Kinetic Studies of Isopeptidase T: Modulation of Peptidase Activity by Ubiquitin

Ross L. Stein,* Zhijian Chen, and Francesco Melandri

ProScript, 38 Sidney Street, Cambridge, Massachusetts 02139

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ABSTRACT: We have investigated the specificity of isopeptidase T toward peptide-AMC substrates based on the C-termini of ubiquitin. The substrates investigated were Z-Gly-Gly-AMC, Z-Arg-Gly-Gly-AMC, Z-Leu-Arg-Gly-Gly-AMC, and Z-Arg-Leu-Arg-Gly-Gly-AMC and were hydrolyzed by isopeptidase T with k_c/K_m values of <0.1, 1, 18, and 95 M^{-1} s^{-1}, respectively. In the course of these experiments, we observed that the hydrolytic activity of isopeptidase T toward these substrates is modulated by ubiquitin in a biphasic fashion. While submicromolar concentrations of ubiquitin activate isopeptidase T, higher concentrations are inhibitory. In the activation phase, the extent of stimulation of k_c/K_m varies with substrate and is 8-, 50-, and 70-fold for Z-Arg-Gly-Gly-AMC, Z-Leu-Arg-Gly-Gly-AMC, and Z-Arg-Leu-Arg-Gly-Gly-AMC, respectively. K_d for ubiquitin in this phase is, of course, independent of substrate and equals 0.10 ± 0.03 μM. At higher concentrations, ubiquitin is inhibitory and titrates k_c/K_m with an average K_i value of 3.0 ± 1.3 μM for all three substrates. To explain these observations, we propose a structural model for isopeptidase T that involves two binding sites for ubiquitin. We propose that the two sites are adjacent to one another and are the extended active site that binds two ubiquitin moieties of a polyubiquitin chain for isopeptide bond hydrolysis. We found that the "activation site" requires ubiquitin to have a free carboxy terminus and propose that this is the site that binds the ubiquitin molecule of the polyubiquitin chain that donates Lys^{48} to the isopeptide bond. The "inhibitory site" is adjacent and binds the ubiquitin molecule of the polyubiquitin chain that donates the C-terminal Gly^{76} to the isopeptide bond. Finally, we propose that these observations may have physiological relevance for the regulation of IPaseT activity since high intracellular levels of ubiquitin will inhibit continued hydrolysis of polyubiquitin chains.

Eukaryotic organisms possess a mechanism for intracellular protein degradation that involves initial ubiquitination of the protein substrate followed by 26S proteasome-catalyzed hydrolysis (Ciechanover, 1994; Finley & Chau, 1991; Hershko & Ciechanover, 1992). Ubiquitination is a multistep process that results in ligation of the C-terminal Gly^{76} of the small, heat-stable protein Ub[1] onto ϵ-amino groups of targeted proteins. Additional rounds of ubiquitination occur on Lys^{48} of Ub and ultimately result in the synthesis of polyUb chains on the protein. Degradation of Ub−protein conjugates by the 26S proteasome is thought to occur with the liberation of intact polyUb chains and small polypeptides derived from the hydrolysis of the parent protein substrate. These polyUb chains are then hydrolyzed to Ub monomers by the action of isopeptidase T (Chen & Pickart, 1990; Hadari et al., 1992).

IPaseT is a member of a large family of enzymes that catalyzes the hydrolysis of amides, esters and thioesters of the C-terminal Gly^{76} of Ub (Mayer & Wilkinson, 1989; Pickart & Rose, 1985, 1986; Rose & Warms, 1983; Wilkinson et al., 1986). These enzymes are known as ubiquitin C-terminal hydrolases and share a common catalytic mechanism involving the nucleophilic participation of the thiol group of an active site Cys residue. UCHs can be classified according to molecular weight and substrate specificity. One group of UCHs, with molecular weight around 30 000, hydrolyze small C-terminal derivites. These enzymes are active against thioesters of ubiquitin that *in vivo* are thought to form nonspecifically during the course of protein ubiquitination (Pickart & Rose, 1985, 1986; Wilkinson et al., 1986). Hydrolysis of isopeptides formed from ligation of Ub-Gly^{76} onto the ϵ-amine of Lys residues of either Ub or other proteins is catalyzed by UCHs of higher molecular weight that presumably possess domains that can recognize and bind these much larger derivatives of Ub (Chen & Pickart, 1990; Hadari et al., 1992; Papa & Hochstrasser, 1993; Falquet et al., 1995). Members of this group of UCHs constitute enzymes that cleave the ubiquitin polyprotein precursors of monomeric Ub.

IPaseT is a member of this latter group of high molecular weight UCHs and functions as a 100 kDa monomer (Chen & Pickart, 1990; Hadari et al., 1992). IPaseT is an abundant protein and exists in all mammalian cells where it is thought to hydrolyze the isopeptide linkages of polyUb chains. Thus, IPaseT may play a critical role in the recycling of Ub (Hadari, et al., 1992). It has also been suggested that IPaseT participates in the regulation of the activity of the 26S proteasome and, thus, protein degradation (Hadari et al., 1992). This is based on the observation that addition of IPaseT to reticulocyte extracts increases the rate of degradation of ubiquitinated protein substrates. Hadari and colleagues (1992) speculated that polyUb chains can inhibit the hydrolytic activity of 26S proteasome on Ub−protein conjugate substrates.

As part of a program to develop inhibitors of enzymes of the ubiquitin−proteasome pathway, we have undertaken a series of studies to understand the molecular mechanism of

* To whom correspondence should be addressed.

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[1] Abbreviations: AMC, (aminomethyl)coumoran; DTT, dithiothreitol; FI, fluorescence intensity; IPaseT, isopeptidase T; polyUb, polyubiquitin chains formed through isopeptide linkages of Gly^{76} of one Ub and the ϵ-amine of Lys^{48} of another Ub; Ub, ubiquitin; Ub-H, ubiquitin aldehyde; UCH, ubiquitin C-terminal hydrolases.

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catalysis by IPaseT and the role that this enzyme plays in protein degradation. As a first step toward these goals, we designed a series of low molecular weight substrates for IPaseT that would be appropriate for detailed kinetic and mechanistic studies. We report in this paper that peptide-AMCs based on the C-terminus of Ub are substrates for IPaseT and form the basis of sensitive assays. Furthermore, we observe that the hydrolytic activity of IPaseT toward these substrates is modulated by Ub.

