Ubiquitin Proteasome Pathway–Mediated Degradation of Proteins: Effects Due to Site-Specific Substrate Deamidation

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Purpose. The accumulation, aggregation, and precipitation of proteins is etiologic for age-related diseases, particularly cataract, because the precipitates cloud the lens. Deamidation of crystallins is associated with protein precipitation, aging, and cataract. Among the roles of the ubiquitin proteasome pathway (UPP) is protein surveillance and maintenance of protein quality. The purpose of this study was to determine whether deamidation can alter clearance of crystallins by the UPP.

Methods. Wild-type (WT) and deamidated crystallins were expressed and ^{125}I-radiolabeled. Ubiquitination and degradation were monitored separately.

Results. For βB2 crystallins, rates of ubiquitination and adenosine triphosphate–dependent degradation, both indicators of active UPP, occurred in the order Q70E/Q162E>Q162E> Q70E=WT βB2 using reticulocyte lysate as the source of degradation machinery. Human lens epithelial cell lysates and lens fiber cell lysates also catalyzed ubiquitination but only limited degradation. Supplementation with proteasome failed to enhance degradation. Rates of ubiquitination and degradation of WT and deamidated βB1 crystallins were rapid and showed little relationship to the site of deamidation using N157D and Q204E mutants. γD-Crystallins were not degraded by the UPP. Deamidation altered amine reactivity, circular dichroism spectra, surface hydrophobicity, and thermal stability.

Conclusions. These data demonstrate for the first time that, like mild oxidative stress, deamidation of some proteins makes them preferred substrates for ubiquitination and, in some cells, for UPP-dependent degradation. Failure to properly execute ubiquitination and degrade the ubiquitin-conjugates may explain their accumulation on aging and in cataractogenesis. (*Invest Ophthalmol Vis Sci.* 2010;51:4164–4173) DOI:10.1167/iovs.09-4087

With the world's population aging and incidences of age-related diseases taking heavy tolls on personal life quality and health care budgets, there is increasing urgency to understand the mechanisms of aging. The accumulation of aggregates of cellular proteins modified by oxidation, nitration, glutathiolation, glycation, truncation, and deamidation have been etiologically associated with risk for age-related diseases, including Parkinson's, Alzheimer's and other neurodegenerative diseases, age-related macular degeneration, compromised immune function, and cataract.[1–19] Levels of these altered proteins reflect their rates of formation and their rates of degradation. Thus, the ability of cells to efficiently clear these modified proteins from the intracellular environment is essential for cellular homeostasis. Deamidation of proteins occurs with aging, and deamidated proteins accumulate in cataracts.[19] Surprisingly, little is known about how deamidated proteins are removed from cells.

A major pathway by which modified proteins are cleared from cells is through proteasomal degradation involving two related proteolytic complexes, the 20S and 26S proteasomes.[20–26] The 26S proteasome is formed on the addition to the 20S proteasome of two "cap" components containing subunits for adenosine triphosphate (ATP) binding and hydrolysis and polyubiquitin chain recognition.[27–29] This ubiquitin-dependent degradation pathway is referred to as the ubiquitin proteasome pathway (UPP). In the UPP, ubiquitin, a highly conserved 8500-Da protein present in all eukaryotic organisms, is usually covalently linked to an ε-amino group of a specific lysine on the target protein. This process is catalyzed by a series of enzymes referred to as E1, E2, and E3. Polyubiquitination results in the high molecular weight (HMW) protein-ubiquitin conjugates that are usually recognized for degradation by the 26S proteasome.[30,31] Low molecular weight (LMW) conjugates, with cellular functions other than as degradation substrates, have also been described.[32]

The lens of the eye is one of only two clear organs in the body, and clarity is required for the lens to collect and focus light on the retina. For clarity, proteins must remain soluble. Cataract can be considered a paradigmatic protein conformation or amyloid disease because aggregation and precipitation of proteins from the normally clear soluble lens milieu result in opacification and cataract. This makes it easy to observe in vivo. The three major classes of vertebrate lens proteins are α-, β-, and γ-crystallins, and they are required to maintain refractive capacity or provide chaperone function.[33–39] These proteins are also expressed, albeit at lower levels, in other tissues.[40–44] The β- and γ-crystallins are part of the same superfamily.

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Importantly, in the lens α-, β-, and γ-crystallins are all long-lived proteins, and they accumulate many age-related protein modifications that compromise their function and that are related to insolubilization.[6,45–49] Deamidation of βB2 and βB1 crystallins is associated with age- and cataract-related protein insolubilization.[19]

Motivated to understand why these modified proteins accumulate, we examined the susceptibility of wild-type (WT) and specific deamidated mutants of βB2 and βB1 crystallins to ubiquitination and UPP-mediated degradation in several physiologically relevant proteolytic systems, including human lens epithelial cell (HLEC), bovine lens fiber (LF) cell lysate, and rabbit reticulocyte lysate (RRL). We demonstrate that WTβB1 and the deamidated βB1 crystallins are inherently excellent substrates for ubiquitination and UPP-mediated degradation using RRL. In comparison, WTβB2 crystallin is a poor substrate for ubiquitination and UPP-mediated degradation, but site-specific deamidation dramatically enhances its ubiquitination and degradation by the UPP. Related γD crystallins are refractory to the UPP. Importantly, lens epithelial cell and fiber lysates catalyze ubiquitination effectively but fail to complete proteolysis of the deamidated proteins as efficiently as RRL, even though the lens is known to contain active proteasome.[50] This finding may explain their accumulation on aging in many tissues and, specifically, their cataractogenic potential.

Materials and Methods

Expression and Purification of Recombinant βB Crystallins

WT and deamidated N157D and Q204E βB1 crystallins and WT and Q70E, Q162E and Q70E/Q162EβB2 crystallins were expressed in bacterial cells and purified by successive ion-exchange chromatography, as previously reported.[51–53] Mass spectrometry was performed to definitively identify the desired mutations.

SDS-PAGE

Individual WT and deamidated βB crystallins (5 μg) were examined by SDS-PAGE under denaturing and nondenaturing conditions to confirm appropriate monomeric MWs and to check for expected changes in charge/mass ratio.

