Selective Activation of the 20 S Proteasome (Multicatalytic Proteinase Complex) by Histone H3[†]

Marian Orlowski*

Department of Pharmacology, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, New York 10029

Received August 7, 2001

ABSTRACT: Two distinct activities cleaving bonds after hydrophobic amino acids have been identified in the bovine pituitary 20 S proteasome. One, expressed by the X subunit, that cleaves bonds after aromatic and branched chain amino acids was designated as chymotrypsin-like (ChT-L).[1] The second, expressed by the Y subunit, that cleaves bonds after acidic amino acids was designated as peptidylglutamyl-peptide hydrolyzing (PGPH) but also cleaves bonds after branched chain amino acids. Low micromolar concentrations of the arginine-rich histone H3 (H3) are shown to induce changes in the specificity of the proteasome by selectively activating cleavages after branched chain and acidic amino acids while inhibiting cleavage of peptidyl−arylamide bonds in synthetic substrates. H3 activates 15-fold cleavage after leucine but not phenylalanine residues in model synthetic substrates. The activation is associated with a decrease in K_m and an increase in V_{max}, suggesting positive allosteric activation. H3 activates more than 60-fold degradation of the oxidized B-chain of insulin, by cleaving mainly bonds after acidic and branched chain amino acids, and accelerates the degradation of casein and lysozyme, the latter in the presence of dithiothreitol. The degradation of lysozyme in the presence of H3 generates fragments that differ from those in its absence, indicating H3-induced specificity changes. H3 inhibits cleavage of the Trp3−Ser4 and Tyr5−Gly6 bonds in gonadotropin releasing hormone, bonds cleaved by the ChT-L activity in the absence of H3. The results suggest H3-selective activation of the Y subunit and specificity changes that could potentially affect proteasomal function in the nuclear compartment.

Early work on the activities of the 20 S proteasome led to the identification of three distinct catalytic activities cleaving bonds after hydrophobic, acidic, and basic amino acids (*1*−*3*). This led to the proposed name "multicatalytic proteinase complex", currently more widely referred to as the 20 S "proteasome" (*4*). The three activities were designated as chymotrypsin-like (ChT-L),[1] peptidylglutamyl-peptide hydrolyzing (PGPH), and trypsin-like (T-L), based on the nature of the amino acid providing the carbonyl group to the scissile bond. Specificity and inhibitor studies provided evidence that each of the activities is expressed by a distinct subunit of the proteasome. Subsequent experiments have shown that exposure of the proteasome to 3,4-dichloroisocoumarin (DCI), an acylating agent that leads to virtually complete inactivation of the three activities described above, does not eliminate the activity toward peptide bonds between two adjacent amino acids in a series of natural and synthetic peptides (*5*, *6*). Indeed, such treatment led to activation of degradation of casein, the oxidized B-chain of insulin, several bioactive peptides, and up to a 10-fold acceleration of degradation of such synthetic substrates as Z-GPAL*A-pAB and Z-GPAL*G-pAB (the asterisks indicating the site of cleavage) (*6*). The preference of cleavage after branched chain amino acids found both in natural and in synthetic peptides suggested the designation as "branched chain amino acid preferring" or for short "BrAAP". Although attempts have been made to credit most of this activity to the regular ChT-L activity (*7*), this was incompatible with a series of findings. Thus, for example, exposure of the proteasome to DCI that rapidly inactivates the ChT-L activity by acylation of the hydroxyl group of the N-terminal threonine in the active center of the X-subunit (known to express the ChT-L activity) causes activation of degradation of casein and a series of synthetic and natural peptides (*8*). Also, a number of inhibitors of the ChT-L activity were shown to have little effect on the BrAAP activity, and concentrations of lactacystin that inhibited the ChT-L activity had no effect on this activity. Furthermore, several proteins, including the heat shock protein 90 and bovine serum albumin, while inhibiting the ChT-L activity had no effect on the BrAAP activity (*9*).

Mutational studies in the yeast *Saccharomycys cerevisiae* led to the identification of those subunits expressing the three classical activities of the proteasome referred to above. Thus, the β1, β2, and β5 subunits were shown to be necessary for expression of the PGPH, T-L, and ChT-L activities, respectively (*10*). This was confirmed and extended in crystallographic studies of the yeast proteasome with demonstration

[†] This work was supported by Grant DK25377 from the NIH (to M.O.).

* Correspondence author. Tel: (212) 659-1706. Fax: (212) 831-0114. E-mail: marian.orlowski@mssm.edu.

[1] Abbreviations: BrAAP, branched chain amino acid preferring; Cdk, cyclin-dependent kinase; ChT-L, chymotrypsin-like; DCI, 3,4-dichloroisocoumarin; GnRH, gonadotropin releasing hormone; HPLC, high-pressure liquid chromatography; MHC, major histocompatibility complex; 2NA, 2-naphthylamide; pNA, *p*-nitroanilide; pAB, *p*-aminobenzoate; PGPH, peptidylglutamyl-peptide hydrolyzing; SDS, sodium dodecyl sulfate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; T-L, trypsin-like; Z, benzyloxycarbonyl. Conventional one- or three-letter abbreviations are used for amino acids.

10.1021/bi0116240 CCC: $20.00
Published on Web 11/22/2001

of binding of a peptidyl−aldehyde inhibitor to the six catalytically active subunits (*11*, *12*). Extrapolation of these results to the proteasome in higher vertebrates led to assignment of these same activities to subunits Y, Z, and X, respectively (*10*). Such assignment was indeed consistent with biochemical studies of proteasomes from higher vertebrates and data obtained from experiments with radioactive inhibitors (*13*−*15*).

