

# Rapid Polyubiquitination and Proteasomal Degradation of a Mutant Form of NAD(P)H:Quinone Oxidoreductase 1

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## ABSTRACT

The NAD(P)H:quinone oxidoreductase 1 (NQO1)\*2 polymorphism is characterized by a single proline-to-serine amino acid substitution. Cell lines and tissues from organisms genotyped as homozygous for the NQO1\*2 polymorphism are deficient in NQO1 activity. In studies with cells homozygous for the wild-type allele and cells homozygous for the mutant NQO1\*2 allele, no difference in the half-life of NQO1 mRNA transcripts was observed. Similarly, in vitro transcription/translation studies showed that both wild-type and mutant NQO1 coding regions were transcribed and translated into full-length protein with equal efficiency. Protein turnover studies in NQO1 wild-type and mutant cell lines demonstrated that the half-life of wild-type NQO1 was greater than 18 h, whereas the half-life of mutant NQO1 was 1.2 h. Incubation of NQO1 mutant cell lines with proteasome inhibitors increased the amount of immunoreactive

NQO1 protein, suggesting that mutant protein may be degraded via the proteasome pathway. Additional studies were performed using purified recombinant NQO1 wild-type and mutant proteins incubated in a rabbit reticulocyte lysate system. In these studies, no degradation of wild-type NQO1 protein was observed; however, mutant NQO1 protein was completely degraded in 2 h. Degradation of mutant NQO1 was inhibited by proteasome inhibitors and was ATP-dependent. Mutant NQO1 incubated in rabbit reticulocyte lysate with MG132 resulted in the accumulation of proteins with increased molecular masses that were immunoreactive for both NQO1 and ubiquitin. These data suggest that wild-type NQO1 persists in cells whereas mutant NQO1 is rapidly degraded via ubiquitination and proteasome degradation.

NAD(P)H:quinone oxidoreductase 1 (EC 1.6.99.2; DT-diaphorase) is a cytosolic flavoenzyme that catalyzes the two-electron reduction of a broad range of substrates. NQO1 is characterized by its ability to utilize either NADPH or NADH as cofactors and is inhibited by the anticoagulant dicumarol (Ernster, 1967). Biochemical and immunohistochemical studies have revealed that NQO1 is widely expressed in human tissues with high levels of protein detected in epithelium and endothelium (Schlager and Powis, 1990; Siegel et al., 1998). NQO1 plays a prominent role in the detoxification of quinones, primarily because of its ability to reduce quinone substrates directly to their hydroquinone derivatives, which can then be conjugated and excreted. Two-electron reduction directly to the hydroquinone bypasses the formation of redox-cycling semiquinones and the generation of reactive oxygen species (Lind et al., 1982). In addition, endogenous quinones, such as  $\alpha$ -tocopherol quinone and coenzyme Q derivatives, have recently been shown to be substrates for NQO1; reduction of these compounds by NQO1

results in the formation of stable hydroquinones with excellent antioxidant properties (Beyer et al., 1996; Siegel et al., 1997). In cellular systems, it is well documented that NQO1 is induced manifold in response to electrophiles and oxidative stress. Diets rich in fruits and vegetables are believed to be chemoprotective and chemopreventive, in part because they contain compounds that induce detoxification enzymes, including NQO1 (Fahey et al., 1997).

In addition to the expression of NQO1 in normal tissues, high levels of NQO1 activity have also been observed in a wide variety of human tumors and cell lines (Schlager and Powis, 1990; Marin et al., 1997; Siegel et al., 1998). Previous work has shown that NQO1 can bioactivate a wide range of antitumor compounds, such as mitomycin C, EO9, streptozotocin, and lapachone (Siegel et al., 1992; Walton et al., 1991; Beall et al., 1996; Pink et al., 2000). The high levels of NQO1 in tumors and the ability to bioactivate a diverse range of quinones suggest that NQO1 may be a useful target for enzyme-directed drug therapy; diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone, a new NQO1-directed antitumor agent, is currently in preclinical development (Winski et al., 1998).

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**ABBREVIATIONS:** NQO1, NAD(P)H:quinone oxidoreductase 1; PAGE, polyacrylamide gel electrophoresis; RIPA, radioimmunoprecipitation assay; RRL, rabbit reticulocyte lysate; ATP $\gamma$ S, adenosine 5'-O-(3-thiotriphosphate); AMP-PNP, 5'-adenylylimidodiphosphate.