Ubiquitination Assays

Ubiquitin conjugation assays of βB crystallins were performed using RRL (Green Hectares, Oregon, WI), SRA 01/04 HLEC, and bovine LF cell lysates as the source of ubiquitinating enzymes. Recombinant proteins were radiolabeled with ^{125}I-Na using chloramine T and purified by column chromatography (G25 Sephadex; GE Healthcare, Little Chalfont, UK).[54] Purified ^{125}I-radiolabeled proteins (~200,000 cpm/assay) were incubated at 37°C for 1 hour in a final reaction volume of 30 μL containing 40 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 40 μM MG132, 2 μM ubiquitin aldehyde, 5 mM ATP, 75 μM ubiquitin, and the ubiquitin conjugating enzyme E2 Ubc4 (2.5 μg) in the presence or absence of cell lysate. Ubiquitination of specific crystallins was further characterized by omitting Ubc4, ATP, or ubiquitin to demonstrate the involvement of UPP. In addition, cell-free ubiquitination assays were performed as described except that mutant ubiquitins unable to efficiently form poly-ubiquitin conjugates (K48R-ubiquitin and methyl-ubiquitin[55,56]) were used instead of WT-ubiquitin in the assays. After incubation, proteins were resolved by 12% SDS-PAGE. The gels were dried and placed with film for autoradiography to visualize ubiquitin-crystallin conjugates. Densitometry of autoradiographs was accomplished using Scion software (Image; Scion Corp., Frederick, MD).

Isolation of Ubiquitin Conjugates and Characterization of Ubiquitinated βB2 Crystallin Moieties

For further corroboration of ubiquitin-crystallin conjugate formation, cell-free ubiquitination assays were performed as described except that 20 μg unlabeled WT and βB2 Q162E crystallin, rather than ^{125}I- labeled protein, was used in the assay in the presence and absence of RRL lysate and $(His)_6$-ubiquitin. After ubiquitination reactions were terminated, washed Ni-resin was added to reaction samples and allowed to incubate at 4°C for 2 hours, after which the resin was collected and washed four times with 10 resin volumes of buffer containing 20 mM followed by 40 mM imidazole. Total ubiquitin conjugates were then recovered from the Ni-resin using Laemmli buffer at 95°C for 5 minutes. The resultant supernatants were resolved by SDS-PAGE, transferred onto nitrocellulose, and probed with an anti-βB2 crystallin antibody (gift from Nicolette Lubsen, Department of Biochemistry, Faculty of Science, Radboud University Nijmegen, Nijmegen, The Netherlands) using enhanced chemiluminescence (ThermoScientific, Rockford, IL).

Degradation Assays

Degradation of crystallins was determined using RRL, HLEC, and LF cell lysates. RRL was obtained commercially from Green Hectares, divided into 200-μL aliquots, and stored at −80°C for the duration of the experiments. Lens outer cortical fiber cells were obtained from 1-year-old bovine eyes, and human lens epithelial cells (SRA 01/04) were a gift from Venkat Reddy (Kellogg Eye Center, University of Michigan). Lysates of lens fiber and lens epithelial cells, previously stored at −80°C, were prepared using hypotonic buffer (10 mM NaCl, 5 mM KCl, 50 mM Tris-HCl, pH 7.6) with incubation on ice for 60 minutes and occasional vortexing. Cellular debris was removed by centrifugation, and the supernatant was recovered and stored at −80°C. Fresh aliquots of lysates were thawed on ice and used on the day of the experiments. For ATP-dependent degradation, purified ^{125}I-radiolabeled protein (~200,000 cpm/assay) was incubated at 37°C for 2 hours in a final reaction volume of 25 μL containing 50 mM Tris-HCl, pH 7.6, 6 mM $MgCl_2$, 1.2 mM DTT, 2.7 mM ATP, 17 mM creatine phosphate, and 4.7 U creatine phosphokinase in the presence or absence of cell lysate, Ubc4, or MG132, a proteasome inhibitor. During the 2-hour reaction, there was no indication of substrate insufficiency, and relative rates of degradation of all the substrates at any time showed the same relationship to data gathered at 2 hours. Interestingly, slow binding of substrate can be inferred.[57] ATP-independent degradation of crystallin proteins was determined as described but in buffer not containing ATP, creatine phosphate, or creatine phosphokinase. After incubation, the degradation reaction was terminated, and TCA/soluble cpm was quantitated using a gamma counter (Cobra II; PerkinElmer, Wellesley, MA). Percentage degradation was calculated as (Experimental Soluble cpm − Buffer Control Soluble cpm/Total cpm − Buffer Control Soluble cpm) × 100.[58]

Amine Reactivity of βB2 Crystallins

Equal amounts of protein in PBS were incubated (Green540; Telechem International Inc., Sunnyvale, CA) for 5 minutes at room temperature, after which unbound dye was removed by column chromatography (Centri-Spin; Princeton Separations, Adelphia, NJ). The samples were then examined by fluorescence spectrometry using a fluorescence plate reader (Cytofluor 4000; MTX Laboratory Systems, Inc., Vienna, VA) at 530-nm excitation and 580-nm emission wavelengths at a gain of 30 to 50. Fluorescence data were expressed as fluorescence of βB2 crystallin minus the control (PBS), with WTβB2 fluorescence set a value of 1. An increase in fluorescence is directly proportional to an increase in amine reactivity of exposed arginine and lysine amino acids and is indicative of protein unfolding.

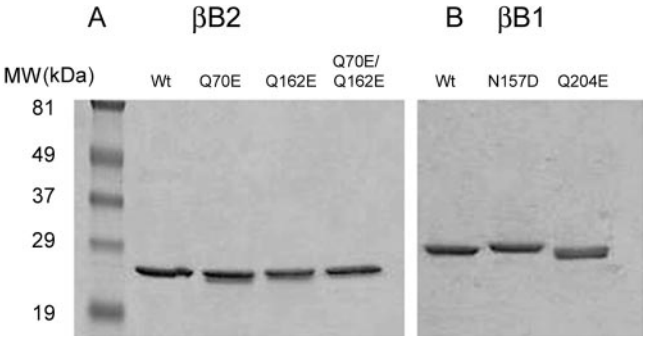

FIGURE 1. Homogeneity of WT and deamidated βB2 and βB1 crystallins. (**A**) βB2 crystallins and (**B**) βB1 crystallins resolved by 15% SDS-PAGE and visualized by Coomassie blue staining.

