Proteasomes Cleave at Multiple Sites within Polyglutamine Tracts

ACTIVATION BY PA28γ(K188E)*

Received for publication, November 14, 2007, and in revised form, January 30, 2008. Published, JBC Papers in Press, March 13, 2008, DOI 10.1074/jbc.M709347200

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Eukaryotic proteasomes have been reported to cleave only once within polyglutamine tracts and then only after the N-terminal glutamine (Venkatraman, P., Wetzel, R., Tanaka, M., Nukina, N., and Goldberg, A. L. (2004) Mol. Cell 14, 95–104). We have obtained results that directly conflict with that report. In the presence of the proteasome activator PA28γ(K188E) human red cell proteasomes progressively degraded fluorescein-GGQ10RR or fluorescein-HPHQ10RR into small fragments as shown by size exclusion chromatography and mass spectrometry. MALDI-TOF mass spectrometry revealed that proteolytic products arose from cleavage after every glutamine in fluorescein-HPHQ10RR, and mass accuracy rules out deamidation of glutamine to glutamic acid as an explanation for peptide degradation. Moreover, degradation cannot be attributed to a contaminating protease because peptide hydrolysis was completely blocked by the proteasome-specific inhibitors, lactacystin and epoxomicin. We conclude that proteasomes cleave repetitively anywhere within a stretch of ten glutamine residues. Thus our results cast doubt on the idea that mammalian proteasomes cannot degrade glutamine-expanded regions within pathogenic polyQ-expanded proteins, such as Huntington.

Trinucleotide expansions, the cause of a number of human diseases, can occur in nontranscribed regions of the genome in transcribed but nontranslated sequences and in coding regions (1). Expansions that result in altered protein structure involve two amino acids, an alanine encoded by GCG and glutamine (Q) encoded by CAG (2, 3). Both polyalanine and polyglutamine expansions lead to the formation of insoluble inclusions, usually nuclear, that often recruit chaperones, ubiquitin, proteasomes, and a variety of other proteins (4, 5). Glutamine tract expansions occur in a disparate set of proteins with no common biochemical function, and the polyglutamine diseases, such as Huntington, Kennedy, and a number of ataxias, usually occur when the glutamine tract exceeds 35 residues in length (6). Whereas most polyQ-expanded proteins are expressed in numerous tissues, the degenerative process is restricted to a subset of neurons specific for each disease (7).

Materials—Suc-LeuLeuValTyr-MCA and cbz-LeuLeuGlu-βNA were obtained from Sigma; boc-LeuArgArg-MCA was

*This work was supported, in whole or in part, by National Institutes of Health Grant NS042892 (to M.R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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purchased from Peptides International (Louisville, KY). Lactacystin was purchased from Calbiochem (EMD Chemicals Darmstadt, Germany), and epoxomicin from A. G. Scientific (San Diego, CA). The peptide aldehydes, Ac-PGPSH-al and Ac-EPFD-al, were gifts from Jennifer Harris of the Burnham Institute for Medical Research (La Jolla, CA). The peptides, Q5, Q3-R2, GG-Q10RR, GG-Q10K188E, and HPH-Q10RR, and HPH-Q10K188E, were synthesized at the University of Utah HSC Core Research Facilities. When employed, carboxyfluorescein was coupled to the N terminus of the peptide while still on the resin. Crude peptides were purified on a C18 column (Grace Vydac, Basel, Switzerland) with an acetonitrile gradient containing 0.1% trifluoroacetic acid. Purity was assessed by re-injection on reversed phase liquid chromatography and by mass spectrometry using either MALDI2 or ESI ionization. Lyophilized samples of the peptides were dissolved by weight in either reticulocyte buffer (40 mM Tris, pH 7.8, 10 mM MgCl2, 20 mM KCl, 2 mM dithiothreitol) or in 10 mM ammonium phosphate, pH 7.8. The concentration of non-fluoresceinated peptides was determined by amino acid analysis, and the concentration of fluoresceinated peptides was assayed by comparison to a fluorescein standard curve.