Using human tumor cell lines devoid of NQO1 activity, we have previously characterized a point mutation in exon 6 of the human NQO1 gene (Traver et al., 1992, 1997). The mutation is a C-to-T base pair substitution at position 609 of the NQO1 cDNA, which codes for a proline-to-serine change at position 187 in the amino acid sequence of the protein. It was further demonstrated that the NQO1 mutant allele mapped to the appropriate site on chromosome 16q22.1 and the distribution of the mutation was consistent with Hardy-Weinberg equilibrium and demonstrated Mendelian transmission (Rosvold et al., 1995). This mutation in human NQO1 has been characterized as a genetic polymorphism (NQO1\*2) and the frequency of the NQO1\*2/\*2 (homozygous mutant) genotype ranges from 4% in white persons to greater than 20% in Chinese populations (Kelsey et al., 1997). In genotype-phenotype studies, we have shown that tissues and cell lines from persons with the NQO1\*2/\*2 genotype have no detectable NQO1 enzymatic activity and only trace levels of immunoreactive NQO1 protein (Traver et al., 1997; Siegel et al., 1999).

The physiological implications of the absence of NQO1 are being investigated using the null polymorphism as a molecular tool. The majority of these studies have been designed to examine the susceptibility to cancer of persons carrying the NQO1\*2/\*2 genotype. The NQO1\*2/\*2 genotype has been associated with an increased risk of urothelial tumors (Schulz et al., 1997), therapy-related acute myeloid leukemia (Larson et al., 1999), cutaneous basal cell carcinomas (Clairmont et al., 1999), and pediatric leukemias (Wiemels et al., 1999). We have also demonstrated that the homozygous NQO1\*2 allele is a significant risk factor for the development of benzene-induced hematotoxicity in exposed workers (Rothman et al., 1997). The purpose of this work was to examine the mechanism(s) whereby cells with the NQO1\*2/\*2 genotype are deficient in NQO1.

## Materials and Methods

**NQO1 Expression Plasmids, Purified NQO1 Proteins, and Antibodies.** The construction and subcloning of Pro187 (wild-type NQO1) from the human H460 NSCLC cell line and Ser187 (mutant NQO1) from the human BE colon carcinoma cell line have been described previously (Traver et al., 1997). Wild-type and mutant human recombinant NQO1 proteins were expressed in *Escherichia coli* and purified by Cibacron Blue affinity chromatography as described previously (Sharkis and Swenson, 1989; Traver et al., 1997). Tissue culture supernatant containing two anti-NQO1 mouse monoclonal antibodies (clones A180 and B771) was used for immunoprecipitation and immunoblot analysis. The monoclonal antibodies used in these studies have demonstrated equal immunoreactivity toward wild-type and mutant NQO1 proteins (Traver et al., 1997).

**Cell Lines.** The HT-29 and Caco-2 human colon carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA) and the BE human colon carcinoma cell line was originally obtained from Dr. N. W. Gibson (University of Southern California, Los Angeles, CA). HT-29, BE, and Caco-2 cell lines were grown as monolayers at 37°C in 5% CO<sub>2</sub> with minimal essential medium supplemented with 10% (HT-29, BE) or 20% (Caco-2) fetal bovine serum, 10 U/ml penicillin/streptomycin, and 2 mM L-glutamine. Genotyping of these cell lines for the NQO1\*2 polymorphism has been described previously (Traver et al., 1997).

**mRNA Stability Studies.** NQO1 mRNA turnover was determined in HT29 and BE cells. Briefly,  $7.5 \times 10^4$  cells were added to 100-mm tissue cultures dishes in 10 ml of complete medium; after

18 h of attachment, the cells were treated with 65  $\mu$ M 5,6-dichloro-benzimidazole riboside (Sigma). Total RNA was isolated (Rneasy; Qiagen, Chatsworth, CA) between 0 and 30 h for Northern analysis (Genovese and Milcarek, 1990). The blot was probed with full-length <sup>32</sup>P-labeled human NQO1 cDNA (DECA prime II; Ambion, Austin TX) then analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Results were normalized to 28S RNA. mRNA half-life determinations were calculated using densitometric analysis (Image Quant, Molecular Dynamics).