RESULTS

βB2 Crystallin Ubiquitin Conjugate Formation and Degradation

The expressed WT and deamidated βB2 and βB1 crystallins used in these studies were at least 95% pure and demonstrated the expected monomeric MWs using SDS-PAGE (Fig. 1), though in vivo they exist as homodimers.[59]

Rate-determining steps in the UPP proteolytic process can involve ubiquitination reactions or processes that are associated with the proteolytic process. When the proteolytic process is rate limiting, which is often the case,[60] HMW ubiquitin conjugates, which are substrates for UPP-dependent proteolysis, are often observed. As indicated in Figure 2A, HMW poly-ubiquitin ^{125}I-crystallin monomers (top) and LMW βB2 crystallin-containing moieties (short exposure, bottom) were enhanced when additional ubiquitin was added to the reaction mixture (Fig. 2, lane 2 vs. lane 1). The Q70E/Q162E double mutant was integrated into more conjugates than the singly deamidated Q162E mutant, and both of these proteins formed more conjugates than the Q70E and WT proteins. These data indicated that deamidated βB2 crystallins were good substrates for ubiquitination using RRL and that deamidation at two sites (lane 5) enhanced this process over the monodeamidation (lane 4 or 3) and the native substrate (lane 2). Because ubiquitin conjugation was increased when ubiquitin was added to the lysates, the data indicated that the RRL system did not have saturating levels of ubiquitin (lane 2 vs. lane 1).

Three experiments were conducted to ensure that βB crystallins were found in ubiquitin conjugates. Only in the presence of ATP and ubiquitin (both of which are absolutely required for ubiquitination and a functional ubiquitin proteolytic pathway) were the highest levels of HMW ^{125}I-labeled Q162E βB crystallins formed in all three cell types (Fig. 3A; lanes 2 vs. 3, 4 vs. 5, 6 vs. 7). ^{125}I-labeled Q162E βB2 crystallin did not show HMW species in the absence of lysate, ATP, or ubiquitin (Fig. 3A, lane 1), suggesting that labeling of the βB crystallins did not produce aggregation that would result in HMW species. Lysines on ubiquitin are required for the formation of multiple ubiquitin adducts that result in the formation of HMW ubiquitin conjugates of substrates. The data in Figure 3B show that when all the lysines on ubiquitin are blocked and ubiquitin polymerization cannot occur, there is no formation of the highest MW forms of ^{125}I Q162E βB crystallins (lane 4). These data indicate that the HMW forms of ^{125}I Q162E βB crystallins noted in lanes 2 and 3 are attributed to poly-ubiquitination. Furthermore, when the K48R ubiquitin variant is included, the extent of formation of the HMW moieties is reduced and higher levels of LMW versions of Q162E βB crystallins are observed (lanes 3). K48 on ubiquitin is required to form the ubiquitin trees that decorate substrates that will be targeted to the proteasome for degradation. Similar results were also reproduced for several of the βB crystallins examined in this study (data not shown). Finally, Ni-resin was used to isolate ubiquitin conjugates derived from ubiquitination assays, which contained $(His)_6$-ubiquitin and unlabeled-βB2 crystallin. The isolated proteins were resolved by SDS-PAGE and blotted using anti–βB2 crystallin antibody. As shown in Figure 3C, mono-ubiquitinated and poly-ubiquitinated βB2 crystallin were observed only in the $(His)_6$-ubiquitin pull-down fraction (left). Interestingly, some of these had MWs indistinguishable from conjugates noted in human lens preparations.[61] No HMW moieties were observed in the absence of added ubiquitin, and no (lowest MW) or few ubiquitin conjugates were observed in the unbound fraction (right). As observed, small fractions of non-ubiquitinated βB2 crystallin monomer and dimer were also detected in the $(His)_6$-ubiquitin pull-down fraction (indicated

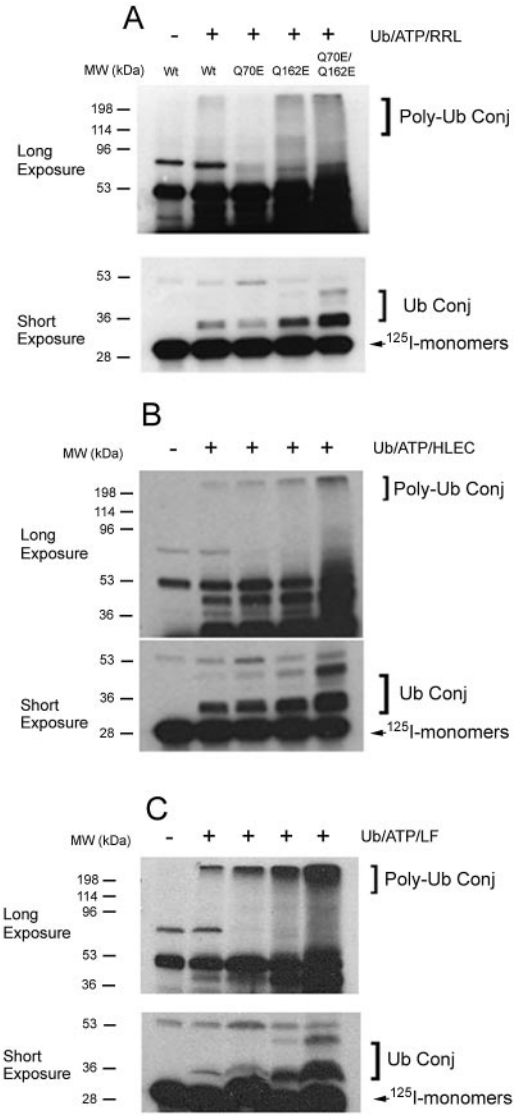

FIGURE 2. Ubiquitin conjugate formation of βB2 crystallins using RRL (**A**), HLEC (**B**), and LF (**C**) cell lysates. WTβB2 and βB2 deamidated mutants, Q70E, Q162E, and Q70E/Q162E were ^{125}I-radiolabeled and examined for ubiquitin-crystallin conjugate formation using cell lysates. Long exposure (*top*) depicted HMW conjugates, whereas short exposure (*bottom*) depicted LMW conjugates. Conjugation reactions were resolved by 12% SDS-PAGE, and conjugate bands were visualized by autoradiography.

