

Evidence for the Existence of a Non-catalytic Modifier Site of Peptide Hydrolysis by the 20 S Proteasome*

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The 20 S proteasome is an endoprotease complex that preferentially cleaves peptides C-terminal of hydrophobic, basic, and acidic residues. Recently, we showed that these specific activities, classified as chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide-hydrolyzing (PGPH) activity, are differently affected by Ritonavir, an inhibitor of human immunodeficiency virus-1 protease. Ritonavir competitively inhibited the chymotrypsin-like activity, whereas the trypsin-like activity was enhanced. Here we demonstrate that the Ritonavir-mediated up-regulation of the trypsin-like activity is not affected by specific active site inhibitors of the chymotrypsin-like and PGPH activity. Moreover, we show that the mutual regulation of chymotrypsin-like and PGPH activities by their substrates as described previously by a "cyclical bite-chew" model is not affected by selective inhibitors of the respective active sites. These data challenge the bite-chew model and suggest that effectors of proteasome activity can act by binding to non-catalytic sites. Accordingly, we propose a kinetic "two-site modifier" model that assumes that the substrate (or effector) may bind to an active site as well as to a second non-catalytic modifier site. This model appears to be valid as it describes the complex kinetic effects of Ritonavir very well. Since Ritonavir partially inhibits major histocompatibility complex class I restricted antigen presentation, the postulated modifier site may be required to coordinate the active centers of the proteasome for the production of class I peptide ligands.

The 20 S proteasome was purified 20 years ago as a cation-sensitive neutral endopeptidase from bovine pituitary tissue and was originally named multicatalytic proteinase complex (MCP) (1–3). This nomenclature was chosen because this 700-kDa protease complex was able to cleave fluorogenic peptide substrates C terminus of hydrophobic, basic, and acidic residues. Therefore the three activities that could be selectively silenced with different inhibitors were referred to as chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide-hydro-

lyzing (PGPH).¹ The structural basis for the peptidolytic properties of the 20 S proteasome has meanwhile been elucidated by mutagenesis experiments and x-ray crystallography on 20 S proteasomes from archaebacteria (4, 5), yeast (6, 7), and mammalian cells (8, 9). The 20 S proteasome has the shape of a barrel composed of four stacked rings. The outer two rings each consist of seven different subunits of the α -type, whereas the inner two rings are formed from seven different subunits of the β -type. Three of the seven β -subunits in each ring, designated delta (β 1), MB1 (β 5), and Z (MC14, β 2) bear the peptidolytic active sites of the eukaryotic 20 S proteasome as they possess threonine residues at their N termini, the γ -hydroxy groups of which act as nucleophiles in the attack of peptide bonds. The site-directed mutagenesis of each of these subunits in *Saccharomyces cerevisiae* has shown that the chymotrypsin-like, the trypsin-like, and the PGPH activity can be assigned to the subunits β 5, β 2, and β 1, respectively. According to mutagenesis and inhibitor experiments, the same assignment is valid for mammalian proteasomes, although the substrate specificity of proteasome subunits seems to be less stringent in mammals (8–10). Active sites other than those at the N termini of β 1, β 2, and β 5 are unlikely to exist in the proteasome, since the peptide aldehyde inhibitor *N*-acetyl-leucyl-leucyl-norleucinal, which interferes with all peptidolytic activities of the proteasome, binds exclusively to these subunits (7).

The x-ray crystallographic structure of the *S. cerevisiae* proteasome revealed that the α and β subunits of the proteasome make intimate and numerous molecular contacts within and in between the α and β rings. This may explain why regulators of the 20 S proteasome, like the 19 S regulator (PA700) (11) or the 11 S regulator (PA28) (12), which bind to the α -end plates of the 20 S proteasome, differentially affect the catalytic activities residing on the different β -type subunits of the proteasome. Consistently, evidence has been obtained that the 20 S proteasome is a cooperative enzyme. The hydrolysis of the substrates Suc-LLVY-MCA (MCA, 7-amido-4-methylcoumarin) and (Z)-LLE- β NA, which are frequently used to monitor the chymotrypsin-like and PGPH activities of the proteasome, show sigmoidal kinetics with a Hill coefficient of about 2, suggesting that there is an allosteric regulation between two active centers of the same substrate specificity (13–17). A detailed kinetic analysis of the chymotrypsin-like activity of the proteasome suggested that the proteasome has two cooperative active sites for the Suc-LLVY-MCA substrate and that binding to one active site induces an interconversion between two conformers of the 20 S proteasome (18). Interestingly, Kisselev *et al.* (19)

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¹ The abbreviations used are: PGPH, peptidylglutamyl peptide-hydrolyzing; Ac, acetyl; Boc, *t*-butyloxycarbonyl; MCA, 7-amido-4-methylcoumarin; MHC, major histocompatibility complex; β NA, β -naphthylamide; pNA, *para*-nitroanilide; 4mna, 4-methoxy- β -naphthylamide; (Z) benzoyloxycarbonyl; Bz, benzoyl; Suc, succinyl.

recently obtained evidence that substrates of one active center can influence the activity at a different active center. They found that the PGPH activity is enhanced in the presence of substrates of the chymotrypsin-like activity and that, conversely, the chymotrypsin-like activity is inhibited in the presence of substrates for the PGPH activity. Since the rate of enhancement or inhibition at one site correlated with substrate saturation at the other site, it was proposed that a mutual allosteric regulation between the active centers responsible for the chymotrypsin-like and the PGPH activity exists. A cyclical bite-chew model was proposed in this study (19), which implies that the chymotryptic activity initially cleaves substrates (to bite), which stimulates the PGPH activity for the performance of subsequent cleavages in the generated fragments (to chew). As long as the PGPH site is occupied, further "bites" at the chymotryptic site are blocked to prevent a supply of new fragments before the PGPH site is free again.