**In Vitro Transcription/Translation.** Human wild-type and mutant NQO1 coding regions were subcloned into the pSP6polyA expression vector (Promega, Madison, WI) by polymerase chain reaction amplification of the full coding region using oligomers 5'-CCCaaagcttATGGTCGGCAGAAGAGCA-3' and 5'-TGCTctagaT-CATTTTCTAGCTTTGATCTG-3' containing the *Hind*III and *Xba*I restriction sites, respectively. NQO1 in vitro transcription/translation assays were performed using the SP6 Quick Coupled Transcription/Translation System (Promega) with [<sup>35</sup>S]methionine at 30°C for 2 h. Analysis of translation products was performed on 12% SDS-PAGE (minigel; Bio-Rad, Hercules, CA). Following electrophoresis gels were fixed, amplified (En<sup>3</sup>hance; NEN, Boston, MA), dried and exposed to film for 12 h at -80°C.

**NQO1 Protein Stability Studies.** NQO1 protein turnover was determined in HT-29 and BE cell lines. Approximately  $20 \times 10^6$  cells in 60 mm plates were treated with 50  $\mu$ g/ml cycloheximide. At regular intervals as indicated, the medium was removed and the cells were washed with PBS, lysed in 1 ml of RIPA buffer (Roche Molecular Biochemicals, Summerville, NJ) then centrifuged at 15,000g for 10 min. To this supernatant, 200  $\mu$ l of anti-NQO1 monoclonal antibody was added for 1 h on ice followed by 30  $\mu$ l of protein A/G conjugated agarose (Calbiochem, San Diego, CA) for an additional 30 min. The protein A/G agarose was collected by centrifugation and washed three times with 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% (v/v) Nonidet P-40. The protein A/G agarose was then resuspended in 2 $\times$  Laemmli SDS sample buffer and heated to 90°C for 5 min. Immunoprecipitated proteins were separated by 12% SDS-PAGE (minigel) then transferred to 0.4  $\mu$ m polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol at 110 V for 1 h. Membranes were blocked overnight in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.2% (v/v) Tween-20, and 5% (w/v) nonfat dry milk (blocking buffer) at 4°C. Hybridoma tissue culture supernatant was diluted 1:40 with 20 ml of blocking buffer and added to each membrane for 1 h. Membranes were then washed extensively in blocking buffer followed by the addition of horseradish peroxidase conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:5,000 in 20 ml of blocking buffer for 30 min. Protein visualization was performed using enhanced chemiluminescence as described by the manufacturer (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Mutant NQO1 protein half-life was calculated using densitometric analysis (Gel Doc 2000; Bio Rad).

**Cell Lines and Proteasome Inhibitors.** Cell lines were grown to 85% confluence in 60-mm dishes in complete minimal essential medium. Fresh medium (5 ml) containing either 25  $\mu$ M MG132 (BIOMOL Research Laboratories, Plymouth Meeting, PA) or 10  $\mu$ M clasto lactocystin  $\beta$ -lactone (Calbiochem) was added to each cell line and repeated 3 h later. After 6 h, cells were harvested in RIPA buffer, protein concentration was determined by the method of Lowry et al. (1951) and 50 to 100  $\mu$ g of total protein was analyzed for NQO1 by immunoblot analysis as described above. Quantification was performed using densitometric analysis (Gel Doc 2000; Bio Rad).

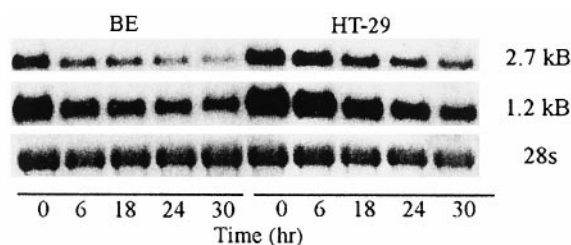
**Optimized SDS-PAGE.** Wild-type and mutant recombinant NQO1 proteins were separated by optimized SDS-PAGE (Leith and Begleiter, 1998). Proteins were solubilized in 2 $\times$  SDS-sample buffer then separated on a standard 16-cm SDS-PAGE gel (4% stacking, 12% separating) at 25 mA constant current for 20 h. The running buffer was chilled to 4°C and recirculated throughout the experi-

ment. Protein transfer and NQO1 immunoblot analysis was performed as described above.

