oligomerization mediated by non-covalent bonds. Thus, the C145S mutation stabilized the hexamerization of NDPK-A and further enhanced its bioactivity including transferase and DNase. These results supported that the cross-linkage of redox-sensitive cysteine residues may be an important negative factor in the functional regulation of NDPK-A.

All the three cysteine residues in NDPK-A may participate in the formation of DBs under oxidative condition. From this study and previous data (*5*), we summarize that fully reduced NDPK-A is a hexamer with strongest activity. In response to the strength of oxidative stress, NDPK-A will firstly form the intramolecular DB between Cys4 and Cys145, and partially lost its activity. If the oxidative stress is so strong that all molecules are cross-linked by the intermolecular disulphides between Cys109 and Cys109, the dissociation of the hexamer into dimmer is unavoidable and the protein will be totally inactivated.

Oxidative stress, associated with reactive oxygen species, can cause damage to cells during pathological and physiological processes such as apoptosis, aging, tumor and neurodegenerative diseases *in vivo* (*26*–*28*). It has been reported that some proteins play important roles in the repair system of organisms through conformational change during oxidative stress (*3*). NDPK-A is very sensitive to oxidants from dissolved oxygen in aqueous solution to hydrogen peroxide and change its conformation and bioactivity gradually according to the strength of oxidative stress. Some proteins must be structure-stabilized or bioactivity-sustained by the DB between cysteines (*29*). However, DBs in NDPK-A act to be a negative regulator of its bioactivity. A reasonable deduction is that different oxidized and reduced forms of NDPK might protect the organism from damage caused by oxidative stress *in vivo*. Thus, NDPK-A in cellular redox equilibrium might have significant impact on its cellular function that deserve further investigation, and the C145S mutant prepared here shed light on the exploring of NDPK-A's multiple function.

Acknowledgements

We thank Dr Xiangping Meng and Mr Zenwei Huang for their contribution to this study.

Funding

The National Natural Science Foundation of China (30873082, 30400071); the Ministry of Science and Technology of China (2008BAI63B05); the Program for New Century Excellent Talents in University (NCET-07-0376); the 211 Project of Jinan University.

Conflict of interest

None declared.

References

1. Narayanan, R. and Ramaswami, M. (2003) Regulation of dynamin by nucleoside diphosphate kinase. *J. Bioenerg. Biomembr.* **35**, 49–55
2. Berg, P. and Joklik, W.K. (1953) Transphosphorylation between nucleoside polyphosphates. *Nature* **172**, 1008–1009
3. Song, E.J., Kim, Y.S., Chung, J.Y., Kim, E., and Chae, S.K. (2000) Oxidative modification of nucleoside diphosphate kinase and its identification by matrix-assisted

