

Interaction of the Molecular Chaperone Hsp70 with Human NAD(P)H:Quinone Oxidoreductase 1*

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NAD(P)H:quinone oxidoreductase 1 (EC 1.6.99.2; DT-Diaphorase, NQO1) is predominantly a cytosolic flavoenzyme that catalyzes a two-electron reduction. Using human tumor cell lines devoid of NQO1 enzymatic activity, we have previously identified a single nucleotide polymorphism (NQO1*2 allele) in the human NQO1 gene. This mutation has been characterized as a genetic polymorphism (NQO1*2), which leads to greatly diminished levels of protein due to rapid degradation of the NQO1*2 protein by the ubiquitin proteasomal pathway (UPP). In an attempt to decipher the mechanism responsible for the differential stability of wild-type NQO1*1 and mutant NQO1*2 proteins, we have investigated the interactions of these proteins with molecular chaperones of the Hsp family. Using co-immunoprecipitation studies (co-IPs), no association was observed between Hsp90 and either wild-type NQO1*1 or mutant NQO1*2 proteins. Hsp70, however, was found to associate with NQO1*1 protein in cells when co-IPs were performed with an anti-NQO1 antibody followed by immunoblotting with an anti-Hsp70 antibody or vice versa. Hsp40 could also be detected in the immunoprecipitated protein complex. Experiments were also performed using either the NQO1*1 or NQO1*2 coding regions in an *in vitro* transcription/translation system employing rabbit reticulocyte lysates (RRLs). Consistent with the cellular data, co-IP experiments in RRLs demonstrated an association of Hsp70 with wild-type NQO1*1 protein but not with NQO1*2 protein. To further elucidate the role of the association of Hsp70 with the NQO1*1 protein, site-directed mutagenesis was used to modify a proposed Hsp70 binding site near the N terminus of the NQO1 protein. We generated a plasmid containing an NQO1*1 coding region with a mutated Hsp70 binding site (isoleucine to aspartic acid at position 8, NQO1*1/I8D). In contrast to the NQO1*1 protein translated in RRLs, the NQO1*1/I8D protein did not associate with Hsp70, as demonstrated by co-IP, was catalytically inactive, and was degraded by the UPP. These data suggest that the association of Hsp70 with NQO1*1 may play an important role in the stability and functionality of the NQO1 protein.

NAD(P)H:quinone oxidoreductase 1 (EC 1.6.99.2; DT-Diaphorase, NQO1)¹ is a cytosolic flavoenzyme that catalyzes the

two-electron reduction of quinones and a wide variety of substrates. NQO1 has relevance for both chemoprotection and chemotherapy, since it can deactivate potentially toxic quinones found in the environment but can also bioactivate anti-tumor quinones such as mitomycin C (1–3). NQO1 gene expression has also shown to be induced in response to xenobiotics, antioxidants, oxidants, heavy metals, UV light, and ionizing radiation, and recently NQO1 has been reported to stabilize p53 (4–6). In addition to the expression of NQO1 in normal tissues, a high level of NQO1 activity has been documented in a wide ranging spectrum of human tumors and cell lines (7–10).

Using human tumor cell lines devoid of NQO1 activity our laboratory has previously characterized a point mutation in exon six of the human NQO1 gene (11). The mutation is a C to T base pair substitution at position 609 of the NQO1 cDNA that codes for a proline to serine change at position 187 of the amino acid sequence of the NQO1 protein. This mutation in humans has been characterized as a genetic polymorphism (NQO1*2), and the frequency of the NQO1*2/*2 (homozygous mutant) genotype ranges from 4% in Caucasians to greater than 20% in Asian populations (12). Low levels of NQO1 activity associated with the NQO1*2 polymorphism has been shown to be associated with an increased risk of benzene-induced hematotoxicity (13), an increased risk of acute leukemia in adults (14) and children (15), and an increased risk of secondary leukemia after treatment with chemotherapeutic agents (16).

Genotype-phenotype studies of the NQO1*2 allele have been performed, and no measurable NQO1 activity or protein could be detected in cell lines or saliva samples from individuals with the NQO1*2/*2 genotype. In agreement, no NQO1*2 mutant protein could be detected in NQO1*2/*2 tissue samples using immunohistochemistry (17). A small amount of NQO1*2 protein could be detected in NQO1*2/*2 cell lines by immunoblot analysis with enhanced chemiluminescence detection and prolonged exposure times (17). These cell lines, however, had no measurable NQO1 activity, since the mutant NQO1*2 protein has very low catalytic activity as compared with the wild-type NQO1*1 protein (11).

We have recently demonstrated that the lack of NQO1*2 protein in tissues and cells from individuals carrying the homozygous NQO1*2/*2 polymorphism is due to rapid degradation of the mutant protein by the ubiquitin proteasomal pathway (UPP) (18). Degradation of the mutant NQO1*2 protein by the UPP could be observed in both cells and cell-free systems, while the wild-type NQO1*1 protein demonstrated prolonged stability in both systems. The mechanisms underlying the dif-

tase 1; UPP, ubiquitin-proteasomal pathway; RRL, rabbit reticulocyte lysate; MG132, *Z*-Leu-Leu-Leu-CHO; LC, *clasto*-lactacystin- β -lactone; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; PMSF, phenylmethylsulfonyl fluoride; RIPA, radioimmune precipitation; PBS, phosphate-buffered saline.

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¹ The abbreviations used are: NQO1, NAD(P)H:quinone oxidoreduc-

ferential stability of NQO1*1 and NQO1*2 proteins, however, remain to be characterized. Heat shock proteins (Hsp) and particularly the Hsp70 protein chaperone family play a key role in catalyzing protein folding. Hsp70, in association with Hsp40, binds hydrophobic regions of unfolded proteins, and through cycles of ATP-dependent binding and release by the Hsp70/40 complex, the nascent protein is manipulated into an active conformation (19). The present investigation was initiated to examine the potential association of Hsp protein chaperones in the modulation of activity and stability of wild-type NQO1*1 and mutant NQO1*2 proteins.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—TNT-RRLs (SP6, Quick Coupled Transcription/Translation System) and untreated RRLs were purchased from Promega (Madison, WI). Apyrase (EC 3.6.1.5), E-64, PMSF, cycloheximide, bovine serum albumin, and 2,6-dichlorophenol-indophenol were purchased from Sigma. Protein A/G-agarose and *clasto*-lactacystin- β -lactone were obtained from Calbiochem. MG132 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). RIPA (protease inhibitor mixture) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Human recombinant NQO1 standard was purified from *Escherichia coli* by Cibacron blue affinity chromatography as described previously (11, 20). All other chemicals and reagents used in this study were of the highest purity grade available commercially.

