20S Proteasomal Degradation of Ornithine Decarboxylase Is Regulated by NQO1

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Summary

Ornithine decarboxylase (ODC), a key enzyme in the biosynthesis of polyamines, is a very labile protein. ODC is a homodimeric enzyme that undergoes ubiquitin-independent proteasomal degradation via direct interaction with antizyme, a polyamine-induced protein. Binding of antizyme promotes the dissociation of ODC homodimers and marks ODC for degradation by the 26S proteasomes. We describe here an alternative pathway for ODC degradation that is regulated by NAD(P)H quinone oxidoreductase 1 (NQO1). We show that NQO1 binds and stabilizes ODC. Dicoumarol, an inhibitor of NQO1, dissociates ODC-NQO1 interaction and enhances ubiquitin-independent ODC proteasomal degradation. We further show that dicoumarol sensitizes ODC monomers to proteasomal degradation in an antizyme-independent manner. This process of NQO1-regulated ODC degradation was recapitulated in vitro by using purified 20S proteasomes. Finally, we show that the regulation of ODC stability by NQO1 is especially prominent under oxidative stress. Our findings assign to NQO1 a role in regulating ubiquitin-independent degradation of ODC by the 20S proteasomes.

Introduction

Protein degradation plays a central role in the regulation of many fundamental cellular processes (Hershko and Ciechanover, 1998). Proteasomes are widely recognized as the major machinery responsible for intracellular protein degradation. The ubiquitin pathway mediates selective degradation of many short-lived proteins by the proteasomes. Ubiquitin is covalently attached to target proteins forming polyubiquitin chains that are recognized by the proteasome as a degradation signal.

One of the most studied short-lived proteins is the tumor suppressor p53. p53 undergoes degradation by ubiquitin-dependent and -independent pathways. In the ubiquitin-dependent pathway, p53 is monoubiquitinated on several lysine residues by the E3 ubiquitin-ligase Mdm2 (Haupt et al., 1997; Kubbataut et al., 1997). The monoubiquitinated residues of p53 are then polyubiquitinated either by p300 (Grossman et al., 2003) or by elevated levels of Mdm2 (Li et al., 2003), leading to its proteasomal degradation. Recently, two additional E3 ubiquitin ligases Pih2 (Leng et al., 2003) and COP1 (Dornan et al., 2004) were demonstrated to polyubiquitinate p53 and induce p53 degradation. The ubiquitin-independent pathway is regulated by NAD(P)H quinone oxidoreductase 1 (NQO1) (Asher et al., 2001; Asher et al., 2002b; Asher et al., 2003; Asher et al., 2004), a ubiquitous flavoenzyme that catalyzes two-electron reduction of various quinones, utilizing NADH or NADPH as electron donors. NQO1 physically interacts with p53 (Anwar et al., 2003; Asher et al., 2003) and stabilizes p53 (Asher et al., 2002a). NQO1 knockdown with a specific siRNA or its inhibition with dicoumarol [3-3’-methylene-bis(4-hydroxycomarin)], a specific inhibitor that competes with NADH or NADPH (Hosoda et al., 1974), induces ubiquitin-independent degradation of p53 (Asher et al., 2002b).

Ornithine decarboxylase (ODC), one of the most labile cellular proteins, is the most notable exception to the ubiquitin-proteasome degradation pathway (Coffino, 2001; Verma and Deshaies, 2000). ODC is the first rate-limiting enzyme in polyamine biosynthesis. In its active form, ODC is a homodimer with two enzymatic active sites (Tobias and Kahana, 1993). ODC degradation is mediated by interaction with a polyamine-induced protein, termed antizyme (Murakami et al., 1992a; Mamroud-Kidron et al., 1994; Coffino, 2001). Binding of antizyme to ODC subunits results in the disruption of ODC homodimers and the formation of enzymatically inactive ODC antizyme heterodimers (Mamroud-Kidron et al., 1994). Studies performed both in vivo and in vitro revealed that ODC degradation by the 26S proteasome requires interaction with antizyme but not ubiquitination (Glass and Gerner, 1987; Murakami et al., 1992a; Mamroud-Kidron et al., 1994). Interestingly, ODC has a short half-life even in the absence of antizyme, but the presence of antizyme further decreases ODC stability (Zhang et al., 2003). Employment of specific ODC mutants revealed that formation of an ODC antizyme heterodimer and enhancement of ODC degradation are two distinct events (Mamroud-Kidron et al., 1994; Chen et al., 2002). The first event involves dissociation of ODC homodimers and the second event presents ODC to the 26S proteasomes. Recognition of ODC antizyme heterodimers by the 26S proteasomes requires the C-terminal region of ODC (Murakami et al., 1992a; Murakami et al., 1992b; Mamroud-Kidron et al., 1994; Zhang et al., 2003).

In the present study, we have investigated the possibility that ODC degradation is also regulated by NQO1. We show that NQO1 regulates ubiquitin-independent proteasomal degradation of ODC via a mechanism that is different from that mediated by antizyme. NQO1 bound to ODC and stabilized it, whereas the NQO1 inhibitor dicoumarol disrupted this association and stimulated ODC degradation by the 20S proteasomes. We further show that this pathway regulates ODC level under oxidative stress.