The finding of a second chymotrypsin-like activity, different from that expressed by the X subunit, showing preferential cleavage of bonds after branched chain amino acids, posed the question of its origin. Experiments in yeast mutants showing that activity toward a model substrate such as Z-GPAL*A-pAB is absent in mutants deficient of the PGPH activity (*10*) pointed to the β1 (Pre3, Y) as its source. This assignment is supported by observations that peptidyl aldehydes with selectivity for inhibition of the BrAAP activity were also inhibiting the PGPH activity (*16*) and observations that the BrAAP activity was also cleaving bonds between two amino acids in which the glutamate residue provides the carbonyl group (*17*). Collectively, these and other results strongly affirm the presence in the proteasome of an activity that preferentially cleaves bonds after branched chain amino acids and indicate the involvement of the PGPH activity (Y subunit) in cleaving bonds after both acidic and branched chain amino acids (*18*).

Here we report that low micromolar concentrations of the arginine-rich histone H3 inhibit the three classical activities of the 20 S proteasome as measured with synthetic peptidyl−arylamide substrates. H3 activates 15-fold cleavage of bonds after leucine residues in the synthetic substrates Z-GPAL*A-pAB and Z-GPAL*G-pAB but not degradation of an identical substrate in which the Leu residue is replaced by a phenylalanine. H3 also activates the degradation of casein and lysozyme and more than 60-fold the degradation of the B-chain of insulin, the latter by mainly cleaving bonds both after acidic and branched chain amino acids. Changes in specificity induced by H3 are also evident by the inhibition of cleavage of the Tyr5−Gly6 and Trp3−Ser4 bonds in gonadotropin releasing hormone (GnRH), a main cleavage site that is catalyzed in the absence of H3 by the ChT-L activity. These findings suggest selective activation of the Y subunit with little effect or inhibition of the activity expressed by the X subunit. The possible significance of these findings for the catalytic function of the proteasome in the nuclear compartment is discussed.

MATERIALS AND METHODS

Synthetic Subsrates. Z-GPALG-pAB, Z-GPALA-pAB, and Z-GPAFG-pAB and the peptidyl−arylamide substrates used for measuring the ChT-L, T-L, and PGPH (Z-LAF-pAB, Z-dALR-2NA, and Z-LLE-2NA) activities were synthesized as described previously (*1*, *3*, *6*, *9*, *19*−*21*).

Isolation of Histone H3 from Calf Thymus. Crude histone H3 was isolated from calf thymus by method 2 described by Johns (*22*). A crude histone H3 preparation (140 mg) was obtained from processing of 50 g of bovine calf thymus tissue (obtained from Pel Freeze Inc., Rogers, AR). HPLC has shown that the preparation contained several histone fractions, with the predominant fraction eluting as peak 3. This component and two other smaller peaks with a shorter retention time were isolated from 42 mg of the crude material by HPLC, using a C18 Delta-Pak 5 μm column (7.9 × 150 mm). Two milligram aliquots of the preparation were injected on the column, and peak 3 was eluted with a 120 min linear gradient established between 30% and 60% acetonitrile, each containing 0.1% of trifluoroacetic acid at a flow rate of 3 mL/min. The material obtained from chromatographic runs was pooled and evaporated to dryness under vacuum. It was then dissolved in 1 mM hydrochloric acid. HPLC of the isolated material on a C18 Delta-Pak column (5 μm, 3.9 × 150 mm) at a flow rate of 1 mL/min, and the same gradient as above, gave a single peak with a retention time of 85.6 min. The yield was 8.4 mg. Mass spectrometry showed the presence of a single peak with a molecular mass of 15270 Da, consistent with bovine histone H3. Amino acid sequencing of the first 11 N-terminal amino acids gave a sequence of ARTKQTARKST, identical with bovine histone H3 (*23*, *24*). The two other peaks (yield 1 mg each) were subjected to HPLC on the same column as above and a linear acetonitrile−TFA gradient between 2% and 60% acetonitrile in 60 min and a flow rate of 1 mL/min. The retention time of the first peak was 53 min, and mass spectrometry gave a molecular mass of 15138 Da. The second peak gave a retention time of 55.3 and a mass of 15146 Da. Under the same conditions H3 gave a retention time of 55.6 min. Amino acid sequencing of the first ten N-terminal amino acids gave the same sequence as that obtained for the main fraction described above. This indicates that the two peaks could be minor variants of H3, probably differing either by deletion of a C-terminal amino acid residue or by containing only one rather than the two usual cysteine residues, as previously described for H3 preparations (*25*). No further analysis of these fractions was carried out.

Other Reagents. The oxidized B-chain of insulin, GnRH, lysozyme, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Determination of Enzyme Activity. The ChT-L, T-L, PGPH, and BrAAP activities were determined with Z-LAF*-pAB, Z-dALR*-2NA, Z-LLE-*2NA, and Z-GPAL*G-pAB, respectively, at the corresponding substrate concentrations of 1.0, 0.4, 0.64, and 1 mM (asterisks indicate the site of cleavage by the enzyme). Reaction mixtures contained 0.05 M Tris-HCl buffer, pH 8.0, and 0.25−0.5 μg of highly purified bovine pituitary MPC in a final volume of 0.2 mL. Incubations were at 37 °C for 30 min. The reaction was stopped by addition of 20 μL of 50% trichloroacetic acid, and the amount of product formed was determined by a modification of the diazotization procedure previously described (*26*). This was done by addition of 40 μL of a 0.5% solution of sodium nitrite following after 3 min of 40 μL of a 2.5% ammonium sulfamate solution and after 2 min of 250 μL of a 0.2% *N*-(naphthyl)ethylenediamine dihydrochloride solution in 95% ethanol. The absorbance was determined at 555 nm for pAB and 580 nm for 2NA. The BrAAP activity was determined in a two-stage reaction. The reaction was stopped after 30 min incubation by boiling the incubation mixture for 2 min. After being cooled to room temperature, 20 μg of aminopeptidase N was added, and the mixture was further incubated for 30 min to release free pAB from the reaction product G-pAB or Ala-pAB. The amount of the aromatic amine was then determined after diazotizition, as described above. Controls without the proteasome and

