Molecular Cloning and Characterization of a Tumor-Associated, Growth-Related, and Time-Keeping Hydroquinone (NADH) Oxidase (tNOX) of the HeLa Cell Surface†

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ABSTRACT: NOX proteins are growth-related cell surface proteins that catalyze both hydroquinone or NADH oxidation and protein disulfide interchange and exhibit prion-like properties. The two enzymatic activities alternate to generate a regular period length of about 24 min. Here we report the expression, cloning, and characterization of a *t*umor-associated *N*ADH *ox*idase (tNOX). The cDNA sequence of 1830 bp is located on gene Xq25-26 with an open reading frame encoding 610 amino acids. The activities of the bacterially expressed tNOX oscillate with a period length of 22 min as is characteristic of tNOX activities in situ. The activities are inhibited completely by capsaicin, which represents a defining characteristic of tNOX activity. Functional motifs identified by site-directed mutagenesis within the C-terminal portion of the tNOX protein corresponding to the processed plasma membrane-associated form include quinone (capsaicin), copper and adenine nucleotide binding domains, and two cysteines essential for catalytic activity. Four of the six cysteine to alanine replacements retained enzymatic activity, but the period lengths of the oscillations were increased. A single protein with two alternating enzymatic activities indicative of a time-keeping function is unprecedented in the biochemical literature.

Both animals and plants exhibit one or more hormone-responsive external plasma membrane hydroquinone oxidases (*1*) that use NADH as substrate (*N*ADH *ox*idase = NOX) and carry out protein disulfide−thiol interchange (*2*). At least two forms of NOX activities have been distinguished on the basis of response to capsaicin and certain anticancer drugs. A tumor-associated NOX (tNOX)[1] is drug responsive (*3*). The constitutive NOX (CNOX) is refractory to drugs (*3*). The drug-responsive tNOX activity has been found on the surface of solid tumors (*4*), on cancer cell lines in culture (*5*), in cell culture media conditioned by growth of cancer cells (*6*), and in sera of cancer patients (*7*, *8*) where the 34 kDa processed form of the protein is shed into the circulation.

The drug-inhibited tNOX activity is absent from sera of healthy volunteers or patients with diseases other than cancer but is widely distributed among tissues and sera of patients with both cellular (leukemia and lymphoma) and solid cancers (carcinomas and sarcomas) (*7*, *8*). CNOX, on the other hand, is present at the surface of all eukaryotic cells and tissues including noncancer, cancer, plant, and animal as well as in sera and conditioned culture media (*2*). CNOX responds to hormones and growth factors (*4*) but not to anticancer drugs (*3*). The CNOX protein is unusual in that the oxidative (NADH or hydroquinone) and protein disulfide−thiol interchange activities alternate to generate a 24 min period (*2*).

The tNOX protein exhibits both oxidative (NADH and hydroquinone) and protein disulfide−thiol interchange activities of the CNOX proteins. The oxidative and interchange activities of the tNOX protein also alternate but generate a 22 min rather than a 24 min period (*9*). Inhibition by vanilloids such as capsaicin (*3*) and by antitumor sulfonylureas (*10*) distinguishes tNOX from other NADH oxidases. Other unusual characteristics exhibited by tNOX include resistance to proteases, resistance to cyanogen bromide digestion, and an ability to form amyloid filaments closely resembling those of transmissible spongiform encephalopathies (*11*). Additionally, tNOX imparts protease resistance to a normally protease-resistant protein (*11*) as is characteristic of PrP^{sc} (PrP^{res}), the presumed infective and proteinase K-resistant form of the scrapie prion.

Here we report the cloning of a tumor-associated NOX protein. The bacterially expressed protein exhibits oscillating patterns of NADH oxidation and protein disulfide−thiol interchange with a period length of 22 min indistinguishable from those of cell surface tNOX. Capsaicin and the antitumor

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[1] Abbreviations: BCIP, 5-bromo-4-chloro-3-indoyl phosphate; capsaicin, 8-methyl-*N*-vanillyl-6-noneamide; CNOX, constitutive and drug-unresponsive cell surface NADH oxidase; DTDP, dithiodipyridine; DTT, dithiothreitol; EGCg, (−)-epigallocatechin-3-gallate; LY181984, *N*-(4-methylphenylsulfonyl)-*N*′-(4-chlorophenyl)urea; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NBT, nitroblue tetrazolium; PMSF, phenylmethylsulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; tNOX, tumor (cancer)-associated and drug- (capsaicin-) responsive cell surface NADH oxidase.

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sulfonylurea LY181984 completely inhibit the activity to confirm its identity as tNOX.

EXPERIMENTAL PROCEDURES

Generation of the Monoclonal Antibody. The antigen for generation of the monoclonal antibody was isolated and characterized from pooled sera of cancer patients (*12*). Two BALB/c mice were immunized with tNOX protein and boosted three times at 3 week intervals. Hybridomas were screened both by enzymatic activity assay and by Western blot analysis. Antibody-generating clones with the following characteristics were selected: (1) able to block completely the drug-responsive NOX activity of cancer cells and sera of cancer patients, (2) able to immunoprecipitate the protein with capsaicin-inhibited NADH oxidase activity from the surface of cancer cells and of sera pooled from cancer patients, (3) having no effect on the NADH oxidase activity of sera from healthy volunteers, and (4) reactive on Western blots with a 34 kDa cell surface protein of HeLa cells and sera of cancer patients. One such clone designated 12.1 was characterized extensively (*13*) and used for immunoscreening.

Isolation of the cDNA Clones. Monoclonal ascites (1:100 dilution) were used for immunoscreening of a Uni-Zap XR HeLa cDNA library (Stratagene, La Jolla, CA) as described (*14*). From a total of ca. 8×10^5 plaques, five positive clones were identified through three additional rounds of screening. The complete nucleotide sequence of clone 1, the longest cDNA, was obtained by gene walking using ten 17 bp synthetic primers (DNA Sequencing Service, Tufts University, Boston, MA). Searches within the NCBI/GenBank database were with nucleotide sequences and the amino acid sequences predicted from the longest open reading frame.