EXPERIMENTAL SECTION

Buffer salts were purchased from Sigma Chemical Co. Peptide substrates were custom synthesized by Enzyme System Products (Dublin, CA) at a purity greater than 95%. IPaseT was purified from rabbit reticulocytes according to the method of Chen and Pickart (1990) and was stored at −80 °C as a concentration between 0.3 and 1.0 mg/mL. des-$Gly^{75}Gly^{76}$-ubiquitin was prepared by the method of Wilkinson (Mayer & Wilkinson, 1989; Wilkinson et al., 1986).

Kinetic Methods. In a typical kinetic run, 2.00 mL of assay buffer (20 mM HEPES, 0.5 mM EDTA, pH 7.8, containing 1 mg/mL ovalbumin and 10 mM DTT) was added to a 3 mL fluorescence cuvette and the cuvette placed in the jacketed cell holder of a Hitachi 2000 fluorescence spectrophotometer. Reaction temperature was maintained at 25.0 ± 0.02 °C by a circulating water bath. After the reaction solution had reached thermal equilibrium (~5 min), 1−10 μL of the stock enzyme solution was added to the cuvette. The reaction solution was incubated an additional 30 min to allow DTT-mediated activation of IPaseT before the addition of 10 μL of substrate solution in DMSO. Reaction progress was monitored by the increase in fluorescence emission at 440 nm (λ_{ex} = 380 nm) that accompanies cleavage of AMC from peptide-AMC substrates. For each kinetic run, 200−1000 data points, corresponding to (time, FI) pairs, were collected by a microcomputer interfaced to the fluorescence spectrophotometer. Velocities were calculated from steady-state regions of reaction progress curves by linear least-squares fitting.

RESULTS

Hydrolysis of Peptide-AMCs by Isopeptidase T. In an attempt to develop a continuous assay for IPaseT, we synthesized Z-Gly-Gly-AMC, Z-Arg-Gly-Gly-AMC, Z-Leu-Arg-Gly-Gly-AMC, and Z-Arg-Leu-Arg-Gly-Gly-AMC and tested them as substrates for IPaseT. In preliminary experiments at [peptide-AMC] = 40 μM, we found that the latter three peptide-AMCs are, in fact, substrates for IPaseT. To characterize the kinetics of these reactions, steady-state velocities, v_{ss}, were measured from reaction progress curves that were recorded at 12 μM < [S] < 400 μM and 2 nM < [IPaseT] < 50 nM. The dependencies of v_{ss} on substrate concentration for the three active peptide-AMCs are shown in Figure 1, and the steady-state kinetic parameters that were calculated from these data are summarized in Table 1. For Z-Arg-Gly-Gly-AMC, and Z-Leu-Arg-Gly-Gly-AMC, the dependence of v_{ss} on [S] is linear and, thus, does not allow calculation of k_c and K_m values. In contrast, for Z-Arg-Leu-Arg-Gly-Gly-AMC, the dependence of v_{ss} on [S] is hyperbolic and allows us to calculate these parameters (Table 1).

Effect of Ubiquitin on the Activity of Isopeptidase T. In the course of our experiments, we found that submicromolar concentrations of Ub were able to activate the IPaseT-catalyzed hydrolysis of Z-Leu-Arg-Gly-Gly-AMC. Data from a typical experiment is shown in Figure 2A, where we see that activation by Ub occurs with a maximal steady-state velocity that is ~40-fold greater than unactivated IPaseT. In this experiment, a Ub concentration of roughly 50 nM provided half-maximal activation.

Additional studies demonstrated that further increases in Ub concentration result in partial inhibition of IPaseT activity. This is illustrated in Figure 2B, where we see inhibition of IPaseT by Ub with an IC_{50} of ~5 μM.

These results are consistent with the kinetic mechanism that is shown in Scheme 1. According to this scheme, IPaseT in the absence of Ub hydrolyzes Z-Leu-Arg-Gly-Gly-AMC via a mechanism that is governed by Michaelis constant K_{se}[2]

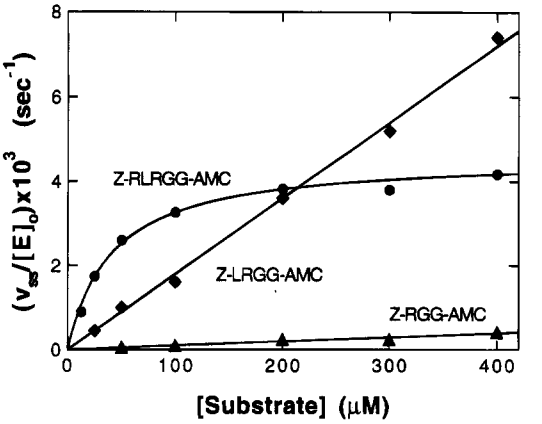


FIGURE 1: Dependence of steady-state velocity on substrate concentration for IPaseT-catalyzed hydrolyses of Z-Arg-Gly-Gly-AMC, Z-Leu-Arg-Gly-Gly-AMC, and Z-Arg-Leu-Arg-Gly-Gly-AMC. Reactions were conducted at 25.0 ± 0.02 °C in an assay buffer of 20 mM HEPES, 0.5 mM EDTA, pH 7.8, containing 1 mg/mL ovalbumin and 10 mM DTT. Steady-state velocities were determined at the indicated substrate concentrations with [IPaseT] = 100, 15, and 5 nM for Z-Arg-Gly-Gly-AMC, Z-Leu-Arg-Gly-Gly-AMC, and Z-Arg-Leu-Arg-Gly-Gly-AMC, respectively. The solid lines through the data points were drawn using the Michaelis−Menten equation and the parameters of Table 1.

Table 1: Steady-State Kinetic Parameters for IPaseT-Catalyzed Hydrolyses of Peptide-AMC Substrates[a]

substrates	K_m (μM)	$10^3 k_c$ (s^{-1})	k_c/K_m (M^{-1} s^{-1})
Z-Gly-Gly-AMC			<0.1
Z-Arg-Gly-Gly-AMC	>2000	>2	1
Z-Leu-Arg-Gly-Gly-AMC	>2000	>36	18
Z-Arg-Leu-Arg-Gly-Gly-AMC	42	4	95

[a] Reactions were conducted at a 20 mM HEPES solution buffered at pH 7.8 containing 0.5 mM EDTA, 1 mg/mL ovalbumin, and 10 mM DTT. Temperature was regulated at 25.0 ± 0.02 °C.