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Figure 1. Regulation of ODC Degradation by NQO1

(A) 293 HEK cells were transiently transfected with pEFIRE-ODC together with pEFIRE (empty vector) or with pEFIRE-NQO1.
(B and C) COS-1 cells were treated without (−) or with 200 or 400 μM dicoumarol for 5 hr, cell extracts were analyzed by SDS PAGE (B), and by ODC enzymatic activity assay (C). To determine the specificity of the ODC activity, the reaction was performed without or with 5 mM DFMO (Tobias et al., 1993).
(D) ODC activity was determined in 293 HEK cells treated with 100, 200, or 400 μM dicoumarol for 5 hr.
(E) COS-1 cells were transiently transfected with pSUPER-X as a control together with pE-H2B-GFP (−) or with pSUPER-NQO1 expressing NQO1 specific siRNA together with pE-H2B-GFP (+). GFP-positive cells were sorted and collected with FACSVantageSE (Becton Dickinson) and cell extracts were analyzed by SDS PAGE.
(F) ODC activity was determined in 293 HEK cells transfected with increasing concentrations of pEFIRE-NQO1. The level of NQO1 expression was analyzed by SDS PAGE.
(G) ODC activity was determined in 293 HEK cells that were transiently transfected with empty vector (−) or with (+) pEFIRE-ODC and treated without (−) or with 100, 200, or 400 μM dicoumarol for 5 hr.
(H) 293 HEK cells that stably express wild-type mouse ODC were treated without (−) or with (+) 200 μM dicoumarol and without (−) or with (+) 25 μM MG132 for 5 hr.
(I) 293 HEK cells that stably express wild-type mouse ODC were treated without (−) or with (+) 300 μM dicoumarol for 2.5 hr and 5 hr.
(J) A31N-ts20 and A31N BALB/c mouse cells were incubated for 24 hr at the permissive (32°C) or restrictive (39°C) temperature and cell extracts were analyzed by SDS PAGE.
(K) A31N and A31N-ts20 BALB/c mouse cells that stably express wild-type mouse ODC were incubated at 39°C for 24 hr and then treated without (−) or with (+) 300 μM dicoumarol without (−) or with (+) 50 μM MG132 for 5 hr.

Results

Regulation of ODC Proteasomal Degradation by NQO1

To examine whether ODC is stabilized by NQO1, we transfected 293 human kidney (293 HEK) cells with ODC, either alone or together with NQO1, and determined the level of ODC. Significant increase in the ODC level was observed in the presence of NQO1 (Figure 1A). Dicoumarol is a potent and specific inhibitor of NQO1 (Hosoda et al., 1974). Treatment of COS-1 cells with increasing concentrations of dicoumarol resulted in a dose-dependent decrease in the level of endogenous ODC as determined by Western blot analysis (Figure 1B) and in ODC enzymatic activity (Figure 1C). Similar results were obtained with 293 HEK cells (Figure 1D). Reduction of the NQO1 level upon expression of NQO1 siRNA (Asher et al., 2002b) resulted in a significant decrease in the endogenous ODC level (Figure 1E). In a reciprocal experiment, NQO1 overexpression resulted in a dose-dependent increase in the level of endogenous ODC activity (Figure 1F). As expected, under conditions of NQO1 overexpression, dicoumarol was less efficient in induction degradation of endogenous ODC...
Dicoumarol Induces ODC Degradation in the Absence of the E1 Ubiquitin-Activating Enzyme

To examine whether dicoumarol-induced ODC degradation is ubiquitin dependent, we utilized A31N and A31N-ts20 BALB/c mouse cell lines. A31N-ts20 cells harbor a temperature-sensitive E1 enzyme, the only ubiquitin-activating enzyme in mammalian cells (Chowdary et al., 1994). When A31N-ts20 cells are cultured at the restrictive temperature (39°C), the E1 enzyme is inactive and protein ubiquitination and degradation is repressed. Under these conditions and in agreement with our previous finding (Asher et al., 2002b), p53 is Mdm2 refractory and accumulates (Figure 1J), confirming inactivation of E1 and lack of significant ubiquitination at 39°C in A31N-ts20 cells. Addition of dicoumarol to A31N or A31N-ts20 cells 24 hr following their transfer to 39°C, resulted in an efficient degradation of ODC in both cell lines (Figure 1K). Here again, dicoumarol-induced ODC degradation was inhibited in the presence of the proteasome inhibitor MG132. These results suggest that dicoumarol-induced ODC degradation is independent of E1 activity and, therefore, does not require ubiquitination.