Expression of tNOX in Escherichia coli. tNOX cDNA from clone 1 was expressed in *E. coli* [BL21(DE3)] as a full-length form (beginning at M1, 71 kDa), as a truncated form (ttNOX) (beginning at M220, 46 kDa), as a fusion protein of truncated tNOX with six histidines fused to the amino terminus (ttNOX-his, 46 kDa), or as a processed tNOX (beginning at G327, 34 kDa). The open reading frame of ttNOX and processed tNOX cDNAs and nucleotides of the 3′ untranslated region were amplified by PCR and digested with *Nde*I and *Bam*HI, followed by ligation into the protein expression vector pET-11b. Primers for PCR amplification of ttNOX were 5′-GAGTGTAAACAGCATATGCTAGCCAGA-3′ (forward) and 5′-TTTCTATGCTTGTCCAACACATAT-3′ (reverse). Primers for the processed form of tNOX were 5′-GGAGATATACATATGGGAATTCTCATTCAA-3′ (forward) and 5′-TTTCTATGCTTGTCCAACACATAT-3′ (reverse). Primers for the histidine-tagged ttNOX were 5′-GATATACATATGCATCATCATCATCATCATCTAGCCAGAGAGGAGCGCCAT-3′ (forward) and 5′-TTTCTATGCTTGTCCAACACATAT-3′ (reverse) with the forward primer designed to incorporate six histidine residues at the amino terminus. *E. coli* [BL21(DE3)] and plasmid pET-34b(+) were from Novagen, Inc. (Madison, WI).

The processed tNOX (34 kDa) also was expressed as a fusion protein with a cellulose-binding domain. The open reading frame of the processed tNOX cDNA and nucleotides of the 3′ untranslated region were amplified by PCR and digested with *Kpn*I and *Bam*HI, followed by ligation into the protein expression vector pET-34b(+). Primers were 5′-CCAGATCTGGGTACCGATGACGACGACAAGGGAATTCTCATTCAATTT-3′ (forward) and 5′-TTTCTATGCTTGTCCAACACATAT-3′ (reverse). All primers were synthesized in the Laboratory for Macromolecular Structure, Purdue University. The amplification utilized an initiation step of 94 °C, 90 s of annealing at 55 °C, and 90 s of extension at 72 °C for 29 cycles.

After ligation of the tNOX cDNAs into the pET-11b or pET-34b(+), *E. coli* [BL21(DE3)] were transfected and grown in LB medium containing ampicillin (100 μg/mL for pET-11b) or kanamycin [50 μg/mL for pET-34b(+)] for 16 h at 25 °C. DNA sequences of the ligation products were confirmed by DNA sequencing. Cells were harvested by centrifuging at 4000*g* for 6 min and resuspended in 20 mM Tris-HCl, pH 7.8, containing 1 mM PMSF, 100 μM benzamidine hydrochloride, and 100 μM 6-aminocaproic acid. Proteins were extracted by French press (SLM Aminco French pressure cell press, three passages at 20000 psi). All forms of the recombinant tNOX proteins were extracted as soluble proteins but became insoluble after ammonium sulfate precipitation (20% saturation). The insoluble proteins were refolded according to the Protein Refolding Kit provided by Novagen (Madison, WI). Briefly, the recombinant proteins were washed with 1% Triton X-100 for 1 h at 25 °C, solubilized with 50 mM CAPS, pH 11, containing 1 mM DTT and 0.3% *N*-lauroylsarcosine sodium salt for 1 h at 25 °C, and refolded by dialysis at 4 °C for 16 h. Dialysis was performed with 20 mM Tris-HCl, pH 8.5, containing 0.1 mM cysteamine and 0.01 mM cystamine, 1 mM PMSF, 100 μM 6-aminocaproic acid (three changes), and 20 mM Tris-HCl, pH 7.8, containing 1 mM PMSF, 100 μM benzamidine hydrochloride, and 100 μM 6-aminocaproic acid (one change). Expression of all forms of tNOX was confirmed by SDS−PAGE with silver staining and immunoblotting. Immunoblot analysis was with anti-tNOX monoclonal antibody and alkaline phosphate-conjugated anti-mouse antibody. Detection was with BCIP and NBT.

Generation of Peptide Antisera. Peptide antisera to the tNOX C-terminus containing the putative adenine-binding site (KQEMTGVGASLEKRW) were generated by Covance Research Products Inc. (Dever, PA). The antisera were diluted 1:300 before use.

Generation of Polyclonal Antisera. The recombinant tNOX proteins expressed in *E. coli* were precipitated with 20% ammonium sulfate and resolved on a 12% SDS−PAGE and stained with Coomassie blue. The protein bands corresponding to the expressed tNOX were excised and chopped into fine pieces. The protein then was mixed with 0.5 mL of complete Freund's adjuvant and injected into two rabbits. Three boosts of antigen in incomplete Freund's adjuvant were given at intervals of 3 weeks. The antisera were diluted 1:300 before use.

Western Blot Analysis. Following separation on 12% SDS−PAGE, proteins were electroblotted to nitrocellulose and incubated overnight at 4 °C with 1:250 diluted polyclonal antibody to tNOX. Detection was with alkaline phosphatase-conjugated antibody diluted 1:5000 followed by incubation with NBT−BCIP.

N-Terminal Amino Acid Sequencing of Expressed tNOX. For partial amino acid sequencing, recombinant truncated

tNOX (ttNOX) proteins from *E. coli* extracts were precipitated with 20% ammonium sulfate, electrophoresed on 12% SDS−PAGE, and transferred to poly(vinylidene difluoride) membranes. Membranes were stained with Coomassie blue, and protein bands were excised and sequenced by automated Edman degradation (Applied Biosystems, Procise 492) by the Laboratory for Macmolecular Structure, Purdue University.