Antibodies—Anti-ubiquitin (rabbit polyclonal) was purchased from Sigma. Hsp-40 (SC-1800, goat polyclonal) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Hsp70 (SPA-810, mouse monoclonal) was a product of Stressgen Biotechnologies Corp. (Victoria, British Columbia, Canada), and Hsp90 (Ab-1, rabbit polyclonal) was obtained from Neo Markers (Union City, CA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Hybridoma tissue culture supernatant (RPMI 1640 with 10% (v/v) fetal bovine serum) containing anti-NQO1 mouse monoclonal antibody clones A180 and B771 (IgG) mixed 1:1 were used in the study. Both A180 and B771 anti-NQO1 monoclonal antibodies have demonstrated immunoreactivity toward NQO1*1 and NQO1*2 proteins (11).

Cell Lines—HT-29 (human colon carcinoma) and MDA-MB 231 (human breast carcinoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown as monolayers at 37 °C in 5% CO₂ with minimal essential medium supplemented with 10% (v/v) fetal bovine serum, 10 units/ml penicillin/streptomycin and 2 mM L-glutamine.

Plasmids—Human wild-type and mutant NQO1 coding regions were subcloned into the pSP64poly(A) expression vector (Promega) by polymerase chain reaction (PCR) amplification of the full coding region using oligomers 5'-ccaagcttATGGTCGGCAGAAGAGCA-3' and 5'-tgctctagatCATTTTCTAGCTTTGATCTG-3' containing *Hind*III and *Xba*I restriction sites, respectively.

Site-directed Mutagenesis—Plasmid pwtSP64poly(A) containing the wild-type NQO1*1 coding region of human NQO1 served as a template. The 822-bp fragment was amplified by PCR. The sequence of oligonucleotides that served as primers to introduce the isoleucine \rightarrow aspartic acid mutation (I8D) into the human NQO1 coding region at amino acid position 8 are as follows: Primer 1, 5'-ccaagcttATGGTCGGCAGGAAAGAGCACTGACGTAGCTGGCTCAC-3'; Primer 2, 5'-tgctctagatCATTTTCTAGCTTTGATCTG-3'.

The altered bases are underlined and encode the mutated amino acid. The temperature cycles used for this PCR amplification were as follows: 95 °C for 5 min, 25 cycles of 95 °C for 1 min, 50 °C for 1 min, followed by 72 °C for 1 min. After the final cycle the reaction was kept at 4 °C. The resulting mutated product was then cut with *Hind*III and *Xba*I followed by subcloning into pSP64poly(A). The construct was verified by digestion and sequencing. This construct is referred henceforth as NQO1*1/I8D.

In Vitro Transcription/Translation—NQO1 *in vitro* transcription/translation was carried out using SP6 Quick Coupled Transcription/Translation System (TNT-RRL; Promega) according to the manufacturer's instructions using 1 μ g of plasmid DNA. Reactions (50 μ l) were carried out for 1 h at 32 °C, after which a small aliquot of reaction mixture was removed, and NQO1 protein translation was monitored by SDS-PAGE (21) followed by immunoblot analysis (see below). Prior to immunoprecipitation, apyrase (EC 3.6.1.5, 5 units) was added and the TNT-RRLs placed on ice for 10 min. The reaction was terminated by the

addition of 200 μ l of RIPA buffer, and immunoprecipitation was carried out using the appropriate antibodies. Proteasome-mediated degradation of NQO1*1/I8D was assayed as described previously in untreated RRL (18). NQO1*1/I8D was generated by *in vitro* transcription/translation (as described above), and 5 μ l of reaction mixture was added to untreated RRLs to measure degradation (18).

SDS-PAGE, Immunoprecipitation, and Immunoblot Analysis—Exponentially growing cells were seeded at $\sim 2 \times 10^6$ in 60-mm plates, and 12 h later the cells were harvested. For immunoprecipitation studies, medium was removed, and cells were washed with phosphate-buffered saline (PBS, pH 7.4), lysed on ice in RIPA buffer (250 μ l), then centrifuged at 13 K for 10 min. To this supernatant 200 μ l of anti-NQO1 monoclonal antibody or 10 μ g of anti-HSP antibody were added, and the mixture was gently rotated at 4 °C for 16 h. Protein A/G-agarose (40 μ l) was then added, and the incubations were continued for an additional 90 min. The protein A/G-agarose was collected by centrifugation and the beads washed three times with 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1% (v/v) Nonidet P-40. The protein A/G-agarose beads were then suspended in 2 \times Laemmli SDS sample buffer and heated to 90 °C for 5 min. Immunoprecipitated proteins were analyzed by SDS-PAGE (12%, minigel) followed by transfer to a polyvinylidene difluoride membrane (0.4 μ m) 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 8.0, 125 mM NaCl, 0.2% (v/v) Tween 20 containing 5% (w/v) nonfat dry milk (TBST-M5). Immunoblot analysis of polyubiquitinated NQO1 was performed using anti-ubiquitin antibodies diluted at 1:100. Immunoblot analysis of Hsp proteins (Hsp40, Hsp70, and Hsp90) was performed using the following antibody dilutions; Hsp40 and Hsp90, 1:250; Hsp70, 1:1,500. Antibodies were diluted in 10 ml of TBST-M5 and incubated with membranes for 1 h at 27 °C. Horseradish peroxidase-labeled secondary antibodies were diluted at 1:5,000 in 20 ml of TBST-M5 and incubated for 30 min. For determinations of cellular levels of NQO1 and Hsp proteins, cells were scrapped, washed with PBS, collected in 200 μ l of ice-cold RIPA buffer, and then lysed by sonication on ice. The supernatant was cleared by centrifugation at 13 K for 5 min, and the protein concentration was determined by the method of Lowry (22). Protein bands were visualized using enhanced chemiluminescence as described by the manufacturer (PerkinElmer Life Sciences). Densitometric analysis of the membranes was performed using GelDoc 2000 (Bio-Rad).

Cell Lines and Inhibitor Studies—Cells were grown to 85% confluence in 60-mm dishes in complete minimal essential medium. Fresh medium (5 ml) containing various inhibitors MG132, *clasto*-lactacystin- β -lactone (LC), E-64, and PMSF were added to each plate, and the medium replaced with fresh inhibitor 3 h later. After 6 h of incubation with inhibitor, cells were washed with PBS and then lysed in ice-cold RIPA buffer. Protein concentrations were determined (22), and NQO1 levels were analyzed by immunoblot analysis.