Degradation Assays—The 20 S proteasome was purified from outdated human red blood cells as previously described (27, 28), and recombinant PA28γ and PA28γ(K188E) were purified as outlined in Realini et al. (29). Assays of fluorogenic peptide substrates were carried out in 100-μl reactions in reticulocyte buffer (20 mM Tris, pH 7.8, 5 mM MgCl2, 10 mM KCl, and 1 mM dithiothreitol). Pre-mixes (50 μl) were prepared containing 0.3 μg of proteasome or 0.3 μg of proteasome plus 1 μg of PA28γ(K188E). Reactions were initiated by adding 50 μl of 200 μM fluorogenic peptidase in reticulocyte buffer. After incubation, samples were quenched with 200 μl of ETOH, and fluorescence was measured in a PerkinElmer LS5 spectrofluorimeter with excitation/emission at 380/440 nm for MCA substrates and excitation/emission at 335/410 nm for βNA substrates. Assays involving fluoresceinated peptides were equivalent to those just described except that the concentrations of fluorescein-GGQ10RR and fluorescein-HPHQ10RR peptide were 42 μM and 125 μM, respectively, and the proteasome concentration was 1 to 4 μg depending upon the experiment. Also reactions were stopped by freezing on dry ice prior to analysis by gel filtration or mass spectrometry. Sizing chromatography was performed on a 10/30 Superdex Peptide column (GE Healthcare Life Science). Samples (25 μl) were injected onto the column at 0.5 ml per minute in a buffer of 25 mM Tris, pH 7.5 containing 125 mM NaCl. After discarding the initial 10 ml of eluate, 25 1-ml fractions were collected, and their fluorescence was measured at 485 nm excitation and 520 nm emission. Mass spectrometry was carried out in the Proteomics Core Facility of the University of Utah. All of the mass spectral data shown were collected using delayed ion extraction mode on a PerSeptive Biosystems Voyager-DE™ STR MALDI/TOF mass spectrometer. Peptide samples were spotted using the dried-droplet method using a fresh solution of saturated α-cyano-4-hydroxycinnamic acid matrix in 50:50 water/acetonitrile 0.1% trifluoroacetic acid. MALDI spectra were acquired in reflector mode, operating at 10,000 resolution over a mass range from 400 to 5000 Da.

RESULTS

PolyQ Peptides Ionize Poorly and Are Underestimated by Mass Spectrometry—We were surprised by the report from Venkatraman et al. (26) that proteasomes do not cleave beyond the first one or two residues within a polyQ tract, because a previous survey of proteasome specificity showed that glutamines at the P2-P4 positions of fluorogenic tetrapeptides markedly enhanced cleavage after the P1 amino acid (30). Mass spectrometric analysis of the degradation products arising upon proteasomal hydrolysis of the substrates biotin-KKQ14KK and biotin-KKQ20KK constituted some of the evidence, leading to the conclusion that polyQ tracts are resistant to proteasome hydrolysis (26). Reaction mixtures containing rabbit 20 S proteasomes and the proteasome activator PA28αβ were reported to cleave bKKQ14KK at a single site generating bKKQ and Q4KK; with the longer substrate bKKQ20KK ions corresponding to Q4KK and larger peptide products were abundant in the proteasome digest, whereas smaller products were judged to be absent. However, the mass spectrum presented in Fig. 3B of Venkatraman et al. (26) indicated that numerous smaller products might well be present. Furthermore the low ion abundance at masses expected for smaller products could result from proteasomal removal of the readily ionizable lysine residues from each end of the 20-residue glutamine tract in KKQ20KK. If peptides containing only glutamine residues ionize poorly, they would be underestimated by mass spectrometry. We tested this possibility by mass spectrometry of two synthetic peptides, Q4 and Q4R2. When the peptides were mixed at equal molar ratios and subjected to mass spectrometry, the peptide containing arginine residues, Q4R2, was at least 10-fold more abundant in the resulting spectrum (Fig. 1). Moreover, whereas Q4R2 was present mainly as a protonated