Mutagenic Oligonucleotides and Site-Directed Mutagenesis. Ten sets of oligonucleotides were designed to replace amino acid residues potentially involved in tNOX function by site-directed mutagenesis as described (*15*). Cysteines C505, C510, C558, C569, C575, and C602 were replaced by alanines. A methionine of the putative drug-binding site was replaced by alanine (M396A). A glycine in the potential adenine nucleotide-binding site was replaced by valine (G592V). Histidines in the conserved copper-binding domain were replaced by alanines (H546A, H562A). Oligonucleotides for mutagenesis were as follows: C505A, 5′-GAAAAGGAAAGCGCCGCTTCTAGGCTGTGTGCC-3′ (forward) and 5′-GGCACACAGTCTAGAAGCGGCGCTTTCCTTTTC-3′ (reverse); C510A, 5′-GCTTCTAGGCTGGCCGCCTCAAACCAGGATAGCG-3′ (forward) and 5′-CGCTATCCTGGTTTGAGGCGGCCAGCCTAGAAGC-3′ (reverse); C558A, 5′-GCAAGCATTGAATACATCGCTTCCTACTTGCACCGTCTTG-3′ (forward) and 5′-CAAGACGGTGCAAGTAGGAAGCGATGTATTCAATGCTTGC-3′ (reverse); C569A, 5′-CGTCTTGATAATAAGATCGCCACCAGCGATGTGGAGTG-3′ (forward) and 5′-CACTCCACATCGCTGGTGGCGATCTTATTATCAAGACG-3′ (reverse); C575A, 5′-CCAGCGATGTGGAGGCCCTCATGGGTAGACTCC-3′ (forward) and 5′-GGAGTCTACCCATGAGGGCCTCCACATCGCTGG-3′ (reverse); C602A, 5′-GAAAAGAGATGGAAATTCGCTGGCTTCGAGGGCTTGAAG-3′ (forward) and 5′-CTTCAAGCCCTCGAAGCCAGCGAATTTCCATCTCTTTTC-3′ (reverse); M396A, 5′-GTCTGATGATGAAATAGAAGAAGCGACAGAAACAAAAGAAACTGAGG-3′ (forward) and 5′-CCTCAGTTTCTTTTGTTTCTGTCGCTTCTTCTATTTCATCATCAGAC-3′ (reverse); G592V, 5′-CAGGAAATGACTGGAGTTGTGGCCAGCCTGGAAAAGAG-3′ (forward) and 5′-CTCTTTTCCAGGCTGGCCACAACTCCAGTCATTTCCTG-3′ (reverse); H546A, 5′-GGATTATCTCCACATTCCTTGCGGTTCACCCATTTGGAGC-3′ (forward) and 5′-GCTCCAAATGGGTGAACCGCAAGGAATGTGGAGATAATCC-3′ (reverse); H562A, 5′-GAATACATCTGTTCCTACTTGGCCCGTCTTGATAATAAGATCTGC-3′ (forward) and 5′-GCAGATCTTATTATCAAGACGGGCCAAGTAGGAACAGATGTATTC-3′ (reverse). The mutagenesis was with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The correctness of the amino acid replacements was confirmed by DNA sequencing.

5′ Rapid Amplification of the cDNA End (5′ RACE). 5′ RACE System for Rapid Amplification of cDNA Ends, version 2.0, was from GIBCO (Gaithersburg, MD). Two gene-specific primers (GSP) were synthesized. GSP1, 5′-CAGACAGATACAGGGCTTTG-3′ (antisense sequence corresponding to tNOX nucleotides 571−552); GSP2, 5′-GGTAAAATTGGTTGTCCGGC-3′ (antisense sequence corresponding to tNOX nucleotides 204−185). HeLa total RNA (5 μg) and 2.5 pmol of GSP1 were used for first-strand cDNA synthesis. When cDNA synthesis was completed, RNAs in the reaction mixture were digested using RNase H and RNase T1 followed by cDNA purification using a GlassMax DNA isolation spin catridge (GIBCO). The purified cDNA was then used in the TdT (terminal deoxynucleotidyl transferase) dC tailing reaction. The dC-tailed cDNA was employed as template for PCR using a bridged anchor primer 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′ and GSP2 primer sets. All PCR products were sequenced.

Fractionation of OVCAR-3 Cells. Cells of the human ovarian cancer cell line OVCAR-3 were obtained from the ATCC (Rockville, MD) and fractionated as described by Chang and Pastan (*16*). Approximately 1×10^8 cells grown to near confluence were rinsed with PBS, harvested by scraping, and then pelleted and washed once in PBS. The cells were resuspended in 5 mL of 10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, and 1 mM EDTA. After 10 min at 4 °C, the cells were homogenized by strokes of a tight Dounce homogenizer. Nuclei, unbroken cells, and debris were removed by centrifugation at 200*g* for 10 min at 4 °C. The membrane-rich fraction was collected by centrifugation at 100000*g* for 90 min.

To solubilize the CAK1 protein, 10% octyl glucoside in 0.25 M sodium phosphate, pH 7.5, 0.02% sodium azide, 0.05 M EDTA, 0.1% SDS, 1% β-mercaptoethanol, and 1 mM PMSF was added at a final detergent concentration of 1% and incubated with agitation for 2 h at 4 °C. Both pellet and supernatant were adjusted to equal volumes prior to analysis by SDS−PAGE.

Enzyme Assays. NADH oxidation was measured spectrophotometrically from the disappearance of NADH measured at 340 nm (*3*). Protein disulfide−thiol interchange was determined either by restoration of activity to scrambled and inactive RNase prepared from native RNase A as described with cCMP as the RNase substrate (*17*) or by spectrophotometric means from the cleavage of dithiodipyridine (DTDP) (*18*). Proportionality of enzymatic activity to protein concentration with the purified ttNOX or with the bacterial extracts was verified for all three assays.

Proteins were estimated by the bicinchoninic acid (BCA) procedure (*19*) with bovine serum albumin as standard.

Determination of Copper. tNOX protein from sera (the antigen sources for the monoclonal antibody) and recombinant tNOX were analyzed for copper content by atomic absorption spectroscopy (Perkin-Elmer AS-90). Standards were diluted from 1 g/L metal stock (Aldrich, Milwaukee, WI). tNOX and $CuCl_2$ were preincubated for 24 h at 4 °C at a 10-fold molar ratio of excess $CuCl_2$. Samples were then dialyzed for 48 h against four changes of 20 mM Tris-HCl, pH 7.8. Samples analyzed contained between 50 and 200 μg/mL bound copper and were within the linear range of the assay.

RESULTS

Molecular Cloning. Monoclonal antibody screening of a HeLa cell cDNA library generated five clones. Restriction enzyme digestions were consistent with the derivation of all five clones from a single primary phage clone. Automated nucleotide sequencing confirmed that all five clones were inserts of different lengths of the same DNA. The largest clone contained a 3.8 kb insert and an open reading frame of 1830 bp (from nucleotides 23−1852).