NQO1 Protein Turnover Studies—NQO1 protein stability was determined in the presence and absence of proteasomal inhibitor MG132. Approximately 2×10^7 cells were plated into 100-mm plates, and 12 h later fresh medium was added containing cycloheximide (50 μ g/ml). At the indicated intervals, cells were scraped and pelleted by centrifugation. The cell pellet was washed with ice-cold PBS, pH 7.4, and then lysed in 500 μ l of ice-cold RIPA buffer. Supernatant was collected by centrifugation at 13 K for 10 min. To this supernatant, 200 μ l of anti-NQO1 monoclonal antibody was added, and the incubation was carried out for 1 h on a shaker at 4 °C, followed by the addition of 30 μ l of protein A/G-conjugated agarose for an additional hour. Agarose beads were washed and processed for immunoblot analysis as described above. To assess NQO1 turnover in the presence of proteasomal inhibitor MG132, cells were treated with MG132 (10 μ M) and cycloheximide (50 μ g/ml) simultaneously. Proteasomal inhibitor and cycloheximide were present during the entire duration of the experiment.

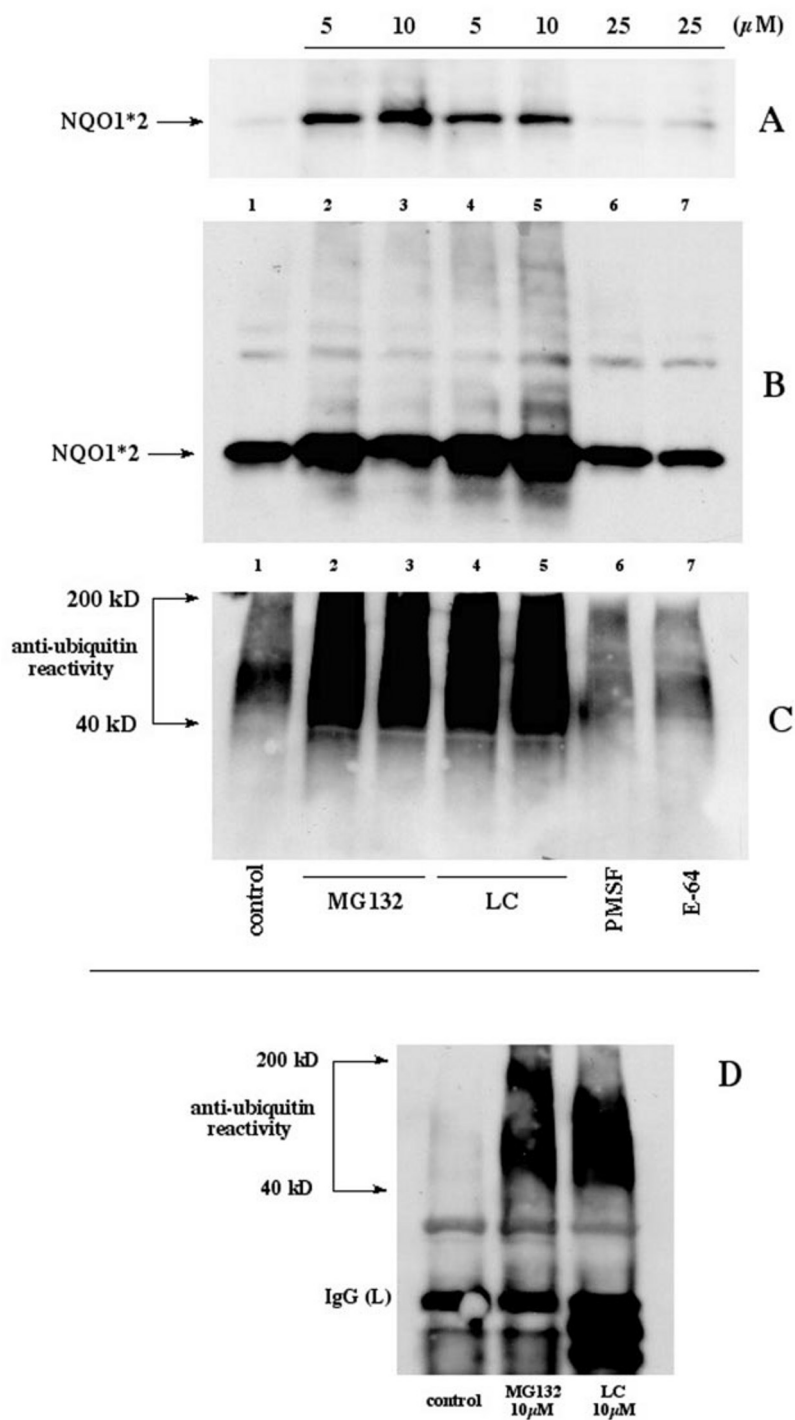
Determination of NQO1 Activity—Aliquots (5 μ l) of TNT-RRL reactions were diluted with 10 μ l of ice-cold 25 mM Tris-HCl, pH 7.4, containing 250 mM sucrose and 5 μ M FAD. NQO1 activity was measured spectrophotometrically using 2,6-dichlorophenol-indophenol as the terminal electron acceptor as described previously (23).

RESULTS

NQO1*2 Protein Is Degraded via the UPP—The effect of UPP inhibitors MG132 and *clasto*-lactacystin- β -lactone or non-proteasomal inhibitors PMSF (serine protease inhibitor) and E-64 (cysteine protease inhibitor) on NQO1 stability was examined in MDA-MB 231 human breast carcinoma cells, which have previously been genotyped as homozygous for the NQO1*2

FIG. 1. Inhibition of mutant NQO1*2 protein degradation in MDA-MB 231 breast cancer cells.