Antizyme Enhances Dicoumarol-Induced ODC Degradation

Two regions of ODC are essential for its antizyme-mediated proteasomal degradation (Figure 2A). The first, encompassing amino acids 117–140, serves as the antizyme binding site (Li and Coffino, 1992). The second, containing the most C-terminal 37 amino acids (Ghoda et al., 1989; Rosenberg-Hasson et al., 1991), forms with antizyme an efficient 26S-proteasome degradation signal (Zhang et al., 2003). To test whether interaction with antizyme is required for the dicoumarol-induced degradation of ODC, we utilized a mutant mouse ODC, termed Del6, whose antizyme binding site was replaced with the corresponding region of trypanosome ODC, which does not bind antizyme, generating an enzymatically active and stable protein (Mamroud-Kidron et al., 1994). Cells transfected with wild-type ODC or the MTM mutant were treated with dicoumarol, and the level of ODC was monitored. A significant degradation of wild-type ODC, but not of the MTM mutant, was observed following dicoumarol treatment (Figure 2B). Similar results were obtained in the presence of the protein synthesis inhibitor cycloheximide (CHX) (Figure 2C), ruling out the possibility that the resistance of the MTM mutant to dicoumarol is due to its rapid synthesis. Control experiments revealed that dicoumarol is active in the presence of CHX and could induce degradation of wild-type ODC (data not shown) and of another ODC mutant (see below). These results indicate that the integrity of the antizyme binding region is important for dicoumarol-induced ODC degradation.

To further evaluate the requirement of antizyme, we examined the ability of dicoumarol to induce ODC degradation under conditions whereby antizyme level is reduced (Rom and Kahana, 1994). COS-1 cells were cultured in the presence of DFMO, a mechanism-based inhibitor of ODC, to reduce cellular polyamines and, as a result, to decrease the level of endogenous antizyme (Figure 2D). Under these conditions the dicoumarol-induced ODC degradation was less efficient (Figure 2E). These results further suggest that antizyme enhances dicoumarol-induced ODC degradation.

The ODC C-Terminal Destabilizing Region Is Dispensable for Dicoumarol-Induced ODC Degradation

Next, we asked whether the dicoumarol-induced ODC degradation requires the C-terminal destabilizing region of ODC. To this end, we utilized the C terminus-truncated ODC mutant lacking the last 37 amino acids, termed Del6 (Figure 2A), (Rosenberg-Hasson et al., 1991). Remarkably, the Del6 mutant that is refractory to antizyme-mediated degradation (Mamroud-Kidron et al., 1994) was degraded in dicoumarol-treated cells as efficiently as wild-type ODC (Figure 2F). Furthermore, similar to wild-type ODC (Figure 1I), degradation of the Del6 mutant was completed within 3 hr following dicoumarol addition (Figure 2G, lanes 5 and 6). In addition, dicoumarol induced degradation of the Del6 mutant even in the presence of CHX, ruling out the involvement of any de novo synthesized protein in this process (Figure 2G, lanes 8–10). In agreement with our previous report (Mamroud-Kidron et al., 1994), overexpression of antizyme resulted in enhanced degradation of wild-type ODC but not of the ODC Del6 mutant (Figure 2H). The requirement of the C-terminal destabilizing signal for antizyme-mediated degradation but not for the dicoumarol-induced ODC degradation, suggests that antizyme and dicoumarol regulate proteasomal degradation of ODC via two distinct pathways.
Figure 2. Dicoumarol-Induced ODC Degradation Is Independent of the C-Terminal Destabilizing Region of ODC and Is Enhanced by Antizyme

(A) Schematic representation of wild-type mouse ODC, MTM ODC, and Del6 ODC mutants.

(B) 293 HEK cells were transiently transfected with pEFires-ODC or pEFires-ODC-MTM. 24 hr posttransfection cells were untreated (−) or treated (+) with 300 μM dicoumarol for 5 hr.

(C) 293 HEK cells were transiently transfected with pEFires-ODC-MTM and 24 hr posttransfection cells were incubated for 1, 3, or 5 hr with 20 μg/ml cycloheximide (CHX) without (−) or with (+) 300 μM dicoumarol.

(D) COS-1 cells were cultured without (−) or with (+) 5 mM DFMO for four successive passages, and cell extracts were analyzed by SDS PAGE.

(E) COS-1 cells were cultured without (−) or with (+) 5 mM DFMO for four successive passages and then treated without (−) or with (+) 300 μM dicoumarol for 5 hr.

(F) 293 HEK cells were transiently transfected with pEFires-ODC or pEFires-ODC-Del6. 24 hr posttransfection cells were treated without (−) or with (+) 300 μM dicoumarol for 5 hr.

(G) 293 HEK cells were transiently transfected with pEFires-ODC-Del6, and 24 hr posttransfection cells were incubated for 1, 3, or 5 hr with 20 μg/ml cycloheximide (CHX), 300 μM dicoumarol, or both 20 μg/ml CHX and 300 μM dicoumarol.

(H) 293 HEK cells were transiently transfected with pEFires-ODC or pEFires-ODC-Del6, together with either pEFires (empty vector) or with pEFires antizyme. 24 hr posttransfection cells were treated without (−) or with (+) 300 μM dicoumarol for 5 hr. Immunoblot analysis (IB) was carried out with mouse monoclonal anti-ODC antibody, rabbit anti-antizyme antibody, and mouse monoclonal anti-Actin antibody.

nor forms homodimers (see below). Degradation of the MTM-G387D double, but not of the MTM, mutant was enhanced in the presence of dicoumarol (Figure 3B).