Table 2: Effect of Ubiquitin on Catalysis by Isopeptidase T[a]

substrate	k_{es}/K_{se} (M^{-1} s^{-1})	k_{ues}/K_{seu} (M^{-1} s^{-1})	k_{uesu}/K_{seuu} (M^{-1} s^{-1})	K_{ue}' (μM)	K_{uue}' (μM)
Z-RGG-AMC	1	8	5	0.13	4.4
Z-LRGG-AMC[b]	18 ± 13	846 ± 54	184 ± 49	0.06 ± 0.01	2.9 ± 0.8
Z-RLRGG-AMC	59	4100	610	0.10	1.8

[a] Reactions were conducted in a 20 mM HEPES solution buffered at pH 7.8 containing 0.5 mM EDTA, 1 mg/mL ovalbumin, and 10 mM DTT. Temperature was regulated at 25.0 ± 0.02 °C. [b] These parameters are the mean and standard deviation for three independent experiments.

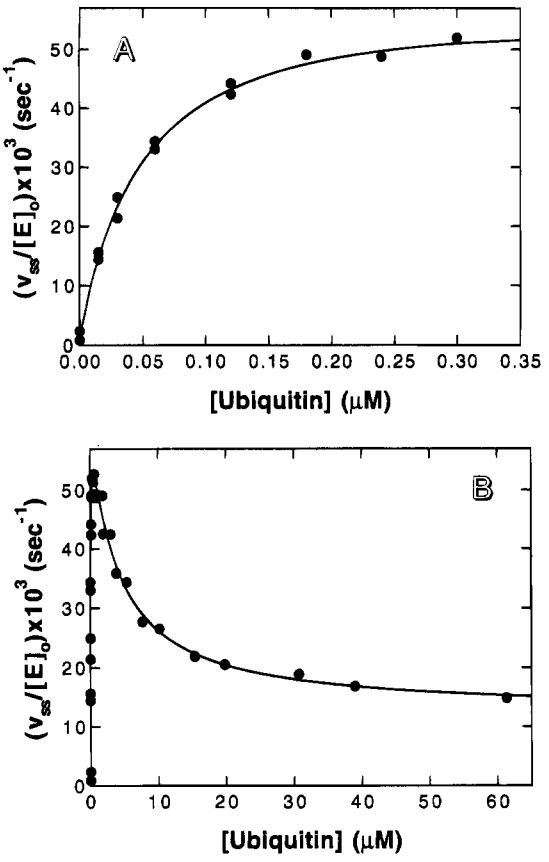
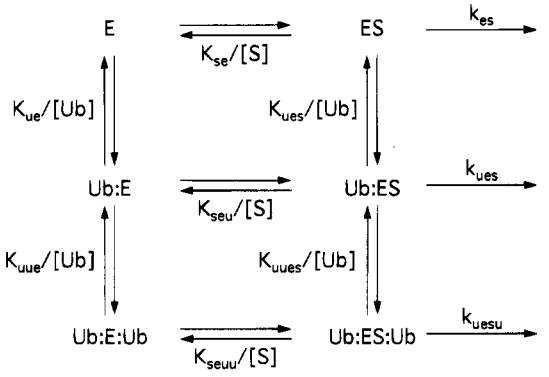


FIGURE 2: Ub concentration dependence of steady-state velocity for the IPaseT-catalyzed hydrolysis of Z-Leu-Arg-Gly-Gly-AMC. In these experiments, [S] = 80 μM and [E] = 5 nM. Reactions were conducted at 25.0 ± 0.02 °C in an assay buffer of 20 mM HEPES, 0.5 mM EDTA, pH 7.8, containing 1 mg/mL ovalbumin and 10 mM DTT: (A) activation of IPaseT activity by Ub in the concentration range of 0.02−0.30 μM; (B) partial inhibition of IPaseT activity by Ub in the concentration range of 0.5−62 μM. The data shown in this figure are from two independent experiments and were fit to eq 1 to provide the following values: $(k_{es}/K_{se})[S] = (1.5 \pm 0.9) \times 10^{-3}$ s^{-1}, $(k_{ues}/K_{seu})[S] = (63.0 \pm 1.5) \times 10^{-3}$ s^{-1}, $(k_{uesu}/K_{seuu})[S] = (12.2 \pm 1.1) \times 10^{-3}$ s^{-1}, K_{ue}' 0.0538 ± 0.0048 μM, and $K_{uue}' = 3.85 \pm 0.46$ μM. These values and eq 1 were used to draw the solid lines in both panels of this figure.

Scheme 1: Minimal Kinetic Mechanism for Isopeptidase T Catalysis



and catalytic constant k_{es}. Addition of Ub results in the formation of the binary complex, Ub−E, with dissociation constant, K_{ue}. Ub−E is an activated form of IPaseT that hydrolyzes substrate with constants K_{seu} and k_{ues}. At still higher concentrations of Ub, the ternary complex Ub−E−Ub forms with dissociation constant, K_{uue}. Ub−E−Ub hydrolyzes substrate with constants K_{seuu} and k_{uesu}.

The rate equation that describes the mechanism of Scheme 1 is given in eq 1

$$\frac{v_{ss}}{[E]_o} = \frac{(k_{es}/K_{se})[S]}{1 + \frac{[Ub]}{K_{ue}'} + \frac{[Ub]^2}{K_{ue}'K_{uue}'}} + \frac{(k_{ues}/K_{seu})[S]}{\frac{K_{ue}'}{[Ub]} + 1 + \frac{[Ub]}{K_{uue}'}} + \frac{(k_{uesu}/K_{seuu})[S]}{\frac{K_{ue}'K_{uue}'}{[Ub]^2} + \frac{K_{uue}'}{[Ub]} + 1} \quad (1)$$

where

$$K_{ue}' = K_{ue}\frac{1 + [S]/K_{se}}{1 + [S]/K_{seu}} \quad (2)$$

$$K_{uue}' = K_{uue}\frac{1 + [S]/K_{seu}}{1 + [S]/K_{seuu}} \quad (3)$$

When the data of Figure 2B are fit to this equation by nonlinear least squares, the following best-fit parameters are obtained: $k_{es}/K_{se} = 19 \pm 13$ M^{-1} s^{-1}, $k_{ues}/K_{seu} = 787 \pm 19$ M^{-1} s^{-1}, $k_{uesu}/K_{seuu} = 153 \pm 14$ M^{-1} s^{-1}, $K_{ue}' = 0.0538 \pm 0.0048$ μM, and $K_{uue}' = 3.85 \pm 0.46$ μM.

