Mechanism of Action of Papain: Aryldehydroalanines as Spectroscopic Probes of Acyl Enzyme Formation†

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ABSTRACT: The α,β-unsaturated aromatic amino acids phenyldehydroalanine (PDA) and styryldehydroalanine (SDA) were synthesized and used as sensitive spectroscopic probes to study the acylation of papain by specific substrates and inhibitors. The spectral changes observed upon acylation of the enzyme with peptides containing these amino acids are large red shifts of the absorption maxima (λ_{max}) of the chromophores. The magnitudes of the absorption shifts were 49 nm (from 277 to 326 nm) for PDA peptide and 59 nm (from 318 to 377 nm) for SDA peptides. The following specific substrates were synthesized: Ac-Phe-PDA-OEt, Ac-Phe-PDA-NH_2, Ala-Ala-Phe-SDA-OMe, Ala-Ala-Phe-SDA-NH_2, Lys-Ala-(*o*-benzyl)tyrosyl-SDA-OMe, and Lys-Ala-(*o*-benzyl)-tyrosyl-SDA-NH_2. Similarly, the specific competitive inhibitors Ac-Phe-PDA ($K_i = 5.3 \times 10^{-6}$ M), Z-Phe-SDA ($K_i = 5.6 \times 10^{-5}$ M), Ala-Ala-Phe-SDA ($K_i = 2.9 \times 10^{-5}$ M), and Lys-Ala-(*o*-benzyl)tyrosyl-SDA ($K_i = 1.1 \times 10^{-5}$ M) were prepared. An additional chromophore was used to follow the noncovalent association of an inhibitor or substrate with papain, independently from the acylation or deacylation steps. This chromophore, which was introduced into the peptides at position P_2, is *p*-(*p'*-dimethylaminophenylazo)phenylalanine (DAP). The light absorption spectrum of DAP is dependent on its environment. The substrates Ala-Ala-DAP-SDA-OMe and Ala-Ala-DAP-SDA-NH_2 and the competitive inhibitor Ala-Ala-DAP-SDA ($K_i = 2.5 \times 10^{-6}$ M) were prepared. The noncovalent binding of these peptides to the active site of papain was followed either by the increase in the absorption at 480 nm or the decrease at 550 nm. With these peptides the acylation and deacylation reactions could be followed simultaneously at 377 nm. The extent of acyl enzyme formation was found to decrease in a sigmoidal way with increasing pH, with a transition point around pH 5.5.

The β-arylacrylic acids exhibit characteristic intense light absorption spectra that are sensitive to chemical substitutions at the carboxyl group of these compounds (Bender et al., 1962; Bender and Brubacher, 1964; Bernhard et al., 1965; Oliver et al., 1967; Hinkle and Kirsch, 1970). Because of this property, β-arylacrylic acids have been used as spectroscopic probes to study the chemical nature of the active sites of various enzymes. Generally, these chromophoric reporter molecules were introduced into the catalytic sites using reactive acylating derivatives of the arylacrylic acids (e.g., arylacryloyl imidazole) to acylate the catalytically reactive nucleophiles in the active site (Bender et al., 1961, 1962; Bender and Kaiser, 1962; Bender and Brubacher, 1964; Bernhard et al., 1965; Brubacher and Bender, 1966; Oliver et al., 1967; Malhotra and Bernhard, 1968, 1973; Hinkle and Kirsch, 1970). This approach provides an experimentally convenient way to introduce spectroscopic probes into the active site of enzymes which are otherwise nonchromophoric and to study the chemical nature of the acyl enzyme complex and other intermediates formed during the catalysis. However, in the case of papain, the small arylacryloyl residue cannot form the various noncovalent interactions with the extended active site of the enzyme that *are formed* with the native substrates of papain, oligo- or polypeptides. Therefore, the effect of a multipoint attachment of the enzyme to its substrates on the acylation and deacylation steps cannot be studied using arylacryloyl acylating reagents. A multipoint attachment may be essential for obtaining a high noncovalent association energy which may be required to induce the appropriate conformational changes of the enzyme and to "force" the peptide bond, which is to be cleaved, into the optimal angle and location, relative to the catalytic functional groups in the active site. Therefore, the kinetic and thermodynamic parameters of the various steps of the interaction of papain with its substrates and inhibitors may be dependent on the amino acid sequence of the peptides.