Dicoumarol induces degradation of p53 in reticulocyte lysates (Asher et al., 2002b). In the present study, we conducted similar experiments with in vitro translated [35S]methionine-labeled MTM and MTM-G387D ODC mutants. Dicoumarol efficiently induced in vitro degradation of the MTM-G387D double mutant, but not of the MTM mutant (Figure 3C, compare lanes 4–6 with lanes 10–12). Furthermore, under these conditions, the C-terminal destabilizing region of ODC was dispensable for dicoumarol-induced degradation as demonstrated by the ability of dicoumarol to induce degradation of the Del6-MTM-G387D triple mutant in vitro (Figure 3D, compare lanes 1–3 with lanes 4–6). These results indicate that dicoumarol induces preferential degradation of ODC monomers and that the C-terminal destabilizing region of ODC is dispensable for this process.

To substantiate this model, we further mutated the Del6 mutant to generate Del6-MTM and Del6-G387D ODC double mutants (Figure 3A). The Del6-MTM double mutant is an antizyme-insensitive homodimer. Remarkably, this mutant was much less sensitive to dicoumarol-induced degradation, compared to the Del6 mutant. Conversely, the Del6-G387D monomeric double mutant displayed enhanced degradation in the presence of dicoumarol (Figure 3E). These results further indicate that dicoumarol preferentially enhances degradation of ODC monomers.

In Vitro Degradation of ODC Monomers by the 20S Proteasomes Is Inhibited by NQO1

The observation that dicoumarol induces degradation of ODC monomers that neither bind antizyme nor contain the C-terminal destabilizing region, was rather surprising and called for the presence of an alternative pathway for ODC degradation. To address this possi-
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Figure 3. ODC Monomerization Is Required for Dicoumarol-Induced Degradation

(A) Schematic representation of wild-type and different ODC mutants.

(B) 293 HEK cells were transiently transfected with pEFires-ODC-MTM or with pEFires-ODC-MTM-G387D. 24 hr post-transfection cells were treated without (−) or with (+) 300 μM dicoumarol for 5 hr.

(C) In vitro translated [35S]methionine-labeled MTM and MTM-G387D were incubated in a reticulocyte lysate degradation mixture at 37°C for 90 and 180 min without (−) or with (+) 300 μM dicoumarol.

(D) In vitro translated [35S]methionine-labeled Del6-MTM-G387D was incubated in a reticulocyte lysate degradation mixture at 37°C for 90 and 180 min without (−) or with (+) 300 μM dicoumarol. The level of the different ODC mutants was determined by autoradiography as described in Experimental Procedures.

(E) 293 HEK cells were transiently transfected with pEFires-ODC-Del6-MTM, with pEFires-ODC-Del6, or with pEFires-ODC-Del6-G387D. 24 hr posttransfection cells were treated without (−) or with (+) 300 μM dicoumarol for 5 hr. Immunoblot analysis (IB) was carried out with mouse monoclonal anti-ODC antibody and mouse monoclonal anti-Actin antibody.

bility, we performed in vitro degradation experiments using purified components. Remarkably, the monomeric ODC-G387D mutant but not wild-type ODC was efficiently degraded (about 70%) by highly purified 20S proteasomes (Figure 4A). As expected, both proteins were not degraded by 26S proteasomes in the absence of antizyme (data not shown). The monomeric nature of the ODC-G387D mutant was determined by cross-linking analysis (Tobias et al., 1993). As expected, unlike wild-type ODC, the ODC-G387D mutant failed to homodimerize (Figure 4B). The degradation of the ODC-G387D mutant by the 20S proteasomes did not require ATP (Figure 4C) and was inhibited by MG132 (Figure 4D).

The MTM mutant that was dicoumarol-refractory in vivo was also resistant to degradation by the 20S proteasomes in vitro (Figure 4E, lanes 1 and 2). However, this mutant was sensitized to 20S-mediated degradation once its capacity to homodimerize was eliminated in the MTM-G387D double mutant (Figure 4E, lanes 3 and 4). To further demonstrate that ODC monomerization is essential for degradation by the 20S proteasomes, we examined the Del6 mutant and the Del6-G387D double mutant. Unlike the Del6 mutant (Figure 4F, lanes 1 and 2), the Del6-G387D double mutant was susceptible to degradation by the 20S proteasomes (Figure 4F, lanes 3 and 4). Finally, we utilized a set of ODC mutants that carry deletions in the ODC homodimerization region. Out of three such deletion mutants, two failed to homodimerize, whereas the Δ423–448 deletion mutant was as effective as wild-type ODC in homodimerization (Figure 4G). Interestingly, Δ423–448 was also the only deletion mutant that resisted degradation by 20S proteasomes (Figure 4H, lanes 7 and 8). These data indicate that ODC monomers are susceptible to degradation by the 20S proteasomes.

As demonstrated above, NQO1 stabilized and dicoumarol destabilized ODC in cells (Figure 1). To examine whether this is also the case in the degradation of ODC by the purified 20S proteasomes, the in vitro degradation assay was repeated in the presence or absence of NQO1. Remarkably, NQO1 inhibited 20S proteasomal degradation of the ODC-monomeric G387D mutant (Figure 4I), and this inhibition was relieved by dicoumarol (Figure 4J). The specificity of the protective effect of NQO1 on ODC degradation was demonstrated by the inability of NQO1 to inhibit degradation of a control protein (p73β) by the 20S proteasomes (data not shown). Together, our data strongly suggest that NQO1 stabilizes ODC by inhibiting 20S proteasomal degradation of ODC monomers. Our recent finding that NQO1 is in physical association with the 20S proteasomes (Asher et al., 2005) supports this possibility.