2 The abbreviations used are: MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; ESI, electrospray ionization; PGP, postglutamyloferring; T, trypsin-like; CT, chymotrypsin-like; Fl, fluorescein; MCA, 7-amino-4-methylcoumarin; βNA, β-naphthalamide.
peptide, the Q3 peptide was almost evenly distributed among sodium, potassium, and protonated ions, thereby making its detection even less efficient. Clearly mass spectrometry does not provide a quantitative measure of products arising upon proteasomal digestion of polyQ peptides. Therefore the low abundance of ions corresponding to small peptides arising from KKQ28KK cannot be taken as evidence for polyQ resistance to proteasome degradation.

Proteasomes Cleave Anywhere within a Tract of Contiguous Glutamine Residues—Because mass spectrometry is not suitable for quantifying proteolysis, we synthesized a fluorescein (Fl)-labeled peptide, Fl-HPHQ10RR, to assay degradation by size exclusion chromatography. The sequence His-Pro-His was placed next to fluorescein on the assumption that the ionizable His residues would enhance detection of N-terminal products resulting from cleavage after each of the ten glutamines were clearly evident in the mass spectrum, and their identification was unequivocal given the accuracy of mass measurement (see Table 1). The absence of products containing only glutamine residues was expected in light of their poor ionization (see Fig. 1). However it was surprising that, with the single exception of Q8R, none of the major products contained arginine. It appears that removal of the two C-terminal basic residues is an early event in the proteolytic digests of Fl-HPHQ10RR.

The results in Fig. 2 and Table 1 are in direct conflict with the conclusions that proteasomes cleave only once within polyQ tracts. Conceivably the presence of fluorescein at the N terminus of HPHQ10RR altered substrate degradation by proteasomes such that multiple cleavages in the polyQ tract now occur. To test this possibility, an additional peptide GGQ7RR was synthesized with or without fluorescein at its N terminus. The two peptides were incubated with proteasomes and a mutant proteasome activator PA28γ(K188E), and samples were analyzed by mass spectrometry. Results of this analysis, presented in Table 2, show a clear effect of fluorescein on the distribution of products arising from GGQ7RR. The presence of fluorescein resulted in products lacking C-terminal arginine residues. By contrast, proteasomal degradation of GGQ7RR generated a wider spectrum of peptides some of which contained one or both C-terminal arginine residues. More importantly, the ions listed in Table 2 indicate that hydrolysis occurred after almost every glutamine in the non-fluoresceinated GGQ7RR substrate. So while an N-terminal fluorescein can affect substrate processing by the proteasome, the enzyme cleaves after multiple glutamines within polyQ tracts even in non-fluoresceinated peptides. Thus, in contrast to the results of Venkatraman et al. (26), we find that human red cell proteasomes cleave anywhere within a stretch of seven or ten contiguous glutamine residues.

Degradation of Fl-HPHQ10RR and Fl-GGQ7RR Is Markedly Stimulated by the Mutant Proteasome Activator PA28γ(K188E)