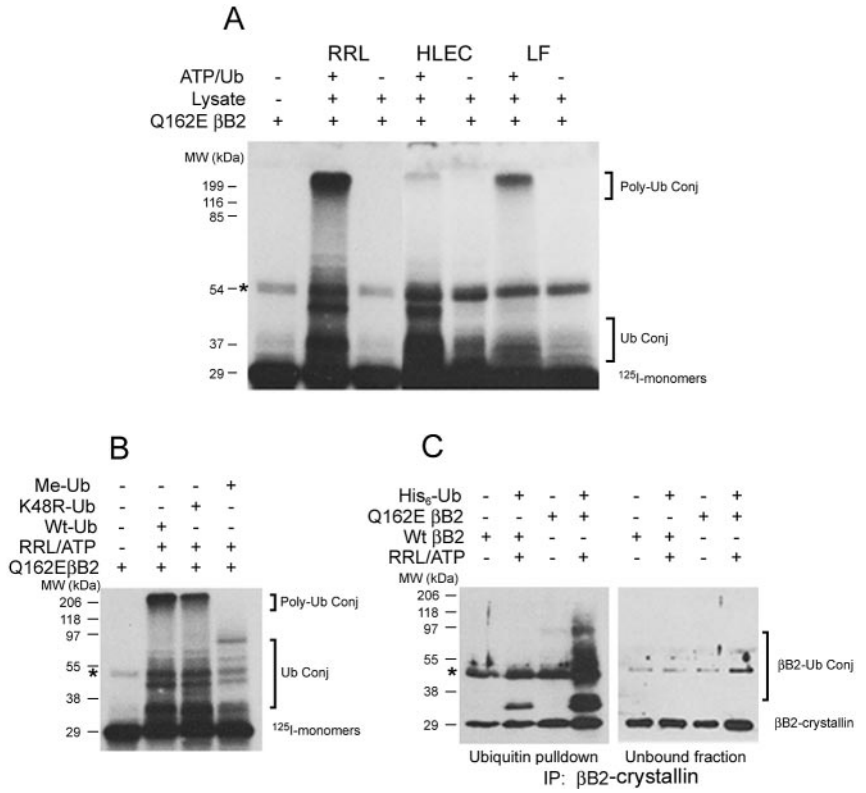

FIGURE 3. In vitro formation of HMW and LMW species of deamidated Q162E βB2 crystallin with cell lysates requires ATP and ubiquitin. (**A**) Conjugate formation of ^{125}I-labeled Q162E βB2 crystallin was examined with RRL, HLEC, and LF lysates in the presence and absence of ATP and ubiquitin. Results are representative of three independent experiments. (**B**) Conjugate formation of ^{125}I-labeled Q162E βB2 crystallin was examined with RRL lysate using the mutant ubiquitins K48R-ubiquitin (K48R-Ub) and methyl-ubiquitin (Me-Ub). Results are representative of two independent experiments. (**C**) Conjugate formation of WT and Q162E βB2 crystallin was examined with RRL lysate using His_6-ubiquitin (His_6-Ub) and Ni-resin isolation of ubiquitin conjugates followed by Western blot analysis with an anti–βB2 crystallin antibody of the pull-down and unbound fractions. Results are representative of two independent experiments. *Nonspecific bands of βB crystallin dimers that adhered to the Ni matrix.

by asterisks). The dramatic enrichment of ubiquitinated species of βB2 crystallin in the $(His)_6$-ubiquitin pull-down fraction versus unbound fraction confirmed that WT and Q162E βB crystallins were ubiquitinated. Furthermore, the levels of the βB crystallin-ubiquitin conjugates appeared to be higher for the Q162E variant, consistent with its more rapid degradation. The very HMW poly-ubiquitinated conjugates were not observed using this technique, probably because the multiple ubiquitins on the substrate prevented binding of the anti–βB-crystallin antibody or because of the lower sensitivity of Western blot analysis compared with autoradiography. Taken together, these data provide strong evidence that βB crystallins are ubiquitinated and are not simply polymerized substrate.

Because ubiquitination is a prerequisite for UPP-dependent degradation, we next sought to determine the relationship between ubiquitination and degradation of these deamidated proteins through the UPP. A requirement for ATP is a hallmark of the UPP. It is clear that all the proteins are degraded in the presence of ATP (Fig. 4A). In all cases, degradation was markedly decreased or not detected when exogenous ATP was not provided. In corroboration that degradation of these proteins occurs through the UPP, the relative rates of degradation are the same as the relative rates of ubiquitination: WT≈Q70E<Q162<Q70E/Q162E. The data clearly indicate that most of the degradation of these proteins is UPP-dependent and that susceptibility to degradation is related to the site or extent of deamidation and ubiquitination. The extent of βB2 crystallin degradation of the double mutant, 35% in the presence of additional ATP, is one of the highest rates of degradation of the physiologic substrates investigated.

Corroboration of UPP-dependent degradation of these proteins was sought by incorporating the proteasome inhibitor MG132 and a ubiquitin-conjugating enzyme Ubc4 with previously identified roles in the degradation of altered proteins.[62] As indicated in Figure 4B, addition of the proteasome inhibitor markedly decreased the extent of UPP-dependent degradation of these substrates. This is the minimal estimate of ATP- and, therefore, UPP-dependent degradation because we made no effort to deplete endogenous ATP and because inhibition of the proteasome by MG132 is incomplete. Taken together, these data indicate that normally stable βB2 crystallin protein is converted to a rapidly degraded protein on deamidation and informs about previously unknown roles of environmental and epigenetic influences, specifically those that affect deamidation rates and regulation of protein stability. Surprisingly, when exogenous Ubc4 was added to the reaction mixtures, there was no enhancement of degradation, indicating that Ubc4 is not a limiting factor for the degradation of deamidated crystallins.

Because the deamidation of βB2 crystallin is known to be associated with cataract formation in the lens, we also determined the extent to which WT and the deamidated βB2 crystallins were susceptible to ubiquitination and UPP-mediated degradation using HLEC and LF lysates. Consistent with results using RRL, both HMW and LMW ubiquitin conjugates of βB2 crystallins were readily formed using HLEC and LF lysates. As observed with RRL, conjugation to ubiquitin was most efficient in the order Q70E/Q162E>Q162E>Q70E≈WT (Figs. 2B, 2C), corroborating previous indications that lens and reticulocytes share many common developmental and metabolic phenomena. Densitometric analyses indicated that there were similar levels of βB2 crystallin in HMW conjugates in the HLEC lysate compared with RRL. Interestingly, the levels of Q70E/Q162E βB2 crystallin in HMW conjugates in the LF lysate was approximately 3- to 5-fold greater than with RRL or HLEC (Table 1). For all proteins examined, conjugation was enhanced in the presence of exogenous ATP and ubiquitin (Figs. 2B, 2C, lane 2 vs. lane 1).