NQO1 Physically Interacts with ODC

NQO1 physically interacts with p53 (Anwar et al., 2003; Asher et al., 2003) and blocks p53 proteasomal degradation. To examine the possibility that NQO1 physically interacts with ODC, both proteins were transiently expressed either alone or together in 293 HEK cells, and...
**Figure 4. In Vitro Degradation of ODC Monomers by the 20S Proteasomes Is Inhibited by NQO1**

(A) In vitro translated [35S]methionine-labeled ODC or ODC-G387D were incubated without (−) or with (+) purified 20S proteasomes at 37°C for 1 hr or (B) were subjected to crosslinking analysis (Tobias et al., 1993). (C) In vitro translated [35S]methionine-labeled ODC-G387D was incubated without (−) or with (+) purified 20S proteasomes at 37°C for 1 hr in the absence or presence of 20 mM 2-deoxy-D-glucose and 20 μg/ml hexokinase (ATP depletion). (D) In vitro translated [35S]methionine-labeled ODC-G387D was incubated without (−) or with (+) purified 20S proteasomes without (−) or with 50 μM MG132 at 37°C for 1 hr. (E) In vitro translated [35S]methionine-labeled ODC-MTM or ODC-MTM-G387D were incubated without (−) or with (+) purified 20S proteasomes at 37°C for 1 hr. (F) In vitro translated [35S]methionine-labeled ODC-Del6 or ODC-Del6-G387D were incubated without (−) or with (+) purified 20S proteasomes without (−) or with (+) purified 20S proteasomes at 37°C for 1 hr. (G) In vitro translated [35S]methionine-labeled ODC-Del6-G387D were incubated without (−) or with (+) purified 20S proteasomes at 37°C for 1 hr. (H) In vitro translated [35S]methionine-labeled ODC-G387D, ODC-Del6-G387D, and wild-type ODC were subjected to crosslinking analysis. (I) In vitro translated [35S]methionine-labeled ODC-G387D was incubated in the presence of 1 mM β-NADH without (−) or with (+) purified 20S proteasomes without (−) or with (+) in vitro translated [35S]methionine-labeled NQO1 at 37°C for 1 hr. (J) In vitro translated [35S]methionine-labeled ODC-G387D was incubated in the presence of 1 mM β-NADH without (−) or with (+) purified 20S proteasomes without (−) or with (+) purified 20S proteasomes at 37°C for 1 hr. (K) In vitro translated [35S]methionine-labeled ODC-G387D was incubated in the presence of β-NADH without (−) or with (+) purified 20S proteasomes without (−) or with (+) dicoumarol at 37°C for 1 hr. The level of ODC and NQO1 was determined by autoradiography. The 20S proteasomes were detected by immunoblot analysis (IB) with rabbit anti-C9 antibody.

Next, we tested whether NQO1 and antizyme might interfere with one other binding to ODC. Our results indicated that ODC association with antizyme was not affected by NQO1 (Figure 5C, lanes 12 and 13), and ODC association with NQO1 was not affected by antizyme (Figure 5C, lanes 11 and 13). These results rule out the possibility that NQO1 stabilized ODC by blocking the ODC-antizyme complex formation.

**NQO1-ODC Association Is Disrupted by Dicoumarol**

It is possible that the protective effect of NQO1, observed both in vivo and in vitro, is due to its direct interaction with ODC. In such a case, the destabilizing effect of dicoumarol may result from preventing and/or
Figure 5. NQO1 Binds ODC Both In Vivo and In Vitro

(A) 293 HEK cells were transiently transfected with (+) or without (−) pEFIRE-ODC with (+) or without (−) pEFIRE-NQO1 (TOTAL). Immunoprecipitation of ODC was performed as described in Experimental Procedures, using mouse monoclonal anti-ODC antibody (IP:ODC). 

(B) In vitro translated [35S]methionine-labeled ODC and NQO1 were incubated alone or mixed together (TOTAL) and ODC was immunoprecipitated using mouse monoclonal anti-ODC antibody (IP:ODC). [35S]methionine-labeled ODC and NQO1 were detected by autoradiography. 

(C) 293 HEK cells were transiently transfected with (+) or without (−) pEFIRE-ODC or pEFIRE-ODC-Del6, with (+) or without (−) pEFIRE-NQO1, and with (+) or without (−) pEFIRE-antizyme (TOTAL). Immunoprecipitation of ODC was carried out using mouse monoclonal anti-ODC antibody (IP:ODC). Immunoblot analysis (IB) was carried out using mouse monoclonal anti-ODC antibody, goat anti-NQO1 antibody, and rabbit anti-antizyme antibody.

disrupting the interaction between NQO1 and ODC. To examine this possibility, NQO1 and ODC were coimmunoprecipitated from cell extracts and the beads were washed without or with dicoumarol before elution. NQO1 was no longer detectable in the coimmunoprecipitate following wash with dicoumarol (Figure 6A). Similar results were obtained in cells with the Del6 ODC mutant (Figure 6B). Furthermore, addition of dicoumarol to an in vitro translated ODC and NQO1 mixture also prevented ODC-NQO1 complex formation (Figure 6C). These results suggest that dicoumarol dissociates ODC-NQO1 complexes and inhibits ODC-NQO1 complex formation.