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012

laser desorption/ionization time-of-flight mass spectrometry. *Biochemistry* **39**, 10090–10097
4. Kim, Y.I., Park, S., Jeoung, D.I., and Lee, H. (2003) Point mutations affecting the oligomeric structure of Nm23-H1 abrogates its inhibitory activity on colonization and invasion of prostate cancer cells. *Biochem. Biophys. Res. Commun.* **307**, 281–289
5. Xiong, S., Qian, C.W., Wang, Y.F., Huang, L., Li, J.X., Yan, J.F., Wang, X.N., Zhang, X.W., and Bi, Z.G. (2005) Isomerization and its mechanism of nucleoside Diphosphate kinase A. *Chinese J Biochem Mol Biol* **21**, 815–821
6. Erent, M., Gonin, P., Cherls, J., Tissier, P., and Raschella, G. (2001) Structural and catalytic properties and homology model ling of the human nucleoside Diphosphate kinase C, product of the DR nm23 gene. *Eur. J. Biochem.* **268**, 1972–1981
7. Mesnildrey, S., Agou, F., Karlsson, A., Bonne, D.D., and Veron, M. (1998) Coupling between catalysis and oligomeric structure in nucleoside diphosphate kinase. *J. Biol. Chem.* **273**, 4436–4442
8. Georgescauld, F., Mocan, I., Lacombe, M.L., and Lascu, I. (2009) Rescue of the neuroblastoma mutant of the human nucleoside diphosphate kinase A/nm23-H1 by the natural osmolyte trimethylamine-N-oxide. *FEBS Lett.* **583**, 820–824
9. Mitchell, K.A.P., Szabo, G., and Otero, A.D.S. (2009) Direct binding of cytosolic NDP kinase to membrane lipids is regulated by nucleotides. *Biochim. Biophys. Acta* **1793**, 469–476
10. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
11. Bottoni, P., Giardina, B., Vitali, A., Boninsegna, A., and Scatena, R. (2009) A proteomic approach to characterizing ciglitazone-induced cancer cell differentiation in Hep-G2 cell line. *Biochimica et Biophysica Acta* **1794**, 615–626
12. Jin, L., Liu, G., Zhang, C.H., Lu, C.H., and Xiong, S. (2009) Nm23-H1 regulates the proliferation and differentiation of the human chronic myeloid leukemia K562 cell line: a functional proteomics study. *Life Sciences* **84**, 458–467
13. Sambrook, J., Fristsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn., Cold Spring Harbor Laboratory Press, New York
14. Yamaguchi, T. and Adachi, K. (2002) Hemoglobin equilibrium analysis by the Multiangle Laser Light-scattering method. *Biochem. Biophys. Res. Commun.* **290**, 1382–1387
15. Bartkowski, R., Kitchel, R., Peckham, N., and Margulis, L. (2002) Aggregation of recombinant bovine granulocyte colony stimulating factor in solution. *J. Protein Chem.* **21**, 137–143
16. Zhong, X.W., Huang, Q.Q., and Xi, T.F. (2006) Determination of molecular weight and molecular weight distribution of chitosan by malls in conjunction with refractive index detector. *Chin. J. Pharm. Ana.* **26**, 1258–1260
17. Qian, C.W., Wang, Y.F., Luo, Y., Zhang, M.Y., and Xiong, S. (2005) Determination of recombinant human NDPK - a activity by HPLC. *Chinese Journal of Health Laboratory Technology* **15**, 390–391
18. Beresford, P.J. (2001) Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. *J. Biol. Chem.* **276**, 43285
19. Zhang, D., Shankar, P., and Xu, Z. (2003) Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic. *Blood* **101**, 226
20. Krondiris, J.V. and Sideris, D.C. (2002) Intramolecular disulde bonding is essential for betanodavirus coat protein conformation. *J. Gen. Virol.* **83**, 2211–2214
21. Parks, R.E. and Agarwal, R.P. (1973) Nucleoside diphosphokinases In *The Enzymes* (Boyer, P.D., ed.) Vol. 8, pp. 307–334, Academic Press, New York
22. Fan, Z., Beresford, P.J., and Oh, D.Y. (2003) Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell* **112**, 659–672
23. Erent, M., Gonin, P., Cherls, J., Tissier, P., and Raschella, G. (2001) Structural and catalytic properties and homology modelling of the human nucleoside Diphosphate kinase C, product of the DR nm23 gene. *Eur. J. Biochem.* **268**, 1972–1981
24. Mesnildrey, S., Agou, F., Karlsson, A., Bonne, D.D., and Veron, M. (1998) Coupling between catalysis and oligomeric structure in nucleoside diphosphate kinase. *J. Biol. Chem.* **273**, 4436–4442
25. Lascu, I., Schaertl, S., Wang, C., Sarger, C., and Giartosio, A. (1997) A point mutation of human nucleoside diphosphate kinase A found in aggressive neuroblastoma affects protein folding. *J. Biol. Chem.* **272**, 15599–15602
26. Berlett, B.S. and Stadtman, E.R. (1997) Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **272**, 20313–20316
27. Stadtman, E.R. (1992) Protein oxidation and aging. *Science* **257**, 1220–1224
28. Jacobson, M.D. (1996) Reactive oxygen species and programmed cell death. *Trends Biochem. Sci.* **21**, 83–86
29. Wakabayashi, K., Nakagawa, H., and Tamura, A. (2007) Intramolecular Disulfide Bond Is a Critical Check Point Determining Degradative Fates of ATP-binding Cassette (ABC) Transporter ABCG2 Protein. *J. Biol. Chem.* **282**, 27841–27845

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012