Degradation of WT and deamidated βB2 crystallins was also observed using HLEC and LF lysates (Figs. 4C, 4D). Consistent with HMW conjugates being better substrates, and as observed with RRL, Q70E/Q162E βB2 crystallin was the most susceptible to degradation. However, although there was extensive formation of HMW ubiquitin conjugates in these lens cell or lens tissue-derived lysates degradation was limited (~2%-6%)

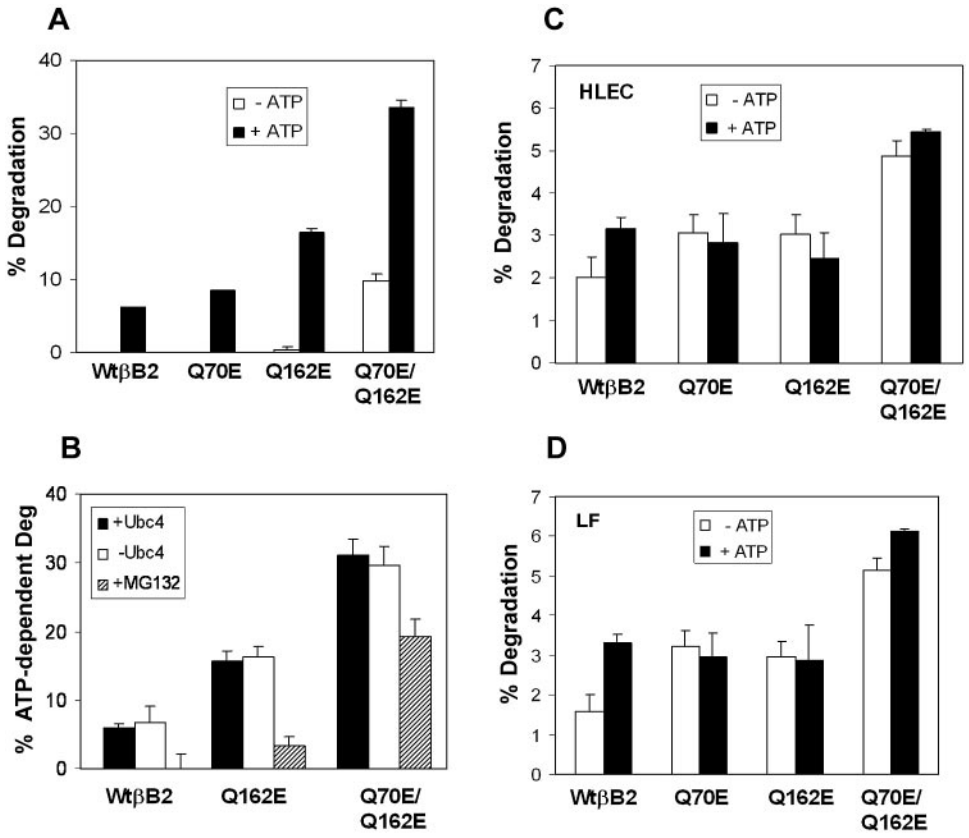

FIGURE 4. Deamidation of WTβB2 crystallin enhances its susceptibility to UPP-dependent degradation in RRL. (**A**) ATP-dependent degradation of WTβB2, and deamidated mutants Q70E, Q162E, and Q70E/Q162E. (**B**) Proteasome-dependent and Ubc4-independent degradation of WTβB2 and deamidated mutants. (**C**) Degradation of βB2 crystallins using HLEC. (**D**) Degradation of βB2 crystallins using LF. Degradation assays were performed in triplicate in four or five independent experiments.

compared with the robust rates of degradation of these proteins using RRL. In addition, there were no significant differences between ATP-supplemented and ATP-unsupplemented assays with respect to degradation of the βB2 crystallins. Assays for proteasome activity indicate that the increased relative amount of HMW ubiquitin conjugates and the decreased rates of proteolysis observed in the LF lysate were likely the result of diminished activity of the 26S proteasome (unpublished data, 2008).

Between 5% and 25% of the crystallin was found in LMW conjugates (Figs. 2B, 2C, lower; Table 1). Proteins to which only individual ubiquitins are attached are often substrates for intracellular transport, relocalization, or regulation. They may also be intermediates which are en route to becoming HMW moieties. At this time it is not possible to assign function to these LMW βB2 crystallins.

βB1 Crystallin Ubiquitin Conjugate Formation and Degradation

To explore the generalizability of the enhancement of ubiquitination or degradation on deamidation, we examined additional WT and deamidated crystallins. The amino acid sequence of βB1 crystallin is 55% identical, 75% homologous, and has a βB core sequence domain similar to that of βB2 crystallin. βB1 and βB2 crystallins differ in that βB1 has an N-terminal extension relative to βB2 (see Figs. 7A, 8).

TABLE 1. Quantitation of HMW (poly-ubiquitinated) and LMW (mono- and di-ubiquitinated) Ubiquitin-βB Crystallin Conjugates

	βB2				βB1		
Lysate	**WT**	**Q70E**	**Q162E**	**Q70E/Q162E**	**WT**	**N157D**	**Q204E**
RRL							
HMW	0.2*	0.2	2.8	3.2	6.5	0.6	3.0
LMW	7.6	5.7	12.5	24.4	21.8	27.1	31.3
Monomer†	92.3	94.1	84.7	72.3	71.7	72.3	65.7
HLEC							
HMW	0.3	0.8	0.8	2.1	1.6	0.1	0.3
LMW	17.4	19.1	25.7	40.7	23.3	13.0	15.9
Monomer	82.4	80.1	73.5	57.3	75.0	86.8	83.9
LF							
HMW	2.0	5.5	7.8	10.6	0.8	0.3	0.3
LMW	6.6	7.4	19.2	26.6	9.1	4.0	4.6
Monomer	91.4	87.1	73.0	62.8	90.1	95.7	95.1

* Percentages of ^{125}I-labeled crystallin proteins, as determined by densitometry.
† Monomeric-labeled crystallin proteins.