The IPaseT-catalyzed hydrolyses of Z-Arg-Gly-Gly-AMC and Z-Arg-Leu-Arg-Gly-Gly-AMC are similarly modulated by Ub (data not shown). Data from these experiments were analyzed according to eq 1 and provide the kinetic parameters that are summarized in Table 2 along with averaged results from several independent experiments for Z-Leu-Arg-Gly-Gly-AMC.

Effect of Ubiquitin on the Activity of Isopeptidase T: Dependence of Steady-State Velocity on Substrate Concentration at Fixed Ubiquitin Concentration. The previous series of experiments established that Ub can modulate the catalytic activity of IPaseT and led to the minimal mechanism of Scheme 1. In these initial experiments, we worked at relatively low concentrations of substrate, and thus, we were able to accurately determine k_{es}/K_{se}, k_{ues}/K_{seu}, and k_{uesu}/K_{seuu}. These parameters are second-order rate constants equivalent to the k_c/K_m values for the three parallel pathways of Ub-dependent IPaseT-catalyzed hydrolysis.

It is of course of some interest to know not only the effect of Ub concentration on the apparent steady-state constant, k_c/K_m, but also the effect of Ub concentration on the apparent constants k_c and K_m. Such an analysis would ultimately lead to an approximation of all the mechanistic parameters of Scheme 1. To this end, we determined the substrate concentration dependence of v_{ss} at three fixed concentrations of Ub: 0, 0.5, and 100 μM. For the mechanism of Scheme 1, the following expressions, which were obtained from rearrangement of eq 1, relate observed steady-state kinetic

[2] In this paper, rate constant nomenclature is according to the following rules: (1) First-order catalytic constants have subscripts that correspond to the complex that is turning over. Thus, k_{es} is the first-order rate constant for the turnover of the Michaelis complex E−S. (2) Dissociation constants have subscripts that correspond to the complex that is dissociating; the first letter of the subscript refers to the substance that is dissociating from the enzyme complex. Thus, K_{seuu} is the dissociation constant for the breakdown of Ub−ES−Ub to Ub−E−Ub and S.

Table 3: Effect of Ubiquitin Concentration on Apparent Steady-State Kinetic Parameters for Catalysis by Isopeptidase T

	[Ub] (μM)	K_m (μM)	10^3k_c (s^{-1})	k_c/K_m (M^{-1} s^{-1})	K_{is} (μM)	10^3k_{ic} (s^{-1})
Z-RGG-AMC	0	>2000	>2	0.9 ± 0.2		
	0.5	>2000	>16	8.1 ± 1.3		
	100	>2000	>12	4.6 ± 0.5		
Z-LRGG-AMC	0	>2000	>50	30 ± 7		
	0.5	177 ± 58	90 ± 13	508 ± 43		
	100	161 ± 64	45 ± 7	280 ± 32		
Z-RLRGG-AMC	0	41 ± 3	5 ± 1	121 ± 13		
	0.5	314 ± 43	568 ± 170	1,810 ± 140	6 ± 2	1.6 ± 0.7
	100	420 ± 48	155 ± 17	370 ± 15	266 ± 60	(0)

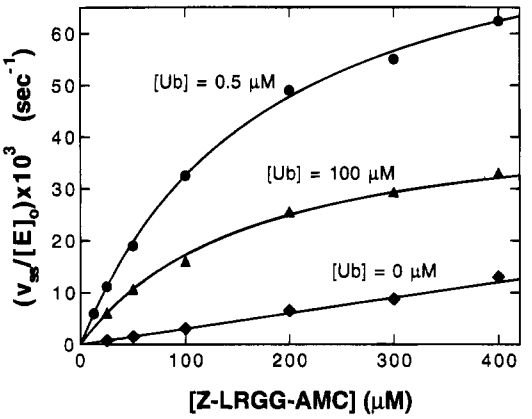


FIGURE 3: Dependence of steady-state velocity on substrate concentration for IPaseT-catalyzed hydrolysis of Z-Leu-Arg-Gly-Gly-AMC as a function of Ub concentration. In these experiments, [E] = 15 nM, 10 μM ≤ [S] ≤ 400 μM, and Ub was held constant at the indicated concentration. Reactions were conducted at 25.0 ± 0.02 °C in an assay buffer of 20 mM HEPES, 0.5 mM EDTA, pH 7.8, containing 1 mg/mL ovalbumin and 10 mM DTT. Solid lines through the data points were drawn according to the Michaelis−Menten equation and the parameters of Table 3.

parameters to mechanistic parameters:

$$\frac{k_c}{K_m} = \frac{k_{es}}{K_{es}}\left\{\frac{1+\alpha\frac{[Ub]}{K_{ues}}+\beta\frac{[Ub]^2}{K_{ues}K_{uues}}}{1+\frac{[Ub]}{K_{ue}}+\frac{[Ub]^2}{K_{uc}K_{uue}}}\right\} \quad (4)$$

$$k_c = k_{es}\left\{\frac{1+\alpha\frac{[Ub]}{K_{ues}}+\beta\frac{[Ub]^2}{K_{ues}K_{uues}}}{1+\frac{[Ub]}{K_{ues}}+\frac{[Ub]^2}{K_{ues}K_{uues}}}\right\} \quad (5)$$