In the present work, we present data that are difficult to rationalize by allosteric interactions among the various active sites and, thus, require an alternative mechanistic explanation. These experiments are based on a previously published observation that an inhibitor of the human immunodeficiency virus-1 protease, named Ritonavir, modulated proteasome activity in that the chymotrypsin-like activity was inhibited, whereas the trypsin-like activity was enhanced (20, 21). A satisfactory quantitative description of the rather complex and seemingly erratic kinetic data on the effect of Ritonavir of the various specific activities of the 20 S proteasome can be obtained by using a two-site modifier model, which assumes that Ritonavir may bind to both an active site and to a second, non-catalytic modifier site. Obviously, this kinetic model cannot make structural inferences whether the modifier site represents a second active site or a novel independent modifier binding site of the proteasome. We addressed this question by utilizing the proteasome inhibitors lactacystin (22), which has been shown to covalently modify the N-terminal threonine residue of the $\beta 5$ subunit (7, 22, 23) and the caspase-1 inhibitor Ac-YVAD-CHO, which selectively inhibits the proteasomal PGPH activity (19). The up-regulation of the trypsin-like activity through Ritonavir remained unchanged in the presence of these inhibitors, indicating that Ritonavir exerts the stimulating effect on the trypsin-like activity by interaction with a site that is not identical with the chymotrypsin-specific or PGPH-specific active site. Intriguingly, the above-mentioned kinetic effects reported by Kisselev *et al.* (19) as basis for their bite-chew model were not affected in the presence of these inhibitors, indicating that these phenomena are not due to a mutual allosteric control of the active centers being in charge of the PGPH and chymotrypsin-like activity. Hence, our data challenge the bite-chew model and suggest that the kinetics of peptide hydrolysis by the 20 S proteasome is brought about by a modulation of the various specific activities by a so-far unidentified modifier site.

MATERIALS AND METHODS

Purification of 20 S Proteasomes—A new and optimized protocol for the purification of 20 S proteasomes from mouse liver was used. 15 mouse livers were minced on ice and homogenized in a blender with 1 volume of buffer A (100 mM KCl, 5 mM MgCl₂, 10 mM Hepes, pH 7.2) supplemented with 0.1% Triton X-100. The homogenate was centrifuged at 30,000 $\times g$ for 30 min, and the supernatant was bound to a 20-ml packed volume of DEAE-Sephacel (Amersham Pharmacia Biotech) at 4 °C for 12 h under agitation. After washes with 5 column volumes of buffer A, elution was performed with buffer B (500 mM KCl, 5 mM MgCl₂, 10 mM Hepes, pH 7.2) and 2-ml fractions were collected. The fractions with high protein content were pooled and precipitated with ammonium sulfate at 35% saturation under agitation on ice. The precipitate was pelleted at 17,000 $\times g$ for 20 min, and the supernatant was loaded onto a phenyl-Sepharose column and eluted on a fast

protein liquid chromatography system (Amersham Pharmacia Biotech) using a gradient of ammonium sulfate in buffer A from 35% to 0% saturation. The fractions were tested for hydrolysis of the (Z)-GGL-MCA substrate, and active fractions were pooled and brought to 80% saturation with ammonium sulfate on ice. The precipitate was pelleted at 12,000 $\times g$ for 10 min, and the pellet was redissolved in 4 ml of buffer A and loaded onto a 15% to 40% sucrose gradient. After centrifugation in a SW28 rotor at 28,000 rpm for 36 h (10¹² $\omega^2 t$ (ω is the angular velocity)), 600- μ l fractions were collected, and fractions with (Z)-GGL-MCA-hydrolyzing activity were pooled and diluted 1:5 in buffer A before they were loaded onto a 6-ml resource Q column. For elution a salt gradient of 0.1 M to 1 M KCl in 5 mM MgCl₂/10 mM Hepes, pH 7.2, was applied. Fractions with (Z)-GGL-MCA-hydrolyzing activity contained 20 S proteasome at apparent homogeneity both on Coomassie and silver-stained SDS-polyacrylamide gel electrophoresis. The average yield was determined by A_{280} (extinction coefficient of 1.0 cm²/mg) to be 7 mg of proteasome/15 mouse livers.

Assays for Proteasome Activity—The 20 S proteasome was purified from mouse liver as described above. The fluorogenic peptide substrates Bz-VGR-MCA, Boc-LGR-pNA, Z-GGL-MCA, Suc-LLVY-MCA, and Suc-FLF-4mna (Bachem, Bubendorf, Switzerland) were diluted from 10 mM frozen stock solutions in *N,N*-dimethylformamide. For the (Z)-LLE- β NA substrate, a stock solution was prepared freshly each time in Me₂SO. Assays were performed at 37 °C in a total volume of 100 μ l of buffer S (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA) containing 1 μ g of purified 20 S proteasome when using Boc-LGR-pNA or 250 ng of 20 S proteasome for all other substrates. Fluorescence was determined with a SpectraFluor Plus plate reader (TECAN, Grödig, Austria) at 30, 60, and 90 min after initiation of the reaction using the wavelengths 360 nm (emission)/465 nm (excitation) for MCA and 340 nm (emission)/405 nm (excitation) for β NA and 4mna. Release of the pNA group was analyzed with the same instrument by measuring the absorbance at 405 nm. In the concentration range used, the measurements of pNA, β NA, and 4mna do not interfere with the detection of MCA. Values presented have been obtained after 60 min and were in the linear range of the reaction; triplicates were measured for all data points.

The inhibitor Ac-YVAD-aldehyde (Bachem) was dissolved in Me₂SO, and lactacystin was dissolved in phosphate-buffered saline. Ritonavir (Abbott) was diluted with CH₃OH to a concentration of 50 mM and further diluted with Me₂SO. The inhibitors were added simultaneously with the substrates at the initiation of the reaction.

RESULTS

Previously we found that the human immunodeficiency virus-1 protease inhibitor Ritonavir (24) modulated the activities of the 20 S proteasome in an unprecedented fashion. Although the chymotrypsin-like activity of the proteasome, as measured by the hydrolysis of the substrate Suc-LLVY-MCA, was inhibited by Ritonavir with an IC₅₀ value of 3 μ M, the trypsin-like activity measured with the substrate Bz-VGR-MCA was enhanced in a dose-dependent fashion (21). Consistent with a competitive inhibition of the chymotrypsin-like activity, we found that the subunit $\beta 5$ (MB1 or LMP7) was selectively protected from covalent modification by a radioactively labeled vinyl sulfone inhibitor of the proteasome (21). To determine the kinetic parameters of this inhibition and to better understand the enhancement of the trypsin-like activity through Ritonavir, we attempted to create a kinetic model of Ritonavir-mediated modulation of proteasome activity.