**Discussion**

We show here that NQO1 regulates the proteasomal degradation of ODC. Both the NQO1-regulated and the antizyme-mediated ODC degradation pathways are ubiquitin-independent; nevertheless, these pathways are substantially distinct. In the presence of antizyme, ODC-antizyme heterodimers are formed to expose discrete ODC C-terminal destabilizing epitopes that are recognized by the 26S proteasomes (Zhang et al., 2003). The new pathway of ODC degradation described here is distinct from the antizyme-dependent ODC degradation by the 26S proteasomes because it does not require the C-terminal proteasome recognition elements of ODC. Degradation via this pathway is blocked by NQO1 and can be enhanced in the presence of dicoumarol, an inhibitor of NQO1.

Several findings suggest that this degradation pathway requires ODC monomers. The MTM mutant is a homodimer even in the presence of antizyme. This mutant is sensitized to dicoumarol-induced degradation, both in vivo and in vitro, only when a point mutation (G387D) that prevents homodimerization is introduced.

NQO1 Regulates ODC Level under Oxidative Stress

NQO1 plays a role in cellular response to oxidative stress (Ross and Siegel, 2004). We therefore examined whether oxidative stress affects ODC level. To this aim, cells were treated with H2O2, and ODC activity was determined. Treatment of COS1 cells with H2O2 resulted in a significant decrease in ODC activity (Figure 7A). Similar results were obtained with 293 HEK cells and A31N-ts20 cells (Figure 7A). By employing MG132, a proteasome inhibitor, we further show that the H2O2-mediated reduction in ODC level is the result of proteasomal degradation (Figure 7B). Remarkably, no significant increase in antizyme level was observed following oxidative stress, raising the possibility that under these conditions, antizyme plays a minor role in accelerating ODC degradation (Figure 7B). This conclusion was further confirmed by data obtained with the ODC-Del6 mutant lacking the C-terminal destabilizing region of ODC, a region that is required for antizyme-mediated degradation of ODC. The activity of both wild-type ODC and the ODC-Del6 mutant was reduced to the same extent following H2O2 treatment (Figure 7C). These data suggest that under oxidative stress, the C-terminal destabilizing region of ODC is not required for ODC degradation. To examine the possibility of NQO1 involvement in this process, we performed coimmunoprecipitation experiments to determine whether the binding of NQO1 to ODC is affected under oxidative stress. Binding of ODC to NQO1 was reduced following treatment with H2O2 (Figure 7D), a condition that enhances ODC degradation by the 20S proteasomes. In agreement with this possibility, overexpression of NQO1 protected ODC from degradation under oxidative stress (Figure 7E). Based on these data, we suggest that under oxidative stress, NQO1 plays an important role in regulating the ODC level.
Figure 6. Dicoumarol Induces Dissociation of NQO1 from ODC Both In Vivo and In Vitro

293 HEK cells were transiently transfected with (+) or without (−) pEFires-ODC in the absence (−) or presence (+) of pEFires-NQO1 (TOTAL) (A), or with (+) or without (−) pEFires-ODC-Del6 in the absence (−) or presence (+) of pEFires-NQO1 (TOTAL) (B). Immunoprecipitation of ODC was performed using mouse monoclonal anti-ODC antibody (IP:ODC). Following washes with NP 40 buffer, the beads were washed without (−) or with (+) 300 μM dicoumarol (Dic. Wash) and then eluted as described in Experimental Procedures. Immunoblot analysis (IB) was carried out using mouse monoclonal anti-ODC antibody and goat anti-NQO1 antibody. (C) In vitro translated [35S]methionine-labeled ODC and NQO1 were incubated alone or mixed together without (−) or with (+) 300 μM dicoumarol (TOTAL), and ODC was immunoprecipitated using mouse monoclonal anti-ODC antibody (IP:ODC). [35S]methionine-labeled ODC and NQO1 were detected by autoradiography.

Similarly, dicoumarol-induced degradation of the antizyme-refractory ODC Del6 G387D mutant is more efficient compared to the Del6 mutant. In addition, studies carried out with these mutants and other ODC deletion mutants (Tobias et al., 1993) show a direct correlation between ODC degradation by the 20S proteasomes and the inability of ODC to form homodimers. Finally, antizyme that monomerizes ODC stimulates the dicoumarol-induced ODC degradation.

Our results show that NQO1 protects ODC from proteasomal degradation both in vivo and in vitro. Overexpression of NQO1 stabilizes endogenous ODC, and conversely, NQO1 knockdown or its pharmacological inhibition with dicoumarol destabilizes endogenous ODC, suggesting that NQO1 protects ODC from proteasomal degradation under basal conditions. The mechanism of ODC protection by NQO1 is not completely solved, but it requires their physical association. NQO1 binding does not interfere with antizyme binding, ruling out a simple displacement model to explain ODC stabilization by NQO1. The findings that dicoumarol dissociates ODC-NQO1, and also p53-NQO1 (Asher et al., 2003) complexes, lend further support to the impor-
tance of the physical interaction between NQO1 and the target protein. Furthermore, we show that dicoumarol not only prevents NQO1 binding to ODC but also dissociates the already established NQO1-ODC complexes. Notably, the finding that an inhibitor of NQO1 that competes for NAD(P)H binding dissociates the complex, suggests that NAD(P)H may play a regulatory role in NQO1 binding to these target proteins.