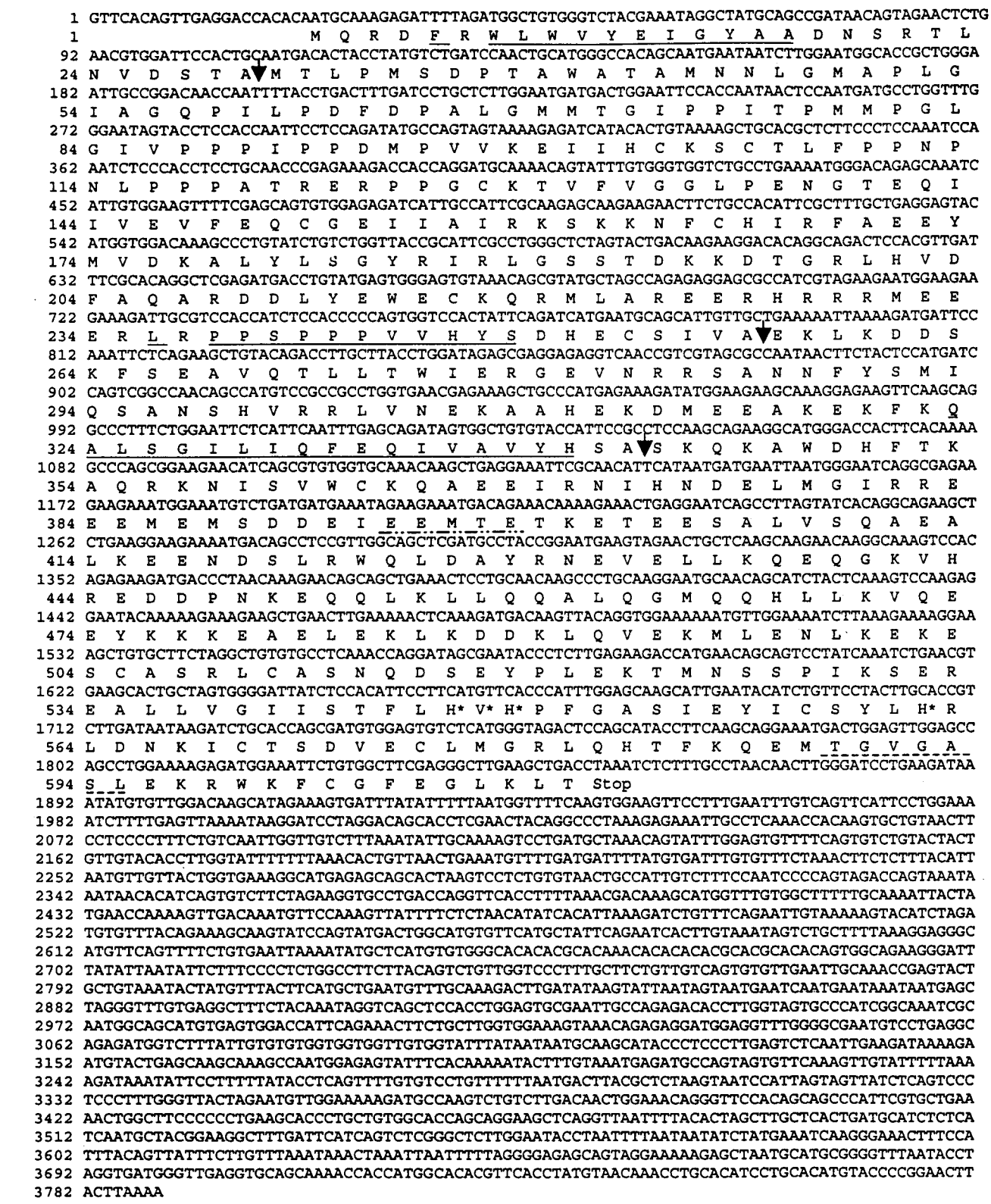

FIGURE 1: Nucleotide and deduced amino acid sequences of the tNOX cDNA. The first potential translation initiation site is indicated at nucleotides 23−25 (ATG) with termination at 1855−1857 (TAA). Putative signal peptides are underlined, and the signal peptide cleavage sites are indicated by arrows. The putative quinone-binding sequence, E394EMTE, is denoted by a long dash−dot dot line. The H546VH and downstream H562 copper sites are shown by asterisks. The adenine nucleotide (NADH) binding sequence, T589GVGASL, is underlined by dashes. The sequence data are available from GenBank under Accession No. AF207881.

The full-length cDNA yielded an open reading frame for a deduced amino acid sequence of a protein of 610 amino acids, with a predicted molecular mass of 70.1 kDa (Figure 1, GenBank Accession No. AF207881). A typical Kozak sequence (AXXATG) (*20*) was at nucleotide 20. A putative initiator methionine at nucleotide 23 was followed at F5 by a sequence of 12 hydrophobic residues in the position of a signal sequence. A typical polyadenylation signal (AATAAA) at nucleotide 3625 followed the termination codon at nucleotide 1853. On the basis of available genomic informa-

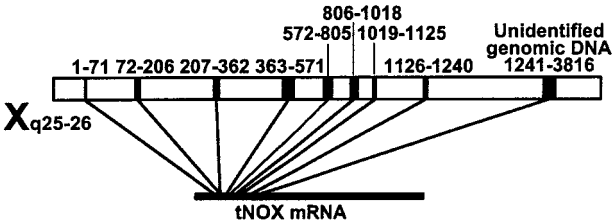

FIGURE 2: Exon−intron organization of the coding for the human tNOX gene located on chromosome Xq25-26. Closed boxes in the genomic DNA map represent the identified protein-coding exons. At least nine exons have been identified within the genomic information available (*21*).

tion (*21*), this particular tNOX cDNA is comprised of multiple exons (at least nine) in just the N-terminal portion of the full-length precursor (Figure 2). 5′ RACE under several different conditions and with RNA from both HeLa and BT-20 (human mammary carcinoma) cells revealed no nucleotides upstream beyond nucleotide base 1 of the cDNA of tNOX. Thus, the tNOX cDNA contained an intact exon 1 and a 5′ untranslated sequence of 22 bases.

The C-terminal portion of the derived amino acid sequence corresponded to the mature, processed molecular mass of 34 kDa (ca. 33.5 kDa from serum) that is refractory to enzymatic or chemical cleavage (*11*). An incompletely processed 38.5 kDa protein from the HeLa cell surface amenable to N-terminal sequencing, however, yielded a sequence confirming that found in recombinant tNOX (*22*). Unambiguous assignments in 11 of 18 cycles yielded a sequence identical to that found in recombinant tNOX of similar molecular mass:

1 DXEXXIVAXKLKDDXKXS 18
(HeLa 38.5 kDa tNOX)

249 DHECSIVAEKLKDDSKFS 266
(recombinant tNOX)

The protein cross-reacted with the tNOX-specific monoclonal antibody, exhibited drug-responsive tNOX activity, and was converted to an immunoreactive and drug-responsive 34 kDa protein with tNOX activity upon digestion with proteinase K.

Protein Expression in E. coli and Enzymatic Activities of the Expressed Protein. Recombinant forms of tNOX were expressed in *E. coli* including the full-length 70.1 kDa form, a truncated 46 kDa form beginning at M220 (ttNOX), a 46 kDa histidine-tagged ttNOX, and a 34 kDa truncated tNOX beginning at G327. The 34 kDa truncated tNOX also was expressed as a fusion protein with a cellulose-binding domain (CBD-ttNOX). The entire sequence of the subcloned cDNA expressed in *E. coli* was confirmed by resequencing. tNOX proteins were identified by reaction with the tNOX-specific monoclonal antibody MAB 12.1 (Figure 3B). The apparent molecular masses of the ttNOX, the ttNOX with the histidine tag, and the processed tNOX were 48, 48, and 42 kDa, respectively, and direct amino acid sequencing confirmed that the expressed proteins purified from bacterial extract were derived from the tNOX cDNA. The induced bacterial extract exhibited a NADH oxidase activity with a 22 min period (arrows in Figure 4A). Neither the induced bacterial extracts when measured in the presence of 1 or 100 μM capsaicin (open circles in Figures 4A and 5) nor the uninduced extracts (Figure 4B) had periodic activity. The addition of 1 μM antitumor sulfonylurea LY181984 or 1 μM (−)-epigallocatechin-3-gallate (EGCg) completely inhibited the activity as well (not shown).