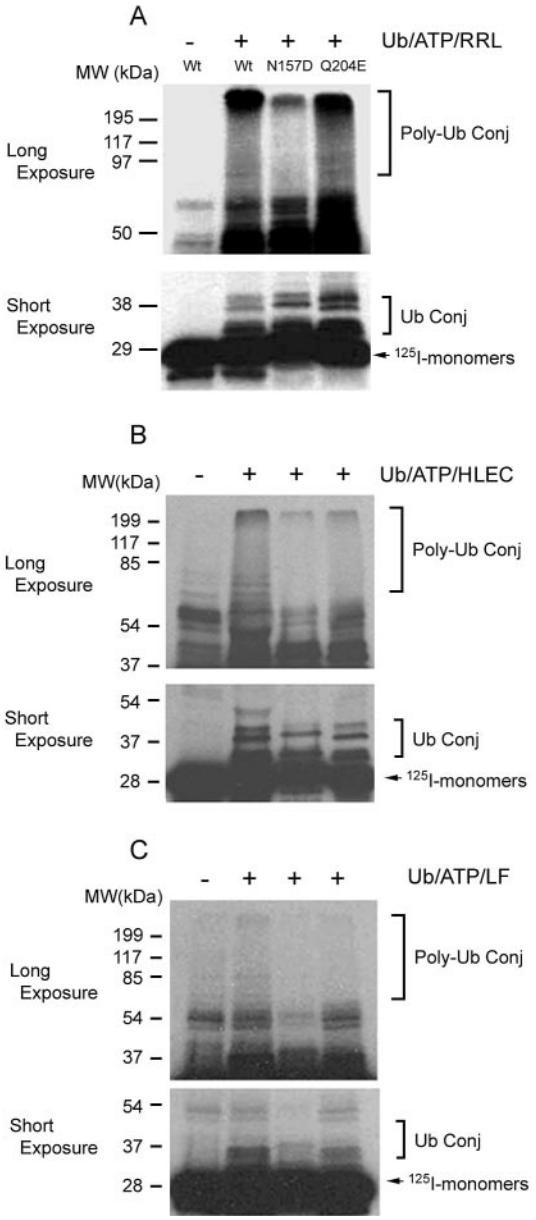

FIGURE 5. Ubiquitin conjugate formation of βB1 crystallins using RRL (**A**), HLEC (**B**), and LF (**C**) cell lysates. WTβB1 and βB1 deamidated mutants N157D and Q204E were ^{125}I-radiolabeled and examined for ubiquitin-crystallin conjugates using cell lysates. Long exposure (*top*) depicted HMW conjugates while short exposure (*bottom*) depicted lower MW conjugates. Conjugation reactions were resolved by 12% SDS-PAGE, and conjugate bands were visualized by autoradiography.

Surprisingly, the proportion of WTβB1 crystallin found in HMW ubiquitin conjugates was far greater (≈33-fold that seen with WTβB2 crystallin) than the usual low proportion of a protein found in HMW ubiquitin conjugates in the RRL system (compare Figs. 5A and 2A, lane 2; Table 1). Interestingly, the Q204E crystallin also showed high levels of HMW ubiquitin conjugates, though they were slightly lower than what was observed with the WT protein (Fig. 5A, top, lanes 4 and 1; Table 1).

Consistent with the expectation that the high levels of HMW ubiquitin conjugates formed by βB1 crystallins would be associated with very rapid degradation rates, the βB1 crystallins were degraded very rapidly—approximately 28% to 46% using RRL (Fig. 6A)—and all the degradation was ATP-dependent and was inhibited almost completely by incorporation of the proteasome inhibitor MG132 (Fig. 6A). Consistently, N157D βB1 crystallin, which is ubiquitinated less efficiently than the other two βB1 crystallins shown here, was also degraded less rapidly than those two proteins. The relationship between the extent of ubiquitination, a requirement for ATP, and inhibition by a proteasome inhibitor established that the degradation of βB1 crystallin was UPP-dependent. As with βB2 crystallins, this degradation was independent of exogenous Ubc4 (Fig. 6B). The data also suggest that, as in many other aging and neurodegenerative cell types, in lens cells and tissues, rates of formation of apparently proteolytically competent (see Discussion) ubiquitin conjugates exceed proteolytic capacity.

HLEC and LF lysates were also able to generate both HMW and LMW ubiquitin-βB1 crystallin conjugates (Figs. 5B, 5C), albeit with far lower efficiency than RRL. As in the RRL system, both deamidated βB1 crystallins were less efficiently integrated into conjugates than the WT protein in the lens conjugation systems. HLEC and LF cell lysates also supported the degradation of WT and deamidated βB1 crystallins. However, degradation, similar to incorporation into HMW conjugates, was less efficient (<2%; Figs. 6C, 6D). A role for ATP is generally indicated.

LMW conjugates are also indicated for the WTβB1 crystallin (Fig. 5A, lane 2 vs. lane 1). However, compared with relatively higher ratios of LMW to HMW ubiquitin conjugates observed for WTβB2 crystallins (≈38-fold) in the RRL system, relative levels of the LMW to HMW ubiquitin-βB1 crystallin conjugates were far lower (≈4-fold). For the double-deamidated βB1 and βB2 mutants, ratios of LMW to HMW ubiquitin conjugates were high in all three systems (Figs. 2B, 2C, 5B, 5C, lower panels; Table 1).

Some of the hypotheses regarding associations between protein ubiquitination and degradation were tested by determining whether WT or the cataractogenic deamidated γD crystallin mutants Q54E and Q143E were also ubiquitinated and degraded similarly to what was observed for the βB crystallins. γD crystallins are smaller members of the same superfamily as β crystallins and are 33% identical with βB2 crystallin but do not share significant structural homology to βB2 crystallin (Fig. 7A). Important for putative substrates for the UPP, γD crystallins have an amino terminal amino group, and the penultimate amino acid is lysine, serine, or cysteine, all potential sites for ubiquitination. Interestingly, WT and the deamidated γD crystallins, which are usually destabilized and less structured and might be anticipated to be more available to degradation,[48] were not integrated into HMW ubiquitin conjugates, nor were they substrates for the RRL UPP (data not shown).