**Rabbit Reticulocyte Lysate Ubiquitination Assays.** Purified human recombinant wild-type and mutant NQO1 proteins (100 ng) were incubated at 37°C in 45  $\mu$ l of rabbit reticulocyte lysate (untreated RRL; Promega) supplemented with 5 mM  $MgCl_2$ , 10 mM creatinine phosphate, 100  $\mu$ g/ml creatinine kinase, and 2 mM ATP (final volume, 90  $\mu$ l). At the times indicated in the figure legends, a 10- $\mu$ l aliquot of the reaction mixture was removed and diluted with 24  $\mu$ l of 2 $\times$  Laemmli SDS sample buffer and a 15- $\mu$ l aliquot (5 ng of NQO1) was analyzed by SDS-PAGE (12%, minigel) and NQO1 immunoblot analysis (see above). ATP analogs, adenosine 5'-O-(3-thiotriphosphate) (ATP $\gamma$ S; Sigma) and 5'-adenylylimidodiphosphate (AMP-PNP, Sigma) were dissolved in distilled water. Immunoprecipitation studies of ubiquitination of mutant NQO1 in RRL were performed as described above except reactions contained 25  $\mu$ M MG132. RRL reactions were terminated by the addition of 200  $\mu$ l of RIPA buffer, after which a sample (10  $\mu$ l) was removed for NQO1 immunoblot analysis (see above). To the remaining RRL reaction, 200  $\mu$ l of hybridoma tissue culture supernatant was added overnight at 4°C. Immunoprecipitates were collected on protein A/G agarose beads (1 h) and washed extensively with 100 mM Tris-HCl, pH 8.2, 500 mM NaCl, 0.75% (v/v) Triton-X100, and 10 mM EDTA. Immunoprecipitates were then separated by SDS-PAGE (12%, minigel), transferred to polyvinylidene difluoride membranes, as described above (except that protein transfer was carried out at 40 V for 12 h), and immunoblot analysis was performed using 20 ml of blocking buffer containing anti-ubiquitin polyclonal antibodies (1:100; Sigma) for 1 h at 27°C. Secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) were diluted 1:5000 in 20 ml of blocking buffer and added for 30 min. Protein visualization was performed using enhanced chemiluminescence.

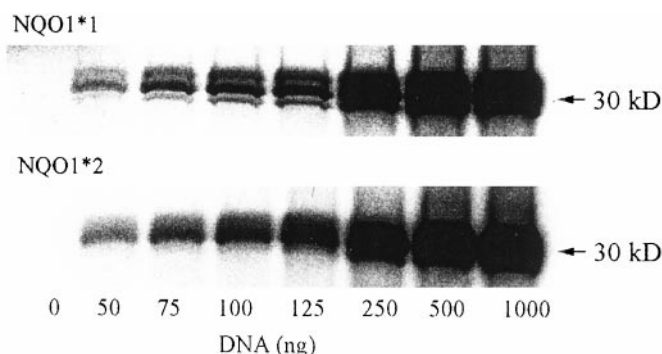
## Results

We have reported previously that cell lines and tissues from persons homozygous for the NQO1\*2 polymorphism have only trace levels of immunoreactive NQO1 protein and no enzymatic activity (Traver et al., 1997; Siegel et al., 1999). To establish a mechanism underlying the NQO1-null phenotype, we examined a number of biochemical parameters related to NQO1 gene expression and protein synthesis in cell lines that have been genotyped as either homozygous wild-type (HT-29, H460) or homozygous (BE, Caco-2) for the NQO1\*2 polymorphism. Previous work has shown that the mutant NQO1 gene could be fully transcribed, because homozygous mutant cell lines were found to have high levels of NQO1 mRNA relative to NQO1 activity (Traver et al., 1992). We extended this work to examine NQO1 mRNA half-life in wild-type and mutant cell lines using RNA synthesis inhibition and Northern blot analysis (Fig. 1). The half-life of the



**Fig. 1.** NQO1 mRNA half-life studies in HT-29 and BE cell lines. NQO1 mRNA transcript (1.2- and 2.7-kilobase) half-life was measured by Northern blot analysis after RNA synthesis inhibition in HT-29 (wild-type) and BE (mutant) cell lines. These data are representative of four separate analyses.

