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Inhibitors Can Activate Proteases To Catalyze the Synthesis and Hydrolysis of Peptides

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ABSTRACT: Competitive inhibitors can activate proteases (papain, trypsin, and cathepsin S) to catalyze the synthesis of peptide bonds and accelerate the hydrolysis of poor substrates (from 1 to 99%). Reaction mixtures contained intermediate molecules that were formed by the coupling of the inhibitor with the poor substrate. This and other findings suggest the following chain of events. Part of the binding energy of formation of the enzyme−inhibitor complex was used to activate the inhibitor, i.e., to form acyl−enzyme species with a high-energy bond (e.g., a thioester bond in the case of papain) required for coupling the inhibitor with the substrate to form the intermediate molecule. The latter was subjected to successive reactions which led to a stepwise degradation of the substrate, as well as to the regeneration of the inhibitor. One mole of the inhibitor could catalyze rapid hydrolysis of at least 53 mol of substrate. The intermediate molecules were the species undergoing rapid hydrolysis. Therefore, 1 mol of inhibitor was involved in the synthesis of 53 mol of intermediate molecules; i.e., the inhibitor functioned as a cofactor that catalyzed the synthesis of peptides. Thus, the binding energy of formation of the enzyme−inhibitor complex can be utilized to catalyze the synthesis of peptide bonds in the absence of an exogenous energy source (e.g., ATP).

The possibility that protease inhibitors can activate enzymatic activity stems from earlier studies on mapping of the active site of papain. It was shown that papain has a large active site (25 Å long) capable of accommodating seven amino acid residues of the substrate (*1*) and that specificity was determined by a hydrophobic subsite, S2 (*2*). The map of the active site enabled rational design of competitive inhibitors in which P2 was a hydrophobic residue (e.g., BocPheAla)[1] (*2*, *3*) and allowed determination of the binding energy of amino acid side chains in various subsites. For example, Δ^2F values were 0.8 kcal/mol for the α-methyl of L-Ala in subsite S1 and 0.9 and 3.1 kcal/mol for the benzene group of L-Phe in S1 and S2, respectively (*3*, *4*). The strict stereospecificity of S2, S1, and S1′ is evident from X-ray crystallography (*5*, *6*) and three-dimensional model building (*3*, *7*) of EI and ES complexes. The α-hydrogens of P2, P1, and P1′ (L-optical isomer) pointed toward the enzyme and the side chain away from it. Therefore, substitution of an L-residue with a D-residue would place the side chain in the position of the α-hydrogen, causing steric interference, disruption of hydrogen bonds, and loss of favorable contacts of the side chain with the enzyme (*3*, *7*). It was found that replacement of L-Ala with D-Ala at P1 of the inhibitor reduces the binding energy by 2.8 kcal/mol (*7*). This strong reduction in Δ^2F could be explained if the P1 α-carboxyl of the inhibitor forms a thioester with the active site Cys-25 of papain. In this case, the P1 α-hydrogen of the inhibitor would be in contact with the enzyme's surface. Consequently, replacement of an L-residue with a D-residue would markedly reduce the binding energy. Accordingly, we proposed that competitive inhibitors would form a thioester bond with Cys-25, i.e., a covalent acyl−enzyme species as obtained with substrates (*3*, *7*). The demonstration that these inhibitors are bound by active papain (Cys-25 with free SH) but not by inactive papain (modified SH of Cys-25) (*8*, *9*) and X-ray data of papain EI complexes indicating the possibility of formation of an acyl−enzyme species (*5*, *6*) further support the formation of acyl−enzyme species. Direct evidence for the formation of acyl−papain species with competitive inhibitors containing a hydrophobic residue at P2 and a free α-carboxyl at P1 was obtained by using specially designed inhibitors (containing α,*β*-unsaturated aromatic residues in P1) that exhibited spectral changes upon thioester bond formation (*10*). Both acylation (k_2) and deacylation (k_{-2}) rate constants revealed that most of the papain-bound inhibitor was in the form of covalent acyl−enzyme species (*11*). Here we demonstrate that acyl−enzyme species formed by inhibitors can catalyze the synthesis of peptide bonds and accelerate the hydrolysis of poor substrates.

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[1] Abbreviations: Ac, acetyl; Boc, butyloxycarbonyl; EI, enzyme−inhibitor complex; ES, enzyme−substrate complex; Int, intermediate molecule; Suc, succinyl.