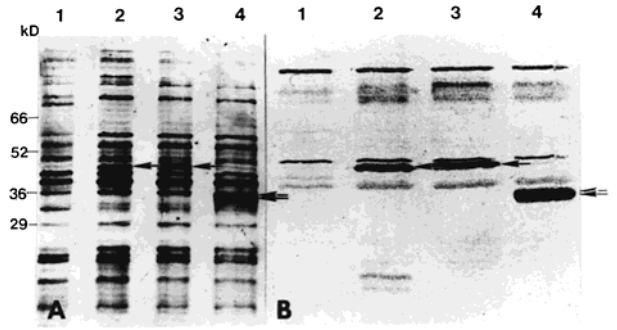

FIGURE 3: Expression of truncated 46 kDa tNOX (ttNOX), histidine-tagged ttNOX, and truncated 34 kDa tNOX. tNOX proteins were expressed in *E. coli* and analyzed by SDS−PAGE with silver staining (A) and by Western blot analysis (B). For Western blot analysis, proteins were reacted with the anti-tNOX monoclonal antibody MAB 12.1 (*13*) used for cloning and alkaline-phosphatase conjugated anti-mouse antibody. Detection was with BCIP and NBT. Expressed tNOX proteins were recognized by MAB 12.1. Arrows indicate expressed proteins of apparent molecular mass 48 kDa corresponding to the expressed protein of 46 kDa and to the processed protein of 34 kDa, respectively. The unspecific bands of lane 1 result from reaction of bacterial proteins with the second antibody and/or alkaline phosphatase substrate in the absence of MAB 12.1. Lane 1: vector alone. Lane 2: ttNOX. Lane 3: histidine-tagged ttNOX. Lane 4: processed tNOX.

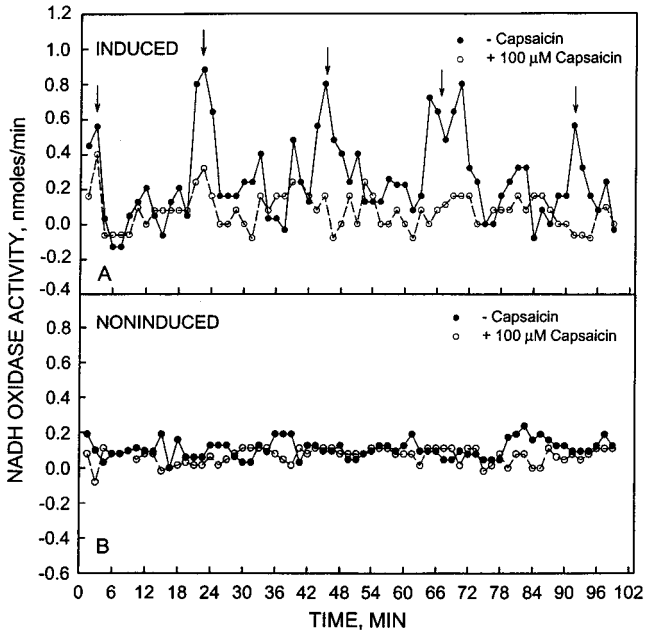

FIGURE 4: Rate of oxidation of NADH as a function of time over 100 min showing five maxima (arrows). (A) Enzyme activity of the bacterial extract expressing truncated tNOX by the addition of IPTG. (B) Enzyme activity of the bacterial extract expressing no truncated tNOX. The induced extract exhibited periodic NADH oxidase activity which was inhibited by 100 μM capsaicin. The estimation of NADH oxidation is based on the decrease in A_{340} as described (*3*).

Illustrated in Figure 5 for the recombinant protein is one of the unique features of NOX proteins where the maximum rates of the hydroquinone (NADH) oxidase activity and the maximum rates of protein disulfide−thiol interchange activity [dithiodipyridine (DTDP) cleavage] alternate. As the rate of

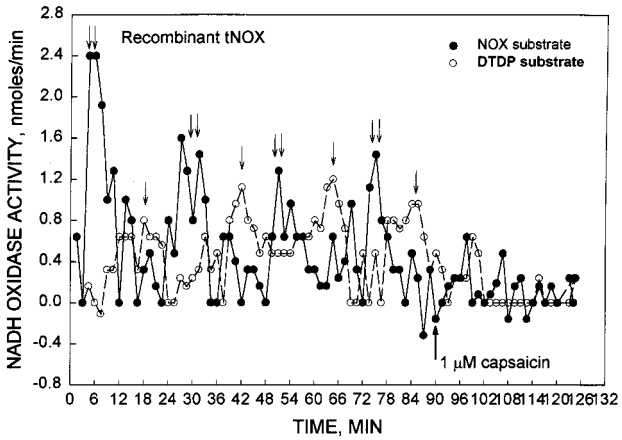

FIGURE 5: Simultaneous measurement of NADH oxidation (solid symbols and lines) and protein disulfide−thiol interchange (dotted curve, open symbols), the latter using the dithiodipyridine (DTDP) substrate. As indicated by the arrows denoting rate maxima (double arrows for NADH oxidation, single arrows for protein disulfide−thiol interchange), the rate maxima of the two activities alternated. Capsaicin (1 μM) inhibited both enzymatic activities.

oxidation of NADH declined, the rate of DTDP cleavage increased so that DTDP cleavage was at a maximum when NADH oxidation was at a minimum. Both had approximately the same period length of 22 min that was 2 min shorter than the constitutive NOX period length of normal cells of 24 min. Both activities were inhibited when 1 μM capsaicin was added (arrow, Figure 5).

The two alternating activities were exhibited by purified recombinant CBD tNOX as well (Figure 6A). Maxima and minima for each activity were significantly different ($p < 0.001$). The maxima in rates of NADH oxidation (double arrows) were separated clearly from the maxima in rates of protein disulfide−thiol interchange (single arrows), both with a period length of about 22 min. The periodic activity was inhibited by the tNOX-specific antibody (MAB 12.1) used in expression cloning (Figure 6B). Inhibition was delayed until the beginning of a new cycle of oxidative activity. Protein disulfide−thiol interchange activity of the recombinant protein was confirmed by its ability to restore activity to denatured and inactive (scrambled) ribonuclease (Figure 6C).