Biochemical and Biophysical Parameters Associated with Ubiquitination and Degradation

Given that site-specific deamidation of crystallins is related to altered ubiquitination of βB2 crystallins and is known to alter protein structure, we sought to determine whether differences in susceptibility to ubiquitination and degradation are systematically related to deamidation-induced changes to the βB crystallins. Such changes would include exposing sequestered amine-reactive arginine and lysine residues. The primary amino acid sequence alignment of βB2, βB1, and γD crystallins, including the specific deamidation sites for arginine and lysine residues, is shown in Figure 7A. As shown in Figure 7B, deamidation of βB2 crystallin resulted in increased amine reactivity in the order WT<Q70E<Q162E<Q70E/Q162E, and it is tempting to hypothesize that changes in protein conformation that result in elevated levels of revealed lysines (or other residues used for ubiquitination) are causally related to the enhanced degradation of these βB2 crystallins (Figs. 4A, 4B).

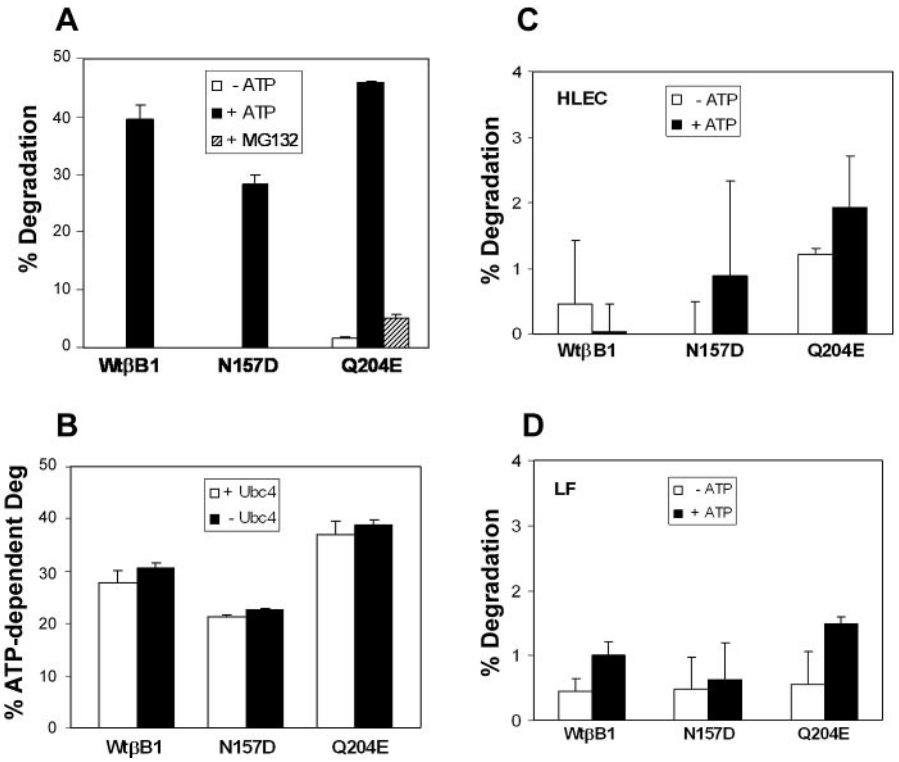

FIGURE 6. The degradation of WT and deamidated βB1 crystallins using RRL is ATP- and proteasome-dependent but Ubc4-independent. (**A**) ATP- and proteasome-dependent degradation of WTβB1 and deamidated mutants N157D and Q204E. ATP-independent degradation and degradation inhibited by MG132 were not observed for WTβB1 and N157D. (**B**) Ubc4-independent degradation of WTβB1 and deamidated mutants. (**C**) Degradation of βB1 crystallins using HLEC lysates. (**D**) Degradation of βB1 crystallins using LF. Degradation assays were performed in triplicate in at least two independent experiments.

This hypothesis is supported by the observations that WT and the N157D and Q204E βB1 crystallins, which are more rapidly degraded than βB2 crystallins, have higher amine reactivities than WTβB2 crystallins (data not shown). Interestingly, Q70 and Q162 are at the protein-protein interfaces between the two monomers of βB2 crystallin that dimerize in vivo to form the native protein (Fig. 8).[52] If on deamidation the βB2 protein structure is "opened" and lysines K67 and K75 or K167 and K171 are made more accessible, they could enter into conjugation reactions leading to enhanced proteolysis. This would further suggest that we have identified lysines that encode ubiquitination and UPP-mediated degradation of βB2 crystallin, but such analysis is beyond the scope of this study.

To probe further into structural changes that accompany deamidation, we performed surface hydrophobicity measurements on the WT and deamidated βB crystallins. Deamidated mutants of βB2 crystallin, demonstrated significantly increased (~10- to 38-fold) hydrophobicity compared with WTβB2 (data not shown). Because the ubiquitination and UPP-mediated degradation of Q162E and Q70E/Q162E deamidated mutants were significantly enhanced compared with WTβB2 (Figs. 2, 4), these data suggest that increased surface hydrophobicity may be correlated to both the observed increase in ubiquitination and the susceptibility to UPP-mediated degradation. In comparison with the positive relationships between hydrophobicity and rates of ubiquitination or proteolysis observed with WT and deamidated βB2 crystallin, WTβB1 crystallin demonstrated higher hydrophobicity than but similar rates of proteolysis to both the deamidated mutants. Thus, it would appear that additional elements of protein structure contribute to the susceptibility of βB1 crystallin to ubiquitination and UPP-dependent degradation. Additional stability measures, including thermostability and urea-induced denaturation/renaturation, and spectrometric techniques such as circular dichroism and fluorescence spectrometry also failed to definitively show a systematic relationship between site-specific deamidation of βB crystallins and ubiquitination and degradation observed using the RRL system.