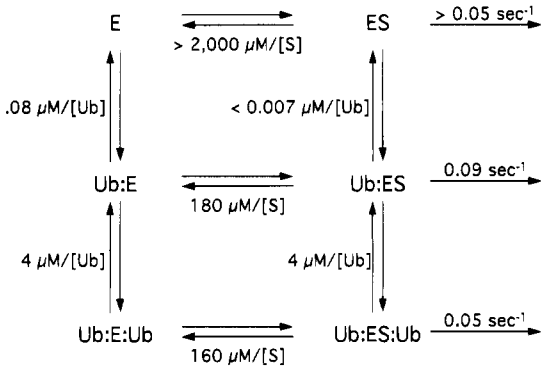
$$K_m = K_{es}\left\{\frac{1+\frac{[Ub]}{K_{ue}}+\frac{[Ub]^2}{K_{ue}K_{uue}}}{1+\frac{[Ub]}{K_{ues}}+\frac{[Ub]^2}{K_{ues}K_{uues}}}\right\} \quad (6)$$

$$\alpha = k_{ues}/k_{es} \quad (7)$$

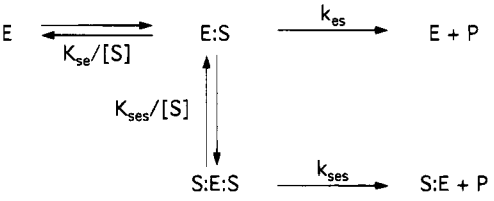
$$\beta = k_{uesu}/k_{es} \quad (8)$$

For the IPaseT-catalyzed hydrolysis of Z-Arg-Gly-Gly-AMC, the dependencee of v_{ss} on [S] (12 μM ≤ [S] ≤ 400 μM) is

Scheme 2: Kinetic Mechanism for the Isopeptidase T-Catalyzed Hydrolysis of Z-Leu-Arg-Gly-Gly-AMC



Scheme 3: Kinetic Mechanism for Simple Substrate Inhibition



linear at all three concentrations of Ub (data not shown). This allows us to calculate k_c/K_m but not individual values of k_c and K_m (see Table 3).

For Z-Leu-Arg-Gly-Gly-AMC, the dependence of v_{ss} on [S] is linear at [Ub] = 0, but hyperbolic at [Ub] = 0.5 and 100 μM (see Figure 3). Thus, at these latter concentrations, we are able to calculate k_c and K_m as well as k_c/K_m (see Table 3). These results together with the relationships of eqs 4−8 allowed us to calculate the mechanistic parameters of Scheme 1; these are shown in Scheme 2. These results suggest that, for Z-Leu-Arg-Gly-Gly-AMC, the increase in k_c/K_m that accompanies increases in Ub concentration is due primarily to a decrease in K_m since k_c is not much influenced by Ub concentration.

The situation becomes more complex for Z-Arg-Leu-Arg-Gly-Gly-AMC (see Figure 4) and, in fact, represents a significant departure from the mechanism of Scheme 1. At zero concentration Ub, the dependence of steady-state velocity on [Z-Arg-Leu-Arg-Gly-Gly-AMC] is hyperbolic and allows us to calculate a K_m of 40 μM and a k_c of 5 × 10^{-3} s^{-1}. However, when the Ub concentration is raised to 0.5 or 100 μM, simple Michaelis−Menten kinetics are no longer observed. These data require us to consider more complex mechanisms and high-order, phenomenologic kinetic equations of the general form that has been described

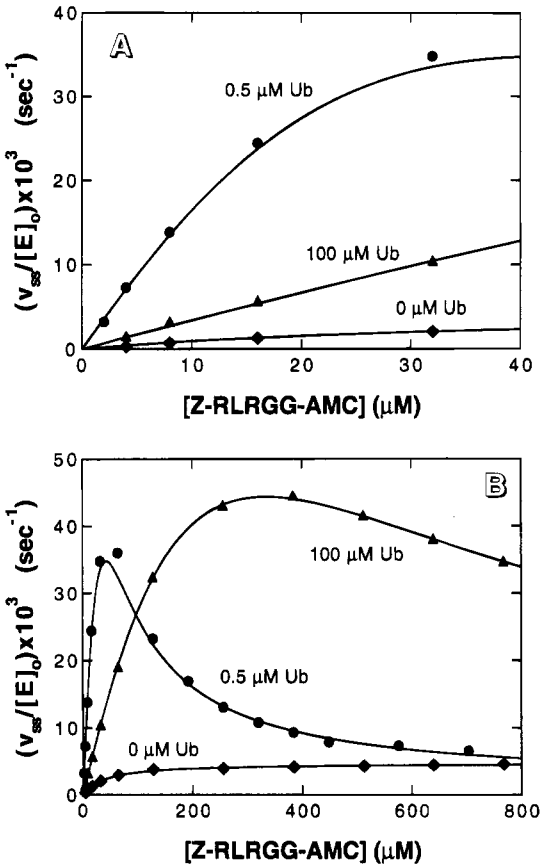


FIGURE 4: Dependence of steady-state velocity on substrate concentration for IPaseT-catalyzed hydrolysis of Z-Arg-Leu-Arg-Gly-Gly-AMC as a function of Ub concentration. In these experiments, [E] = 5 nM, 2 μM ≤ [S] ≤ 780 μM, and Ub was held constant at the indicated concentration. Reactions were conducted at 25.0 ± 0.02 °C in an assay buffer of 20 mM HEPES, 0.5 mM EDTA, pH 7.8, containing 1 mg/mL ovalbumin and 10 mM DTT. Solid lines through the data points were drawn according to eq 11 and the parameters of Table 3.

by Bardsley (Bardsley et al., 1980):

$$\frac{v_{ss}}{[E]} = \frac{\alpha_1[S] + \alpha_2[S]^2 + ... + \alpha_n[S]^n}{1 + \beta_1[S] + \beta_2[S]^2 + ... + \beta_n[S]^n} \quad (9)$$

The simplest form of eq 9 that can account for the data of Figure 4 occurs with $n = 2$:

$$\frac{v_{ss}}{[E]} = \frac{\alpha_1[S] + \alpha_2[S]^2}{1 + \beta_1[S] + \beta_2[S]^2} \quad (10)$$

When the data set for [Ub] = 0.5 μM were fit to this equation by nonlinear least-squares analysis, the follow best-fit parameters were obtained: $\alpha_1 = (1.81 \pm 0.05) \times 10^{-3}$ μM^{-1} s^{-1}, $\alpha_2 = (0.90 \pm 0.12) \times 10^{-6}$ μM^{-2} s^{-1}, $\beta_1 = (3.2 \pm 1.6) \times 10^{-3}$ μM^{-1}, and $\beta_2 = (0.57 \pm 0.24) \times 10^{-3}$ μM^{-2}.