Kinetic Model

For the interaction of the proteasome with small peptide substrates in the presence of an effector, a two-site modifier model was applied that assumes the existence of two distinct binding sites, an active site and a modifier site. Both the peptide substrate and the effector may competitively bind to these two sites. Cleavage of the peptide requires its binding to the active site. The cleavage rate is determined by the occupation state of the modifier site. Since the modifier site may be empty or occupied either by a second peptide substrate or by the effector, one has to distinguish three different cleavage rates V_i ($i = 1, 2, 3$) as depicted in the scheme in Fig. 1.

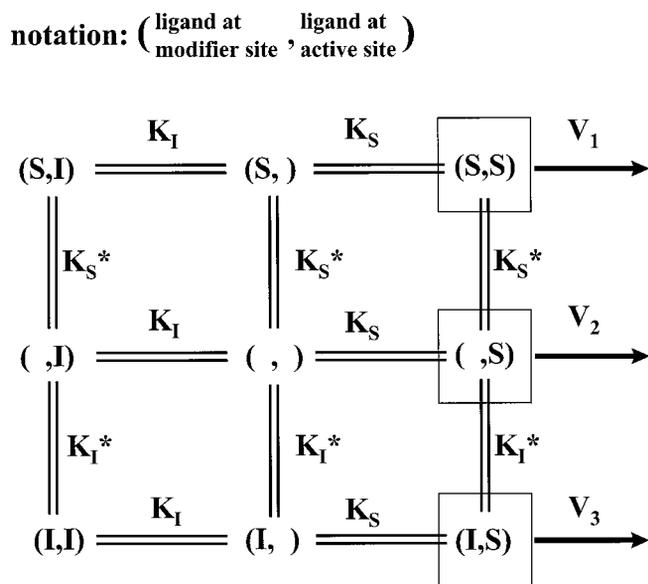


FIG. 1. **Binding scheme for the two-site modifier model used in this study.** Both the peptide and the effector may competitively bind to a modifier site (*left*) or the active site (*right*). The dissociation constants for binding of the peptide and the effector are denoted as K_S^* and K_I^* for binding to the modifier site and as K_S and K_I for binding to the active site. Depending on the occupation of the modifier site, the cleavage rates are denoted V_1 , V_2 , or V_3 .

In this scheme the possible binding states of the enzyme are shown in parenthesis and are separated by a comma; the left symbol refers to the occupation of the modifier site, and the right symbol refers to the occupation of the active site (if a site is unoccupied, there appears no symbol). The dissociation constants for the binding of the peptide and the effector are denoted by K_S^* and K_I^* for the binding to the modifier site and by K_S and K_I for the binding to the active site. For simplicity, it is assumed that the occupation state of the modifier site may only affect the activity of the enzyme without altering the affinity of the active site. Then the steady-state rate equation reads,

$$V = \frac{(V_1 + V_2 \frac{[S]}{K_S^*} + V_3 \frac{[I]}{K_I^*}) [S]}{(1 + \frac{[S]}{K_S} + \frac{[I]}{K_I}) (1 + \frac{[S]}{K_S^*} + \frac{[I]}{K_I^*})} \quad (\text{Eq. 1})$$

Depending on the relative proportion between the rate constants V_1 , V_2 , and V_3 , the model may describe various types of $V(S)$ characteristics observed on experiments with small fluorogenic peptides (Fig. 2).

Case I ($I = 0$, Absence of an Effector)—The ratio $V_1:V_2$ determines whether the $V(S)$ characteristics are hyperbolic ($V_1 > V_2$; Fig. 2A), sigmoid-monotone ($V_2 > V_1$; Fig. 2, C and D), or non-monotone, *i.e.* displaying substrate inhibition ($V_1 > V_2$; Fig. 2B).

Case II ($I > 0$, Presence of an Effector)—Upon binding to the modifier site, the effector may act as activator (Fig. 2A) or inhibitor (Fig. 2, B and C); upon binding to the active site, it competes with the substrate and, thus, acts as a competitive inhibitor. The modes of action exerted upon binding to the active site and the modifier site may be controversial. For example, if $V_2 > V_1$ and $V_3 > V_2$ (Fig. 2D), binding of the effector to the active site results in a competitive inhibition, whereas occupation of the modifier site activates the enzyme. For high concentrations of the effector, superposition of these two antagonistic effects may give rise to a $V(S)$ characteristic, which intersects the $V(S)$ curve obtained in the absence of the effector (*dotted* and *solid* curves in Fig. 2D).