Protein expression was optimized to produce at least 50 mg of recombinant proteins/200 mL of media, and the refolded proteins were 85−90% pure as determined from SDS−PAGE and silver staining.

Identification of Functional Motifs. Several potential functional motifs required for the activities associated with the NOX protein were located in the 34 kDa C-terminal portion of the expressed protein. The sequence E394-E-M-T-E forms a putative sulfonylurea- and/or quinone-binding site with four of five amino acids occurring in at least one other putative quinone site in the same relative positions (Table 1). The correctness of this assignment is supported by analyses of a M396A replacement where full activity was retained but the response to capsaicin was lost (Table 2). C505 and C569 provided potential active site cysteines for the protein disulfide−thiol interchange activity based on site-directed mutagenesis (Table 2). Also indicative of involvement of C569 in the protein disulfide−thiol interchange activity was inhibition of the enzymatic activity by antisera to a C-X-X-X-X-X-C-containing peptide (LAILPACATPATCNPD) corresponding to C569-X-X-X-X-X-C575.

Table 1: Comparison of Amino Acid Sequences within the Known Quinone- and Sulfonylurea-Binding Sites of Several Proteins

protein	sequence
Q_B protein[b]	S A M H G
L/M subunit[b]	L A M H G
acetolactate synthetase (tobacco)[b,c]	L G M H G
pyruvate oxidase[b]	A T M H W
D_1 *Synechococcus*	E T M R F
NADH (ubiquinone) dehydrogenase	G E M R E
bovine serum albumin	E T M R E
human serum albumin	A T L R E
acetolactate synthetase (*Brassica*)	E D L R E

[a] Binds quinone. [b] Binds sulfonylurea.

Table 2: Effect of Site-Directed Mutagenesis of ttNOX on NADH Oxidase Enzyme Activity, Period Length, and Inhibition by Capsaicin

replacement[a]	enzymatic activity[b]	period length (min)	inhibition by 1 μM capsaicin
none	+	22	+
C505A	−		
C510A	+	36	+
C558A	+	42	+
C569A	−		
C575A	+	36	+
C602A	+	36	+
M396A	+	22	−
H546A	−		
H562A	−		
G592V	−		

[a] Resequencing confirmed the expected DNA sequences for each of the indicated amino acid replacements. [b] Absolute specific activities were determined from detailed kinetic analyses of both NADH oxidation and protein disulfide−thiol interchange (*47*) for each mutant. For example, with C505A and C569A replacements both NADH oxidase and protein disulfide−thiol interchange activities were absent.

The sequence T589-G-V-G-A-S-L together with E605 forms a putative binding site for the adenine portion of NADH. In this motif, five of seven amino acids are conserved with mitochondrial adenine-binding proteins (*23*). The H546-V-H motif conserved in periplastic copper oxidases together with His562 form a copper-binding ligand potentially important to oxygen binding. In addition, the H546-V-H-P-F-G motif is conserved in the copper-binding site of both human and chicken superoxide dismutases (*24*). Both the G592V replacement in the adenine-binding domain and the H546A replacement in the putative copper site lacked enzymatic activity when expressed in *E. coli* (Table 2). Copper analyses by atomic absorption spectroscopy revealed ca. 1 mol of copper/mol of 34 kDa processed tNOX purified from sera of cancer patients, 0.7 ± 0.1 mol of copper/mol of recombinant tNOX, and 1.3−1.5 mol of copper/mol of tNOX for recombinant tNOX loaded with excess copper followed by dialysis. No zinc or iron were detected in fully active tNOX from either source.

Potential N-glycosylation sites (NXS/T) were at positions 138, 358, 418, and 525. Potential O-glycosylation sites were at T38, T71, S35, and S240. tNOX purified from HeLa cells contained trace amounts of galactose, glucosamine, glucose, and mannose (*22*). The electrophoretic mobility of the protein was unaffected by deglycosylation with peptide *N*-glycanase F and/or desialylation with neuraminidase.

Membrane Association. NOX proteins are membrane-associated but not transmembrane. Three putative signal sequences and cleavage sites were identified as potentially membrane targeting near the N-terminus. The first was 11 amino acids long. The second, also 11 amino acids long, was near M220 and would yield a 45.6 kDa protein containing all of the identified functional motifs above. The third and longest potential signal sequence (17 amino acids) near M314 would yield a 34 kDa protein also containing all of the identified functional motifs.

The membrane association of the 34 kDa processed form of tNOX appears to be as a non-lipid-linked, extrinsic protein of the external plasma membrane surface (*5*, *25*). It is released by incubation at pH 5 (*26*). The hydropathy plot of the derived amino acid sequence of tNOX does not predict for membrane-spanning domains (*25*).

The deduced amino acid sequence of the tNOX protein showed homology over part of its length with the deduced amino acid sequence of a cDNA previously designated as APK1 antigen (from K357 to T610 of tNOX) (*16*; GenBank Accession No. 572094). However, neither the cell surface-derived, serum-derived, nor the recombinant tNOX shared significant characteristics with the K-1 antigen. A high titer polyclonal antibody to the recombinant tNOX reacted with both unprocessed (70 kDa) and processed (34 kDa) forms of tNOX expressed by OVCAR cells but failed to show any reactivity in portions of the gel corresponding to molecular masses of the 30 kDa APK1 antigen or the 40 kDa CAK1 either in detergent-solubilized (Figure 7) or in unsolubilized fractions. The CAK1 protein, rather than tNOX, was subsequently identified as the protein reactive with the K1 antibody (*16*, *27*). The CAK1 protein is expressed primarily in cell lines of mesothelial origin (*27*) and is anchored in the membrane by a glycosidic phosphatdylinositol (GPI) anchor. tNOX is more widely distributed and lacks a GPI anchor (*25*).

Response of ttNOX Period Length to Cysteine Replacements. ttNOX proteins with six different cysteine to alanine replacements were expressed in *E. coli*. Of these, C505A and C569A exhibited neither NADH oxidase nor protein disulfide−thiol interchange activity. The four other cysteine replacements retained both NADH oxidase and protein disulfide−thiol interchange activities, but the period lengths were increased from 22 to 36 min for C510A, C575A, and C602A and to 42 min for C558A (Table 2).