Table 1: Effect of Histone H3 on the Catalytic Activities of the Bovine Pituitary Proteasome[a]

catalytic activity	enzyme (μg)	substrate (mM)	additions (histone, μg)	activity (μmol mg^{-1} h^{-1})	activation/inhibition (+/−%)
ChT-L	0.25	Z-LAF-pAB (1.0)	none	47.2 ± 3.6	
ChT-L	0.25	Z-LAF-pAB (1.0)	5.0	6.3 ± 0.9	−87
T-L	0.75	Z-dALR-2NA (0.4)	none	3.35 ± 0.22	
T-L	0.75	Z-dALR-2NA (0.4)	5.0	1.95 ± 0.2	−43
PGPH	0.5	Z-LLE-2NA (0.64)	none	24.4 ± 0.8	
PGPH	0.5	Z-LLE-2NA (0.64)	4.0	4.52 ± 1.3	−81
BrAAP	0.5	Z-GPALG-pAB (1.0)	none	5.44	
BrAAP	0.5	Z-GPALG-pAB (1.0)	3.0	56.1	+1000

[a] Incubation mixtures contained the indicated substrate concentrations, Tris-HCl buffer (0.05 M, pH 8.0), and, where indicated, histone H3 in a final volume of 0.2 mL. Reactions were started by the addition of the enzyme, and incubations were at 37 °C for 30 min. Activities were measured in the absence and presence of 1−5 μg of histone H3 in the incubation mixtures. Values are means from four independent determinations ± SE, except for the BrAAP activity which represents the average of two experiments. The table shows data obtained at histone concentrations giving either maximal inhibition or maximal activation.

without aminopeptidase were also carried through the procedure.

The ChT-L activity was measured with Z-Leu-Ala-Phe*-pAB as the substrate. The amino acid composition of the substrate was chosen because previous experiments have shown that sequences containing a small neutral amino acid flanked by hydrophobic residues, of which a phenylalaninal residue is present in the P1 position, constitute good inhibitors of the ChT-L activity (*8*). Other advantages of this substrate are its relatively good solubility at a mild alkaline pH optimum for activity, and its high rate of degradation with V_{max} values of about 100 μmol (mg of enzyme)$^{-1}$ h^{-1}, more than 10 times higher than those usually obtained with previously used substrates such as Z-GGL-pNA or Z-GGF-pAB.

Degradation of the Oxidized B-Chain of Insulin. Incubation mixtures in control samples (final volume 200 μL) contained 10 nmol of the oxidized insulin B-chain, 2.5 μg of the pituitary proteasome, and 155 μL of Tris-HCl buffer (0.05 M, pH 8.0). Experimental samples contained in addition 17.5 μg of histone H3. Incubations were at 37 °C, and 40 μL aliquots of the samples were withdrawn from the control samples at time 0, 1, 2, and 4 h and treated with 5 μL of glacial acetic acid. Experimental samples were treated in the same manner, but they were withdrawn at time 0, 10, 20, and 30 min. The samples were subjected to HPLC on a C18 Delta-Pak column (5 μm,; 3.9 × 150 mm) using a linear 30 min gradient between 2% and 40% acetonitrile, each containing 0.1% trifluoroacetic acid, at a flow rate of 1 mL/min. The rate of degradation was calculated from the decrease in the peak height of the oxidized insulin B-chain. The amino acid sequences of the main proteolytic products were deduced from the masses of degradation products determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Peptides were identified by comparison of the measured molecular masses to the molecular masses of all possible peptides resulting from fragmentation of the oxidized B-chain of insulin using the mass spectrometry data analysis program Paws (Version 6.6.1 for Macintosh computers).

Degradation of β-Casein. Equal volumes (15 μL each) of Tris-HCl buffer (0.2 M, pH 8.0), the proteasome (7.5 μg), and histone H3 (52.5 μg) were preincubated at 26 °C for 20 min. Thirty microliters of the mixture was then transferred to an assay mixture at 37 °C containing 200 μg of β-casein and Tris-HCl buffer (pH, 8.0) in a final volume of 200 μL. Control samples contained the same amount of enzyme, casein, and buffer but not histone H3. Forty microliters of the reaction mixtures was then withdrawn at time 0, 1, 2, and 4 h, and the reaction was terminated by addition of 5 μL of glacial acetic acid. Twenty microliter aliquots were then subjected to HPLC on a C18 Delta-Pak column (5 μm, 3.9 × 150 mm). Elution was carried out with a 30 min linear gradient between 2% and 50% acetonitrile, each containing 0.1% acetonitrile, at a flow rate of 1 mL/min. The emerging peaks were monitored at 210 nm. The amount of casein degraded was calculated from the decrease in the height of the casein peak.

Degradation of Lysozyme. Incubation mixtures contained 20 μL of a 0.6% solution of lysozyme (53300 units/mg of protein; lot no. 118H8657; Sigma Chemical Co.) from chicken egg white in 0.05 M Tris-HCl buffer, pH 7.5, 2.5 μg of proteasome, 10 mM DTT, 8.5 μg of histone H3, and 0.05 M Tris-HCl buffer in a final volume of 60 μL. Incubations were for 4 h at 37 °C. Reactions were stopped by addition of SDS to a final concentration of 1%, 2-mercaptoethanol (final concentration 5%), and bromophenol blue as marker. Control samples without histone or DTT and/or proteasome were also carried through the procedure. Aliquots containing 20 μg of lysozyme were subjected to electrophoresis as described below.

SDS−PAGE. Dissociating PAGE was carried out in 12% gels of a 11 cm length and 1.5 mm thickness using a Hoeffer Scientific vertical gel apparatus.

Other Conditions. Other conditions are given in the legends to the corresponding tables and figures.