MDA-MB 231 cells were treated with proteasome inhibitors (MG132, LC) or non-proteasomal inhibitors (PMSF, E-64) at the indicated concentrations for 6 h. *A*, crude sonicate (50 μ g) was subjected to SDS-PAGE and immunoblot analysis. Membranes were probed with anti-NQO1 antibodies. Treatment of MDA-MB 231 cells with proteasome inhibitors generates higher molecular weight NQO1 immunoreactive products. *B*, experimental conditions were identical as described in *A*, except 100 μ g of crude sonicate was analyzed by SDS-PAGE and immunoblot analysis, and film was exposed for 1 h. Treatment of MDA-MB 231 cells with proteasome inhibitors generates higher molecular weight ubiquitin immunoreactive products. *C*, experimental conditions were identical as described in *A*, except membranes were probed with anti-ubiquitin antibodies. *D*, MDA-MB 231 cells were treated with either MG132 or LC (10 μ M) for 6 h. Samples were then immunoprecipitated with anti-NQO1 antibodies followed by SDS-PAGE and immunoblot analysis with anti-ubiquitin antibodies.



allele (17). NQO1 degradation was inhibited by the addition of MG132 or LC (Fig. 1A). In contrast, PMSF or E-64 had no effect on the degradation of NQO1. Several higher molecular weight, anti-NQO1 immunoreactive proteins could be observed after pretreatment with UPP inhibitors but not after pretreatment with PMSF or E-64 (Fig. 1B). The presence of higher molecular weight products after pretreatment with UPP inhibitors was consistent with the accumulation of polyubiquitinated forms of NQO1. Confirmation of the build up of high molecular weight polyubiquitinated proteins in samples treated with proteasomal inhibitors was demonstrated using an anti-ubiquitin antibody (Fig. 1C). The abundance of polyubiquitinated species was greatly enhanced when UPP was blocked by MG132 or LC (Fig. 1C, lanes 2–5). No accumulation of polyubiquitinated proteins

could be detected following treatment with PMSF or E-64 (Fig. 1C, lanes 6 and 7). In agreement with UPP-mediated degradation of NQO1*2, immunoprecipitation analysis using an anti-NQO1 monoclonal antibody followed by anti-ubiquitin immunoblot analysis confirmed the presence of polyubiquitinated NQO1*2 proteins in sonicates from MDA-MB 231 cells treated with proteasome inhibitors (Fig. 1D).

NQO1*2 Protein Is Short-lived in Cells—The half-life of mutant NQO1 was investigated in MDA-MB 231 cells. Cells were pretreated with cycloheximide to inhibit *de novo* protein synthesis followed by treatment with LC to inhibit the UPP, and cell lysates were monitored for the disappearance of the NQO1*2 protein. Because of the low levels of NQO1*2 protein in MDA-MB 231 cells, we utilized NQO1 immunoprecipitation

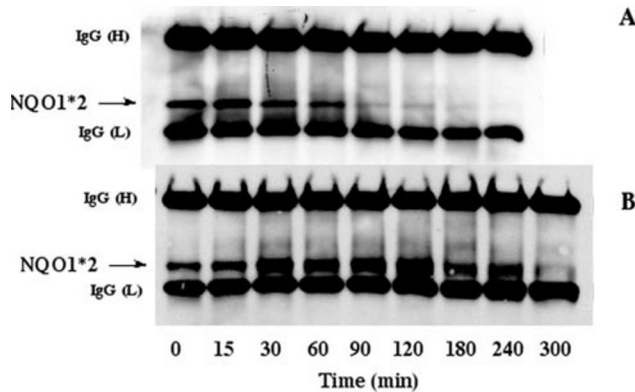


FIG. 2. The effect of proteasome inhibitor MG132 on the stability of mutant NQO1*2 protein in MDA-MB 231 cells. At the indicated time following cycloheximide treatment, cells were processed for immunoprecipitation, SDS-PAGE, and immunoblot analysis. Membranes were probed with anti-NQO1 antibodies. *A*, in the absence of proteasome inhibitor MG132. *B*, in the presence of MG132 (10 μ M). IgG (H), immunoglobulin heavy chain; IgG (L), immunoglobulin light chain.

followed by NQO1 immunoblot analysis to increase the sensitivity of this assay. The half-life of the NQO1*2 protein was determined to be \sim 1.5 h in MDA-MB 231 cells (Fig. 2A). This observation is in agreement with the short half-life of NQO1*2 protein in BE colon carcinoma cells, which are also homozygous for the NQO1*2 allele (18). In contrast to the rapid degradation of the NQO1*2 protein, the wild-type NQO1*1 protein exhibits considerable stability and has a half-life of greater than 18 h in HT-29 cells (18).

NQO1*2 Protein Half-life Increases in Response to UPP Inhibitors—Since the NQO1*2 protein is efficiently degraded by the UPP, inhibitors of the UPP such as MG132 would be predicted to increase the half-life of mutant NQO1. MDA-MB 231 cells were treated with both cycloheximide and MG132, and the rate of NQO1 degradation was followed by immunoprecipitation and immunoblot analysis (Fig. 2B). Treatment with MG132 led to increased stability of the NQO1*2 protein in MDA-MB 231 cells, and there was an approximately 4-fold increase in the half-life of the NQO1*2 protein as determined by the densitometric analysis of the blots (Fig. 2, A and B).

Wild-type NQO1*1, but Not Mutant NQO1*2, Protein Associates with the Molecular Chaperone Hsp70 in Cells—To examine the potential role of the molecular chaperone Hsp70 in the differential stability of NQO1*1 and NQO1*2 proteins, we investigated whether an interaction between the different forms of NQO1 and Hsp70 could be observed. For this purpose, cell sonicates from HT-29 (NQO1*1/*1) and MDA-MB 231 (NQO1*2/*2) cells were analyzed by immunoprecipitation followed by immunoblot analysis. Immunoprecipitation was carried out using anti-NQO1 monoclonal antibodies, and the blots were probed with anti-Hsp70 monoclonal antibodies. In HT-29 cells an Hsp70-NQO1*1 protein complex could be co-immunoprecipitated using anti-NQO1 antibodies (Fig. 3A). No Hsp70-NQO1 complex was observed in MDA-MB 231 cells. To further confirm these observations, we used anti-Hsp70 antibodies to first carry out the immunoprecipitation, and the membrane was then probed with anti-NQO1 antibodies (Fig. 3B). Using this reverse immunoprecipitation, an association of Hsp70 could be observed with the NQO1*1 protein but not with the mutant NQO1*2 protein. Furthermore, increasing the cellular levels of mutant NQO1*2 protein in MDA-MB 231 cells following treatment with UPP inhibitors did not result in immunoprecipitation of an Hsp70-NQO1 complex (Fig. 4A). Treatment of HT-29 cells with UPP inhibitors had no effect on the levels of NQO1-Hsp70 complex (Fig. 4A). These experiments confirm that the molecular chaperone Hsp70 associates with