To successfully fit the data set for [Ub] = 100 μM, we had to constrain α_2 to zero. This was required since we were unable to collect data at sufficiently high concentrations of substrate to accurately define α_2. The best-fit parameters are as follows: $\alpha_1 = (0.371 \pm 0.021) \times 10^{-3}$ μM^{-1} s^{-1}, $\alpha_2 = 0$, $\beta_1 = (2.4 \pm 1.1) \times 10^{-3}$ μM^{-1} and $\beta_2 = (0.0089 \pm 0.0034) \times 10^{-3}$ μM^{-2}.

Equation 10 can be rearranged to produce the rate expression for the familiar mechanism of substrate inhibition shown in Scheme 3:

$$\frac{v_{ss}}{[E]} = \frac{k_{es}[S] + (k_{ses}/K_{ses})[S]^2}{K_{se} + [S](1 + [S]/K_{ses})} \quad (11)$$

The relationships between the parameters of eq 10 and eq 11 are

$$\alpha_1 = k_{es}/K_{se} \quad (12)$$

$$\alpha_2 = k_{ses}/K_{es}K_{ses} \quad (13)$$

$$\beta_1 = K_{se}^{-1} \quad (14)$$

$$\beta_2 = (K_{se}K_{ses})^{-1} \quad (15)$$

These expressions and the estimates for α_1, α_2, β_1, and β_2 were used to calculate the mechanistic parameters that are summarized in Table 3.

Note that we observe substrate inhibition only for Z-Arg-Leu-Arg-Gly-Gly-AMC and only in the presence of Ub (see Figure 4B and Table 3). These results tell us that when [Ub] = 0.5 μM and only the "activation" site of IPaseT is occupied by Ub, Z-Arg-Leu-Arg-Gly-Gly-AMC can bind to the activated Michaelis complex, Ub−ES, with a dissociation constant, K_{ses}, of 6 μM. This value is 50-fold smaller than the K_m for Ub−ES. The ternary complex that forms, Ub−ES−S, turns over with a first-order rate constant, k_{ses}, that is only 0.3% of k_c and is essentially equal to zero. Although of great interest, it is completely unclear what the structure of Ub−ES−S might be. When the Ub concentration is raised to 100 μM and both the "activation" and "inhibition" sites of IPaseT are occupied by Ub, substrate inhibition is still observed, suggesting that Z-Arg-Leu-Arg-Gly-Gly-AMC can also bind to Ub−ES−Ub, and reduce turnover to zero. Again, the structure of the resultant complex is unknown.

Modulation of IPaseT by des-Gly^{75}Gly^{76}-Ubiquitin. Finally, we examined the effect of the Ub analog, des-Gly^{75}-Gly^{76}-Ub, on catalysis by IPaseT. These data are shown in Figure 5 and, when fit to eq 1, provide the following parameter estimates: $k_{es}/K_{se} = 20 \pm 3$ M^{-1} s^{-1}, $k_{ues}/K_{seu} = 338 \pm 48$ M^{-1} s^{-1}, $k_{uesu}/K_{seuu} = 173 \pm 9$ M^{-1} s^{-1}, $K_{ue}' = 4.16 \pm 1.15$ μM, and $K_{uue}' = 5.05 \pm 3.01$ μM. As a positive control in this experiment, the effect of Ub on IPaseT was examined. These data are also shown in Figure 5 and, when fit to eq 1, provide the following: $k_{es}/K_{se} = 5 \pm 19$ M^{-1} s^{-1}, $k_{ues}/K_{seu} = 893 \pm 38$ M^{-1} s^{-1}, $k_{uesu}/K_{seuu} = 241 \pm 14$ M^{-1} s^{-1}, $K_{ue}' = 0.086 \pm 0.012$ μM, and $K_{uue}' = 2.61 \pm 0.51$ μM. These results are in excellent agreement with those previously summarized (see Table 2).

DISCUSSION

Peptide-AMC Substrates for Isopeptidase T. In this paper, we describe the first fluorogenic substrates for IPaseT: Z-Arg-Gly-Gly-AMC, Z-Leu-Arg-Gly-Gly-AMC, and Z-Arg-Leu-Arg-Gly-Gly-AMC. In designing these substrates, we reasoned that IPaseT might recognize N-blocked peptides that are based on the C-terminus of Ub. These peptide-AMCs are in fact substrates for IPaseT and form the basis of continuous assays for precise mechanistic studies or microtiter plate assays for high-throughput inhibitor screening. Other assays have of course been described for IPaseT.

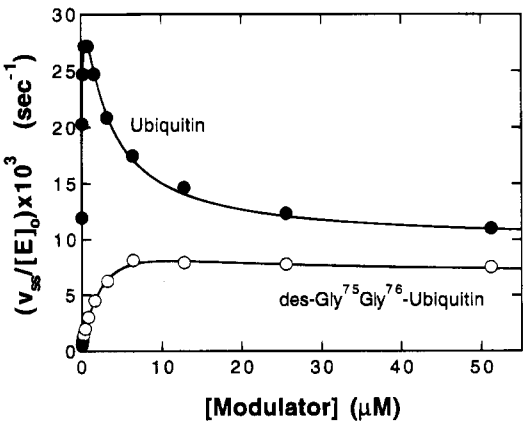


FIGURE 5: Dependence of steady-state velocity for the IPaseT-catalyzed hydrolysis of Z-Leu-Arg-Gly-Gly-AMC on the concentration of Ub or des-Gly^{75}Gly^{76}-Ub. In these experiments, [S] = 40 μM and [E] = 8 nM. Reactions were conducted at 25.0 ± 0.02 °C in an assay buffer of 20 mM HEPES, 0.5 mM EDTA, pH 7.8, containing 1 mg/mL ovalbumin and 10 mM DTT. The data were fit to eq 1 and the following parameters, along with this equation, were used to draw the lines through the data. Ubiquitin: $(k_{es}/K_{se})[S] = (0.21 \pm 0.92) \times 10^{-3}$ s^{-1}, $(k_{ues}/K_{seu})[S] = (35.7 \pm 1.5) \times 10^{-3}$ s^{-1}, $(k_{uesu}/K_{seuu})[S] = (9.64 \pm 0.56) \times 10^{-3}$ s^{-1}, $K_{ue}' = 0.086 \pm 0.0012$ μM, and $K_{uue}' = 2.61 \pm 0.51$ μM. des-Gly^{75}Gly^{76}-Ubiquitin: $(k_{es}/K_{se})[S] = (0.80 \pm 0.11) \times 10^{-3}$ s^{-1}, $(k_{ues}/K_{seu})[S] = (13.5 \pm 1.9) \times 10^{-3}$ s^{-1}, $(k_{uesu}/K_{seuu})[S] = (6.92 \pm 0.36) \times 10^{-3}$ s^{-1}, $K_{ue}' = 4.16 \pm 1.15$ μM, and $K_{uue}' = 5.05 \pm 3.01$ μM.