RESULTS

Effect of Histone H3 on Catalytic Activities of the Bovine Pituitary Proteasome. The catalytic activities of a highly purified preparation of the pituitary proteasome in the presence and absence of low concentrations of histone H3 are shown in Table 1. Over 80% inhibition of the ChT-L and PGPH activities was obtained in the presence of 4−5 μg (1.3−1.6 μM) of H3 in reactions started without preincubation with the enzyme. A similar concentration was only half as effective in inhibition of the T-L activity. Further increases of H3 concentration did not result in an increase of the extent of inhibition, suggesting the possibility that a small fraction of the enzyme activity was resistant to histone inhibition. Unlike the ChT-L, PGPH, and T-L activities, the presence of H3 strongly activated the degradation of Z-GPAL*G-pAB, a substrate cleaved on the carboxyl side

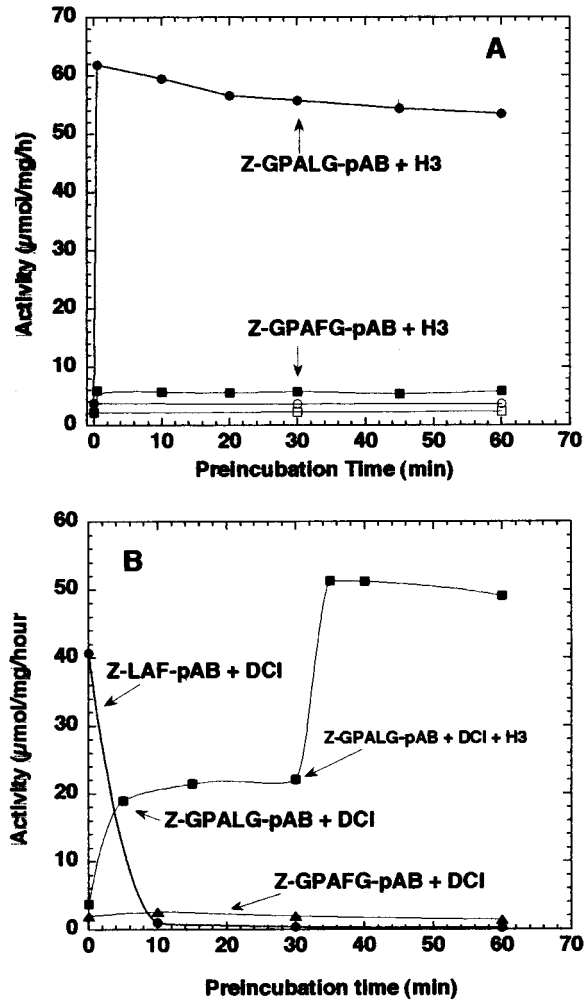

FIGURE 1: Selective activation of the branched chain preferring activity by histone H3 and 3,4-dichloroisocoumarin. Panel A: Preincubation mixtures (final volume 60 μL) contained the proteasome (10 μg), Tris-HCl buffer (0.066 M, pH 8.0), and histone H3 (84 μg). Controls contained the same amounts of enzyme and buffer but no H3. Preincubations were at 26 °C, and aliquots (3 μL) were withdrawn at the indicated times for determination of activity with Z-GPALG-pAB and Z-GPAFG-pAB (filled and open circles or squares represent experimental and controls, respectively). Panel B. The proteasome (50 μg) was preincubated with DCI (final concentration 25 μM), and aliquots (1 μL, 0.5 μg of enzyme) were withdrawn at the indicated times and used for determination of activity toward three different substrates, indicated in the panel. After 30 min exposure to DCI, 3.3 μg of H3/μg of proteasome was introduced in the preincubation mixture, and the effect on the activities was measured.

of the Leu residue. Activation was concentration dependent, with more than a 10-fold activation being obtained at 3 μg (1 μM) of histone in the presence of 0.5 μg of the proteasome. A further increase of histone concentration caused a decrease in activation. Examination of the reaction products showed exclusive cleavage of the Leu−Gly bond. To test the preference for the Leu residue in the P1 position, we examined the effect of preincubation of the proteasome with H3 on cleavage of Z-GPAFG-pAB, a substrate in which the Leu residue in Z-GPALG-pAB was replaced by a phenylalanine. This substrate, like the previous one, was again cleaved exclusively on the carboxyl side of the Phe residue. However, as shown in Figure 1 (panel A), preincubation of the substrates with H3 accelerated the degradation of the leucine containing substrate by more than 15-fold, whereas the rate of degradation of the Phe-containing substrate was hardly affected. A similar negative result was also obtained with a substrate in which the Leu residue was replaced by a Gly (Z-GPAGG-pAB; not shown).

The activation by H3 of cleavage on the carboxyl side of a Leu residue could be interpreted as the result of involvement of the ChT-L activity, a catalytic component of the proteasome known to cleave bonds after both aromatic and branched chain amino acids. The finding, however, that the activation occurs even when the ChT-L activity of the proteasome was completely inactivated argues against this assumption (Figure 1, panel B). Thus, the exposure of the proteasome to 25 μM 3,4-dichloroisocoumarin (DCI), an irreversible inhibitor of the ChT-L (known to be expressed by the X subunit), led to a rapid and complete inactivation of the ChT-L activity determined with Z-LAF*-pAB as substrate but resulted in activation of cleavage of the Leu-containing substrate, Z-GPAL*G-pAB. The possibility, however, could not be excluded that the activation by DCI could be a consequence of inactivation of the ChT-L activity. Such inactivation could promote the cleavage of the substrate by a catalytic activity of the proteasome other than the X subunit, for example, the Y subunit. This function of the Y subunit could replace the ChT-L activity and preserve thereby the ability of the proteasome to cleave bonds after hydrophobic amino acids even in the presence of an inactivated X subunit. The failure to increase by DCI the degradation of the Phe-containing substrate (Z-GPAF*G-pAB) observed under the same conditions (Figure 1, panel B) further indicated involvement of a catalytic activity different from that expressed by the ChT-L activity. Of interest is the finding that introduction of H3 into the incubation mixture containing DCI-inactivated ChT-L activity induced a further increase in the degradation rate of the Z-GPALG-pAB substrate. This suggests that inactivation of the ChT-L activity by DCI could be responsible for only a part of the activation of cleavage after Leu and that the activation by H3 is independent of the presence of a catalytically intact ChT-L component (Figure 1, panel B).