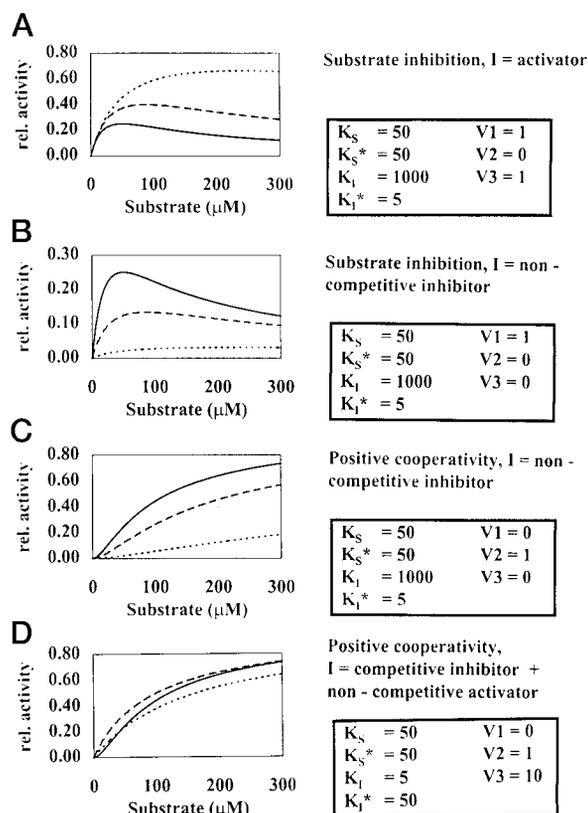


FIG. 2. **Theoretical activities obtained by the two-site modifier model for substrate inhibition or positive cooperativity.** The model parameters K_S , K_S^* , K_I , K_I^* , V_1 , V_2 , and V_3 that were used for the generation of theoretical curves (*left*) are listed to the right of each set of curves. The absence of an inhibitor (or modulator) ($I = 0 \mu\text{M}$) is shown by *solid lines*, and the presence of an inhibitor (modulator) at $I = 10 \mu\text{M}$ is shown by *dashed lines* and, at $I = 100 \mu\text{M}$, by *dotted lines*. A, the substrate displays self-inhibition; Ritonavir acts as an activator upon binding to the modifier site ($V_3 = 1$) but does not compete with the substrate at the active site. The higher the concentration of Ritonavir, the more active is the proteasome. B, the substrate displays self-inhibition; Ritonavir acts as an inhibitor upon binding to the modifier site ($V_3 = 0$). The higher the concentration of Ritonavir, the more inhibited is the proteasome. C, there is positive cooperativity between the active sites ($V_2 = 1$); Ritonavir and substrate act as inhibitors upon binding to the modifier site ($V_1 = 0$, $V_3 = 0$). Enhancing the concentration of Ritonavir leads to an increasingly inactive proteasome. D, the active sites display positive cooperativity ($V_2 = 1$), and the inhibitor can act as a competitive inhibitor as well as a non-competitive activator ($V_3 = 10$). For high concentrations of the effector, superposition of these two antagonistic effects may give rise to a $V(S)$ characteristics that intersect the $V(S)$ curve obtained in the absence of the effector.

Estimation of Model Parameters for Four Fluorogenic Peptides

Numerical values for the seven unknown parameters (four dissociation constants and three velocities) of the rate equation 1 have been estimated by fitting the rate equation to kinetic data obtained with four different fluorogenic peptides as reported in Schmidtke *et al.* (21). Both types of kinetics (*i.e.* varying the substrate concentration at fixed concentrations of Ritonavir (Fig. 3) and varying the concentration of Ritonavir at fixed concentrations of substrate (Fig. 4)) have been simultaneously involved in the regression analysis for a certain peptide substrate. Adjustment of the model to the experimental data by non-linear regression analysis was performed by using the software package SIMFIT (25). The obtained parameter combinations are shown in Table I, and the associated theoretical curves (plotted as *lines*) and experimental data (*filled symbols*) are depicted in Fig. 3 (substrate titration) and Fig. 4 (Ritonavir titration).

FIG. 3. Comparison of experimental data and theoretical values obtained by the two-site modifier model for the influence of substrate concentration on proteasome modulation by Ritonavir. The experimental data are taken from Fig. 5 of Schmidtke *et al.* (21) with the experimental concentrations of Ritonavir: 0 μM (squares), 1 μM (triangles), 10 μM (diamonds), and 100 μM (circles). The theoretical curves were obtained by the two-site modifier model with the fitted parameters for K_S , K_S^* , K_I , K_I^* , V_1 , V_2 , and V_3 for A–D as shown in Table I and the same effector concentrations as applied experimentally: 0 μM (solid lines), 1 μM (dotted + dashed line), 10 μM (dashed line), and 100 μM (dotted line).

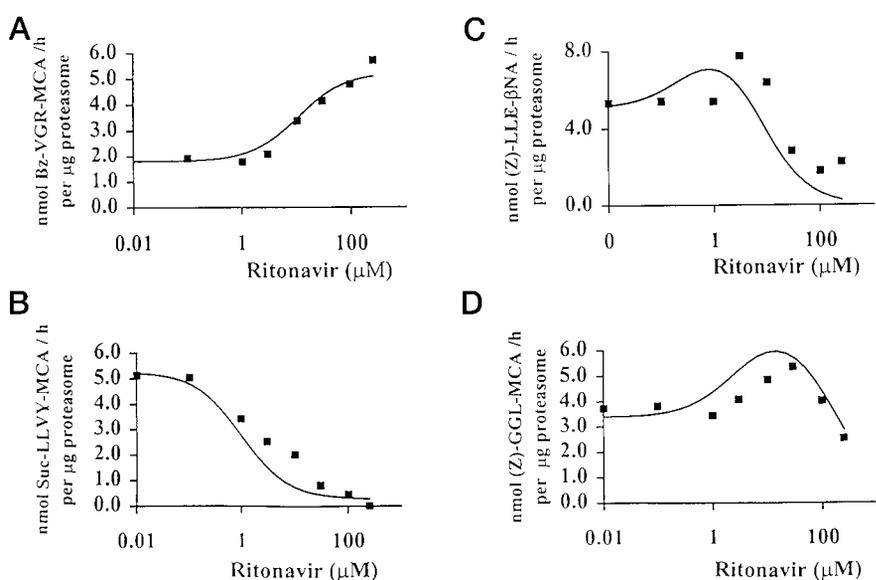


FIG. 4. Comparison of experimental data and theoretical values obtained by the two-site modifier model for the Ritonavir (effector)-mediated modulation of proteasomal hydrolysis of four fluorogenic peptides. The experimental data are taken from Fig. 4 of Schmidtke *et al.* (21) (filled boxes), and the theoretical curves (solid line) were obtained with the same substrate concentrations as applied experimentally, and fitted parameters for K_S , K_S^* , K_I , K_I^* , V_1 , V_2 , and V_3 for A–D are as shown in Table I.

TABLE I
Estimates for kinetic parameters of the modulation of proteasome activity by Ritonavir

P	Substrate	K_S	K_S^*	K_I	K_I^*	V_1^a	V_2^a	V_3^a
		μM	μM	μM	μM			
A	Bz-VGR-MCA	71.09	9.10	1×10^5	2.89	0.0	0.95	2.02
B	Suc-LLVY-MCA	358.22	44.56	0.41	181.91	6.49	0.0	71.02
C	(Z)-LLE- β NA	No estimate	No estimate	0.24	8.06	0.016	5.4×10^{-5}	0.17
D	(Z)-GGL-MCA	75.92	141.09	1.56	129.38	0.0	3.25	118.5

^a The reaction velocities are given in nmol of product/h/ μg of proteasome. The listed values were used for the generation of theoretical curves in Figs. 3 and 4. For the substrate (Z)-LLE- β NA, no values for K_S and K_S^* could be estimated because of lacking substrate saturation. Therefore, values could only be estimated for the ratios V_1/K_S , $V_2/K_S/K_S^*$, and $V_3/K_S/K_I^*$.