1.2- and 2.7-kilobase mRNA transcripts isolated from either wild-type HT-29 or homozygous mutant BE cell lines was estimated to be 31 h (HT-29) and 30 h (BE; results from four independent experiments). In addition, *in vitro* transcription/translations assays demonstrated that NQO1 coding regions cloned from either H460 or BE cell lines were transcribed and translated into full-length NQO1 protein with equal efficiency (Fig. 2). These data suggest that lack of NQO1 protein in mutant cells does not arise from substantial differences in transcription or translation efficiencies of the NQO1 gene or mRNA, respectively. Studies were then performed to examine the turnover of NQO1 protein in wild-type and mutant cell lines. Protein stability studies were carried out using cycloheximide to inhibit protein synthesis, followed by immunoprecipitation and immunoblot analysis. In these studies, the half-life of wild-type NQO1 protein was determined to be greater than 24 h, whereas mutant NQO1 was 1.2 h (Fig. 3). Additional studies using HT-29 cells and [ $^{35}$ S]methionine labeling with NQO1 immunoprecipitation confirmed that the half-life of wild-type NQO1 was greater than 24 h, whereas in the BE cell line, no radio-labeled NQO1 protein could be detected (data not shown). Treatment of mutant cell lines with proteasome inhibitors MG132 or *clasto* lactocystin  $\beta$ -lactone resulted in an increase in immunoreactive mutant NQO1 protein of 1.5- and 2-fold, respectively, in BE cells and 1.3- and 2-fold, respectively, in Caco-2 cells (Fig. 4). Proteasome inhibitors MG132 and *clasto* lactocystin  $\beta$ -lactone bind to the 26S-proteasome and prevent degradation of short-lived polyubiquitinated proteins (Lee and Goldberg, 1996). These data suggest that the ubiquitin/proteasome pathway may be involved in mutant NQO1 protein degradation. To study the proteolytic degradation of mutant NQO1 in a cell-free system, we used purified recombinant NQO1 proteins as model substrates. In Fig. 5, we observed the difference in migration of purified recombinant wild-type and mutant NQO1 proteins under optimized SDS-PAGE and the corresponding migration of wild-type and mutant NQO1 proteins synthesized *in vivo*. These data demonstrate the effect of the Pro187-to-serine amino acid substitution on NQO1 mobility in SDS-PAGE and also demonstrate that migration of recombinant NQO1 proteins and NQO1 proteins synthesized *in vivo* was identical. Under normal SDS-PAGE conditions, both wild-type and mutant NQO1 proteins migrate identically. How-

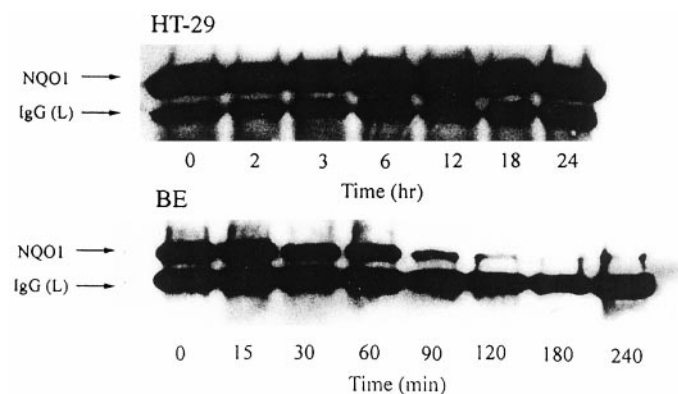


**Fig. 2.** A comparison of *in vitro* transcription/translation of wild-type and mutant NQO1 coding regions. Plasmid DNA constructs (0.05–1  $\mu$ g) containing either wild-type ( $^{609}C$ ) or mutant ( $^{609}T$ ) NQO1 coding regions were incubated with an eukaryotic transcription/translation system including [ $^{35}$ S]methionine. After 1.5 h, reactions were terminated and the resultant transcription/translation products were separated by SDS-PAGE followed by autoradiography.

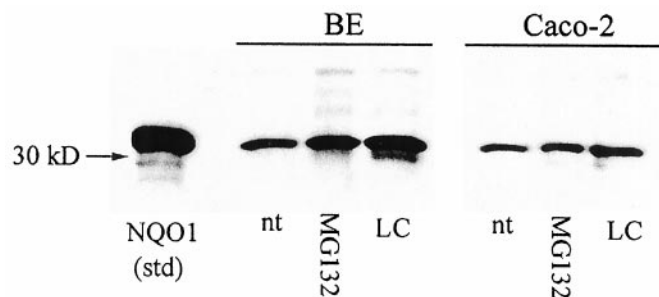


ever, when the proteins are separated at 4°C on a longer gel with a lower applied voltage, wild-type and mutant NQO1 proteins migrate at slightly different rates (Leith and Begleiter, 1998). These data also show that the Pro187-to-serine change induces alterations in NQO1 protein conformation that can be detected in the denatured protein.