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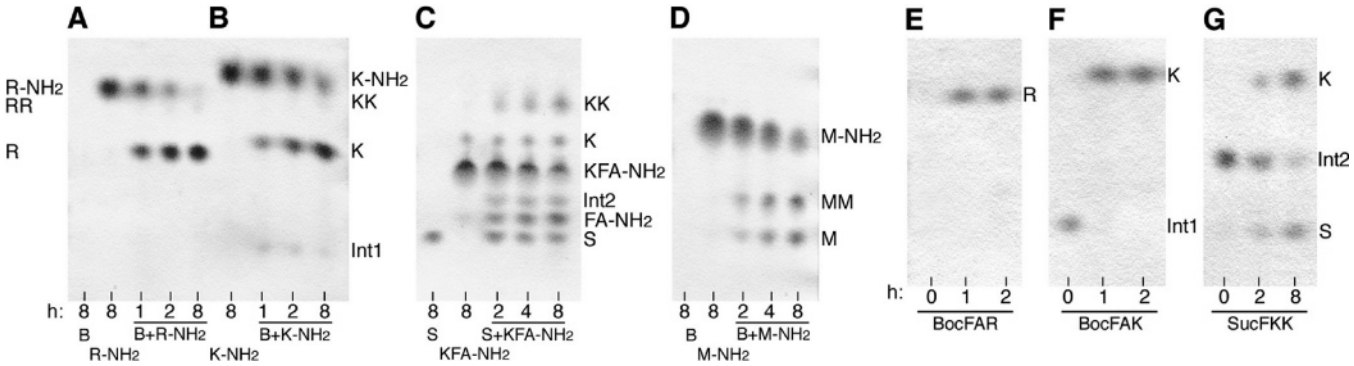

FIGURE 1: Paper electrophoresis data showing acceleration of protease activity by inhibitors (A−D) and enzymatic hydrolysis of intermediate molecules (E−G). (A) Papain (108 μg/mL) with BocPheAla (B, 5 mM) alone, Arg-NH2 (R-NH2, 20 mM) alone, and BocPheAla with Arg-NH2 (B+R-NH2). (B) Papain (108 μg/mL) with Lys-NH2 (K-NH2, 20 mM) alone and BocPheAla with Lys-NH2 (B+K-NH2). (C) Trypsin (87 μg/mL) with SucPheLys (S, 5 mM) alone, LysPheAla-NH2 (KFA-NH2, 20 mM) alone, and SucPheLys with LysPheAla-NH2 (S+KFA-NH2). (D) Cathepsin S (14 μg/mL) with BocPheLeu (B, 5 mM) alone, Met-NH2 (M-NH2, 20 mM) alone, and BocPheLeu with Met-NH2 (B+M-NH2). Components of the reaction mixtures: Arg-NH2 (R-NH2), ArgArg (RR), arginine (R), Lys-NH2 (K-NH2), LysLys (KK), lysine (K), BocPheAlaLys (Int1), LysPheAla-NH2 (KFA-NH2), SucPheLysLys (Int2), PheAla-NH2 (FA-NH2), SucPheLys (S), Met-NH2 (M-NH2), MetMet (MM), and methionine (M). Reaction products (R, RR, K, KK, FA-NH2, M, and MM) were identified by using authentic markers. Intermediate molecules (Int1 and Int2) were isolated by FPLC and characterized by mass spectrometry and enzymatic hydrolysis (see panels E−G). The intermediate molecules BocPheAlaArg (E) and BocPheAlaLys (F) were digested by papain (54 μg/mL), while SucPheLysLys (G) was digested by trypsin (87 μg/mL): Arg (R), Lys (K), BocPheAlaLys (Int1), SucPheLysLys (Int2), SucPheLys (S). Aliquots of the reaction mixture kept at 37 °C for the indicated time (h, hours) were resolved by paper electrophoresis and stained with ninhydrin (*13*).

MATERIALS AND METHODS

Materials and Enzyme Assays. Peptides used as donors and acceptors were described previously (*1*−*3*, *7*, *12*). The dipeptides ArgArg, LysLys, LeuMet, and MetMet (Bachem), papain (twice crystallized, Sigma), bovine trypsin (TPCK-treated, Sigma), and recombinant human cathepsin S (Calbiochem) were purchased from commercial sources. Conditions for the interaction of the enzyme, donor, and acceptor were as follows: for papain, 3.3 mM citric acid/44 mM Na_2HPO_4 (pH 7), 10 mM mercaptoethanol, and 1 mM EDTA; for cathepsin S, 5.7 mM citric acid/35 mM Na_2HPO_4 (pH 6.5), 10 mM mercaptoethanol, and 1 mM EDTA; and for trypsin, 33 mM Tris-HCl (pH 8.0). Reactions were conducted at 37 °C.

Analysis of Reaction Mixtures. Samples (2−5 μL) were loaded on Whatman no. 3 paper, and components of the reaction mixture were separated by paper electrophoresis in 0.5 M formic acid or 0.5 M acetic acid, essentially as described previously (*13*). Spots on the paper sheet were developed with ninhydrin; the colored spots were cut out, dipped in elution solution, and centrifuged, and the color of the clear supernatant was quantified by measuring the absorbance at 570 nm (*14*).

Materials not stained by ninhydrin (BocPheAla and BocPheAlaArg) and intermediate molecules (Int1 and Int2; see Figure 1B,C,F,G) were analyzed by FPLC. Samples of papain digests for FPLC were prepared with 1 mM DTT instead of mercaptoethanol because the later eluted as a relatively high peak (absorbance at 220 nm) near the peak of the intermediate molecules. This modification did not affect the pattern of reaction products and slightly reduced (∼15%) the level of substrate degradation (tested by paper electrophoresis). The reaction mixture (15−40 μL) was loaded on a Superdex Peptide HR 10/30 column (Pharmacia) run with water. The effluent was monitored at 220 nm. Peak fractions were separated, concentrated (1 mL lyophilized and dissolved in 10−20 μL of H_2O) and characterized by enzyme digestion followed by paper electrophoresis and FPLC. The molecular weight of the material in the peak fractions was determined by using MALDI-TOF mass spectrometry (Bruker, reflex3).