In one case, Chen and Pickart (1990) prepared short Ub isopeptide polomers biosynthetically using E2-25k and found that these were substrates for IPaseT. More frequently, investigators use polyUb−protein conjugates as substrates (Chen & Pickart, 1990; Hadari et al., 1992). In all of these cases, reaction progress is followed by monitoring release of Ub monomers using SDS−gel electrophoresis. In earlier studies of a purified UCH that has properties similar to those of IPaseT, Wilkinson et al. (1986) monitored the hydrolysis of Ub-OEt, with HPLC detection of Ub. While these assays are suitable for many purposes, they do not offer the precision and convenience of a continuous, fluorogenic assay.

The IPaseT-catalyzed hydrolyses of the peptide substrates reported herein occur with values of k_c/K_m that increase by at least 3 orders of magnitude as substrate length increases from Z-Gly-Gly-AMC to Z-Arg-Leu-Arg-Gly-Gly-AMC (see Table 1). This observation suggests that IPaseT has an extended active site that can bind peptide substrates. This is a structural feature that is common among proteolytic enzymes (Stein et al., 1987). During catalysis by proteases, and now IPaseT, interaction of a peptide substrate with the enzyme's extended active site liberates free energy which can be utilized for transition-state stabilization. Peptides of critical length and/or sequence interact most favorably and are hydrolyzed with values of k_c/K_m that are frequently orders of magnitude larger than k_c/K_m values for suboptimal peptides (Stein et al., 1987).

Correlation of substrate structure with k_c/K_m defines substrate specificity (Fersht, 1985). For IPaseT, the studies reported herein probe the structural features of small peptides that are required for efficient catalysis by IPaseT. It is, of course, our long-term goal to understand the mechanistic origins of substrate specificity for IPaseT. In these, our initial efforts, we sought only to dissect k_c/K_m and determine how substrate specificity is manifested in the individual parameters, k_c and K_m. The results of this analysis is summarized in Table 1 and provide several insights.

(1) The extension of Z-Gly-Gly-AMC to Z-Arg-Gly-Gly-AMC results in an increase in k_c/K_m that is greater than 10-fold. Thus, occupancy of the S_3[3] subsite by an Arg residue is critical for catalytic efficiency of IPaseT.

(2) Further extension of substrate structure from Z-Arg-Gly-Gly-AMC to Z-Leu-Arg-Gly-Gly-AMC results in a 20-fold increase in k_c/K_m. Since both of these substrates bind very weakly with K_m values greater than 2 mM, this increase in catalytic efficiency is due to an increase in k_c. Thus, the additional binding energy derived from extending Z-Arg-Gly-Gly-AMC to Z-Leu-Arg-Gly-Gly-AMC is used to stabilize catalytic transition states and not to stabilize stable-state structures, such as the Michaelis complex or an acyl-enzyme.

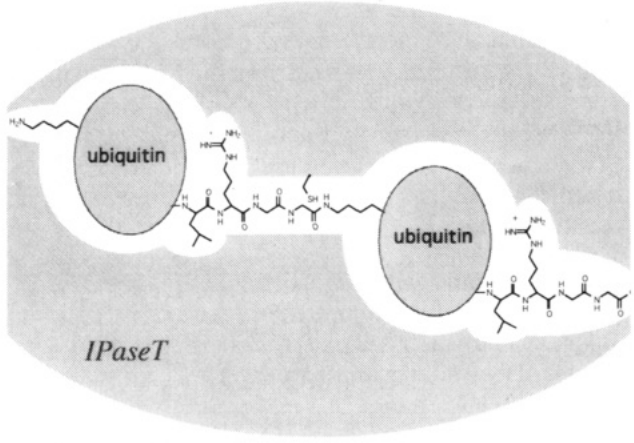
(3) When Z-Leu-Arg-Gly-Gly-AMC is extended to Z-Arg-Leu-Arg-Gly-Gly-AMC, k_c/K_m increases by only a factor of 5. Interestingly, this results from a 10-fold *decrease* in k_c offset by a 50-fold decrease in K_m. This observation is consistent with two mechanisms: (i) nonproductive binding of the longer substrate or (ii) a mechanism in which the free energy that is released when Arg binds to the S_5 subsite is utilized primarily for ground-state stabilization at the expense of transition-state stabilization. It is currently unclear which of these mechanisms obtains for IPaseT.

Modulation of Isopeptidase T Activity by Ubiquitin. In the course of these studies, we discovered that the catalytic activity of IPaseT can be modulated by Ub. For all three substrates, the modulation is biphasic with an activation phase that peaks at Ub concentrations of ~0.5 μM followed by partial inhibition at higher concentrations of Ub (see Figure 2 and Table 2). The simplest kinetic mechanism that can account for these results is shown in Scheme 1 and involves three parallel pathways for conversion of substrate to product. The contribution that each pathway makes to the overall hydrolysis of substrate is modulated by Ub concentration according to eq 1. At least for Z-Leu-Arg-Gly-Gly-AMC, Scheme 1 can adequately account for IPaseT catalysis.