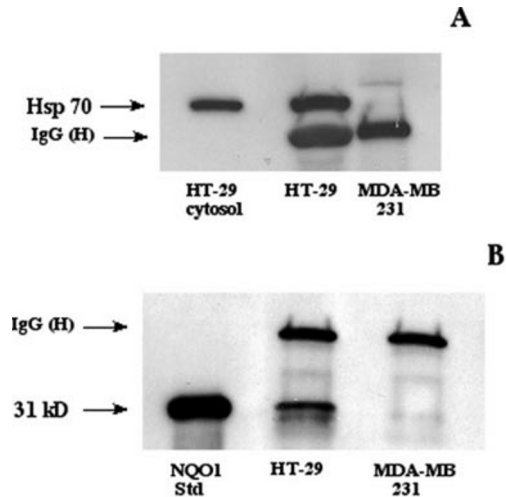


FIG. 3. The association of NQO1 with Hsp70 in cells. Co-immunoprecipitation was carried out to examine sonicates from HT-29 and MDA-MB 231 cells for an association of NQO1 with Hsp70. *A*, sonicated samples were immunoprecipitated with anti-NQO1 antibodies followed by SDS-PAGE and immunoblot analysis with anti-Hsp70 antibodies. HT-29 sonicate (50 μ g) was used as a molecular weight standard for Hsp70. *B*, samples were immunoprecipitated with anti-Hsp70 antibodies followed by SDS-PAGE and immunoblot analysis with anti-NQO1 antibodies. NQO1 Std, purified human recombinant NQO1 (5 ng).

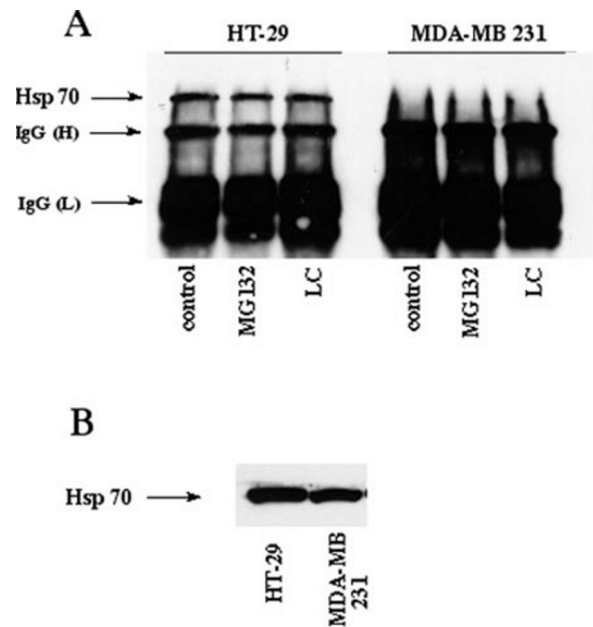


FIG. 4. Effect of proteasome inhibitors on the association of NQO1 with Hsp70 in cells. Co-immunoprecipitation was carried out to examine sonicates prepared from HT-29 and MDA-MB 231 cells pretreated with proteasome inhibitors (MG132, LC, 10 μ M) for an association of NQO1 with Hsp70. *A*, samples were immunoprecipitated with anti-NQO1 antibodies followed by SDS-PAGE and immunoblot analysis with anti-Hsp70 antibodies. *B*, cellular levels of Hsp70 in HT-29 and MDA-MB 231 cells. Sonicates prepared from HT-29 and MDA-MB 231 cells (50 μ g) were examined by SDS-PAGE and immunoblot analysis with anti-Hsp70 antibodies.

the NQO1*1 protein in HT-29 cells but not with mutant NQO1*2 protein in MDA-MB 231 cells. Both HT-29 and MDA-MB 231 cells used in this study have approximately equivalent levels of Hsp70 (Fig. 4B). Since molecular chaperones such as Hsp70 can function as catalysts and are released from interactions with target proteins after the facilitation of correct folding, we investigated the time course of association of NQO1*1 and Hsp70. *De novo* protein synthesis was blocked by

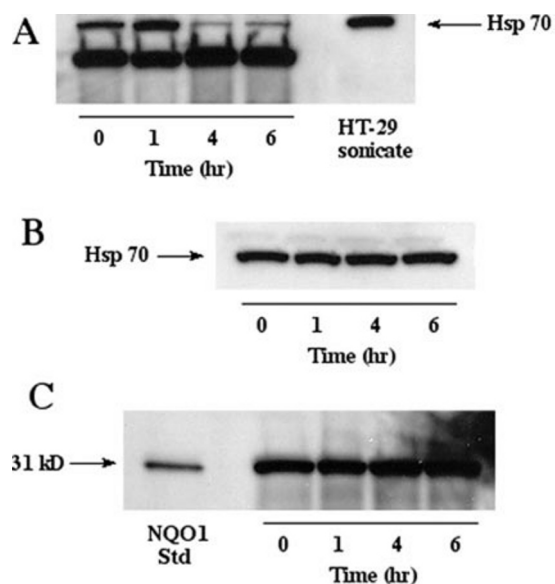


FIG. 5. The effect of *de novo* protein synthesis inhibition on the association of NQO1 with Hsp70. A, HT-29 cells were treated with cycloheximide, and at the indicated times samples were immunoprecipitated with anti-NQO1 antibodies followed by SDS-PAGE and immunoblot analysis with anti-Hsp70 antibodies. HT-29 sonicate (50 μ g) was used as a molecular weight standard for Hsp70. B, prior to immunoprecipitation, sonicate (50 μ g) was removed and analyzed by SDS-PAGE and immunoblot analysis with anti-Hsp70 antibodies. C, the membrane utilized in B was stripped and reprobed with anti-NQO1 antibodies. NQO1 Std, purified human recombinant NQO1 (5 ng).

cycloheximide treatment in HT-29 cells, and samples were analyzed for the Hsp70-NQO1*1 complex. Samples were collected at the indicated times, and immunoprecipitation was carried out using anti-NQO1 antibodies followed by immunoblot analysis with anti-Hsp70 antibodies. Maximal interaction of Hsp70 and NQO1*1 was observed at 1 h; however, at later time points of 4 and 6 h the interaction between Hsp70 and NQO1 was markedly decreased (Fig. 5A). This suggested that the chaperone may interact with unfolded or a partially folded immature form of NQO1*1. Treatment of HT-29 cells with cycloheximide did not have a major effect on cellular levels of either Hsp70 or NQO1 for the duration of the experiment (Fig. 5, B and C). When the membranes were stripped and probed for Hsp40, we were able to detect the presence of Hsp40 in the immunoprecipitated complexes of NQO1*1 and Hsp70, suggesting the formation of a ternary complex of Hsp40, Hsp70, and NQO1 (data not shown, see below).