RESULTS AND DISCUSSION

BocPheAla is a competitive inhibitor of papain that is not cleaved by the enzyme (*2*, *3*). Arginine amide is a poor substrate of the enzyme (1% hydrolysis to arginine after 24 h with papain, 108 μg/mL). However, when BocPheAla and Arg-NH2 were mixed with papain, the Arg-NH2 was rapidly hydrolyzed (Figures 1A and 2A). The pH profile of the reaction was determined (pH 4−8) because binding of the inhibitor to papain is optimal at low pH (*2*, *3*) while nucleophilic attack by Arg-NH2 is optimal at high pH. The results show that the hydrolysis of Arg-NH2 by papain in the presence of BocPheAla is maximal at pH 7 (data not shown).

The time course of the enzymatic hydrolysis of Arg-NH2 at different BocPheAla concentrations is given in Figure 2. At 20 mM Arg-NH2 and 5 mM BocPheAla (4:1 Arg-NH2:BocPheAla ratio), the Arg-NH2 is rapidly hydrolyzed nearly to completion (80% after 2 h and 99% after 6 h). That is, 1 mol of BocPheAla enables papain to rapidly hydrolyze 4 mol of Arg-NH2 (Figure 2B). The level of hydrolysis of Arg-NH2 diminishes with decreasing concentrations of BocPheAla from 5 to 0.1 mM. Yet, even at 0.1 mM BocPheAla, the hydrolysis of Arg-NH2 is still much faster than the hydrolysis without BocPheAla (Figure 2A). The efficacy of BocPheAla in accelerating the hydrolysis of Arg-NH2 is markedly increased with decreasing concentrations of BocPheAla. One mole of BocPheAla enables papain to hydrolyze 3.9, 19.6, 42.6, and 53.6 mol of Arg-NH2 at BocPheAla concentrations of 5, 1, 0.2, and 0.1 mM, respectively (Figure 2B and Table 1). With BocPheAla at 5 and 1 mM, the extent of hydrolysis leveled off because Arg-NH2 in the reaction was completely degraded. At lower BocPheAla concentrations, the hydrolysis of Arg-NH2 did not plateau (Figure 2A), suggesting that, in

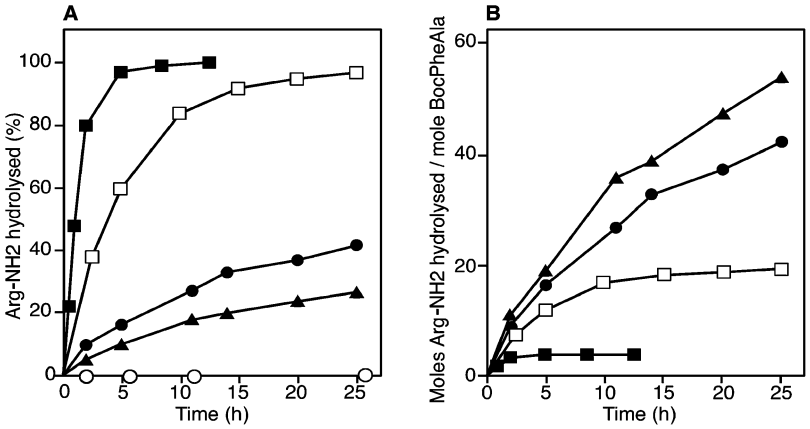

FIGURE 2: Effect of inhibitor concentration on the hydrolysis of arginine amide by papain. (A) Hydrolysis of Arg-NH2 (20 mM) by papain (108 μg/mL) in the presence of BocPheAla at 5 (■), 1 (□), 0.2 (●), and 0.1 mM (▲). Arg-NH2 (20 mM) and papain (108 μg/mL) without BocPheAla (○). (B) Ratio of the moles of Arg-NH2 hydrolyzed per mole of BocPheAla.

Table 1: Hydrolysis of Poor Substrates (Acceptors) in the Tripartite Reaction

donor (mM)	acceptor:donor ratio	moles of poor substrate (acceptor) hydrolyzed per mole of donor[a]				
		papain[b]		trypsin[c]		cathepsin S[d]
5	4	3.9	3.9	3.6	3.2	3.1
2.5	8			5.2	3.9	5.1
1	20	19.6	15.0	9.7	7.6	9.7
0.2	100	42.6	21.2			
0.1	200	53.6				

[a] Hydrolysis of acceptor after 24 h. [b] Papain (108 μg/mL). Donor, BocPheAla at indicated concentrations. Acceptor, 20 mM Arg-NH2 (first value) or Lys-NH2 (second). [c] Trypsin (87 μg/mL). Donor, SucPheLys at indicated concentrations. Acceptor, 20 mM LysPhe-NH2 (first value) or LysPheAla-NH2 (second). [d] Cathepsin S (7 μg/mL). Donor, BocPheLeu at indicated concentrations. Acceptor, 20 mM Met-NH2.

the presence of papain, 1 mol of BocPheAla could catalyze the hydrolysis of more than 53.6 mol of Arg-NH2 (see Figure 2B). Another product of the reaction was the ArgArg dipeptide that was produced in small amounts. Initially, it was barely detectable, but as the reaction proceeded to completion, it comprised ~2.5% of the free arginine (see Figure 1A).