One of the features of the model of Scheme 1 is that it requires IPaseT to bind two molecules of Ub. Independent evidence for two Ub binding sites has been obtained by Hadari et al. (1992), who observed that while treatment of IPaseT with Ub-H has no effect on the equilibrium constant for association of the enzyme with Ub, treatment with Ub-H does decrease the rate constant for the dissociation of the IPaseT−Ub complex. They interpreted this result to suggest that IPaseT has two Ub binding sites: one that can bind Ub-H, and substrate analogs of Ub, and another that can bind free Ub. They speculated that the two sites are adjacent to one another and actually form an extended active site in which two Ub moieties of a polyUb chain bind and undergo isopeptide bond hydrolysis between them. This is shown in Scheme 4, where the Michaelis complex of IPaseT and diubiquitin is shown. According to this model, Ub-H binds to the same site as the N-terminal Ub of diubiquitin and free Ub binds to the adjacent site where the C-terminal and

[3] The nomenclature for the amino acid residues of the substrate (P_n, ..., P_3, P_2, P_1-P_1', P_2', P_3', ..., P_n'; cleavage occurs at P_1−P_1') and the corresponding protease subsites to which they bind (S_n, ..., S_3, S_2, S_1−S_1', S_2', S_3', ..., S_n') is that of Schecter and Berger (1967).

Scheme 4: Proposed Binding Mode of Diubiquitin to the Extended Active Site of Isopeptidase T



diubiquitin binds. It is assumed, that, after binding, Ub-H forms a hemithioacetal with the active site thiol group.

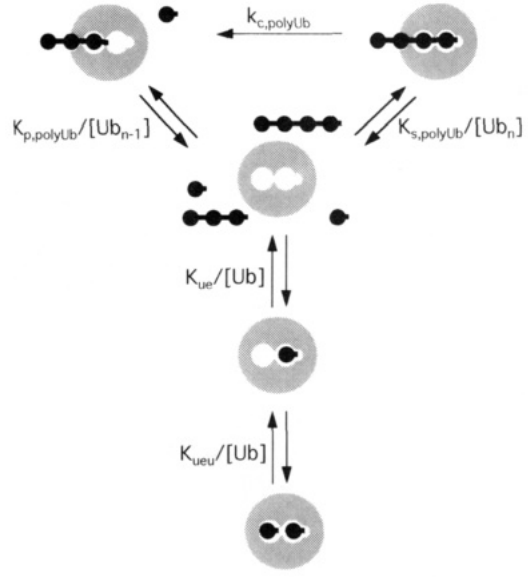
Their results and model are supported by the studies reported herein. Our value of K_{ue} (see Scheme 1) for the binding of Ub to IPaseT is ~0.1 μM (Table 2) and very similar to the K_d of 0.2 μM that we calculate from results that Hadari et al. (1992) presented in their Figure 5. Furthermore, they reported that binding of Ub cannot be competed by des-Gly^{75}Gly^{76}-Ub or a C-terminal alcohol derivative of Ub. This result is consistent with the 40-fold increase in K_{ue} that we observe when Ub is replaced with des-Gly^{75}Gly^{76}-Ub.

The importance of an intact C-termini is also supported by recent experiments of Pickart and Wilkinson (personnel communication), who have studied the hydrolysis of polyUb by IPaseT. In these studies, Pickart and Wilkinson found that whereas $(Ub)_n$ ($n = 2-6$) is efficiently hydrolyzed by IPaseT, the C-terminally modified substrate $(Ub)_{n-1}$-des-Gly^{75}Gly^{76}-Ub is not hydrolyzed at all. Taken together, the work of Hadari et al. (1992), Pickart and Wilkinson, and this study suggest a hydrolytic mechanism for IPaseT in which IPaseT preferentially cleaves off Ub monomers from the C-terminal end of polyUb.[4]

Combined, these results also suggest a structural basis for modulation of the peptidase activity of IPaseT. According to this structural model, when Ub binds to free IPaseT, it binds to the same site as the C-terminal Ub of diubiquitin (see Scheme 4). This is a high affinity site ($K_d \sim 0.1$ μM $= K_{ue}$ of Scheme 1) and, when occupied by Ub, it activates the peptidase activity of IPaseT. At high concentrations of Ub, when Ub binds to IPaseT−Ub to form Ub−IPaseT Ub, it binds to the same site as the N-terminal Ub of diubiquitin (see Scheme 4). This is a low affinity site ($K_d \sim 3$ μM $= K_{uue}$ of Scheme 1) and, when occupied by Ub, inhibits the peptidase activity of IPaseT.

This leads us to the most intriguing question that emerges from our studies: What is the mechanism by which Ub is able to activate IPaseT for hydrolysis of peptide-AMC substrates? We really have no insight into how binding of Ub to IPaseT can cause increased catalytic efficiency other than speculative proposals of Ub-induced conformational changes of IPaseT. This remains an area of investigation in our laboratory.

Scheme 5: Mechanism of Product Inhibition of IPaseT by Ubiquitin



Physiological Relevance of the Binding of Ubiquitin to IPaseT. In this study, we demonstrate that IPaseT can bind Ub at two sites with dissociation constants of 0.1 and 3 μM, respectively. The question we raise here is whether binding of Ub to these sites might represent a regulatory mechanism to control IPaseT activity. Given that the intracellular concentration of Ub is on the order of 10−20 μM (Haas, 1988), we believe that regulation of IPaseT by Ub is a distinct possibility and propose Scheme 5 as our mechanistic hypothesis.

The mechanism of Scheme 5 indicates that as Ub_n is hydrolyzed by IPaseT and levels of Ub rise, K_{ue}/[Ub] and K_{ueu}/[Ub] will become small relative to $K_{s,polyUb}/[Ub_n]$. As a consequence, Ub−E and Ub−E−Ub will accumulate as the predominant forms of IPaseT and hydrolysis of Ub_n will be slowed. When levels of Ub_n again rise, for example, due to hydrolysis of ubiquitinated proteins by the 26S proteasome, $K_{s,polyUb}/[Ub_n]$ will become small relative to K_{ue}/[Ub] and hydrolysis of Ub_n will once more commence.

From this mechanism, it is also apparent that Ub_{n-1} can act as a product inhibitor if it binds nonproductively to IPaseT via the pathway govered by $K_{p,polyUb}$. The contribution that this makes to regulation of IPaseT activity currently cannot be estimated.

[4] Note that Hadari et al. (1992) also found that IPaseT can cleave polyUb−protein substrates internally to produce shortened polyUb*−protein species and shorter polyUb chains. Hadari et al. went on to state that these polyUb chains are "highly susceptible to the action of IPaseT" (step 3 of their Figure 10).

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