The Association of Hsp70 with RRL Generated NQO1 Proteins—The level of the NQO1*2 protein in cells homozygous for the NQO1*2 allele such as MDA-MB 231 cells is low relative to cell homozygous for the wild-type NQO1*1 allele. The inability to observe an association of the NQO1*2 protein with Hsp70 may, therefore, be explained by the low cellular levels of NQO1*2 protein in MDA-MB 231 cells. This explanation seemed unlikely, however, since an interaction between Hsp70 and the NQO1*2 protein could not be detected even in the presence of UPP inhibitors, which increased the cellular levels of the NQO1*2 protein (Fig. 4A). To circumvent this potential problem we generated recombinant NQO1*1 and NQO1*2 proteins using a coupled transcription/translation approach in a cell-free rabbit reticulocyte lysate (TNT-RRL) system. Association of Hsp70 with the wild-type NQO1*1 protein could be observed by immunoprecipitation using anti-NQO1 antibodies followed by immunoblotting with anti-Hsp70 antibodies (Fig. 6A, lane 4). In contrast, very little association of Hsp70 was detected with mutant NQO1*2 protein (Fig. 6A, lane 3). Imme-

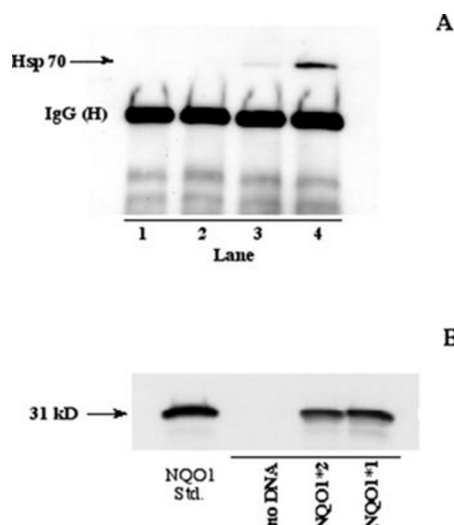


FIG. 6. The association of Hsp70 with NQO1 generated in an *in vitro* transcription/translation system (RRL). A, following *in vitro* transcription/translation of the wild-type (NQO1*1) and mutant (NQO1*2) coding regions, samples were immunoprecipitated with anti-NQO1 antibodies followed by SDS-PAGE and immunoblot analysis with anti-Hsp70 antibodies. Lane 1, RRLs only; lane 2, RRLs plus 1 μ g of purified human recombinant NQO1; lane 3, RRLs plus mutant NQO1*2 coding region; lane 4, RRLs plus wild-type NQO1*1 coding region. B, immunoblot analysis for NQO1 translation products just prior to immunoprecipitation (A). NQO1 Std, purified human recombinant NQO1 (5 ng).

diately prior to immunoprecipitation, a small aliquot of the RRL reaction was removed for NQO1 immunoblot analysis, which demonstrated that plasmids containing coding regions for NQO1*1 and NQO1*2 proteins were transcribed and translated with approximately equal efficiency in the RRL system (Fig. 6B). Importantly, no association of Hsp70 with NQO1 could be detected when mature purified recombinant NQO1*1 protein was substituted for newly synthesized NQO1, suggesting association of Hsp70 with an early nascent form of NQO1 (Fig. 6A, lane 2).

Site-directed Mutagenesis of a Hsp70 Binding Site on NQO1—To further investigate the interaction of Hsp70 with NQO1, we examined the NQO1 amino acid sequence for potential Hsp70 binding sites. Previous work has identified a potential Hsp70 binding motif described by a central hydrophobic core region enriched in leucine and isoleucine and flanked by basic amino acids. In this model, acidic amino acids are excluded from the hydrophobic core and disfavored in flanking regions (24, 25). Analysis of the NQO1*1 amino acid sequence revealed a potential Hsp70 binding site located near the N terminus of the NQO1 protein (Fig. 7A). Using site-directed mutagenesis we introduced an amino acid substitution (aspartic acid for isoleucine) at position 8 in the NQO1 protein. The plasmid encoding mutant NQO1*1/I8D was efficiently transcribed and translated in RRLs with almost equal efficiency to that of wild-type NQO1*1 (Fig. 7B). The mutant NQO1 protein, however, failed to interact with Hsp70 *in vitro* as demonstrated by co-immunoprecipitation studies (Fig. 7C).

Catalytic Activity of RRL-generated NQO1 Proteins—The catalytic activity of *in vitro* transcribed/translated NQO1*1, NQO1*2, and NQO1*1/I8D proteins was measured spectrophotometrically by following the reduction of 2,6-dichlorophenol-indophenol at 600 nm. Aliquots (5 μ l) of RRL reactions were removed at regular intervals over 60 min and assayed for NQO1 catalytic activity. NQO1 protein expression was confirmed by immunoblot analysis as described previously (data not shown). Transcription/translation of the wild-type NQO1*1