In this paper, I show that β-aryldehydroalanines have similar spectral properties to the arylacrylic acids. Two such amino acids were incorporated into specific substrates and inhibitors of papain at the position adjacent to the point of catalysis, toward the amino terminus of the peptides (position P_1) (Berger and Schechter, 1970; Berger et al., 1971). With these peptides it was possible to follow the acylation of papain by substrates and by competitive inhibitors with a free α-carboxyl at position P_1. In this way, the effect of enzyme–substrate (or inhibitor) interactions, remote from the catalytic site, on the acylation and deacylation steps could be studied.

Materials and Methods

Buffers. Buffer A: potassium acetate, 0.05 M; KCl, 0.01 M (pH 4.3). Buffer B: sodium acetate, 0.05 M; NaCl, 0.01 M (pH 4.3).

Stock Solutions of Peptides. These (0.01–0.1 M) were made in freshly distilled and anhydrous dimethylformamide and stored in the dark at −20 °C. The peptides stored under these conditions show no detectable chemical change after 1 year as tested by thin-layer chromatography.

Papain. Enzyme two times crystallized, prepared by the method of Kimmel and Smith (1954), was obtained from Worthington Biochemical Corp. as a suspension (24 or 31 mg/mL) in 0.05 M sodium acetate buffer (pH 4.5). These preparations contained about 50% activitable enzyme, as measured by active-site titration.

Mercuripapain was obtained by further purification of the commercial enzyme by affinity chromatography (Blumberg

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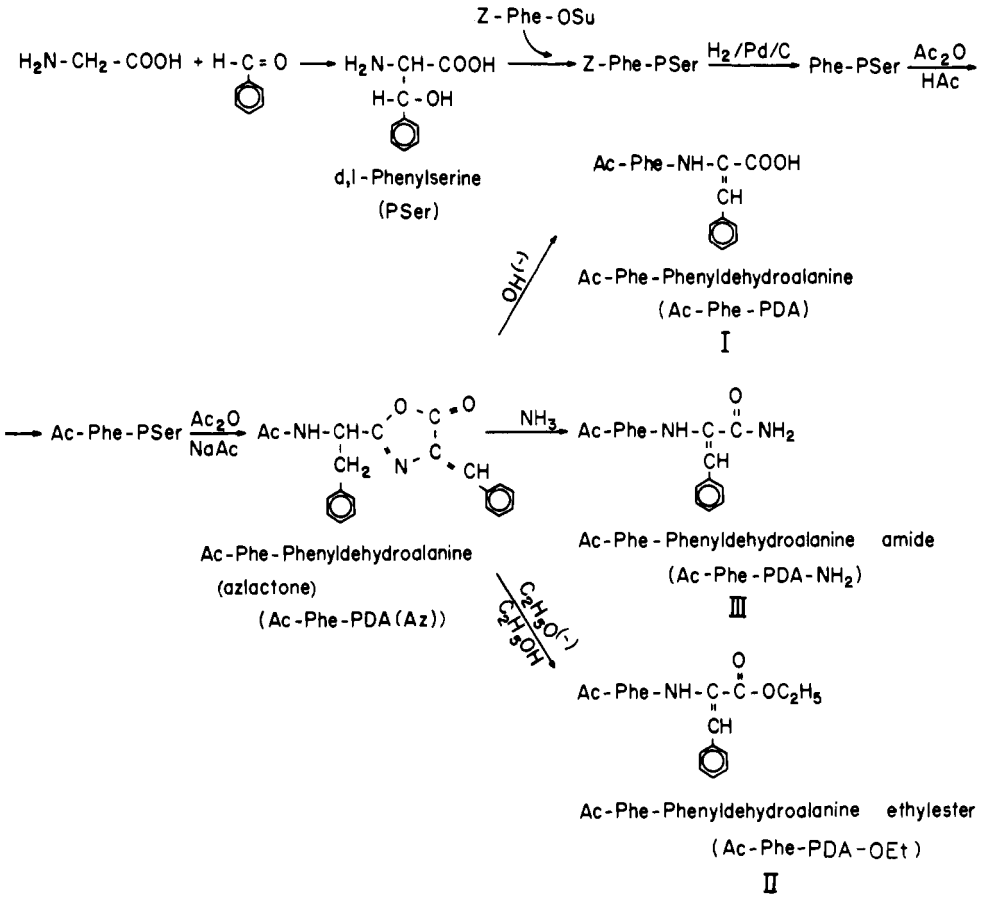


FIGURE 1: Synthesis of the competitive inhibitor Ac-Phe-PDA and of the substrates Ac-Phe-PDA-OEt and Ac-Phe-PDA-NH_2.

et al., 1970). The purified enzyme was mixed with 1 equiv of mercuric chloride and stored at 4 °C.