Generally, a fairly acceptable concordance between experimental data and theoretical curves was obtained indicating the validity of the proposed model. Larger discrepancies only occurred at very high substrate concentrations. For example, the abrupt decline of the rate curve for Suc-LLVY-MCA in the concentration interval between 200 and 300 μM could not be adequately described by the model. Self-inhibition for this substrate appears rather abrupt, thus pointing to a co-operative (*i.e.* non-hyperbolic) binding of the substrate to the modifier site, which is not covered by rate Equation 1. From the parameter estimates obtained for the four different peptides the following conclusions can be drawn.

Bz-VGR-MCA (Figs. 3A and 4A)—Ritonavir may not compete with this substrate at the active site catalyzing the cleavage of this peptide (the estimate $K_I = 100,000 \mu\text{M}$ represents

the upper limit imposed to the search range in the regression procedure). The only effect of Ritonavir is a stimulation of the enzyme upon binding to the modifier site. This binding appears with high affinity ($K_I^* = 2.89 \mu\text{M}$).

Suc-LLVY-MCA (Figs. 3B and 4B)—This peptide exhibits self-inhibition kinetics. In the light of the model used, the self-inhibitory effect is explained by the fact that only the single-ligand state (\cdot, S) is active, whereas occupation of the modifier site by the substrate ($K_S^* = 44.6 \mu\text{M}$) prevents cleavage ($V_2 = 0$). Interestingly, and similar to (Z)GGL-MCA, the presence of Ritonavir at the modifier site is predicted to give rise to a strong activation of the enzyme. This activating effect is, however, completely abolished by the very effective competition of Ritonavir with the peptide at the active site ($K_I = 0.41 \mu\text{M}$). Hence, the net effect of Ritonavir is a monotone inhibition

of the enzyme.

(Z)-LLE-βNA (Figs. 3C and 4C)—The $V(S)$ data do not reveal substrate saturation (except a single measurement at 300 μM in the absence of Ritonavir). Hence, no numerical estimates were assessable for the dissociation constants K_S and K_S^* . In the non-saturating range of substrate concentrations, rate Equation 1 reads, approximated,

$$V = \frac{\frac{V_1}{K_S}[S] + \frac{V_2}{K_S K_S^*}[S]^2 + \frac{V_3}{K_I^* K_S}[I][S]^2}{\left(1 + \frac{[I]}{K_I}\right)\left(1 + \frac{[I]}{K_I^*}\right)} \quad (\text{Eq. 2})$$

so that only apparent rate constants $V_1^* = V_1/K_S$, $V_2^* = V_2/K_S K_S^*$, and $V_3^* = V_3/K_S K_I^*$ can be identified. Ritonavir inhibits the enzyme by competition with the substrate at the active site as well as by occupation of the modifier site resulting in an inactive complex (I,S).

(Z)GGL-MCA (Figs. 3D and 4D)—For this substrate the enzyme displays a strong positive cooperativity, *i.e.* the complex (,S) with substrate bound to the active center is practically inactive ($V_1 = 0$), *i.e.* presence of the peptide at the modifier site is required to achieve the active complex (S,S) ($V_2 = 3.25$). Ritonavir competes with this peptide for binding both binding sites. Ritonavir acts as a strong activator upon binding to the modifier site, *i.e.* the enzyme complex (I,S) possesses an about 40-fold higher activity ($V_3 = 118.5$) than the homomeric complex (S,S). On the other hand, the binding of Ritonavir to the modifier site is weak ($K_I^* = 129.4 \mu\text{M}$) compared with the binding of the peptide ($K_I = 1.56 \mu\text{M}$). High affinity competition at the active site and weak competition at the modifier site under formation of a strongly activated enzyme may mutually compensate, depending on the concentration of Ritonavir applied. This accounts for the non-monotone inhibition kinetics. At moderate Ritonavir concentrations between 1 and 50 μM , the stimulating effect via the modifier site is prevailing, whereas at high Ritonavir concentrations, the competition at the active site becomes increasingly dominant. Although no sizeable net inhibition was observed in the experiments for Ritonavir concentrations up to 250 μM , the model predicts a successively increasing inhibition for higher Ritonavir concentrations (phenomenological half-inhibition constant: $\text{IC}_{50} = 530 \mu\text{M}$).

In conclusion, the two-site modifier model could be nicely fitted to our experimental results and, thus, strongly suggests that Ritonavir binds to a second site in the proteasome that is not the MB1 active site. However, the model makes no predictions whether this second site is one of the other active sites of the 20 S proteasome or whether it is a novel site that may be bound by Ritonavir or other effectors to control proteasome activity. To investigate whether the Ritonavir-mediated enhancement of the trypsin-like activity is exerted via its binding to the active centers of the chymotrypsin-like activity, we first tested whether a fluorogenic substrate cleaved by one site would influence the hydrolysis of a substrate of the other.

The Chymotrypsin-like Activity and the Trypsin-like Activity of the Proteasome Do Not Allosterically Interact with Each Other

In previous experiments (21) we used the substrate Bz-VGR-MCA to monitor the effect of Ritonavir on the trypsin-like activity of the proteasome. To test whether the stimulatory effect of Ritonavir on the trypsin-like activity was also evident for another “tryptic” peptide, we used the Boc-LGR-pNA substrate in these experiments. As shown in Fig. 5A, Ritonavir accelerated the hydrolysis of this substrate by highly purified 20 S proteasomes from mouse liver in a concentration-dependent manner up to 5-fold.