We speculated that the tripartite reaction would involve an intermediate molecule composed of BocPheAla (donor) and Arg-NH2 (acceptor). This putative intermediate was not observed by paper electrophoresis stained with ninhydrin since BocPheAla coupled to Arg-NH2 has no α- or ϵ-amine needed for color development with ninhydrin. Therefore, we studied lysine amide that bears an ϵ-amine.

Lysine amide shows a reaction pattern similar to that obtained with Arg-NH2. Lys-NH2 is a poor substrate (1% hydrolysis after 24 h with papain, 108 μg/mL). The addition of BocPheAla causes rapid hydrolysis of Lys-NH2 to lysine and small amounts of LysLys (optimum pH of 7). Decreasing concentrations of BocPheAla decrease the rate of hydrolysis of Lys-NH2 but markedly increase the "catalytic activity" in terms of moles of Lys-NH2 hydrolyzed per mole of BocPheAla (Table 1). Lys-NH2 hydrolyzes at a slower rate than Arg-NH2 (see Figure 1 and Tables 1 and 2). However, with Lys-NH2, we observed a new ninhydrin positive spot corresponding to the intermediate molecule, which was evident at the beginning of the reaction and then gradually faded (Int1 in Figure 1B).

Table 2: Donor and Acceptor Specificity in the Tripartite Reaction

Influence of Donor Structure		
	hydrolysis (%)	
donor	papain[a]	trypsin[b]
BocPheAla	53	3
BocPheLeu	38	3.2
BocPheGlu	2.7	2.9
BocPheLys	1.6	1.5
BocPheArg	1.4	5.7
AcPheAla	27	2.9
AcPheLeu	24	2.8
AcPheLys	1.8	49.5
SucPheLys	2.6	63.5
Influence of Acceptor Structure		
	hydrolysis (%)	
acceptor	papain[c]	trypsin[c]
Arg-HN2	49.5 (−)	− (−)
Lys-NH2	29.5 (−)	− (−)
Met-NH2	69.5 (−)	
Leu-NH2	29.5 (6)	
Ala-NH2	5.3 (−)	
LysPhe-NH2	17.8 (−)	68 (6.1)
LysPhe	− (−)	− (−)
LysPheAla-NH2	7.2 (−)	52.4 (7.2)
MetMet	− (−)	
LeuMet	− (−)	

[a] Percent hydrolysis of Arg-NH2 (20 mM) by papain (54 μg/mL) in the presence of the indicated donor (5 mM) after 2 h. Hydrolysis of Arg-NH2 by papain without donor was undetectable after 2 h. All the donors were not cleaved by the enzyme after 24 h. [b] Percent hydrolysis of LysPhe-NH2 (20 mM) by trypsin (87 μg/mL) in the presence of the indicated donor (5 mM) after 5 h. The percent hydrolysis of LysPhe-NH2 by trypsin without donor was 4.6% after 5 h. All the donors were not cleaved by the enzyme after 24 h. [c] Percent hydrolysis of the indicated acceptor (20 mM) by papain (54 μg/mL) in the presence of BocPheAla (5 mM) after 2 h or by trypsin (87 μg/mL) in the presence of SucPheLys (5 mM) after 5 h. The percent hydrolysis of acceptor by enzyme without donor is given in parentheses. Dashes denote that the hydrolysis of the acceptor was undetectable.

FPLC of the reaction mixture containing Lys-NH2, BocPheAla, and papain showed a new peak, and mass spectrometry revealed that it contained molecules with a molecular weight of 464, identical to that of BocPheAlaLys. The peak of BocPheAlaLys was isolated and concentrated. Paper electrophoresis showed a spot with electrophoretic mobility identical to that of the new spot (Int1) seen in the complete reaction mixture (data not shown). Digestion with