Two substrates, Z-GPAL*G-pAB and Z-GPAL*A-pAB, each containing a Leu residue in the P1 position but differing in the nature of the amino acid residue in the P1′ position were used to determine the dependence of activation on the H3 concentration and preincubation time. The extent of activation was concentration dependent with 3−4 μg of H3 (1−1.3 μM) producing optimal activation when measured with the Z-GPALG-pAB substrate and 4−6 μg of H3 (1.3−2 μM) being optimal for Z-GPALA-pAB substrate (Figure 2, panel A). At optimal concentrations of H3 up to 15-fold increases in activity were observed. The effect of preincubation of the enzyme with H3 on the activity measured with the above two substrates is shown in Figure 2, panel B. Although activation of the enzyme was almost immediate after addition of H3 to the reaction mixture, some further activity increases were usually observed with the increase in preincubation time. Thus, about 65% of the final activation was already evident after 30 s (average of six experiments), but the maximal activation was usually reached after about 15−20 min when determined with Z-GPALG-pAB as the substrate. When Z-GPALA-pAB was used as the substrate, a further slow increase in activity was seen even after 60

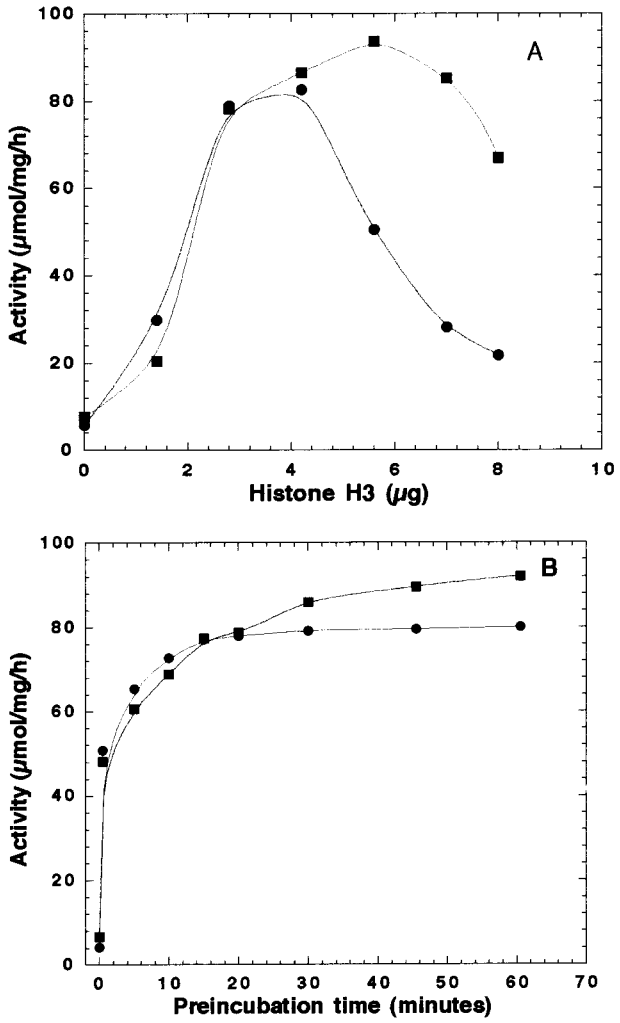

FIGURE 2: Time and concentration dependence of activation of the branched chain amino acid preferring activity by H3. Panel A: Reaction mixtures (final volume 0.2 mL) contained 1.8 nM proteasome and the indicated amounts of histone H3. Activity was determined with Z-GPALG-pAB (solid circles) and Z-GPALA-pAB (solid squares) at a concentration of 1 mM. Data are mean values obtained from two separate experiments. Panel B: Effect of preincubation with histone H3 on the branched chain preferring activity. The preincubation mixture, final volume 60 μL, contained 5 μg of proteasome, 70 μg of histone H3, and 20 μL of Tris-HCl buffer (pH 8.0) in a final volume of 60 μL. Aliquots of the preincubation mixture (3 μL) were withdrawn at the indicated times and tested for activity as in panel A. Preincubations were at 26 °C.

min of preincubation. The activity increase was still present even after 3 h of preincubation.

The kinetic parameters underlying the activation by H3 were examined using Z-GPALG-pAB as the substrate within the concentration range of 0.5−5 mM. Incubation mixtures (final volume 0.2 mL) contained enzyme (0.5 μg), H3 (3 μg), and Tris-HCl buffer (0.05 M, pH 8.0). Activity was measured without preincubation of the enzyme with H3 as described in Materials and Methods. Double reciprocal plots gave straight lines with correlation coefficients of 0.98−0.99. In the presence of histone the V_{max} increased from 26 to 95 μmol mg^{-1} h^{-1}, and the K_m decreased from 2.47 to 0.6 mM (average of four determinations), expressing both increased substrate affinity and a higher catalytic efficiency and resulting in an increase of 15-fold in the specificity constant (k_{cat}/K_m).

Table 2: Effect of H3 on Degradation of Natural Substrates by the 20 S Proteasome[a]

peptide	concn	proteasome (μg)	histone H3 (μg)	activity (nmol mg^{-1} h^{-1})
GnRH	0.05 mM	5.0	none	389 (9)
GnRH	0.05 mM	5.0	17.5	0 (4)
insulin B-chain	0.05 mM	2.5	none	143 (2)
insulin B-chain	0.05 mM	2.5	17.5	8930 (2)
β-casein	200 μg	5.0	none	1810 (2)[b]
β-casein	200 μg	5.0	11.5	5480 (2)[b]

[a] Incubation mixtures contained the indicated amounts of pituitary proteasome, histone H3, GnRH, and Tris-HCl buffer (0.05 M, pH 8.0) in a final volume of 200 μL. Incubations were at 37 °C. Aliquots of the incubation mixtures (40 μL) were withdrawn at 0, 60, 120, 180, or 240 min, and the reaction was stopped after addition of 5 μL of glacial acetic acid. The samples were subjected to HPLC on a C18 Delta-Pak column (3.9 × 150 mm, 5 μm). The column was eluted with a linear gradient established between 2% and 40% acetonitrile, each containing 0.1% trifluoroacetic acid. The rate of degradation was calculated from the decrease of the peak height of GnRH compared with that of the control containing the same reaction components but not histone H3. Degradation of the B-chain of insulin was determined as described in Materials and Methods. Numbers in parentheses indicate the number of independent experiments. [b] Values for casein are given in μg mg^{-1} h^{-1}.