### Table 1

| Degradation product | Adduct | Theoretical mass | Observed mass | % Error | Ion intensity |
|---------------------|--------|------------------|---------------|---------|--------------|
| FL-HPHQ10RR         | H      | 2185.18          | 2185.69       | 0.02    | 55           |
| FL-HPHQ10RR         | K      | 2067.05          | 2067.77       | 0.03    | 17           |
| FL-HPHQ10RR         | H      | 2029.08          | 2028.85       | 0.02    | 40           |
| FL-HPHQ10RR         | K      | 1938.99          | 1938.76       | 0.01    | 17           |
| FL-HPHQ10RR         | H      | 1901.02          | 1900.80       | 0.02    | 60           |
| FL-HPHQ10RR         | K      | 1810.93          | 1810.71       | 0.01    | 20           |
| FL-HPHQ10RR         | H      | 1772.96          | 1772.75       | 0.01    | 57           |
| FL-HPHQ10RR         | K      | 1682.87          | 1682.66       | 0.01    | 30           |
| FL-HPHQ10RR         | H      | 1644.90          | 1644.70       | 0.01    | 83           |
| FL-HPHQ10RR         | K      | 1554.81          | 1554.51       | 0.02    | 27           |
| FL-HPHQ10RR         | H      | 1516.85          | 1516.64       | 0.01    | 100          |
| FL-HPHQ10RR         | K      | 1426.75          | 1426.46       | 0.02    | 17           |
| FL-HPHQ10RR         | H      | 1388.79          | 1388.57       | 0.01    | 47           |
| FL-HPHQ10RR         | K      | 1298.69          | 1298.41       | 0.02    | 14           |
| FL-HPHQ10RR         | H      | 1260.73          | 1260.53       | 0.02    | 43           |
| FL-HPHQ10RR         | K      | 1132.67          | 1132.45       | 0.02    | 23           |
| FL-HPHQ10RR         | H      | 1042.58          | 1042.31       | 0.03    | 22           |
| FL-HPHQ10RR         | K      | 1004.61          | 1004.39       | 0.02    | 94           |
| FL-HPHQ10RR         | H      | 876.55           | 876.35        | 0.02    | 27           |
| Q,R                 | H      | 1199.59          | 1199.64       | 0.00    | 14           |
| Q,R                 | H      | 1071.53          | 1071.57       | 0.00    | 15           |
| Q2                   | H      | 915.43           | 915.30        | 0.01    | 15           |
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**TABLE 2**

| Product | GGQ₇| FI-GGQ₇ | Product | FI-GGQ₉ | Product | FI-GGQ₁₀ | Product | FI-GGQ₁₀ |
|---------|------|---------|---------|---------|---------|---------|---------|---------|
| % Ion intensity | 55 | 4 | 33 | 20 | 19 | 8 | 71 | 12 |
| % Ion intensity | 66 | 38 | 55 | 55 | 41 | 100 | 37 | 22 |
| % Ion intensity | 20 | 22 |

**PA28γ(K188E)—**To determine whether rates of FI-HPHQ₁₀RR degradation could be monitored by gel filtration as well as mass spectrometry, the peptide was incubated with 20 S proteasomes and the mutant activator PA28γ(K188E). Samples were taken after 30, 90, or 270 min for analysis by MALDI-TOF or by gel filtration. Relative ion intensities for the proteolytic products, obtained from spectra like that shown in Fig. 1, are plotted in Fig. 3A. Whereas peptides containing 8, 9, or 10 glutamines were still present at 30 min of incubation, they were much less abundant at 90 min and absent by 4.5 h. The disappearance of larger products was accompanied by an increase in smaller peptides, FI-HPHQ₂₋₄, and the appearance of FI-HPHQ by 90 min of incubation. Gel filtration profiles of the same reaction mixtures, presented in Fig. 3B, correlate with the mass spectrometric analyses at 90 and 270 min and show the progressive shortening of peptide products as the reaction proceeds.

**FIGURE 3.**

**A** Progressive degradation of FI-HPHQ₁₀RR by proteasomes. Two additional samples from the reaction that generated the spectrum in Fig. 2 were taken at 90 and 270 min of incubation and analyzed by MALDI-TOF. A, ion frequency of FI-HPH-peptides containing 1–10 glutamine residues is plotted for samples taken at 30, 90, and 270 min and shows the progressive shortening of peptide products as the reaction proceeds. B, separate timed samples from the same reaction were analyzed by gel filtration on a 10/30 Superdex Peptide column, and the elution profiles show a similar progressive degradation of FI-HPHQ₁₀RR to small peptides over the 270 min of incubation.