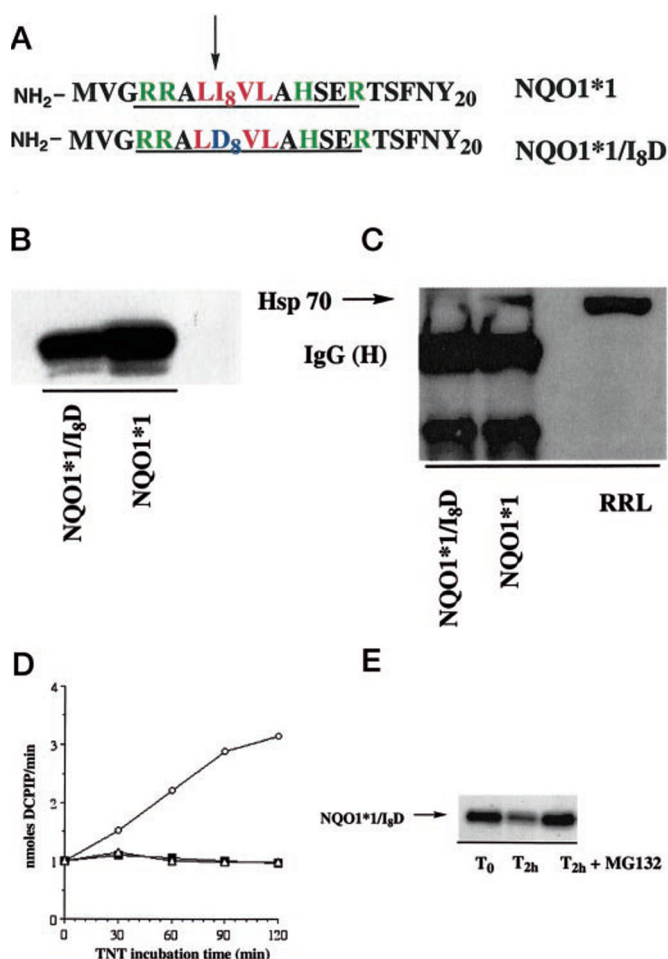


FIG. 7. Mutation of a Hsp70 binding motif in NQO1 results in the generation of a NQO1 protein with no catalytic activity. *A*, diagram representing the Hsp binding motif (underlined) located near the N terminus of the human NQO1 protein. Hydrophobic core amino acids are in red, and basic flanking amino acids are in green. Introduction of an aspartic acid residue (blue) at position 8 is shown in the lower diagram (NQO1*1/I₈D). *B*, immunoblot analysis for NQO1 translation products just prior to immunoprecipitation. *C*, following *in vitro* transcription/translation of wild-type (NQO1*1) and mutated (NQO1*1/I₈D) coding regions, samples were immunoprecipitated with anti-NQO1 antibodies followed by SDS-PAGE and immunoblot analysis with anti-Hsp70 antibodies. RRL (50 μ g) was utilized as a molecular weight standard for Hsp70. *D*, NQO1 catalytic activity was measured at the indicated times in an *in vitro* transcription/translation system (RRLs) supplemented with the following NQO1 coding regions. Open circles, wild-type NQO1*1; open triangles, mutant NQO1*2; closed squares, mutated NQO1*1/I₈D. *E*, immunoblot analysis of RRL-mediated degradation of NQO1*1/I₈D in the presence and absence of 25 μ M MG132. The NQO1*1/I₈D protein was generated in the TNT-RRL system and then transferred into untreated RRLs as described previously (18).

coding region resulted in a time-dependent increase in NQO1 activity (Fig. 7D). Although RRL-mediated transcription/translation of either the NQO1*2 or NQO1*1/I₈D coding regions produced full-length NQO1 products, both proteins failed to demonstrate catalytic activity, suggesting the functional role of Hsp70 interaction with NQO1*1 protein. The NQO1*1/I₈D protein also underwent RRL-mediated degradation, and protein degradation was inhibited by the addition of MG132, suggesting that NQO1*1/I₈D was degraded by the UPP (Fig. 7E).

Hsp40 and Hsp90 Interactions with NQO1*1 and NQO1*2 Proteins—Since Hsp40 and Hsp90 are known to be co-chaperones of Hsp70 in the Hsp family, we also investigated the interactions of these chaperones with the NQO1*1 and NQO1*2 proteins. Co-immunoprecipitation studies were per-

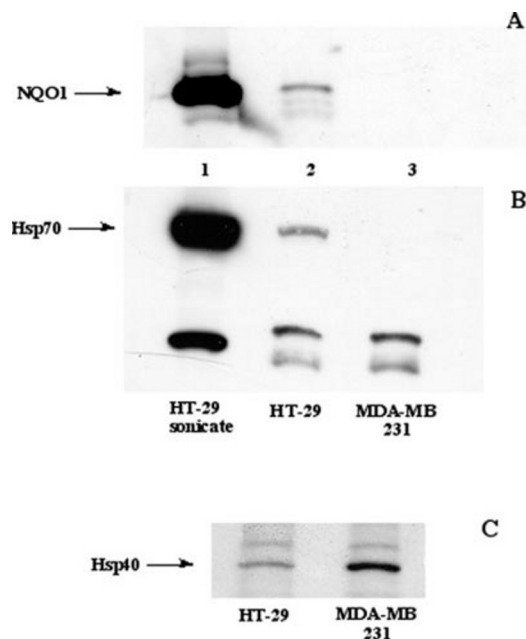


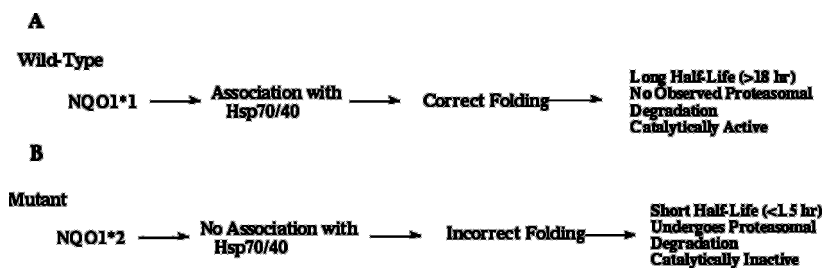
FIG. 8. The association of NQO1 with Hsp40 in cells. Co-immunoprecipitation was used to examine sonicates from HT-29 and MDA-MB 231 cells for an association of NQO1 with Hsp40. *A*, samples were immunoprecipitated with anti-Hsp40 antibodies followed by SDS-PAGE and immunoblot analysis with anti-NQO1 antibodies. HT-29 sonicate (50 μ g) was utilized as a molecular weight standard for NQO1 (lane 1). *B*, membrane in *A* was stripped and reprobed with anti-Hsp70 antibodies. HT-29 sonicate (50 μ g) was utilized as a molecular weight standard for Hsp70 (lane 1). *C*, levels of Hsp40 in HT-29 and MDA-MB 231 cells. Sonicates prepared from HT-29 and MDA-MB 231 cells (50 μ g) were examined by SDS-PAGE and immunoblot analysis with anti-Hsp40 antibodies.

formed in HT-29 and MDA-MB 231 cells. Sonicated samples were immunoprecipitated using anti-Hsp40 antibodies followed by immunoblot analysis with anti-NQO1 antibodies. These co-IP experiments demonstrated an association between wild-type NQO1*1 protein and Hsp40, but no association was observed between Hsp40 and mutant NQO1*2 protein (Fig. 8A). These results are similar to data obtained for the interaction between Hsp70 and wild-type NQO1*1 protein, suggesting that Hsp40 may complex together with Hsp70 in their association with NQO1*1. The ternary complex of NQO1, Hsp40, and Hsp70 was confirmed by stripping the membrane shown in Fig. 8A and re-probing with anti-Hsp70 antibodies. The presence of Hsp70 in the immunoprecipitated complex could be observed in HT-29 cells but not MDA-MB 231 cells (Fig. 8B). The absence of an association of Hsp40 with NQO1 in MDA-MB 231 cells could not be explained by the cellular levels of Hsp40, since greater levels of Hsp40 could be detected in MDA-MB 231 cells than in HT-29 cells (Fig. 8C). Using similar co-IP studies, however, we failed to detect an association of NQO1*1 or NQO1*2 proteins with Hsp90 in HT-29 and MDA-MB 231 cell lines or RRLs (data not shown).