It is widely believed that degradation of the majority of the cellular proteins in mammalian cells is executed by the 26S proteasomes. The 20S particles that serve as the catalytic core are capped at one or both ends by the 19S regulatory particles to form the 26S proteasomes. In this context, the 19S particles are involved in recognizing polyubiquitinated protein substrates, unfolding them (Pickart and Cohen, 2004), removing ubiquitin chains for recycling (Verma et al., 2002; Yao and Cohen, 2002), and opening an axial portal into the 20S chamber (Kohler et al., 2001). Using a cell-free degradation system, it has been demonstrated that purified 26S proteasomes catalyze ODC degradation in the presence of antizyme without requiring ubiquitination (Murakami et al., 1992a). Under these conditions, the most C-terminal region of ODC plays an important role in recognition by the 19S in the context of the 26S proteasomes (Zhang et al., 2003). The lack of requirement for the C-terminal region in the dicoumarol-induced ODC degradation provided the first hint toward the possibility that this process is executed by an alternative proteasome complex. Our cell-free degradation studies confirmed this possibility.

We suggest that ODC monomers undergo degradation in living cells by the 20S proteasomes. This conclusion is based on the observations that degradation of ODC monomers in vivo, and in vitro by the purified 20S proteasomes, obeys the same rules. First, under both conditions, ODC monomers are required. Second, the C-terminal region of ODC that is involved in recognition by the 26S proteasomes is dispensable. Finally, NQO1 binds and inhibits ODC degradation both in vivo and in vitro and dicoumarol dissociates the binding of NQO1 to ODC and eliminates the stabilizing effect of NQO1. Notably, degradation of ODC monomers by the 20S proteasomes was also inhibited in the presence of antizyme (Figure S1 in the Supplemental Data available with this article online). It therefore appears that antizyme targets ODC to degradation by the 26S proteasomes, whereas the “free” ODC monomers are susceptible to degradation by the 20S proteasomes.

The 20S proteasomes are abundant and ubiquitously present in the nucleus and cytoplasm (Wojcik and DeMartino, 2003). They have been widely regarded as being incapable of degrading folded proteins and therefore considered latent proteasomes. However, recent studies suggest otherwise. Several proteins were reported to undergo efficient degradation by the 20S proteasomes without requiring polyubiquitination (Sheaff et al., 2000; Tofaris et al., 2001; Touitou et al., 2001; Liu et al., 2003). In the case of the cyclin-dependent kinase inhibitor p21, it was proposed that degradation of at least a fraction of p21 is regulated by cyclin-D1 and probably by PCNA, which interferes with p21 interactions with the C8α subunit of the 20S proteasomes (Coleman et al., 2003). p21 is a natively unfolded protein, and, therefore, it has been suggested that unstructured proteins may have an intrinsic capacity to open the pore of the 20S particle (Pickart and Cohen, 2004).

Inspection of the ODC sequence revealed that at least a portion of ODC might acquire unstructured configuration (data not shown), and its importance in the degradation process described in this study remains to be clarified.

In the absence of antizyme expression, ODC is unstable and still has a short half-life (Murakami and Hayashi, 1985; Zhang et al., 2003). This suggests that ODC is inherently an unstable protein whose rate of degradation is greatly stimulated by interaction with antizyme. Although the underlying mechanism is not known, pulse-labeling experiments demonstrated that ODC is subjected to very rapid degradation (van Daalen Wetters et al., 1989) before it fully folds and generates the active ODC homodimer. This labile behavior of ODC might be explained by a model that assumes that nascent polypeptides that are in the process of folding may present otherwise cryptic degradation signals (Turner and Varshavsky, 2000). Thus, either the nascent ODC chain or a posttranslational product has to escape this degradation process to ensure the production of a more stable ODC homodimer. It is possible that NQO1 plays a protective role at this early stage of ODC synthesis and maturation. The finding that ODC monomers, formed either by antizyme or by specific mutations, are sensitive to dicoumarol-mediated degradation lends further support to this model.

Our results demonstrate that upon oxidative stress, the ability of NQO1 to protect ODC from 20S proteasomal degradation is compromised. Previously we have reported that overexpression of ODC induces oxidative stress (Erez et al., 2002). Here, we show that under these conditions, the capacity of NQO1 to bind ODC is hampered and ODC is degraded by the 20S proteasomes. Our study, therefore, reveals a negative regulatory feedback loop that regulates ODC level under oxidative stress.