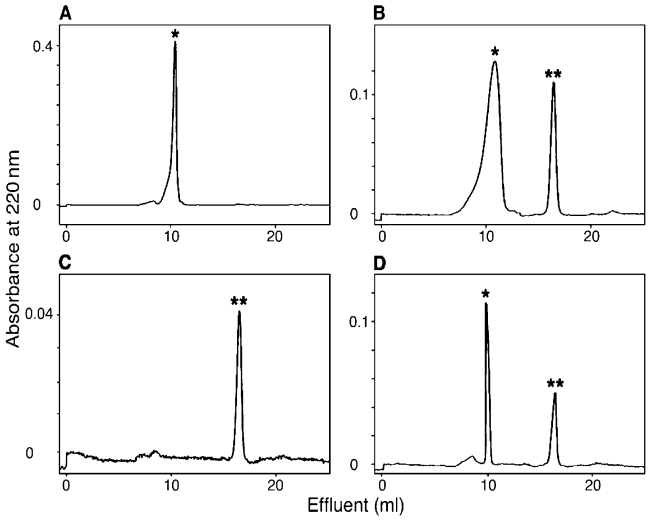

FIGURE 3: FPLC profiles showing formation and degradation of the intermediate molecule BocPheAlaArg. (A) Control of BocPheAla (asterisk) alone. (B) Tripartite reaction of BocPheAla, Arg-NH2, and papain (108 μg/mL) after 0.5 h showing a new peak of BocPheAlaArg (two asterisks). (C) BocPheAlaArg (two asterisks) isolated from the reaction mixture. (D) Purified BocPheAlaArg (two asterisks) and papain (54 μg/mL) after 0.5 h showing formation of BocPheAla (asterisk). The authenticity of BocPheAla and BocPheAlaArg was ascertained by mass spectrometry and hydrolysis of BocPheAlaArg by papain into BocPheAla (D) and arginine (Figure 1E).

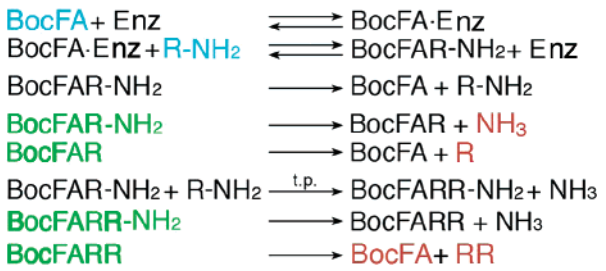

FIGURE 4: Scheme of the tripartite reaction with the enzyme, inhibitor, and poor substrate. Papain (Enz) in the presence of the inhibitor BocPheAla (BocFA) degrades Arg-NH2 (R-NH2) in a stepwise manner to Arg (R), ammonia (NH3), and ArgArg (RR). BocFA.Enz is the acyl−enzyme species in which BocFA is coupled to the active site Cys-25 of papain by a thioester bond. BocPheAlaArg-NH2 (BocFAR-NH2) is the primary intermediate molecule. t.p. means transpeptidation. Light blue denotes reactants, orange reaction products, and green intermediate molecules.

papain shows rapid degradation of BocPheAlaLys (95% after 1 h), the release of lysine [detected by paper electrophoresis (Figure 1F)], and BocPheAla [detected by FPLC (data not shown)]. The intermediate molecule generated by Arg-NH2 and BocPheAla, which was not seen by paper electrophoresis, is readily detected by FPLC as a new peak of molecules with a molecular weight of 492, identical to that of BocPheAlaArg (Figure 3B). It was isolated (Figure 3C), and papain rapidly digested BocPheAlaArg (95% after 1 h) to produce arginine (Figure 1E) and BocPheAla (Figure 3D).

The findings described above suggest consecutive reactions of peptide synthesis and hydrolysis as outlined in Figure 4. The inhibitor (donor) and papain form acyl−enzyme species in which BocPheAla is coupled to Cys-25. The acyl−enzyme species reacts with Arg-NH2 which acts as a nucleophile (i.e., as an acceptor in which the Arg moiety occupies subsite S1′ but not S1 as a substrate) to yield the primary intermediate molecule BocPheAlaArg-NH2. The fate of this primary intermediate is dependent on its mode of binding to the active site of papain. It was previously shown that same peptide can bind to the enzyme in various ways (*1*, *3*, *7*). For example, hexa-L-alanine and papain form four different complexes which hydrolyze at different rates ($k_{cat}/K_m = 10-450$ M^{-1} s^{-1}), and each complex yields different products (Ala5 with Ala, Ala4 with Ala2, etc.) (*3*, *7*). It is likely that most of the BocPheAlaArg-NH2 would be rapidly hydrolyzed to BocPheAla and Arg-NH2 due to interaction of the Phe residue with hydrophobic subsite S2 of papain. However, a small fraction of the intermediate molecule might bind in a different manner where the Phe residue occupies S3 to yield ammonia and BocPheAlaArg which is then hydrolyzed to arginine and BocPheAla. Thus, in the tripartite reaction, papain catalyzes the hydrolysis of Arg-NH2 in a stepwise manner: coupling to the donor, the release of ammonia and then arginine, and regeneration of BocPheAla. In addition, via transpeptidation Arg-NH2 is degraded to yield ammonia and ArgArg (see below and Figure 4).

BocPheAlaArg-NH2 should be a better substrate than BocPheAlaArg because at pH 7 the latter has P1′ bearing a charged carboxylate ion which is bound at S1′ at lower affinity compared to uncharged P1 (*3*, *7*). Accordingly, BocPheAlaArg-NH2 is undetectable. On the other hand, the more stable BocPheAlaArg can be detected and isolated (Figure 3C).