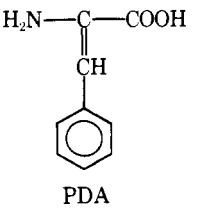
Enzyme Kinetics. Determinations of K_M, V_{max}, and K_i were carried out with a Radiometer automatic titrator (pH stat). In these measurements the purified mercuripapain was used. Inhibition of enzymatic hydrolysis by competitive inhibitors was studied at 36 °C (pH 4.3) under an atomosphere of nitrogen. The reaction mixture contained in 2.5 mL $0.8-1.6 \times 10^{-8}$ M papain, $8-16 \times 10^{-4}$ M DTT,[1] or 5×10^{-3} M cysteine, 2×10^{-3} M EDTA, and 0.33 M KCl. Substrates were BzArgOEt (K_m = 22 mM) or Ala-Ala-Ala-ONb (K_M = 3.3 mM).

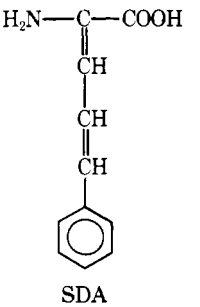
Acylation of Papain by Substrates and Inhibitors. Acylation was studied spectrophotometrically with a Cary 14 spectrophotometer using optical paths of 2-10 mm. The acylation reactions were carried out at 25 or 37 °C in buffer A (unless indicated otherwise) in which were dissolved papain, substrate, or inhibitor, DTT, or cysteine and EDTA. In these experiments, the concentrated commercial suspension of the two times crystallized enzyme was used. It was diluted with buffer A (previously deaerated with N_2) to the appropriate concentration, the pH was adjusted to 4.3, and the solution was clarified by filtration through a 0.45-μm Millipore filter. The enzyme was activated in the cuvette just prior or during the experiment by adding a small volume of a solution of 0.05 M DTT and 0.02 M EDTA (pH 4.3), freshly prepared before use. Substrates and inhibitors were added to the reaction mixtures in minimal volumes of the stock solutions of the peptides in DMF. Final concentration of DMF in the acylation reaction mixtures did not exceed 0.5%.

Syntheses of Amino Acids and Peptides. These syntheses and the chemical analysis of the products are described in the Supplementary Material (see paragraph at the end of this paper) and are shown schematically in Figures 1-3.

Results

Peptide Synthesis. The spectroscopic probes of acyl-papain complex formation that were used in this study are phenyldehydroalanine (PDA) and styryldehydroalanine (SDA).





The peptides were designed according to the specificity of

[1] Abbreviations of amino acid derivatives and peptides are according to the IUPAC-IUB Commission on Biochemical Nomenclature recommendations [*J. Biol. Chem. 247*, 977 (1972)]. Further abbreviations are: Az, azlactone; BzArgOEt, benzoylarginine ethyl ester; DAP, *p*-(*p'*-dimethylaminophenylazo)phenylalanine; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DIPE, diisopropyl ether; DMF, dimethylformamide; Me_2SO, dimethyl sulfoxide; DTT, dithiothreithol; Et_3N, triethylamine; HAc, acetic acid; IPOH, isopropyl alcohol; IR, infrared; MeOH, methyl alcohol; OBT, *o*-benzyltyrosine; OSu, *N*-oxysuccinimide ester; PAP, *p*-aminophenylalanine; PDA, β-phenyldehydroalanine; PIP, *p*-iodophenylalanine; PNP, *p*-nitrophenylalanine; PSer, β-phenylserine; SDA, β-styryldehydroalanine; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; SSer, β-styrylserine; SuOH, *N*-hydroxysuccinimide.