**PA28γ(K188E)** that activates all three catalytic subunits (20). Because proteasomal degradation of fluorogenic tetrapeptides with glutamine at the P1 position was markedly stimulated by PA28αβ, but only barely by PA28γ (30), we suspected that PA28γ(K188E) would increase the rate of FI-HPHQ₁₀RR degradation by proteasomes. As a test of this hypothesis, we incubated the peptide with proteasomes alone or proteasomes plus wild-type or mutant PA28γ, and timed samples were analyzed by gel filtration as described in Fig. 3B. As shown by the results in Fig. 4A, degradation of FI-HPHQ₁₀RR was increased 10-fold more by PA28γ(K188E) than by wild-type PA28γ; equivalent results were obtained with FI-GGQ₁₀RR as substrate (Fig. 4B). Although degradation of both substrates was stimulated slightly by PA28γ, this may reflect opening of the “gate” though the proteasome α-ring (31) rather than activation of proteasome β-subunits by PA28γ. In any event, PA28γ(K188E) mark-
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This is apparent from the mass spectrometry data presented in Fig. 2 as well as Tables 1 and 2. Proteasomal degradation of Fl-HPHQ$_{10}$RR and GGQ$_{7}$RR produced ions corresponding to peptides generated by hydrolysis after each glutamine in Fl-HPHQ$_{10}$RR and five of the seven glutamines in GGQ$_{7}$RR. Equally important, both mass spectrometry (Fig. 3A) and gel filtration (Fig. 3B) demonstrated that degradation was progressive, leading eventually to small peptides that contained only one or two glutamines. We conclude that proteasomes can cleave at multiple sites within a polyQ tract, and they can do so repeatedly. The complete inhibition of peptide breakdown by lactacystin or epoxomicin (Fig. 5, A and B) eliminates the possibility that degradation was due to a contaminating protease.

If glutamine residues in our peptide substrates were to deamidate to glutamates, then peptide degradation might be attributed to the PGPH active site of the proteasome. However, the difference between observed and expected masses for each proteolytic product was generally less than 0.22 Da (Table 1). This high degree of mass accuracy rules out glutamine deamidation as a potential explanation for peptide degradation because replacement of NH by O would increase the mass of products by 1 Da. Furthermore, if proteasome cleavage occurred only after glutamic acid residues generated by deamidation, the responsible catalytic subunit of the proteasome would contain the PGPH active site. Accordingly, one would have expected Ac-EPFD-al to inhibit peptide degradation, but it did not (Fig. 5C).

Our results are in direct conflict with those reported by Venkatraman et al. (26). As mentioned, we believe that poor ionization of peptides containing only glutamine (see Fig. 1) led them to the mistaken conclusion that proteasomes do not cleave within polyQ tracts. However, the discrepancy between our results and theirs might reflect differences in proteasome source or reaction components. Because we both used mammalian proteasomes and because PA28$_{γ}$ (K188E) has activation properties very similar to PA28$_{αβ}$ used in their studies, we do not favor the idea that proteasomes cleave polyQ sequences only under our conditions of assay. Rather, we consider it much more likely that proteasomes are generally capable of degrading polyQ sequences provided they remain soluble. In this regard, we have found that the half-life of ataxin-7(Q$_{86}$)-GFP is the same as ataxin-7(Q$_{10}$)-GFP in HEK cells and that both are degraded by proteasomes.$^3$

As noted under the “Results,” PA28$_{αβ}$ and PA28$_{γ}$ differ in their activation properties with PA28$_{αβ}$ activating all three proteasome catalytic subunits, while PA28$_{γ}$ only activates

\[ \text{V. Ustrell, G. Goellner, G. Pratt, C. Sloan, and M. Rechsteiner, manuscript in preparation.} \]

\[ ^3 \text{V. Ustrell, G. Goellner, G. Pratt, C. Sloan, and M. Rechsteiner, manuscript in preparation.} \]

\[ \text{DISCUSSION} \]