### Experimental Procedures

#### Cells and Reagents

The cell lines used were: 293 human kidney (293 HEK), COS-1 monkey kidney, A31N-ts20, a BALB/c mouse cell line that harbors a temperature-sensitive E1 ubiquitin-activating enzyme, and its parental cell line A31N (Chowdary et al., 1994). Cells were grown in DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin and cultured at 37°C in a humidified incubator with 5.6% CO2. A31N and A31N-ts20 were cultured at 32°C. Transfer of A31N-ts20 cells to 39°C inactivates the E1 ubiquitin-activating enzyme. Transient transfections of 293 HEK cells were carried out by the calcium phosphate method and of COS-1 cells with jetPEI, a cationic polymer transfection reagent, (Poly Transfection) according to the manufacturer’s protocol. Stable transfections of 293 HEK, A31N, and A31N-ts20 BALB/c cells with pEfires-ODC were carried out with jetPEI, and cells were selected for growth in the presence of puromycin (Sigma).

Dicoumarol (Sigma) was dissolved in 0.1N NaOH and MG132 (Sigma) in DMEM. Cycloheximide (CHX), 2-deoxy-D-glucose, hexokinase, β-NADH (Sigma), and α-difluromethylornithine (DFMO) were dissolved in H2O.

#### Plasmids

The plasmids used were: pEfires-NOQ1 or pSG5-NOQ1 encoding wild-type human NOQ1, pSUPER NOQ1 expressing NOQ1 siRNA.
pSUPER X expressing HBV X protein siRNA, pEFIRE-OCD or pGEM-OCD encoding wild-type mouse ODC, pEFIRE-OCD-G387D or pGEM-OCD-G387D, in which glycine 387 was converted to aspartic acid using site-directed mutagenesis, pEFIRE-OCD-Del6 or pGEM-OCD-Del6 encoding a mutant mouse ODC lacking amino acids 423-461 (Rosenberg-Hasson et al., 1991), pEFIRE-OCD-Del6-G387D or pGEM-OCD-Del6-G387D, pEFIRE-OCD-MTM or pGEM-OCD-MTM encoding a mutant mouse ODC whose antizyme binding site (amino acids 117-140) was replaced with the corresponding region of trypanosome ODC (Mamroud-Kidron et al., 1994), pEFIRE-OCD-MTM-G387D or pGEM-OCD-MTM-G387D, pEFIRE-OCD-Del6-MTM, pEFIRE-OCD-Del6-MTM-G387D, pGEM-OCD-J332-375, pGEM-OCD-J375-412, pGEM-OCD-J423-448, pEFIRE-antizyme expressing wild-type mouse antizyme, and p6-H2B-GFP expressing histone H2B GFP.

Immunoblot Analysis
Cells and immunoblot analysis were carried out as described (Asher et al., 2001). The antibodies used were: goat anti-NQO1 (Santa Cruz), mouse monoclonal anti-ODC (Sigma), mouse monoclonal anti-p53 (Pab 240), mouse monoclonal anti-Mdm2 (Pab 4B2), rabbit anti-C9 subunit of the 20S proteasomes, rabbit anti-antizyme (Mamroud-Kidron et al., 1994), and mouse monoclonal anti-Actin (Sigma).

In Vitro Protein Degradation Assay
In vitro translated [35S]methylene-labeled wild-type ODC or ODC mutants were incubated in reticulocyte lysate degradation mixture (40 mM Tris-HCL, [pH 7.5], 5 mM MgCl2, 2 mM DTT, 0.5 mM ATP, 10 mM phosphocreatine [Sigma], 1.6 mg/ml creatine phosphokinase [Boehringer Mannheim], 200 μg/ml ubiquitin [Sigma]) at 37°C for 90 and 180 min. Degradation of in vitro translated [35S]methylene-labeled wild-type ODC or ODC mutants, with 1 μg purified 20S proteasome (from Sigma, prepared by us, or kindly provided by Dr. Y. Reiss) was carried out in degradation buffer (100 mM Tris-HCL [pH 7.5], 150 mM NaCl, 5 mM MgCl2, 2 mM DTT) at 37°C for 1 hr. Samples were mixed with Laemmli sample buffer, heated at 95°C for 5 min, and fractionated by electrophoresis in a 12.5% polyacrylamide-SDS gel. Following electrophoresis, proteins were transferred to cellulose nitrate membranes. In vitro translated [35S]methione-labeled proteins were detected by autoradiography; purified 20S proteasome was detected by immunoblotting with rabbit anti-C9 antibody.

Coimmunoprecipitation Studies
Coimmunoprecipitation experiments were carried out with extracts from cells transiently transfected with ODC, NQO1, or both lysed in RIPA lysis buffer or with in vitro translated [35S]methione-labeled ODC and NQO1 that were incubated separately or mixed together in RIPA lysis buffer. Samples were incubated with mouse monoclonal anti-ODC antibody (Sigma) at 4°C for 12 hr and then incubated with protein A/G agarose beads (Santa Cruz) for an additional 2 hr. The beads were collected by centrifugations and washed four times with NP 40 buffer [50 mM tris-HCL, pH 7.5, 2 mM EDTA, 150 mM NaCl and 1% NP 40]. Laemmli sample buffer was added, and samples were heated at 95°C for 5 min and loaded on polyacrylamide-SDS gel.

Supplemental Data
Supplemental Data include an additional figure and can be found with this article online at http://www.molecule.org/cgi/content/full/1175/645/DC1/.