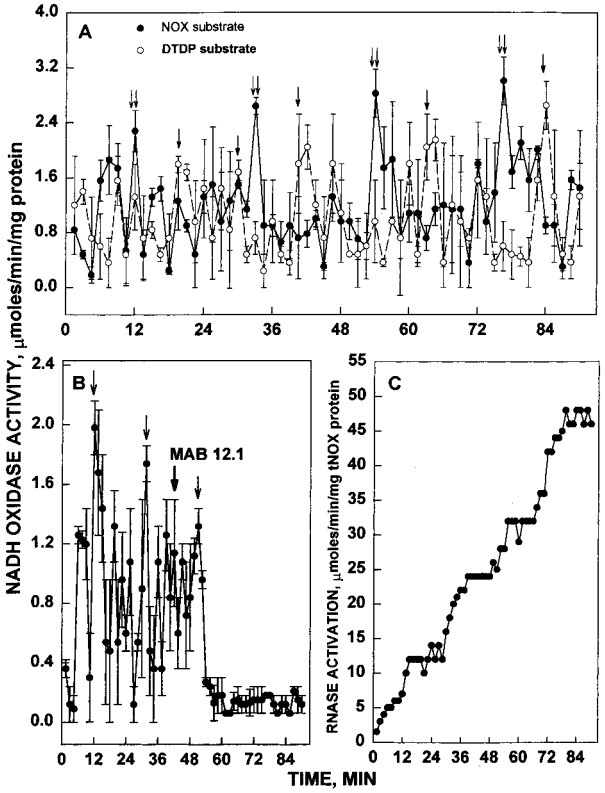

FIGURE 6: NADH oxidation (A, B) and protein disulfide−thiol interchange (A, C) of purified recombinant CBD tNOX. (A) Simultaneous measurement as in Figure 5 of NADH oxidation (solid symbols and lines) and protein disulfide−thiol interchange (dotted curve, open symbols), the latter with the DTDP substrate. Double arrows denote maxima for NADH oxidation. Single arrows denote maxima for protein disulfide−thiol interchange. Maxima and minima were significantly different ($p < 0.001$) for both activities, and the two activities alternated. Results are averages of three experiments ± standard deviations. Each experiment was carried out as for Figure 5 using protein of the same ttNOX preparation and two paired spectrophotometers with NADH substrate with one and DTDP substrate with the other. Each of the three repetitions was then phase fitted to the first, and standard deviations were calculated. (B) NADH oxidation and inhibition by MAB 12.1, the same antibody as used in expression cloning. Inhibition was delayed until the beginning of a new activity cycle and was complete thereafter. Results are averages of three experiments ± standard deviations. Two experiments were carried out simultaneously in two paired spectrophotometers. The third experiment was carried out subsequently in one of the instruments and phase fitted to the other two. (C) Time course of activation of protein disulfide−thiol interchange activity estimated from the activation of scrambled RNase with cCMP as the RNase substrate.

DISCUSSION

Our work identifies a novel protein at the external cell surface of cancer cells (tNOX) with properties of a prion (*11*) that exhibits both hydroquinone (NADH) oxidase and protein disulfide−thiol interchange activities. An unprecedented feature of the protein is that the oxidative and the interchange activities alternate to generate a regular period length of 22 min.

Interest in NOX proteins derives not only from their plasma membrane location but also from their potential roles as time-keeping proteins (*9*) and a relationship between the oscillatory enzymatic activity and cell growth (*2*, *28*). Both tNOX, with a period length of 22 min (*1*, *9*), and CNOX, with a period length of 24 min (*28*, *29*), appear to be members of a family of NADH oxidases with two similar enzymatic activities that cycle to produce regular oscillations where the period lengths are independent of temperature and entrainable (CLOX family of cycling cell surface NADH oxidases).

Several NOX forms belonging to this CLOX family exist on the basis of enzymatic activity measurements. The cDNA of the drug-responsive tNOX is the first to be cloned. tNOX activity differs from the activity of the constitutive CNOX form also present in both cancer and noncancer tissues by being inhibited by capsaicin, EGCg, several anticancer drugs, and thiol reagents and by having a period length of 22 min. The inhibition of tNOX activity by capsaicin was used to guide purification of the tNOX protein from sera of cancer patients, as the basis for the monoclonal antibody selection, and to confirm the identity of the cloned tNOX cDNA.

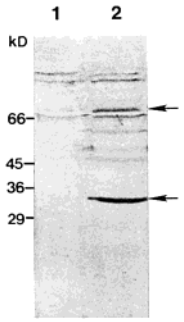

FIGURE 7: Western blot analysis of OVCAR-3 cells using antisera raised from rabbit immunized with expressed tNOX. Lane 1: membrane pellet after octyl glucoside solubilization. Lane 2: supernatant after octyl glucoside solubilization. Arrows indicate immunoreactive unprocessed tNOX (70 kDa) and processed tNOX (34 kDa). The regions of the gel corresponding to APK1 (29 kDa) and mesothelin (40 kDa) lacked immunoreactive material. The identities of the minor bands in the higher molecular weight region of the gel are unknown.

A portion of the tNOX sequence obtained was one previously attributed by Chang and Pastan (*16*) to a cytosolic protein, the APK1 antigen. The sequence was obtained by expression cloning using a K1 antibody produced from the ovarian carcinoma cell line, OVCAR-3, as immunogen. The longest cDNA they obtained contained 2444 bp with a 789 bp open reading frame that encoded a protein of 30.5 kDa. A portion of the cDNA of tNOX appears to be the same as that of tNOX except that their sequence contained one extra T at nucleotide 929 and one less G at nucleotide 1092 (at the nucleotides 83 and 247 of their sequence). These differences generated an incorrect reading frame. The two errors were confirmed by Sugano et al. (*30*).

The protein reactive with the K1 antibody, originally identified as CAK1, was a membrane-bound protein with a molecular mass of 40 kDa (*16*). Chang and Pastan (*31*) eventually isolated a 2138 bp cDNA that encoded CAK1. The cDNA had an 1884 bp open reading frame encoding a 69 kDa protein. The 69 kDa precursor was processed to the 40 kDa form characteristic of mesothelin. Mesothelin (CAK1) is associated with the cell membrane via a glycosylphosphatidylinositol tail, is not shed into the sera of cancer patients, nor does it appear in conditioned medium supporting the growth of cultured cells (*16*, *31*). No protein sequence homology or overlap of functional characteristics has been found between CAK1 and tNOX.

Appropriate motifs were encountered within the ttNOX sequence to account for the range of functional activities exhibited by tNOX and other NOX proteins. We had previously photoaffinity-labeled the tNOX protein with [^{32}P]-NAD(H), indicating that it contained a pyridine nucleotide-binding site. The typical adenine nucleotide-binding sequence motif (G-X-G-X-X-G) with downstream remote acidic amino acid residues D or E (*23*) was represented by T589-G-V-G-A-S-L and E605 near the C-terminus. This sequence resembled closely the sequence T-G-V-G-A-G-V-G from mitochondrial ATP synthase protein subunit 9 from *Chondrous crispus* (*32*). The inactivity of the tNOX carrying G592V replacement supported this assignment.