Effect of Histone H3 on Degradation of Gonadotropin Releasing Hormone (GnRH) and Other Natural Substrates. The finding that histone H3 inhibits the activities of the pituitary proteasome, as measured with synthetic peptidyl−arylamide substrates, but activates cleavage between adjacent two amino acids after a Leu residue posed the question whether these effects change the specificity of the proteasome toward natural substrates. To test this possibility, we examined the effect of H3 on the degradation of GnRH, a natural peptide known to be degraded mainly by the ChT-L component of the proteasome. Previous work in our laboratory has shown that degradation of GnRH by the pituitary proteasome proceeds mainly by cleavage of the Tyr5−Gly6 and Trp3−Ser4 bonds (*1*). That these cleavages are catalyzed by the ChT-L activity was indicated by results of experiments showing that virtually complete inhibition of these reactions can be obtained by short exposure of the proteasome to DCI. This agent is known to rapidly inactivate the ChT-L activity of the proteasome, at a time and concentration that does not materially affect the PGPH and T-L activities. The main sites of cleavage of GnRH degradation by the pituitary proteasome are shown below (arrows indicating sites of cleavage).

pGlu-His-Trp▼-Ser-Tyr▼-Gly-Leu-Arg-Pro-Gly-NH2

The effect of H3 on the degradation of GnRH is shown in Table 2. At concentrations and under conditions described in the legend, the main site of degradation was cleavage of the Tyr5−Gly6 bond with a slowly following cleavage of the Trp3−Ser4 bond. The reaction proceeded in the absence of H3 at an average rate of 389 nmol mg^{-1} h^{-1}. Introduction into the incubation mixture of H3 abolished the reaction, and no degradation products were obtained even after a 3 h incubation. This indicates that the presence of H3 inhibits the involvement of the ChT-L activity in the degradation of GnRH.

The rate of degradation of the oxidized B-chain of insulin by the pituitary enzyme in the presence and absence of

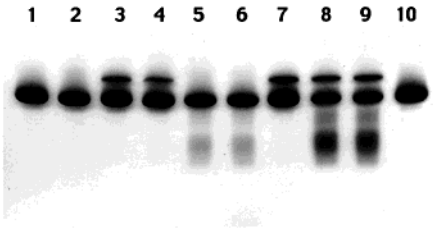

FIGURE 3: Degradation of lysozyme by the 20 S proteasome. Incubation mixtures, final volume 60 μL, contained 120 μg of lysozyme and, where indicated below, 2.5 μg of the 20 S proteasome, 8 μg of histone H3, and 5 mM DTT. After 4 h of incubation the reaction was terminated by addition of sodium dodecyl sulfate (final concentration 1%), 2-mercaptoethanol (2.5%), and bromophenolblue as marker. The mixture was boiled for 3 min, and 10 μL was subjected to PAGE under dissociating conditions. All lanes contained lysozyme. Lanes 3, 4, 7, 8, and 9 contained, in addition, H3. Lanes 5−10 contained DTT, and lanes 2, 4−6, 8, and 9 contained the proteasome.

histone H3 is summarized in Table 2. The untreated enzyme cleaves the peptide very slowly, with little degradation seen even after 3 h of incubation. Introduction of histone H3 into the incubation mixture caused a rapid hydrolysis of the peptide, leading to an almost complete degradation within 30 min. Indeed, the rate of degradation in the presence of the activator increased by more than 60-fold in comparison with that obtained in the absence of H3. The main cleavage products were isolated by HPLC and subjected to mass spectrometry. The cleavage sites deduced from the masses of the products in the presence of histone H3 are shown below: They include peptide bonds on the carboxyl side of two acidic residues, one a cysteic acid residue and the other a Glu residue, five cleavages after branched chain amino acid residues, and one cleavage after a histidine residue. Thus, the presence of H3 promotes mainly cleavages after acidic and branched chain amino acid residues. The cleavage after histidine suggests a possible involvement of the T-L activity in the degradation of the peptide.

F-W-N-Q-H▼-L-▼C$_{SO3H}$-G-S-H-L-▼V-▼E-▼A-L-Y-L-▼V-▼C$_{SO3H}$▼-G-E-R-G-F-F-Y-T-P-K-A

The finding that the presence of histone H3 greatly accelerates degradation of the B-chain of insulin induced us to determine whether protein degradation will also be accelerated by H3. Two proteins, β-casein and lysozyme, were chosen as substrates. Casein is an unfolded protein, known to be degraded by the 20 S proteasome. Degradation of the two proteins was determined as described under Materials and Methods, and the results are summarized in Table 2. The presence of histone H3 accelerated the degradation of casein by more than 3-fold when compared with that obtained in absence of the histone. Lysozyme is a protein resistant to degradation by the enzyme and is indeed commonly used as substrate for following ubiquitin-dependent degradation of proteins by the 26 S proteasome. As shown, however, exposure of this protein to DTT, a thiol reducing agent, makes the protein susceptible to degradation by the 20 S proteasome, apparently because breaking of the disulfide linkages induces unfolding of the protein. The SDS−PAGE pattern obtained after incubation of lysozyme with the 20 S proteasome in the presence and absence of H3 or DTT is shown in Figure 3 (compare lanes 5 and 6 with lanes 8 and 9). Degradation required the presence of DTT and the proteasome and was accelerated by the presence of H3. Densitometry of the results using the NIH IMAGE program (version 1.6 for MacIntosh) has shown that the presence of H3 accelerates the degradation of lysozyme by about 2-fold. No degradation occurred when lysozyme was incubated alone or incubated with the 20 S proteasome in the absence of DTT but in the presence of histone H3. No degradation of H3 by the proteasome was observed either in the absence or in the presence of DTT.