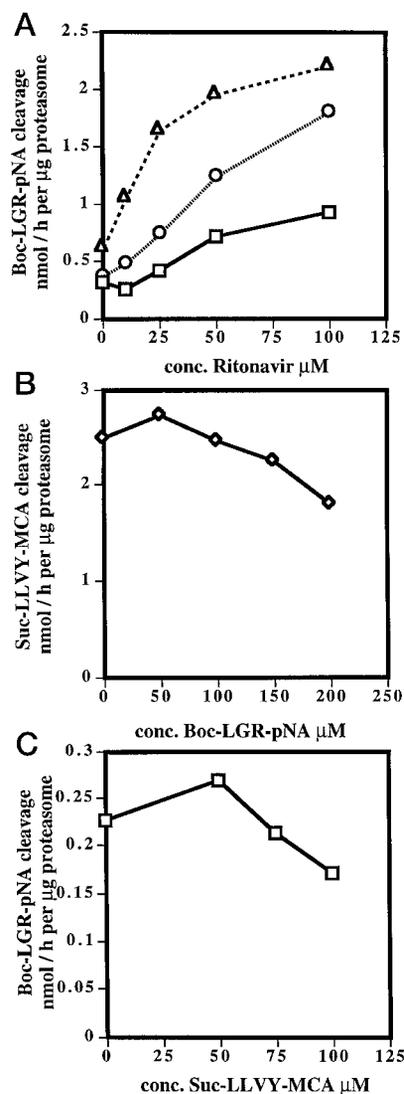


FIG. 5. The activation of the proteasomal trypsin-like activity by Ritonavir. A, the trypsin-like activity of isolated 20 S proteasomes from murine liver was measured by hydrolysis of the chromogenic substrate Boc-LGR-pNA at concentrations of 50 μM (squares), 100 μM (circles), and 200 μM (triangles) in the presence of titrated concentrations of Ritonavir. B, hydrolysis of 50 μM Suc-LLVY-MCA by purified proteasomes in the presence of different concentrations of Ritonavir. C, cleavage of 50 μM Boc-LGR-pNA by purified 20 S proteasomes in the presence of different concentrations of Suc-LLVY-MCA. Displayed values are the means of triplicates with S.E. of <5% for all data points.

Next we investigated whether the stimulation of the tryptic activity of the proteasome could be due to an allosteric activation by the chymotryptic site. To this end we monitored simultaneously the tryptic and chymotryptic activity by using the specific peptides Suc-LLVA-MCA and Boc-LGR-pNA. This was possible because *para*-nitroanilide (pNA) and the fluorogenic leaving group 7-amido-4-methylcoumarin (MCA) can be determined without mutual interference in the applied concentration range (data not shown), but due to solubility problems only 100 μM Suc-LLVA-MCA could be measured in the presence of 50 μM Boc-LGR-pNA. As shown in Fig. 5, B and C, the two substrates did not mutually affect their hydrolysis to a significant extent. As these experiments provided no evidence for a mutual allosteric regulation of the tryptic and chymotryptic site, it was unlikely that the up-regulation of the trypsin-like activity was caused by the binding of Ritonavir to the proteasome subunit MB1.

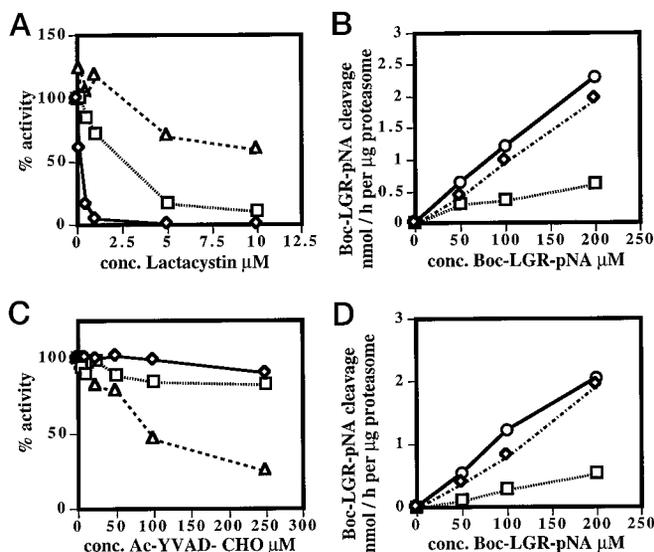


FIG. 6. Ritonavir activates the trypsin-like activity of the proteasome in the presence of different proteasome inhibitors. A, inhibition of proteasome activities by titrated concentrations of lactacystin: *diamonds*, chymotrypsin-like activity (100 μM Suc-LLVY-MCA); *squares*, trypsin-like activity (100 μM Boc-LGR-pNA); and *triangles*, PGPH activity (100 μM (Z)-LLE- β NA). B, hydrolysis of the Boc-LGR-pNA substrate in the absence of inhibitors (*squares*), in the presence of 50 μM Ritonavir (*circles*), and in the presence of 1 μM lactacystin and 50 μM Ritonavir (*diamonds*). C, inhibition of proteasome activities by titrated concentrations of caspase-1 inhibitor (Ac-YVAD-CHO): *diamonds*, chymotrypsin-like activity (100 μM Suc-LLVY-MCA); *squares*, trypsin-like activity (100 μM Boc-LGR-pNA); and *triangles*, PGPH activity (100 μM (Z)-LLE- β NA). D, hydrolysis of the Boc-LGR-pNA substrate in the absence of inhibitors (*squares*), in the presence of 50 μM Ritonavir (*circles*), and in the presence of 250 μM caspase-1 inhibitor and 50 μM Ritonavir (*diamonds*). The values are the means of triplicates with S.E. of <5% for all data points.

Selective Inhibition of the Proteasomal PGPH and Chymotrypsin-like Activities Does Not Affect the Ritonavir-mediated Enhancement of the Trypsin-like Activity

To directly test whether Ritonavir needed to bind to the active centers of the chymotrypsin-like and/or the PGPH activity to cause an enhancement of the trypsin-like activity, we tested whether this effect of Ritonavir was blocked in the presence of selective inhibitors of the chymotrypsin-like and PGPH activity. For the selective inhibition of the chymotrypsin-like activity of the proteasome, we used lactacystin (26). As lactacystin reduces the trypsin-like activity at high concentrations (22), we measured its effect on the proteasomal hydrolysis of the substrates Suc-LLVY-MCA (chymotrypsin-like activity), Boc-LGR-pNA (trypsin-like activity), and (Z)-LLE- β NA (PGPH activity) to find a concentration at which the chymotrypsin-like activity is inhibited, although the trypsin-like activity is still intact. We found that at a concentration of 1 μM lactacystin, the chymotrypsin-like activity of the 20 S proteasome is inhibited to 95%, whereas the trypsin-like activity is only diminished by about 20% (Fig. 6A). We then used this concentration of lactacystin to test its impact on the enhancement of the trypsin-like activity in the presence of 50 μM Ritonavir. As can be seen in Figs. 6B, lactacystin did not significantly alter the ability of Ritonavir to enhance the trypsin-like activity, indicating that Ritonavir does not rely on binding to the active sites of the chymotrypsin-like activity for mediating this effect.