DISCUSSION

It is well known that misfolded proteins are substrates for the UPP. Inhibition of the 26 S proteasome results in an increase in the amount of NQO1*2 protein and a build up of higher molecular weight NQO1 immunoreactive species consistent with the formation of polyubiquitinated NQO1 proteins. Treatment of MDA-MB 231 cells with inhibitors of the 26 S proteasome also results in an increase in the half-life of the NQO1*2 protein, clearly demonstrating the role of the UPP in degradation of the mutant NQO1*2 protein. The shortened half-life of the mutant NQO1*2 protein (1.5 h) is in contrast to

FIG. 9. Proposed mechanism for the difference in stability between wild-type NQO1*1 and mutant NQO1*2 proteins.



the stability of wild-type NQO1*1 protein, which has a half-life of greater than 18 h (18).

In an attempt to understand possible mechanisms underlying the differential stability of NQO1*1 and NQO1*2 proteins, we have investigated a potential role for the involvement of molecular chaperones of the heat shock protein family (Hsp). Molecular chaperones have been implicated in a wide variety of cellular processes including protein folding, protein transport, and protein degradation (26–29).

In co-immunoprecipitation studies using cellular systems, we found that Hsp70 associated with the wild-type NQO1*1 protein but not with the mutant NQO1*2 protein. To confirm these observations in a cell-free system, we performed similar experiments employing RRLs in an *in vitro* coupled transcription/translation system. Our results demonstrated that although approximately equivalent amounts of NQO1*1 and NQO1*2 proteins were generated in RRLs, interaction of Hsp70 could only be observed with wild-type NQO1*1 but not with mutant NQO1*2 protein. Hsp70-catalyzed protein folding requires ATP hydrolysis and often involves the presence of co-chaperones such as Hsp40 and Hsp90 (19, 30). In the co-immunoprecipitated complex of NQO1 and Hsp70, Hsp40 could also be detected, suggesting the presence of a ternary complex. No interaction, however, could be observed between NQO1*1 or NQO1*2 proteins and Hsp90, demonstrating that there is some degree of specificity to the interactions of NQO1 and the proteins of the Hsp family.

In protein folding interactions, molecular chaperones such as Hsp70 are believed to bind with the target substrate, facilitate correct folding, and then dissociate from the complex (19, 28). In HT-29 cells, time course studies demonstrated that the Hsp70-NQO1 complex was greatly reduced after 4 h following the inhibition of protein synthesis. In addition, RRL experiments using *in vitro* transcription/translation demonstrated that Hsp70 did associate with newly synthesized NQO1*1 protein; however, Hsp70 failed to interact with mature recombinant NQO1*1. The association of Hsp70 and NQO1*1 in cells and in cell-free systems, therefore, most probably reflects the association of Hsp70 with a precursor form of the mature NQO1 protein that has not been fully and completely folded (Fig. 9). These results are consistent with the proposed role of Hsp70 in protein folding (30) and are the first demonstration that chaperones interact with NQO1.

Additional evidence linking Hsp70 with NQO1 has come from site-directed mutagenesis studies of a proposed Hsp70 binding motif located near the N terminus of the NQO1 protein. Studies with Dna K, the *E. coli* homologue of human Hsp70, has identified binding motifs in the substrate proteins that associate with Dna K (25, 30). The binding motifs consist of a hydrophobic core of four to five residues enriched in leucine, isoleucine, and valine and two flanking regions enriched in basic residues. Acidic residues are excluded from the core and disfavored in the flanking regions. A search of the human, mouse, and rat NQO1 amino acid sequences revealed the presence of an identical Dna K binding site (R₄R₅A₆L₇I₈V₉L₁₀A₁₁H₁₂) located near the N terminus of the

protein. Site-directed mutagenesis was used in combination with *in vitro* transcription/translation to generate a human NQO1*1 protein where isoleucine was replaced with aspartic acid at position 8 of the amino acid sequence (NQO1*1/I8D). As predicted from the proposed model, the insertion of an acidic residue into the hydrophobic core resulted in the loss of binding of NQO1*1/I8D to Hsp70. The resultant *in vitro* translated NQO1*1/I8D protein had no measurable catalytic activity and was degraded by the UPP system demonstrating the functional role of the interaction of Hsp70 with NQO1. In addition, an examination of the x-ray crystal structure of recombinant human NQO1 has revealed that the Dna K binding site is not directly exposed to the surface in the mature protein (31). This is confirmed by the absence of an association between mature NQO1*1 and Hsp70. These data further implicate a role for Hsp70 in the folding of immature NQO1 into an active protein. It is also possible that introduction of an acidic amino acid residue into the N terminus of the NQO1 amino acid sequence might have interfered with the catalytic function of the enzyme. This seems unlikely, however, since the addition of positively charged residues to the N terminus of NQO1 in the form of a 6×-His-tag did not result in significant loss of enzymatic activity as compared with the NQO1*1 protein (32).² The His-tagged NQO1*1 protein had ~70% of the catalytic activity of the wild-type NQO1*1 protein and was not degraded by the UPP, while in contrast the NQO1*1/I8D protein was a target of UPP-mediated degradation.

The absence of an association between Hsp70/40 and mutant NQO1*2 protein suggests that the proline to serine amino acid substitution in the NQO1*2 protein prevents binding of Hsp70 to the mutant protein. X-ray crystallographic studies have shown that this substitution is located near the transition of a β -sheet and an α -helical bend. The proline to serine substitution may disrupt the confirmation of the NQO1*2 protein preventing recognition and association with Hsp70/40, resulting in misfolding and subsequent UPP-mediated degradation. Alternatively, it has been proposed that the proline to serine amino acid change results in disruption of the central parallel β -sheet, resulting in a decreased affinity of the protein for the FAD cofactor (33). It is conceivable that the inability of NQO1*2 protein to efficiently bind FAD prevents the association of the mutant protein with Hsp70/40. Studies are under way to determine whether an association with Hsp70/40 facilitates incorporation of FAD into NQO1 proteins.

In summary, we have demonstrated an association of Hsp70 and Hsp40 with wild-type NQO1 but not mutant NQO1*2 protein. Given the documented role for Hsp70/40 in protein folding (30, 34), we hypothesize a role for Hsp70/40 in the differential stability of NQO1*1 and NQO1*2 proteins (Fig. 9). This proposed model will allow for the design of future experiments to characterize the structural motifs that govern the interaction of Hsp and NQO1 proteins.

² D. Siegel and J. K. Kepa, unpublished data.

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