The results presented above provide compelling evidence that proteasomes can cleave multiple times within polyQ tracts.

\[ \text{edly enhanced cleavage within polyQ tracts, and this finding has implications for the possible treatment of polyglutamine diseases.} \]

Degradation of Fl-HPHQ$_{10}$RR and Fl-GGQ$_{7}$RR Cannot Be Attributed to a Contaminating Protease—The fact that the proteasome activator PA28$_{γ}$ (K188E) markedly increased the rate of polyQ peptide proteolysis (see Fig. 4) is prima facie evidence that the proteasome is the responsible enzyme. But PA28$_{γ}$ (K188E) is a recombinant protein that could be contaminated by an Escherichia coli protease. To test for a possible contaminating protease, we examined the effect of two specific proteasome inhibitors, lactacystin and epoxomicin, on peptide hydrolysis. The gel filtration profile in Fig. 5A shows that degradation of Fl-HPHQ$_{10}$RR was completely inhibited by lactacystin, and the profile in Fig. 5B demonstrates that Fl-GGQ$_{7}$RR remained intact in the presence of epoxomicin. Furthermore, mass spectroscopy showed that the unblocked peptide, GGQ$_{7}$RR, remained intact after 3 h of incubation with lactacystin- or epoxomicin-inhibited proteasomes. Six unblocked fluorogenic di-peptides or amino acid-MCA substrates were not degraded after 90 min of incubation with uninhibited proteasomes. Combined, these results indicate that our proteasome preparation is devoid of contaminating peptidases and provide clear evidence that the fluoresceinated peptides employed in these studies were degraded by the proteasome.

Our previous survey of proteasome specificity using a combinatorial library of fluorogenic tetrapeptides demonstrated that, in the presence of PA28$_{αβ}$, proteasomes readily cleave after glutamine residues, but they remain incapable of cleaving XXXGly-MCA or XXXPro-MCA bonds (30). That survey also led to the development of two peptide aldehydes, Ac-EPFD-al and Ac-PRQR-al, which target enzyme PGPH and T active sites, respectively (see Table 3). As shown in Fig. 5C, neither compound inhibited degradation of Fl-GGQ$_{7}$RR, indicating that the CT-like active site of the proteasome is mainly responsible for cleavage within polyQ tracts.

\[ \text{FIGURE 4. The mutant proteasome activator PA28$_{γ}$ (K188E) stimulates degradation of peptides containing polyQ tracts. Fl-HPHQ$_{10}$RR and Fl-GGQ$_{7}$RR were incubated with 20 S proteasomes alone (●) or 20 S proteasomes plus PA28$_{γ}$ (■) or PA28$_{γ}$K188E (▲), and degradation was monitored by the analysis of timed samples on a 10/30 Superdex column as described in the legend to Fig. 3B.} \]
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FIGURE 5. Inhibition of polyQ-peptide degradation by the proteasome inhibitors lactacystin and epoxomicin. Reactions containing 20 S proteasome were preincubated with or without proteasome inhibitors for 30 min before adding Fl-polyQ peptide. A, reactions with FI-HPHO<sub>7</sub>RR as substrate were incubated for 6 h before samples were chromatographed on the 10/30 Superdex peptide column. Whereas in the absence of lactacystin FI-HPHO<sub>7</sub>RR was degraded to small fragments ( ), 20 μM of the proteasome inhibitor completely blocked peptide degradation (compare and ). B, peptide FI-HPHO<sub>7</sub>RR was incubated with 20 S proteasome/P<sub>A28γ</sub>(K188E) for 3 h in the presence ( ) or absence ( ) of epoxomicin prior to chromatography. Note that epoxomicin completely blocked proteolysis of FI-HPHO<sub>7</sub>RR.