Similar experiments were performed with the caspase-1 inhibitor (Ac-YVAD-aldehyde) which, when used at 250 μM , inhibits the PGPH activity of the proteasome by 75% while leaving the trypsin-like and chymotrypsin-like activities intact (Fig. 6C). Also, this inhibitor did not significantly change the

Ritonavir effect on the trypsin-like activity, indicating that also the active center of subunit β 1 (δ), which is responsible for the PGPH activity of the proteasome (8), can be ruled out as a modulator site used by Ritonavir to up-regulate the trypsin-like activity. This conclusion is further supported by computer-modeling studies, according to which, Ritonavir does not fit into the substrate binding pocket of the β 1 of the yeast 20 S proteasome (21). Taken together, these data strongly suggest that Ritonavir enhances the trypsin-like activity of the proteasome by binding to a site that is not another active site of the proteasome. It thus appears that the modifier site that was predicted by our kinetic model is not an active site of the proteasome but an independent, non-catalytic site capable of modulating the proteolytic activity of the proteasome.

The Substrate-mediated Rate Modulation of the Proteasomal Chymotrypsin-like and PGPH Activities Is Not Affected by Inhibitors of the Respective Activities

Previously a cyclical bite-chew model of proteasome activity was proposed to explain a mutual regulation between the chymotrypsin-like and PGPH activities of the proteasome (19). The existence of modifier sites in the proteasome, however, is a new paradigm that may provide an alternative explanation for the observed regulatory phenomena. If the bite-chew model, as proposed by Goldberg and co-workers (19), was correct, one would predict that inhibitors of the respective active sites should eliminate the proposed allostereism. We, hence, decided to test the latter prediction experimentally. To this aim, we first attempted to reproduce the substrate-mediated rate modulation described by Kisselev *et al.* (19) by measuring the hydrolysis of the substrate Ac-YVAD-MCA in the presence of Suc-FLF-4mna. As shown in Fig. 7B, the Ac-YVAD-MCA cleavage (PGPH or caspase-like activity) was enhanced in the presence of Suc-FLF-4mna in a dose-dependent manner that was saturated at 50 μM . Conversely, the chymotrypsin-like activity (Suc-LLVY-MCA hydrolysis) was inhibited in a dose-dependent manner in the presence of the (Z)-LLE- β NA substrate (Fig. 7C). These data are in full agreement with the findings of Kisselev *et al.* (19) and further demonstrate that the allosteric effects are the same for 20 S proteasomes from rabbit muscle (see Ref. 19) and mouse liver (Fig. 7).

Next we tested whether this substrate interference was also apparent if the respective active sites are blocked with site-specific inhibitors. This experiment was performed at an optimal concentration of 1 μM lactacystin, where the hydrolysis of Suc-FLF-4mna was inhibited by 90%, whereas the hydrolysis of Ac-YVAD-MCA was not affected (Fig. 7A). As shown in Fig. 7B, the concentration-dependent enhancement of the Ac-YVAD-MCA hydrolysis through Suc-FLF-4mna was not affected by the presence of 1 μM lactacystin. Moreover, the concentration-dependent inhibition of Suc-LLVY-MCA cleavage by (Z)-LLE- β NA was also not affected in the presence of 250 μM caspase-1 inhibitor, which inhibited the cleavage of this substrate by 75% (Fig. 6C). Taken together, these results indicate that the elevation of the PGPH activity through substrates of the chymotrypsin-like activity relies neither on the binding of the Suc-FLF-4mna substrate to its active site nor on the event of hydrolysis at this site. Also substrates of the PGPH activity apparently do not need to be hydrolyzed at their active site to inhibit the hydrolysis of Suc-LLVY-MCA. These data challenge the cyclical bite-chew model and suggest that also this mutual regulation of the PGPH and chymotrypsin-like activity of the 20 S proteasome could be due to the binding of the respective substrates to modulatory sites of the 20 S proteasome.

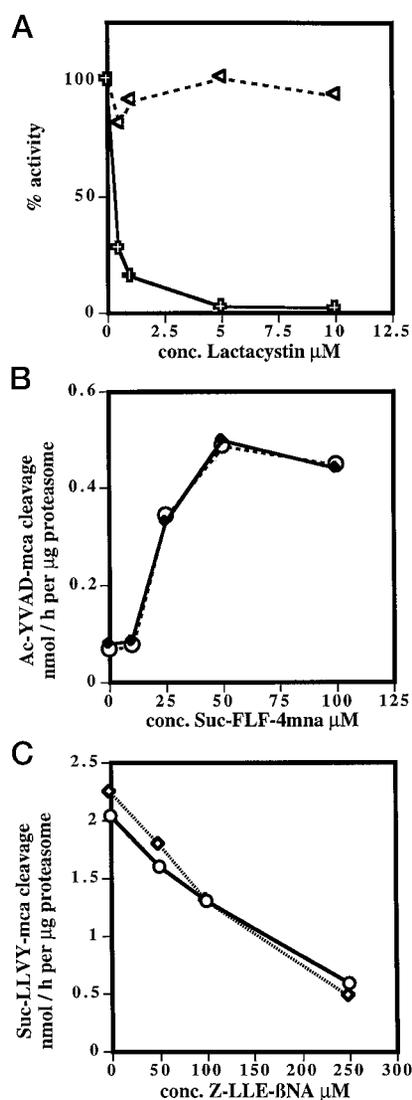


FIG. 7. Substrates for the chymotrypsin-like activity and the PGPH (caspase-like) activity of the 20 S proteasome affect each other's hydrolysis in the presence of their respective active site inhibitors. A, titration of the inhibition of the chymotrypsin like activity (crosses, 100 μM Suc-FLF-4mna) and PGPH activity (triangles, 100 μM Ac-YVAD-MCA) by lactacystin. B, a substrate of the proteasomal chymotrypsin-like activity (Suc-FLF-4mna) enhances the PGPH activity (substrate: 100 μM Ac-YVAD-MCA) in the presence (open circles) or in the absence (closed circles) of 1 μM lactacystin. C, a substrate of the PGPH activity ((Z)-LLE- β NA) suppresses the proteasomal chymotrypsin-like activity (substrate: 100 μM Suc-LLVY-MCA) in the presence (circles) or in the absence (diamonds) of 250 μM caspase-1 inhibitor. The values are the means of triplicates with S.E. of <5% for all data points.