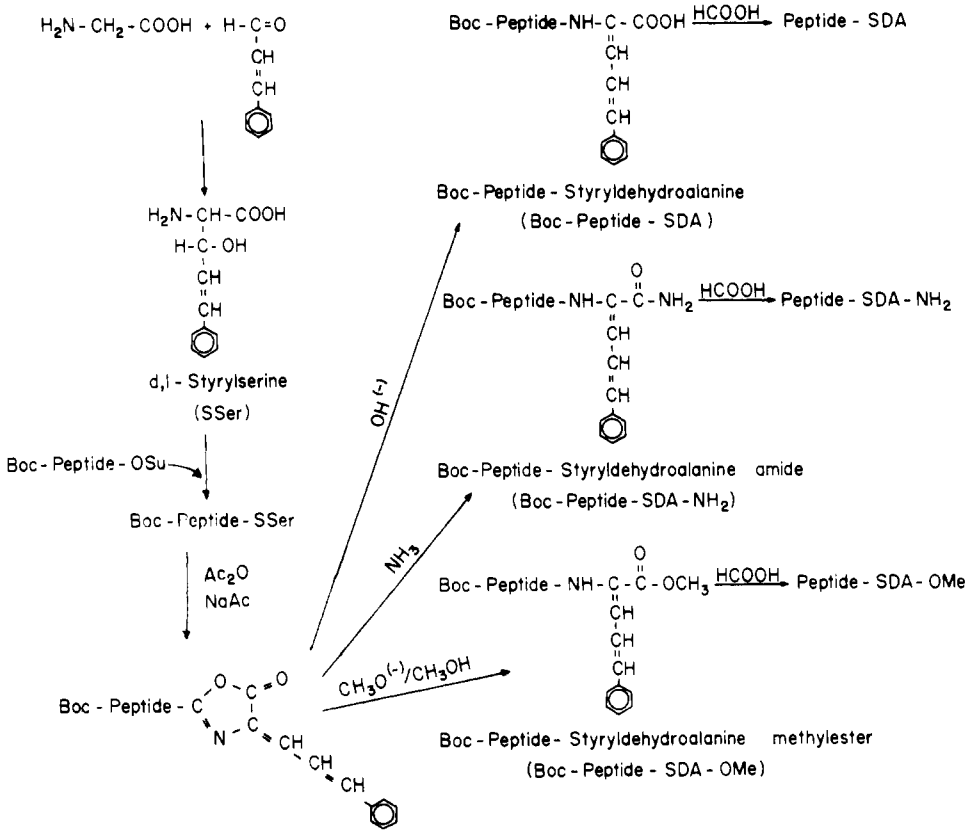
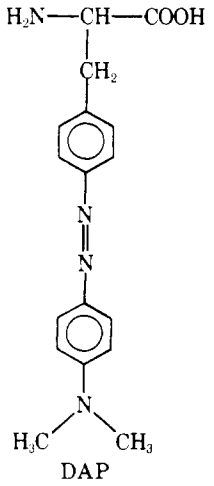


FIGURE 2: Synthesis of peptides with the amino acid SDA at position P_1.

papain, which requires a hydrophobic residue at P_1[2] and a hydrophobic aromatic amino acid at P_2 (Berger and Schechter, 1970, Berger et al., 1971). They were synthesized as shown schematically in Figures 1 and 2. The competitive inhibitors that were prepared by this procedure include: Z-Phe-SDA (IV), Ala-Ala-Phe-SDA (V), and Lys-Ala-OBT-SDA (VI). The substrates that were synthesized by this procedure are: Ala-Ala-Phe-SDA-OMe (VII), Ala-Ala-Phe-SDA-NH_2 (VIII), Lys-Ala-OBT-SDA-OMe (IX), and Lys-Ala-OBT-SDA-NH_2 (X). The spectroscopic probe of the noncovalent binding of peptides to the active site of papain that was used in this study is the amino acid *p*-(*p'*-dimethylaminophenylazo)phenylalanine (DAP). The synthesis of peptides



DAP

[2] Nomenclature of the subsites in the active site of papain and of the respective positions of the amino acids in peptide substrates and inhibitors of the enzyme is given according to Berger and Schechter (1970) and Berger et al. (1971). P_1 is the position adjacent to the point of catalysis, toward the amino terminus of the substrate.

with SDA at position P_1 and with DAP at P_2 is presented schematically in Figure 3. The peptides that were synthesized by this method are the competitive inhibitor Ala-Ala-DAP-SDA (XI) and the substrates Ala-Ala-DAP-SDA-OMe (XII) and Ala-Ala-DAP-SDA-NH_2 (XIII).