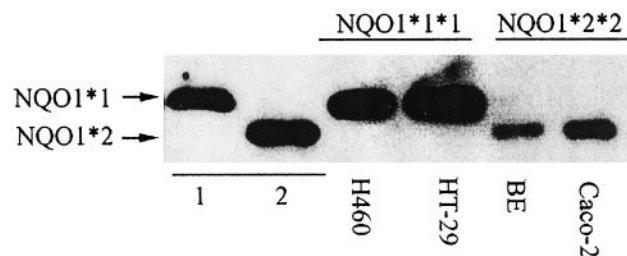
The rapid degradation of NQO1 observed in the mutant BE



**Fig. 3.** NQO1 protein stability in HT-29 and BE cell lines. The stability of NQO1 was examined in NQO1 wild-type (HT-29) and mutant (BE) cells after cycloheximide treatment. At the indicated times after cycloheximide treatment, cells were harvested for NQO1 immunoprecipitation, SDS-PAGE and immunoblot analysis with enhanced chemiluminescence. Membranes were exposed to radiographic film for 10 s (HT-29) and 30 s (BE). IgG (L); mouse immunoglobulin light chain (29 kDa).



**Fig. 4.** Inhibition of mutant NQO1 protein degradation in BE and Caco-2 cell lines by proteasome inhibitors. NQO1 mutant cell lines (BE, Caco-2) were treated with either MG132 (25  $\mu$ M) or *clasto* lactocystin  $\beta$ -lactone (10  $\mu$ M; LC) for 6 h. Crude cytosol (50  $\mu$ g) was subjected to SDS-PAGE and immunoblot analysis. NQO1 std, 5 ng of purified recombinant mutant NQO1; nt; no treatment.

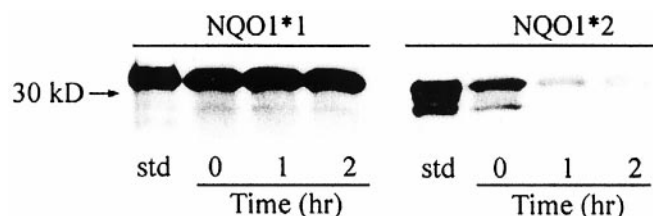


**Fig. 5.** Separation of wild-type and mutant NQO1 proteins by optimized SDS-PAGE and immunoblot analysis. Using optimized SDS-PAGE (see *Materials and Methods*) and immunoblot analysis, we have analyzed the migration of recombinant wild-type and mutant recombinant NQO1 proteins and wild-type and recombinant NQO1 protein synthesized in vivo. In these studies, we confirmed that both wild-type and mutant recombinant NQO1 proteins have identical separation properties to NQO1 proteins synthesized in vivo. Lane 1, 5 ng of recombinant wild-type (Pro187) NQO1; lane 2, 5 ng of recombinant mutant (Ser187) NQO1; wild-type cell lines, 1  $\mu$ g of total cytosolic protein; mutant cell lines, 20  $\mu$ g of total cytosolic protein.

cell line was also seen in studies with recombinant mutant NQO1 protein in RRL. Incubation of recombinant mutant NQO1 protein (Ser187) with RRL and an ATP-generating system resulted in rapid degradation with complete loss of NQO1 by 2 h. When reactions were carried out with recombinant wild-type NQO1 (Pro187), however, no loss of NQO1 protein was observed (Fig. 6). Similar experiments demonstrated that degradation of mutant NQO1 protein in RRL could be prevented by the addition of the proteasome inhibitor *clasto* lactocystin  $\beta$ -lactone (Fig. 7). Cysteine or serine protease inhibitors E64 or phenylmethylsulfonyl fluoride did not prevent RRL-mediated degradation of recombinant mutant NQO1 (data not shown). The degradation of mutant NQO1 in RRL was ATP-dependent; no degradation of mutant protein was observed in the absence of ATP or in the presence of nonhydrolyzable ATP analogs (Fig. 8). ATP hydrolysis is essential for ubiquitin-conjugating reactions and proteolysis via the 26S proteasome (Ciechanover et al., 1980; Armon et al., 1990). Finally, experiments were carried out using mutant NQO1 protein in MG132-treated RRL. MG132 was used to inhibit proteasomal degradation of ubiquitinated proteins. In these experiments mutant NQO1 protein was incubated with MG132-treated RRL and an ATP-generating system, then NQO1-immunoreactive proteins were immunoprecipitated, followed by anti-ubiquitin immunoblot analysis. In these experiments, no ubiquitin immunoreactivity was observed at  $t = 0$ , but time-dependent formation of higher molecular mass ubiquitin immunoreactive products ( $>70$  kDa) with a simultaneous loss of native NQO1 protein was observed (Fig. 9, A and B). Little accumulation of ubiquitinated products could be observed in the absence of MG132, indicating that ubiquitinated NQO1 proteins undergo rapid proteasomal degradation. In these experiments, no ubiquitin immunoreactive products were observed in the absence of mutant NQO1 or with the use of a nonspecific immunoprecipitating antibody. These data suggest that the ubiquitin immunoreactive products detected were NQO1 specific and were polyubiquitinated forms of NQO1.