Formation of the dipeptide ArgArg could be explained as follows. Transpeptidation between BocPheAlaArg-NH2 and Arg-NH2 would generate BocPheAlaArgArg-NH2. The release of ammonia from the latter would form BocPheAlaArgArg that could be readily hydrolyzed (Phe occupies S2) to ArgArg and regeneration of BocPheAla (Figure 4). Thus, the primary intermediate BocPheAlaArg-NH2 is processed in three different manners: (1) hydrolysis to the original reactants, (2) hydrolysis to yield ammonia, and (3) transpeptidation with Arg-NH2 to form BocPheAlaArgArg-NH2 (see Figure 4). The same mechanism can be applied to form LysLys from Lys-NH2.

The influence of donor and acceptor structure on the hydrolysis of the acceptor by papain is summarized in Table 2. Donors with uncharged residues at P1 (Ala and Leu) are more effective catalysts for the hydrolysis of Arg-NH2 than donors with charged residues (Glu, Lys, and Arg). As acceptors, different amino acid amides (occupying S1′) exhibit a wide range of rates of hydrolysis (69.5−5.3%). Lys-NH2, Met-NH2, Leu-NH2, and LysPhe-NH2 are hydrolyzed, while the related free dipeptides (LysPhe, MetMet, and LeuMet) are not, suggesting strong interference by the charged α-carboxyl in S2′. Our limited data demonstrate specificity of subsites S1, S1′, and S2′ toward the donor and acceptor in the tripartite reaction. This specificity may be similar to that found for substrates and inhibitors (*3*), but occasionally, it may differ. X-ray analysis of EI complexes of papain shows that inhibitors which occupy a portion of the active site (subsites S*n*−S1) cause widening of the active site groove by ~1 Å (*6*). Therefore, subsites (S1′−S*n*′) that interact with the acceptor may have different properties in the free enzyme and in the EI complex.

Similar experiments conducted with bovine trypsin and human cathepsin S show reaction patterns like those obtained with papain. The results are briefly described below. Several peptides were screened for the tripartite reaction with trypsin

(optimum pH of 8). Considering the specificity of trypsin, as donors we tested N-blocked dipeptides with Arg or Lys in P1, previously shown to inhibit trypsin (*12*). As acceptors, we tested molecules with an N-terminal Lys or Arg (Table 2) to generate a new susceptible bond in the putative intermediate molecule, as indeed was found. For further analysis, we used the inhibitor SucPheLys (donor) that is not cleaved by the enzyme and LysPheAla-NH2 (acceptor) that is slowly hydrolyzed by trypsin (see Figure 1C and Table 2). The addition of SucPheLys causes rapid degradation of LysPheAla-NH2 to PheAla-NH2, lysine, and significant amounts of the dipeptide LysLys (Figure 1C). One mole of SucPheLys enables trypsin to degrade 7.6 mol of LysPheAla-NH2 (Table 1) and perhaps more since the degradation of LysPheAla-NH2 does not plateau (data not shown). An additional ninhydrin positive spot with electrophoretic mobility consistent with that of an intermediate species was observed (Int2 in Figure 1C). This material was isolated by FPLC, and mass spectrometry yielded a molecular weight of 521, identical to that of SucPheLysLys. Trypsin cleaves SucPheLysLys (50% after 2 h) into lysine and SucPheLys (Figure 1G, FPLC data not shown). These findings suggest that the inhibitor forms an acyl−enzyme species in which SucPheLys is linked to the active site Ser-183 of trypsin by an ester bond. Interaction of the acyl−enzyme species with LysPheAla-NH2 would yield the primary intermediate species SucPheLysLysPheAla-NH2 with two trypsin susceptible bonds. Hydrolysis after the first Lys would yield the initial reactants, and hydrolysis after the second Lys would yield PheAla-NH2 and SucPheLysLys, which is then cleaved to lysine and SucPheLys. These reactions and those leading to the formation of LysLys are similar to those proposed for papain (see Figure 4). In the course of these reactions, LysPheAla-NH2 is degraded in a stepwise manner to PheAla-NH2, lysine, and LysLys, while SucPheLys is regenerated. The tripartite reaction of trypsin, SucPheLys, and another acceptor, LysPhe-NH2, yields the expected products: Lys, LysLys, an identical intermediate molecule (SucPheLysLys), and Phe-NH2 which replaces PheAla-NH2 (data not shown). The hydrolysis of LysPhe-NH2 is faster (Table 2), and 1 mol of SucPheLys catalyzes the hydrolysis of 9.7 mol of LysPhe-NH2 (Table 1).

Cathepsin S is a cystein protease with a hydrophobic S2 subsite similar to papain. Several peptides and amino acids were screened for a tripartite reaction with cathepsin S (optimum pH of 6.5), and two were selected: BocPheLeu (donor) that is not cleaved by the enzyme and Met-NH2 (acceptor) that is barely hydrolyzed by the enzyme. The addition of BocPheLeu causes rapid degradation of Met-NH2 to methionine and the dipeptide MetMet (see Figure 1D). One mole of BocPheLeu enables cathepsin S to degrade 9.7 mol of Met-NH2 (Table 1) and perhaps more since the degradation of Met-NH2 does not plateau (data not shown). FPLC of the reaction mixture revealed overlapping peaks that were not purified for further characterization.