DISCUSSION

The data presented here indicate for the first time that the deamidation of proteins, specifically βB2 crystallins, enhances their recognition and degradation by the UPP, thus mechanistically linking the previously discovered phenomena of in vivo deamidation[63] and protein destabilization. As such, age-related nonenzymatic, enzymatic,[64–66] or mutation-induced site-specific deamidation is analogous to mild oxidation, glutathiolation, and truncation in eliciting enhanced UPP responses.[7,9,67] β-Crystallins are major constituents of the vertebrate lens that are also expressed in other tissues, such as retina, muscle, and kidney, where they appear to function as stress response proteins.[43,44] Interestingly, levels of deamidated proteins are also increased in other protein precipitation or amyloid diseases, including Parkinson's and Alzheimer's.[68–70]

To understand why deamidation is related to the accumulation rather than the timely degradation of the deamidated proteins, we examined the susceptibility of WT and three deamidated mutants of βB2 crystallins to the UPP using RRL and biologically relevant HLEC and LF cell lysates. Whereas WTβB2 crystallin is a poor substrate for UPP-mediated degradation, deamidations at Q70, Q162, and Q70/Q162 make βB2 crystallin a progressively better substrate for ubiquitination and UPP-mediated degradation using RRL. Ubiquitination is also enhanced, but considerably less so, in HLEC and LF cell lysates. It appears that the limited degradation of these substrates in these systems is attributed to compromised proteolytic activity or inefficient ubiquitination or that ubiquitination does not result in proteolytically competent conjugates.[16,71–75] Adding active proteasome was without effect (data not shown). This raises the possibility that inactivation of the proteasome, ineffective ubiquitination, and endogenous inhibitors of the proteasome may also compromise the efficiency of the protein editing machinery. Thus, it would appear that impaired UPP activity is causally related to the accumulation, rather than the timely degradation, of deamidated proteins in several age-related syndromes associated with the accumulation of deami-

dated proteins. These data will be informative about new ways to modulate rates of cataractogenesis by regulation of protein quality control. For example, drugs are being developed to activate the endogenous proteasome.

The only literature regarding relationships between deamidation and UPP-dependent proteolysis include observations of the effects of deamidation on the susceptibility of N-terminal amino acids of protein substrates to N-end rule UPP-dependent processes[76,77] and a single report regarding CNF1 catalyzed deamidation of RhoA/Rac internal residues in relation to altered UPP proteolytic susceptibility.[78] No mechanism links the deamidation of the latter to enhanced ubiquitination and degradation. The present data suggest that similar recognition or degradation machinery is involved in the ubiquitination and degradation of these deamidated crystallins as is used for the degradation of enzymatically deamidated Rac proteins.[78]

Biochemical and biophysical changes were also induced by these deamidations. Amine reactivity is clearly of interest because most frequently ubiquitin becomes covalently attached to lysines before the ubiquitinated protein is recognized by the 26S proteasome. The deamidation-related increase in susceptibility of βB2 crystallins to ubiquitination and increased amine reactivity on deamidation is consistent with a role for the UPP in targeting deamidated proteins for degradation. However, it is not possible to systematically relate susceptibility of a specific protein to the UPP and a set of biophysical measures, including hydrophobicity, amine reactivity, ability to form dimers, heat stability, circular dichroism, and EPR parameters for these proteins.[51,52,59,79–84]

We and others[16,74] have previously demonstrated that insufficient UPP proteolytic activity is associated with many age-related dysfunctions of the eye. Various stresses, including extensive oxidative stress, glycation (Uchiki et al., manuscript submitted), and associated protein aggregation have been associated with resistance of substrates to proteolysis. The loss of proteolytic capacity or aberrant ubiquitination that is not followed by degradation should also be considered among the etiologic factors in the protein precipitation diseases because, before degradation, very HMW ubiquitin conjugates are often formed, and compromised degradation of these components will result in their further accumulation. With stress, these HMW moieties may cross-link and precipitate, forming foci for cataract. Here we show that deamidated proteins are also potentially part of the proteostasis burden. This possibility will be enhanced if the conjugates have longer dwell times. These data are consistent with our previous observations of accumulation of HMW ubiquitin conjugates in the insoluble fraction of aged human lenses, where proteolytic potential is limited.[61]

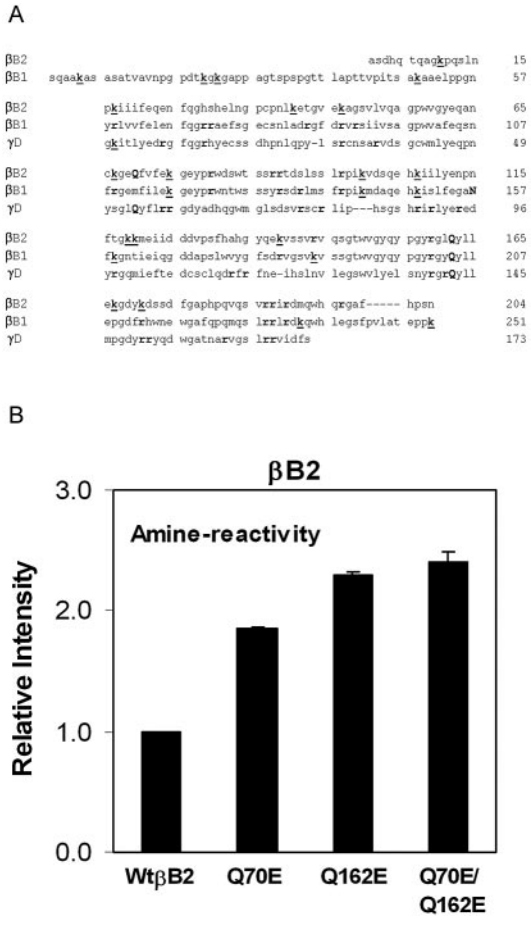

FIGURE 7. Sequence alignment and amine reactivity. (**A**) Primary amino acid sequence of βB2, βB1, and γD crystallins depicting sites of deamidation (**Q**, **N**), lysine (**k**), and arginine (**r**) residues. Amino acid number is indicated at the right. (**B**) Amine reactivity of WT and deamidated βB2 crystallins. Graph represents data obtained from two independent experiments.

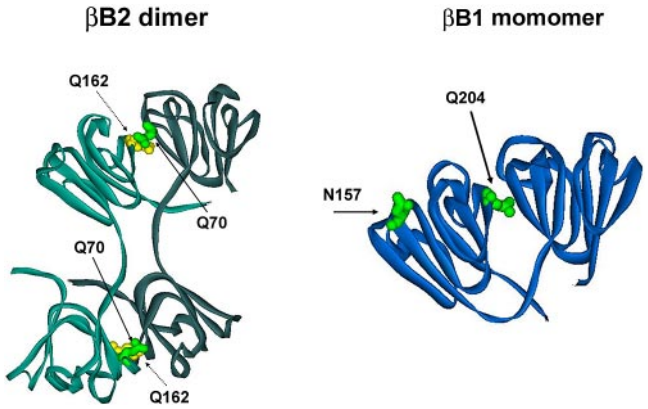

FIGURE 8. Ribbon diagrams for dimeric βB2 and monomeric βB1 crystallins showing sites of deamidation.

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