## Discussion

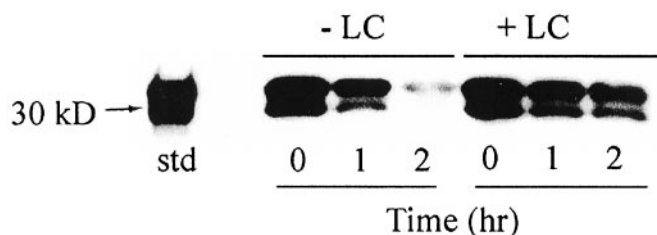
The results presented in this study strongly suggest that the mechanism underlying the absence of NQO1 protein in organisms homozygous for the NQO1\*2 polymorphism is a post-translational event. Because of the Pro187-to-Ser amino acid substitution, mutant NQO1 protein undergoes rapid degradation in cellular systems and in *in vitro* systems using recombinant proteins. Experiments with proteasome inhibitors, ATP analogs and ubiquitin coimmunoprecipitation



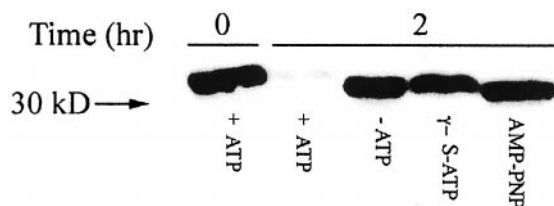
**Fig. 6.** Rabbit reticulocyte lysate mediated degradation of wild-type and mutant NQO1. Recombinant wild-type and mutant NQO1 proteins (100 ng) were incubated in RRL supplemented with an ATP-generating system. At the indicated times reactions were terminated and 5 ng of NQO1 protein was removed and analyzed by SDS-PAGE and immunoblot analysis. std; recombinant mutant NQO1, 5 ng.

strongly implicate the ubiquitin/proteasome pathway in mutant NQO1 degradation.

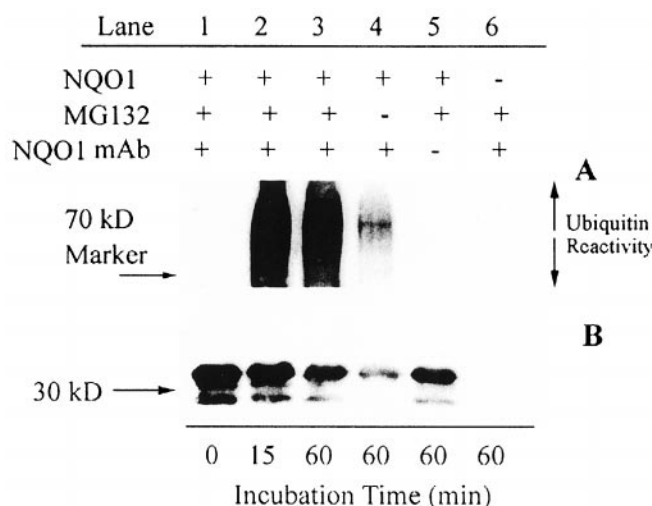
Studies using optimized SDS-PAGE have demonstrated that the Pro187-to-Ser amino acid substitution causes alterations in the structure of the unfolded protein such that the mutant NQO1 protein migrates at a slightly faster rate com-



**Fig. 7.** Effect of proteasome inhibition on mutant NQO1 protein degradation in rabbit reticulocyte lysate. Recombinant mutant NQO1 protein (100 ng) was incubated in RRL supplemented with an ATP generating system in the presence and absence of 10  $\mu$ M clasto lactocystin  $\beta$ -lactone (LC). At the indicated times reactions were terminated and 5 ng of NQO1 protein was removed and analyzed by SDS-PAGE and immunoblot analysis. std; recombinant mutant NQO1, 5 ng.