Concluding Remarks. In the tripartite reactions described here, 1 mol of donor could catalyze rapid hydrolysis of at least 53 mol of the acceptor. The intermediate molecules are the species that mediate rapid hydrolysis. Therefore, 1 mol of the donor is involved in the synthesis of 53 mol of the intermediate species; i.e., the donor functions as a cofactor that catalyzes the synthesis of peptide bonds.

Peptide bond formation is an endergonic process requiring ~3.5 kcal/bond (*15*, *16*). Therefore, for peptide synthesis, the α-carboxyl of the donor is usually activated. In organic synthesis, the α-carboxyl is converted into an acyl chloride, etc. For enzyme-catalyzed peptide synthesis, energy-rich derivatives of the donor (esters or amides) are used (*17*). In protein biosynthesis, the α-carboxyl of the amino acid is activated by ATP to form mixed anhydride with the phosphoryl group of AMP. We propose that in the tripartite reaction part of the binding energy of formation of the enzyme−inhibitor complex is used to activate the donor to form acyl−enzyme species. This species has a high-energy thioester bond (papain and cathepsin S) or ester bond (trypsin) capable of coupling the donor with the acceptor to form the primary intermediate molecule.

The primary intermediate molecules are removed from the reaction mixture by successive enzymatic reactions. Removal of the intermediate molecules causes an increased level of synthesis of these molecules to restore the equilibrium of the donor−acceptor interaction. Thus, more acceptor is incorporated into intermediate molecules. This mediates stepwise degradation of the acceptor and the regeneration of the donor in intact form which then initiates a new reaction cycle.

An important feature of the active site is its size. Multiple points of interaction are essential for obtaining the high association constants necessary for efficient biological function (*1*, *3*). Strong binding of the substrate over a large active site can also contribute to the catalytic process (*3*, *18*). In lysozyme, part of the binding energy is used to "strain" the substrate into the geometry of the transition state (*19*). In carboxypeptidase A, binding induces a conformational change in the enzyme to bring about the correct orientation of the catalytic groups (*20*). Here we demonstrate that the binding energy of formation of the enzyme−inhibitor complex can be utilized to catalyze the synthesis of peptide bonds in the absence of an exogenous energy source (e.g., ATP).

We searched the literature for papers related to this work and came across a paper published more than 65 years ago by O. Behrens and M. Bergmann, entitled Cosubstrates in Proteolysis (*21*). It is interesting to summarize their findings. They showed that the papain-resistant glycine anilide was slowly degraded if horse serum was added and concluded that the serum contained or produced substances that enabled papain to hydrolyze the anilide. To understand the action of the serum, they searched for substances of known structure which exhibit a similar effect. They found that AcPheGly, but not AcPhe, enables papain to hydrolyze glycine anilide and other papain-resistant anilides. From the reaction mixture they isolated glycine, aniline, and AcPheGlyGly-anilide, and they proposed successive reactions of synthesis and degradation. AcPheGly and Gly-anilide combine to form the intermediate molecule AcPheGlyGly-anilide which is then degraded by two hydrolytic steps to release aniline followed by glycine, and the regeneration of AcPheGly. AcPheGly is designated as a cosubstrate since it enables papain to hydrolyze otherwise enzyme-resistant molecules (up to 0.6 mol of resistant molecule per mole of cosubstrate). They mention that "the enzymatic fate of a substance may differ in accordance with the presence or absence of other substances which may act as cosubstrates" (*21*). Clearly, our studies overlap with those of Behrens and Bergmann, yet

they differ in several important aspects. Behrens and Bergmann did not know that the specificity of papain was determined by a hydrophobic subsite S2, that AcPheGly (Phe at P2) was a competitive inhibitor due to interaction with S2, that papain forms covalent acyl−enzyme species, and that the cosubstrate or inhibitor functioned as a cofactor to catalyze many cycles of peptide synthesis and hydrolysis.

Recently, it was proposed that a tetrapeptide performed the nucleophilic attack on an acyl−enzyme species formed during polypeptide degradation in proteasomes. This transpeptidation reaction conceivably generated a new antigenic determinant with a novel amino acid sequence not found in the parent protein (*22*).

What is the relevance of the tripartite reaction to biological processes? The inhibitors described here are not found in nature, yet they form an acyl−enzyme species, the initiator of the tripartite reaction, which is similar to the acyl−enzyme species formed by enzyme−substrate interaction. Acyl−enzyme species in which the acyl moiety is activated by thioester or ester bonds are widespread in nature. They are generated not only by proteases but also in a large variety of other biological processes such as ubiquitination where ubiquitin is coupled by a thioester bond to the E1 and E2 enzymes (*23*), or thioester bonds in lipid metabolism (*24*). Potentially, these acyl−enzyme species can serve as donors for naturally occurring acceptors (i.e., nucleophiles), e.g., short peptides or spermine. Therefore, it is of interest to investigate this issue, because the finding of tripartite reactions in nature would uncover yet unknown biochemical processes. This study and earlier studies (*21*) demonstrate that the enzymatic fate of a substance does not always follow a "consensus" known pattern. In the presence of other substance(s) (donor or acceptor), it may be shifted to alternate pathways leading to the synthesis of new compounds, the intermediate molecules. In the cell, some intermediate molecules may escape degradation due to diffusion to other cellular organelles or interaction with other enzyme−protein molecules and affect some biological process.