the T-like subunit. PA28αβ and PA28γ also differ in their subcellular locations and organ distribution (32). PA28αβ is cytoplasmic, is enriched in immune cells, and is virtually absent in brain. By contrast, PA28γ is nuclear and is highly expressed in neurons. In addition, PA28γ suppresses the proteasome CT-like subunit that is mainly responsible for cleavage within polyQ tracts (see Fig. 5C). These properties led us to speculate that PA28γ might be detrimental to the clearance of polyQ-expanded proteins, and its high level of expression in brain could account for restricting polyglutamine pathologies to that organ (20). Our recent finding that PA28γ status did not affect disease progression in R6/2 mice (21) argues against this hypothesis. Still the ability of proteasomes to cleave within polyQ tracts and PA28γ(K188E) stimulation of polyQ peptide degradation (Fig. 4) raises the possibility of a proteasome-mediated therapy.

A number of approaches have been proposed as potential therapies for polyglutamine diseases (33, 34). Glutamine-expanded proteins often misfold and form intracellular aggregates making molecular chaperones attractive candidates for ameliorating the polyQ disease state. A vast literature has shown that overexpression of heat shock proteins increases the survival of cells expressing polyQ-expanded proteins (see Ref. 35 for a review). If polyQ-expanded proteins are inherently toxic, as seems to be the case (36, 37), a reduction in their levels should also prove beneficial. Strikingly, RNAi targeting of mRNAs encoding human polyQ-expanded SCA1 or huntingtin in mouse models of these diseases produced dramatic improvement in motor coordination (38, 39). Increasing the degradation of polyQ-expanded proteins should have the same effect. In this regard, both autophagy-mediated lysosomal destruction of polyQ aggregates (40), and proteasome-mediated degradation of polyQ-expanded proteins have been demonstrated (41). The studies presented above show that a mutant PA28γ speeds the degradation of peptides containing polyQ tracts. If these results extend to intact proteins, then a small molecule able to bind to wild-type PA28γ and convert its proteasome activation properties to those of PA28γ(K188E) might speed the clearance of polyglutamine-expanded proteins in neurons.

| Fluorogenic peptide | Ac-PRQR-al | Ac-EPFD-al | Lactacystin |
|---------------------|------------|------------|-------------|
| Sac-LLVY-MCA        | –32        | –5         | 97          |
| Boc-LRR-MCA         | 97         | –22        | 63          |
| Cbz-LLE-βRNA        | 2          | 93         | 10          |

TABLE 3 Specificities of Ac-PRQR-al and Ac-EPFD-al as proteasome inhibitors

Reactation mixtures (50 μl) contained 0.32 μg of 20 S proteasome, 1 μg of P<sub>A28γ</sub>(K188E), and either reticulocyte buffer alone or 2 μM Ac-PRQR-al, 2 μM Ac-EPFD-al, or 20 μM lactacystin. After incubation at 37 °C for 30 min, 50 μl of a 200 μM solution of the indicated fluorogenic peptide was added to initiate degradation. Duplicate samples were quenched with 200 μl of ethanol at 10 and 30 min, and fluorescence was measured at ex/em 380/440 nm for MCA substrates and ex/em 335/410 for the βNA substrate Cbz-LLE. The measured fluorescence was divided by the fluorescence obtained in the absence of inhibitor to produce % inhibition. A negative value indicates stimulation of peptide hydrolysis in the presence of the inhibitor. Note that whereas lactacystin completely inhibited hydrolysis of sLLVY-MCA (a substrate for the chymotrypsin-like activity of the proteasome) and bLRR-MCA (a substrate for the trypsin-like subunit), the peptide aldehydes are strikingly specific for just one proteasome active site.
Acknowledgments—We thank Vicenç Ustrell and Carlos Gorbea for helpful suggestions during the course of these studies and for providing assistance in the preparation of the manuscript. We also thank Robert Schackmann for peptide synthesis and Chad Nelson and Parsawar Krishna for help with the mass spectrometry.

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