**Fig. 8.** Inhibition of rabbit reticulocyte lysate mediated degradation of mutant NQO1 by ATP analogs. Recombinant mutant NQO1 protein (100 ng) was incubated in RRL in the presence and absence of a complete ATP generating system or in the presence of ATP analogs. At the indicated times, reactions were terminated and 5 ng of NQO1 protein was removed and analyzed by SDS-PAGE and immunoblot analysis. ATP analogs were added at 5-fold molar excess to ATP. +ATP, complete ATP generating system; -ATP, ATP generating system minus ATP; ATP $\gamma$ S, complete ATP generating system plus ATP $\gamma$ S; AMP-PNP, complete ATP generating system plus AMP-PNP.



**Fig. 9.** Rabbit reticulocyte lysate mediated ubiquitination of mutant NQO1. Recombinant mutant NQO1 (100 ng) was incubated in RRL supplemented with an ATP-generating system and 25  $\mu$ M MG132. At the indicated times, reactions were terminated and subjected to NQO1 immunoprecipitation followed by SDS-PAGE and immunoblot analysis for antiubiquitin immunoreactivity (A). Before NQO1 immunoprecipitation, 5 ng of NQO1 was removed and analyzed by SDS-PAGE and immunoblot analysis (B).

pared with wild-type NQO1 under denaturing conditions. The effect of this mutation on the structure of the folded protein is not clear. Studies with purified recombinant mutant protein have shown that NQO1 catalytic activity is significantly reduced (Traver et al., 1997; Misra et al., 1998). The reduction in catalytic activity of mutant NQO1 was not accompanied by changes in the  $K_m$  values for NADH; however, the  $K_m$  value for menadione increased 2-fold (Chen et al., 1999). NQO1 operates via a ping-pong kinetic mechanism whereby binding of the NAD(P)H cofactor causes conformational changes in the substrate (quinone) binding site (Hosoda et al., 1974). NQO1 is a homodimer made up of two identical monomers. Each monomer contains a cofactor and a substrate binding site in addition to a FAD prosthetic group that is used by the enzyme to transfer electrons from cofactor to substrate. X-ray crystallographic studies have shown that each NQO1 monomer contains two separate domains: a large catalytic domain folded in an  $\alpha/\beta$  secondary structure and a small C-terminal domain (Li et al., 1995). The overall folding resembles a twisted central parallel  $\beta$ -sheet surrounded by connecting helices (Li et al., 1995; Faig et al., 2000). The Pro187-to-Ser amino acid change in mutant NQO1 is located very near the transition of a  $\beta$ -sheet and an  $\alpha$ -helical bend in an exposed loop. It is possible that this mutation destroys the structure of an exposed loop, resulting in misfolding of the protein. Alternatively, the Pro187-to-Ser amino acid change could disrupt the structure of the central parallel  $\beta$ -sheet, resulting in a decreased binding affinity for the FAD prosthetic group (Wu et al., 1998). Alterations in FAD binding to NQO1 could decrease the overall catalytic activity of the enzyme. In addition, the inability of FAD to bind tightly could destabilize NQO1, allowing for unfolding of portions of the protein. Alterations in the structure of NQO1 as a result of the Pro187-to-Ser substitution is supported by preliminary far-Western and coimmunoprecipitation studies demonstrating major differences in the interactions between wild-type and mutant NQO1 proteins and other cytosolic proteins. Studies are currently underway to examine what alterations in NQO1 protein structure are induced by the Pro187-to-Ser substitution and how these changes in protein structure may play a role in targeting the protein for ubiquitination and proteasomal degradation.

These studies describe a biochemical mechanism for the absence of NQO1 activity in organisms homozygous for the NQO1\*2 polymorphism. This mutation does not effect transcription or translation of the NQO1 gene; instead, it results in synthesis of a mutant protein with a single proline-to-serine amino acid substitution. In comparative studies with wild-type protein, the mutant NQO1 protein exhibited a dramatically decreased stability because of ubiquitination and proteasomal degradation. These data support previous genotype-phenotype studies that failed to detect immunoreactive NQO1 protein in tissues from patients homozygous for the NQO1\*2 polymorphism (Siegel et al., 1999) and have relevance for both chemotherapy and studies examining the susceptibility of organisms with the NQO1\*2/\*2 genotype to disease.

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