The HPLC pattern of lysozyme degradation products obtained in the presence and absence of H3 (panels A and B, respectively) is shown in Figure 4. Both the quantity and nature of degradation products is markedly different in the presence than in the absence of H3. It is notable that relatively high molecular fragments of the protein were generated especially in the presence of H3, as indicated by the relatively high retention time of the main degradation fragments. A clear difference is also notable between the nature and retention times of protein fragments generated in the presence of H3 compared with those obtained in its absence. For example, a cluster of at least seven relatively high peaks with close elution times between 32.44 and 37.66 min is visible in panel B but not in panel A. Although the amino acid sequences of these degradation products remain to be examined, the elution time of the fragments and the height of the peaks are sufficiently different to provide support to the conclusion that the presence of H3 causes marked changes in the specificity of the proteasome.

DISCUSSION

The results presented here show that the arginine-rich histone H3 is at low micromolar concentrations a potent and selective activator of the 20 S proteasome. Preliminary examination of crude histone preparations (available from Sigma Chemical Co.) indicated that only those containing arginine-rich histones H3 and H4, but not the lysine-rich histones, share this property. It is notable that none of the three classical activities, the ChT-L, T-L, and PGPH, are activated when assayed with chromogenic, peptidyl−arylamide substrates. Indeed, all of these activities are inhibited to a different extent by the presence of histone H3, the activation being apparently limited to cleavage of bonds only between two adjacent amino acids in both synthetic and natural peptides. It is possible that because of use of peptidyl−arylamide substrates and high concentrations of crude histone preparations the nature and selectivity of the H3 effect could have escaped previous observations (*27*).

A series of polycationic substances, including polylysine, polyarginine, protamine, histone H1, and even Mg^{2+}, have been previously shown (*28*) to activate degradation of casein by the proteasome. The relationship of this effect to that described here for histone H3 cannot be evaluated, if only because the multiple degradation products generated from casein in the presence of polylysine and histone H3 are not known at present, and their analysis is beyond the scope of this report. The effects, however, of polylysine and histone H3 on the proteasome differ in several respects. Thus, preincubation (100 min) of the proteasome at 37 °C with polylysine led to progressive proteolytic inactivation of the enzyme, loss in intensity of staining of proteasome bands in

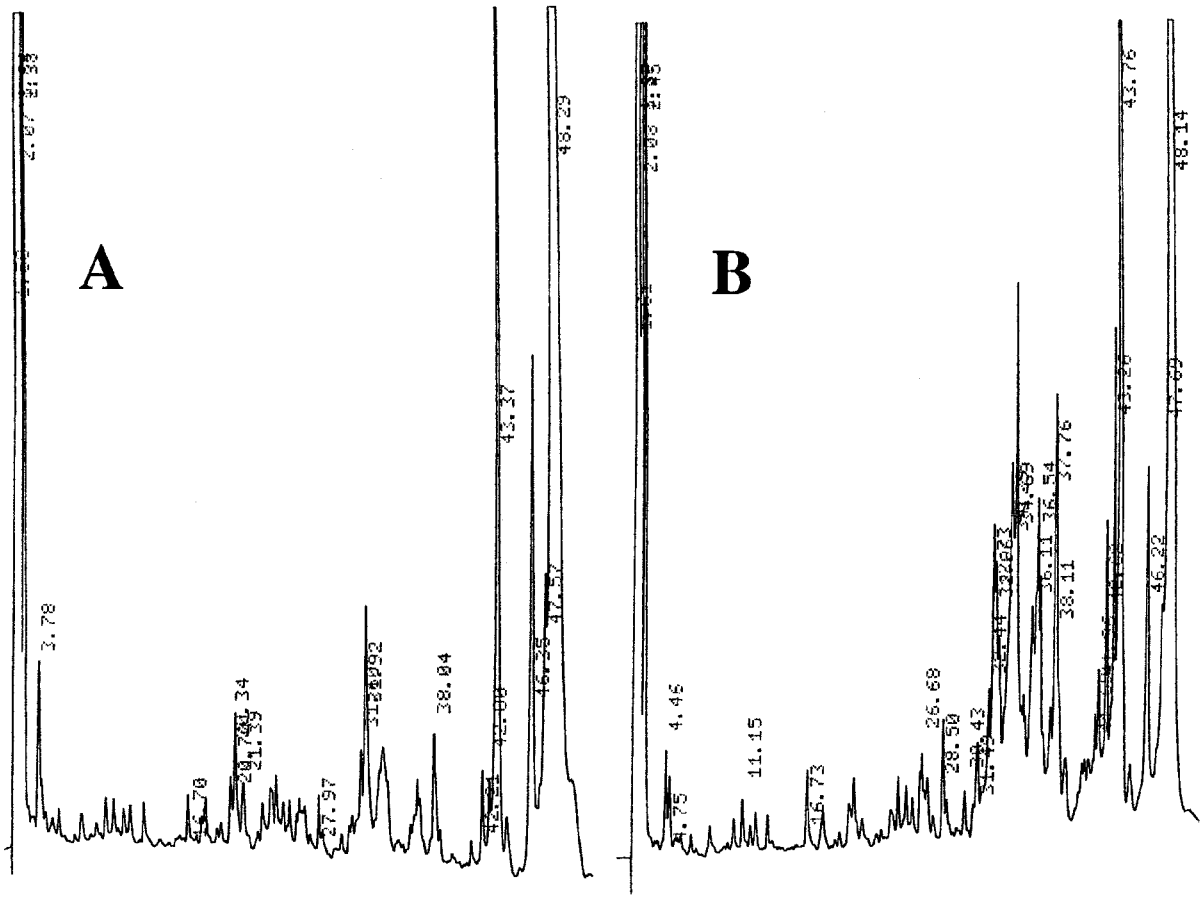

FIGURE 4: HPLC of lysozyme degradation products. Both panel A and panel B contained lysozyme, DTT (10 mM), the proteasome, and buffer at concentrations identical to those in Figure 3. Only panel B contained, in addition, histone H3. Incubations were for 4 h at 37 °C. The reactions were terminated by addition of 5 μL of glacial acetic acid, and 40 μL of the mixtures was subjected to HPLC using a C18 Delta-Pak column (5 μm, 3.9 × 150 mm; Waters). Elution was carried out with a linear gradient between buffer A (0.1% trifluoroacetic acid solution in water) and buffer B (0.085% trifluoroacetic acid in 90% acetonitrile). The concentration of buffer B was increased linearly from 10% to 50% in 50 min at a flow rate of 1 mL/min.