DISCUSSION

The 20 S proteasome harbors six active centers, which contribute to the fragmentation of proteins. How these different active centers, residing on the two copies of subunits $\beta 1$, $\beta 2$, and $\beta 5$ per proteasome are coordinated for protein degradation in general and for the production of MHC class I peptide ligands in particular, is an important and presently unresolved question. Sigmoidal kinetics have been observed for fluorogenic substrates used to monitor the chymotrypsin-like activity and the PGPH activity of the proteasome, suggesting that cooperativity may exist between two subunits of the same substrate specificity (13–18). Evidence for a more complex allostereism involving two active centers of different specificity have recently been obtained (21, 19). In an attempt to understand the complex effects that Ritonavir has on the hydrolysis of four

fluorogenic substrates by the proteasome, we have established a “two-site modifier” model that provides a plausible interpretation and satisfactory quantitative description of the experimental data obtained for the effect of Ritonavir on the kinetics of different peptide substrates. In particular, this model accounts for the self-inhibition kinetics observed for the substrate Suc-LLVY-MCA and for the different effects that Ritonavir had on the hydrolysis of the substrates Suc-LLVY-MCA and (Z)-GGL-MCA, which are expected to be cleaved by the same active site. Our model assumes two binding sites for either an effector or a substrate. One binding site is the active center, which can hydrolyze a given substrate, whereas the other site, designated “modifier site,” is located elsewhere.

From previous experimental data and kinetic models (18, 19), one would assume that the modifier site is located at the substrate binding pocket of another active site. To our surprise, this assumption proved to be incorrect, as the selective inhibition of the chymotrypsin-like and PGPH (or caspase-like) activities by lactacystin and caspase-1 inhibitor did not affect the enhancement of the trypsin-like activity by Ritonavir. Although these results rule out the chymotrypsin-like and PGPH sites as targets, we cannot exclude that Ritonavir binds to one trypsin-like active site (*i.e.* proteasome subunit $\beta 2$ (Z, MC14)) in order to strongly activate hydrolysis of the Boc-LGR-pNA substrate at the other $\beta 2$ subunit in the complex. However, the latter scenario is very unlikely for two reasons. First, Ritonavir protected the subunit MB1($\beta 5$) but not the subunit Z($\beta 2$) from active site modification with the vinyl sulfone inhibitor ^{125}I -NLVS, indicating that Ritonavir did not bind to Z with comparable affinity (21). Secondly, we and others (19) did not obtain evidence that the trypsin-like activity is subject to allosteric regulation, as the substrates are hydrolyzed by the 20 S proteasome according to linear rather than sigmoidal kinetics (Fig. 6B). Therefore, our data strongly suggest that Ritonavir binds to a so-far unidentified modifier site in the 20 S proteasome that is not identical with any of the known active centers of the proteasome.

The existence of one or several of such modifier sites in the proteasome provides a plausible explanation of our experimental results, which are difficult to reconcile with models based on mutual allosteric regulation between active centers. The mutual allosteric regulation of the proteasomal chymotrypsin-like and PGPH activity, for instance, cannot be based on the hydrolysis and binding of substrates to their respective active centers, as suggested by the cyclic bite-chew model, because the mutual regulation persists if hydrolysis of a “regulatory” substrate and binding to its active site is prevented by selective inhibitors. An intriguing alternative to the bite-chew model suggested by our analysis is that control of the proteasomal chymotrypsin-like and PGPH activities is exerted through the binding of substrates to non-catalytic modifier sites. The localization of these putative modifier site(s) is unclear. Hitherto, there are no indications for the binding of model ligands to sites that are different from the catalytically active sites. Active site inhibitors of the proteasome have so far been localized exclusively to the N-terminal threonine residues of the subunits $\beta 1$, $\beta 2$, and $\beta 5$, as elucidated by either x-ray crystallography or biochemical analysis (22, 23, 7). Also, for the proteasome modifier Ritonavir, we have so far been unable to identify binding sites other than the active center of the $\beta 5$ subunit. Biochemical approaches to identify further Ritonavir binding sites do not hold much promise because this molecule binds to the proteasome in a non-covalent and reversible manner (21). Unfortunately also, x-ray crystallography has not allowed much progress because crystals of mammalian 20 S proteasomes are not yet available and attempts to localize Ritonavir in crystals

of *S. cerevisiae* 20 S proteasomes were unsuccessful, most likely because the affinity of Ritonavir for yeast proteasomes was insufficient (21).

An interesting but as of yet speculative issue is the physiological implication of the two-site modifier model and, in particular, the question whether physiological modifiers of proteasome activity exist. One scenario that has been suggested by Kisselev *et al.* (19) is that intermediate fragments of protein breakdown may control proteasome activity to prevent clogging of the substrate binding cavities. On the other hand, numerous low molecular weight effectors of proteasome activity have been described, as for instance fatty acids (27), polylysine (28), cardiolipin (29), polycations (30), sodium dodecyl sulfate (2), or peptide analogs (31). Whether any one of these activators exerts a physiological function is uncertain, but they may mimic some of the effects that are physiologically induced by the three known activator complexes of the 20 S proteasome: PA700, PA28 α/β , and PA28 γ (32).

Irrespective of their structural location, modifier sites of proteasome activity could be of interest as targets of pharmacological intervention. Ritonavir, for example, is a modulator of proteasome activity that has a marked effect on MHC class I restricted antigen presentation *in vitro* and on the generation of cytotoxic immune responses against T cell epitopes of lymphocytic choriomeningitis virus *in vivo* (20). As the proteasome is the main protease in charge of generating peptide ligands for MHC class I molecules (33) it seems likely that the modulation of proteasome activity is the cause for the reduced presentation of lymphocytic choriomeningitis virus epitopes in Ritonavir-treated cells. The generation of different class I peptide ligands requires the cleavage C-terminal of hydrophobic, basic, and acidic amino acids, which must be accomplished by the concerted action of different active sites of the proteasome. Ligands of modifier sites in the proteasome that control the peptide hydrolysis at different active sites of the proteasome could therefore be used to either up- or down-regulate the intracellular production of MHC class I ligands and, thus, control tissue destruction in autoimmune diseases or transplant rejection.

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