The NOX protein binds the antitumor sulfonylurea LY181984 (*33*), and activity is inhibited or stimulated depending on the redox environment of the protein (*34*). Reduced coenzyme Q is readily oxidized by the protein (*1*). Other substances which inhibit the activity such as capsaicin, (−)-epigallocatechin-3-gallate (EGCg), and adriamycin are considered to occupy quinone sites. Ubiquinone protects against the binding and inhibition of activity by the antitumor sulfonylurea LY181984. Thus, the presence in the tNOX sequence of a motif indicative of quinone binding as well as binding of antitumor sulfonylureas and other molecules regarded to occupy quinone sites or cryptic quinone sites was sought on the basis of the suggestion that a site with a methionine−histidine pair represented such a site in pyruvate oxidase (*35*) and by analogy with several quinone-binding proteins of the photosystem II complex of chloroplasts. All known urea and sulfonylyrea herbicide inhibitors of photosystem II are directed to such sites (*36*). On the basis of these considerations, a preliminary consensus sequence for the amino acids surrounding the charged residues critical to the sulfonylurea-binding site was determined to be A-M-H-G or a closely related sequence (Table 1). Apparently arginine can substitute for the critical histidine (*36*). For example, the putative quinone-binding site of the D_1 protein of a cyanobacterium, *Synechococcus*, contains the sequence E-T-M-R-E. A sequence similar to the E-T-M-R-E sequence is present in the NADH−ubiquinone oxidoreductase of chloroplasts. Serum albumins also bind sulfonylureas, and their putative sulfonylurea-binding sites are included in Table 1 as well. We found a sequence, E394-E-M-T-E, as a potential quinone site having neither H nor R in the fourth position but still with a degree of similarity to other putative quinone- and/or sulfonylurea-binding sites. The correctness of identification of E394-E-M-T-E as the drug-binding site is supported by findings from the M396A replacement which retained NADH oxidase activity but where the activity was no longer inhibited by capsaicin (Table 2).

The first demonstrations of the thiol interchange activity for the tNOX protein used as the principal criterion the restoration of activity to reduced, denatured, and oxidized (scrambled) yeast RNase through reduction and refolding under nondenaturing conditions and reoxidation to form a correct secondary structure stabilized by internal disulfide bonds (*37*). The restoration of activity to scrambled yeast RNase was similar to that catalyzed by protein disulfide isomerases of the endoplasmic reticulum (*38*) but was clearly due to an activity of a different protein. The characteristic C-X-X-C motif common to most, if not all, members of the protein disulfide isomerase family of proteins (*39*) and present, as well, in thioredoxin reductase and related proteins where it appears to catalyze the transfer of electrons in conjunction with bound flavin (*40*, *41*) is lacking in tNOX. In addition to lacking C-X-X-C, tNOX does not appear to contain bound flavin nor is its activity dependent upon addition of flavins (FAD or FMN). Thus the protein disulfide−thiol interchange catalyzed by tNOX appears to be unique from that of classic protein disulfide isomerases or thioredoxin reductases.

While the two C-X-X-X-X-C motifs characteristic of flavoproteins are missing from tNOX, the redox-active disulfide of thioredoxin reductase from the malaria parasite *Plasmodium falciparum* contains a motif, C88-X-X-X-X-C93 (*42*), similar to one found in tNOX. Together with a downstream His 509, this motif was shown to be a putative proton donor/acceptor. Either a C88A or the C93A replacement resulted in complete loss of enzymatic activity (*42*). A

C535-X-X-X-X-C540 motif in the same protein was crucially involved in substrate coordination and/or electron transfer (*43*). A C535A replacement did result in diminution of enzymatic activity, but the C5440A replacement did not (*43*). Thus, either or both of the two comparable motifs present in tNOX, C505-X-X-X-X-C510 or C569-X-X-X-X-X-C575, alone or together with downstream histidines, provide potential active sites for protein disulfide−thiol interchange. With tNOX, the C505A and C569A replacements lost activity as with the C535A replacement above for the *P. falciparum* protein, but the C510A and C575A did not as with the above C540A replacement for the *P. falciparum* protein.

Four of the cysteine mutations analyzed exhibited an altered period length for the oscillations in tNOX activity (Table 2). Both NADH oxidation and protein disulfide thiol interchange were affected in parallel. The period length was increased from 22 to 36 min for three of the four replacements and to 42 min for the fourth. The significance of these findings rests in observations that COS cells transfected with tNOX having a period length of 22 min exhibit both 22 and 24 h circadian rhythms whereas COS cells transfected with the tNOX with a period length of 36 min exhibit a 36 h circadian rhythm (60 × tNOX period length). These results are being prepared for publication in a separate report. Of potential interest is the observation that the six amino acid motif M588-T-G-V-G-A of tNOX is shared with the *Drosophila melanogaster* clock *period* protein (*44*).

During the oxidative portion of the NOX activity cycle, the tNOX protein catalyzes the transfer of electrons and protons to molecular oxygen with a stoichiometry of 1 mol of NADH + H^+ oxidized to 1 mol of 1/2 O_2 reduced. Oxygen uptake by plasma membranes prepared from HeLa cells is inhibited by the antitumor sulfonylurea LY181984 with approximately the same dose response (Figure 1 of ref *45*) as are other aspects of tNOX activity (compare to Figure 6 of ref *45*). Therefore, tNOX and NOX proteins in general must bind oxygen. The minimum requirement for an oxygen site would appear to be a metal together with appropriate covalent interactions such as hydrogen bonding (*46*). There are no indications in the tNOX sequence for regions with a typical motif for a 4Fe-4S cluster (C-X-X-C-X-X-C and a remote cysteine followed by a proline). On the other hand, tNOX does contain a copper site conserved with superoxide dismutase (*24*) that is required for activity (Table 2).

The expression of truncated tNOX in *E. coli* has confirmed that the cloned cDNA exhibits fully the characteristics of the tNOX protein. All forms of tNOX (including the truncated and processed forms) were recognized by the tNOX-specific monoclonal antibody used in expression cloning. In addition, the expressed protein exhibited both enzymatic activities associated with NOX proteins (Figures 4−6).

On the basis of analyses of the drug-responsive circulating tNOX activity, the tNOX protein is widely distributed among cancer patients (*7*, *8*). The resistance of tNOX to proteases and chemical degradation and the ability of tNOX to impart protease resistance to normally protease-resistant proteins (*11*) add to the interest in this protein and are among the distinguishing characteristics of prions. Although we have no direct evidence that tNOX plays a role in the dissemination of cancer, this question should remain open.

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