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REFERENCES

1. Schechter, I., and Berger, A. (1967) On the size of the active site in proteases. I. Papain, *Biochem. Biophys. Res. Commun. 27*, 157−162.
2. Schechter, I., and Berger, A. (1968) On the size of the active site of proteases. III. Mapping the active site of papain: Specific peptide inhibitors of papain, *Biochem. Biophys. Res. Commun. 32*, 898−902.
3. Berger, A., and Schechter, I. (1970) Mapping the active site of papain with the aid of peptide substrates and inhibitors, *Philos. Trans. R. Soc. London, Ser. B 257*, 249−264.
4. Schechter, I. (2005) Mapping of the active site of proteases in the 1960s and rational design of inhibitors/drugs in the 1990s, *Curr. Protein Pept. Sci. 6*, 501−512.
5. Drenth, J., Jansonius, J. N., Koekoek, R., and Wolthers, B. G. (1971) The structure of papain, *Adv. Protein Chem. 25*, 79−115.
6. Drenth, J., Kalk, K. H., and Swen, H. M. (1976) Binding of chloromethyl ketone substrate analogues to crystalline papain, *Biochemistry 15*, 3731−3738.
7. Berger, A., Schechter, I., Benderly, H. A., and Kurn, N. (1971) Enzyme-inhibitor interaction as a model for biological recognition, in *10th European Peptide Symposium*, pp 290−309, North-Holland Publishing Company, Amsterdam.
8. Wolthers, B. G., and Kalk, K. H. (1970) Binding of competitive (product) inhibitors to papain in the active and inactive state, *Biochim. Biophys. Acta 198*, 556−568.
9. Blumberg, S., Schechter, I., and Berger, A. (1970) The purification of papain by affinity chromatography, *Eur. J. Biochem. 15*, 92−102.
10. Smolarsky, M. (1978) Mechanism of action of papain: Aryldehydroalanines as spectroscopic probes of acyl enzyme formation, *Biochemistry 17*, 4606−4615.
11. Smolarsky, M. (1980) Mechanism of papain catalysis: Studies of active site acylation and deacylation by the stopped-flow technique, *Biochemistry 19*, 478−484.
12. Zur, M. (1975) Mapping the active site of a serine protease-trypsin, Ph.D. Thesis, The Weizmann Institute of Science, Rehovot, Israel.
13. Schechter, I., and Berger, A. (1966) Peptides of L- and D-alanine. Synthesis and optical rotations, *Biochemistry 5*, 3362−3370.
14. Schechter, I., and Berger, A. (1966) The hydrolysis of diastereoisomers of alanine peptides by carboxypeptidase A and aminopeptidase, *Biochemistry 5*, 3371−3375.
15. Fruton, J. S. (1949) The synthesis of peptides, *Adv. Protein Chem. 5*, 1−82.
16. Borsook, H. (1953) Peptide bond formation, *Adv. Protein Chem. 8*, 127−174.
17. Fruton, J. S. (1982) Proteinase-catalyzed synthesis of peptide bonds, *Adv. Enzymol. 53*, 239−306.
18. Blow, D. M., and Steitz, T. A. (1970) X-ray diffraction studies of enzymes, *Annu. Rev. Biochem. 39*, 63−100.
19. Johnson, L. N., Phillips, D. C., and Rupley, J. A. (1968) The activity of lysozyme: An interim review of crystallographic and chemical evidence, *Brookhaven Symp. Biol. 21*, 120−138.
20. Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Quiocho, F., Bethge, P. A., Ludwig, M. L., Steitz, T. A., Muirhead, H., and Coppola, J. C. (1968) The structure of carboxypeptidase A. VII. The 2.0-Å resolution studies of the enzyme and of its complex with glycyltyrosine, and mechanistic deductions, *Brookhaven Symp. Biol. 21*, 24−90.
21. Behrens, O. K., and Bergmann, M. (1939) Cosubstrates in proteolysis, *J. Biol. Chem. 129*, 587−602.
22. Warren, E. H., Vigneron, N. J., Gavin, M. A., Coulie, P. G., Stroonbant, V., Dalet, A., Tycodi, S. S., Xuereb, S. M., Mito, J. K., Riddell, S. R., and Van den Eynde, B. J. (2006) An antigen produced by splicing of noncontiguous peptides in the reverse order, *Science 313*, 1444−1447.
23. Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983) Components of ubiquitin-protein ligase system, *J. Biol. Chem. 258*, 8206−8214.
24. Volpe, J. J., and Vegelos, P. R. (1973) Saturated fatty acid biosynthesis and its regulation, *Annu. Rev. Biochem. 42*, 21−60.

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