SDS−PAGE, a time-dependent loss of caseinolytic activity, and an associated loss of a 23 kDa subunit (*28*). These findings suggest an autolytic effect of polylysine. In our experiments, a 4 h preincubation of the proteasome with H3 under conditions used for lysozyme degradation generated no loss in staining intensity of the proteasome subunits (in SDS−PAGE), nor was any loss of subunits observed. Furthermore, a 120 min preincubation with H3 did not decrease the activation of the catalytic activity of the enzyme toward Z-GPALG-pAB, a substrate of the BrAAP activity (data not shown).

Examination of degradation products of two synthetic substrates and the oxidized B-chain of insulin showed preferential cleavage after branched chain and acidic amino acids such as glutamic and cysteic acid, both cleavages presumably catalyzed by the Y subunit. That secondary interactions between the enzyme and amino acids beyond those forming the scissile bond are also a factor affecting the nature of cleavage sites is indicated by the finding that not all bonds after acidic and branched chain amino acids are equally susceptible to cleavage. The selectivity of the activation is apparently also responsible for the specificity changes observed in experiments with GnRH and lysozyme. That this is indeed the case is evident from the finding that degradation of GnRH, that in the absence of H3 involves cleavage by the ChT-L activity of bonds after two aromatic amino acids Tyr5 and Trp3, is inhibited in the presence of H3. That the presence of histone H3 causes major changes in the specificity of the proteasome is also evident from examination of the degradation products of lysozyme generated in the presence of H3 (Figure 4). Although detailed identification of the amino acid sequences of these products remains to be determined, the HPLC pattern indicates that products generated in the presence of H3 are different than those in the absence of the histone. Accumulation of degradation products with longer retention times, and apparently higher molecular masses, seems to be characteristic for the presence of H3. Identification of the amino acid sequences of the products generated in the presence of H3 should therefore provide further information on both the primary and secondary specificity changes induced by the presence of H3.

Optimal concentrations of H3 necessary for activation were close to those reported for the PA28 (11 S regulator) activator (*29*). However, unlike this activator which increases the activity of all catalytic components of the proteasome (*30*), the effect of H3 is limited to bonds after branched chain and acidic amino acids. Also, unlike the 11 S regulator, the activation is not limited to small peptide substrates but is also effective toward proteins, such as β-casein and lysozyme and larger peptides, such as the oxidized B-chain of insulin. The association of inhibition of the ChT-L activity by DCI

and histone H3 with activation of cleavages after acidic and branched chain amino acids poses the question whether such inhibition is sufficient to activation. A similar inhibition, however, of the ChT-L activity by heat shock protein 90 was shown not to be associated with either activation or inhibition of the branched chain amino acid preferring activity, nor was the degradation of the oxidized B-chain of insulin activated by heat shock protein 90 (*9*). The selectivity of proteasomal activation by histone H3 suggests conformational changes different from those attributed to heat shock protein 90. Access of peptide or protein substrates to the inner chamber of the proteasome, where the active sites are positioned, is limited to two narrow openings at the two ends of the barrel-shaped complex. The access is partially occluded by N-terminal extension of the α-subunits. Some narrow openings are also present at the interface between the α- and *β*-rings, but their functional significance is not known (*11*). The finding that activation of the proteasome by histone H3 is associated with an increase in V_{max} and a decrease in K_m suggests facilitated access of the substrate to the active sites, probably due to widening of some of the openings leading to the catalytic chamber. The restriction of the activation to cleavage of bonds after acidic and branched chain amino acids suggests the involvment of the Y subunit, the only subunit capable of cleaving both such bonds. It is notable that the presence of H3 activates cleavages after acidic amino acids in the oxidized B-chain of insulin but inhibits cleavage after a glutamate residue in Z-LLE-2NA, a synthetic substrate commonly used for determination of the PGPH activity (Y subunit). This suggests that conformational changes induced by H3 prevent binding of the glutamic acid containing substrate, possibly because of the balkiness of the naphthylamide group.

The ChT-L activity is one of the most active catalytic components of the proteasome (*18*). Its inhibition leads to major functional changes of the MPC, as expressed by the accumulation in cells of a series of short-lived regulatory proteins when cells are exposed to such active site-directed inhibitors as peptidyl aldehydes or lactacystin. Indeed, longer exposure of cells to these inhibitors leads to cell death. The association of inactivation of this activity by DCI with activation of cleavages after branched chain amino acids could therefore be considered as a rescue or replacement mechanism that maintains the ability of the proteasome to hydrolyze bonds after hydrophobic residues.

The localization of histones in the nucleus poses the question of the functional significance of proteasomal activation in this compartment. Although this question remains to be answered, it is notable that proteasomes are found "loosely attached to chromatin", from which they can be released by nucleases (*31*). Although the concentration of proteasomes in the nucleus was reported to be only 10% of that present in cytoplasm, its involvement in degradation of a number of short-lived proteins such as cyclins, oncogene products, and transcription factors and mitosis in general is well documented (*30*) Evidence was also reported that degradation of a protein was more rapid when localized in the nucleus than in the cytoplasm (*32*) and that nuclear antigens are efficiently presented by MHC class I molecules (*33*). Furthermore, a recent study has shown that ubiquitination is not required for the rapid degradation by the proteasome of the Cdk inhibitor $p21^{Cip1}$ in the nucleus (*34*, *35*). These and other findings make it tempting to contemplate that the ubiquitin-independent selectivity of activation by the H3 histone, being restricted only to the nucleus, could represent a mechanism by which functional changes of an enzyme system could be limited only to a specific cellular compartment.

ACKNOWLEDGMENT

I thank Dr. Sherwin Wilk for help and critique during preparation of the manuscript and Charlene Michaud and Xianghan Lu for technical help.

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BI0116240