Observation of a New Light Absorption Band When Specific Substrates or Inhibitors, Containing PDA at P_1, Interact with Activated Papain. Ac-Phe-PDA (I) binds to activated papain in an equimolar ratio (Figure 4) and is a competitive inhibitor with a $K_i = 5.3 \times 10^{-6}$ M. A solution of inhibitor I in buffer A has a light absorption spectrum with a λ_{max} at 277 nm (Figure 5). This light absorption is caused by the chromophoric amino acid PDA and is similar to the absorption spectrum of its analogue cinnamic acid (278 nm, Bender et al., 1962). When Ac-Phe-PDA was mixed with activated papain at pH 4.3, a new absorption band appeared with a λ_{max} at 326 nm, indicating the formation of a new species (Figure 5). The new species was formed only after activation of the enzyme and it disappeared when the enzyme was poisoned by mercury ions, which are known from X-ray analysis to bind to the active thiol residue of Cys-25 (Drenth et al., 1971a). The absorption band at 326 nm disappeared also when Ac-Phe-PDA in the enzyme–inhibitor complex was displaced by an excess of other specific competitive inhibitors like Boc-PIP-Leu ($K_i = 1.6 \times 10^{-6}$ M) or Boc-PIP-Arg ($K_i = 3.3 \times 10^{-6}$ M). This latter inhibitor was found by X-ray analysis to bind specifically to the active site of papain, possibly forming an acyl–enzyme complex (Drenth et al., 1971a). The same results were obtained when the related ester (II) and amide (III) were used (Table I). This new absorption band, with λ_{max} at 326 nm, is similar to the light absorption spectrum of the cinnamoyl–papain complex, which was obtained by acylation of papain by cinnamoylimidazole (λ_{max} 326 nm; Bender and Brubacher, 1964). These findings suggest that the new species formed when inhibitor I or the substrates II and III interact with activated

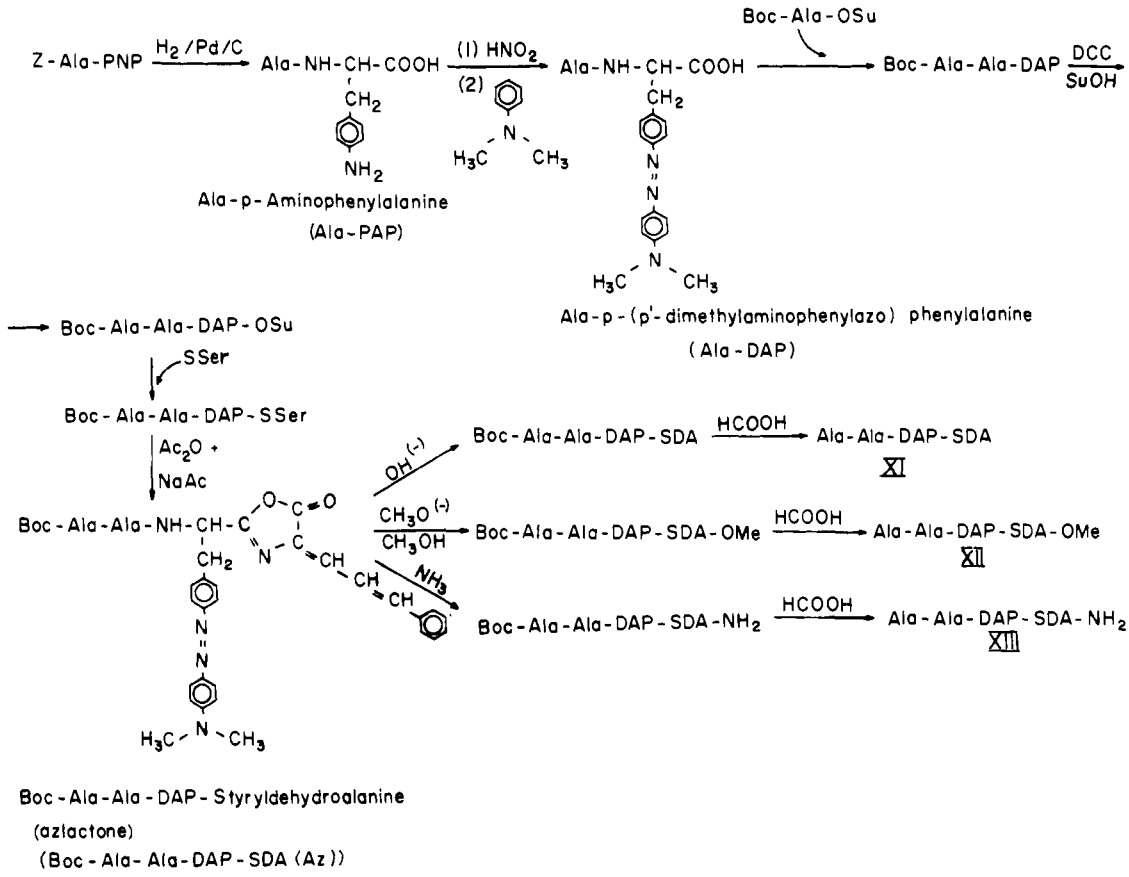


FIGURE 3: Synthesis of peptides with SDA at position P_1 and with DAP at P_2.

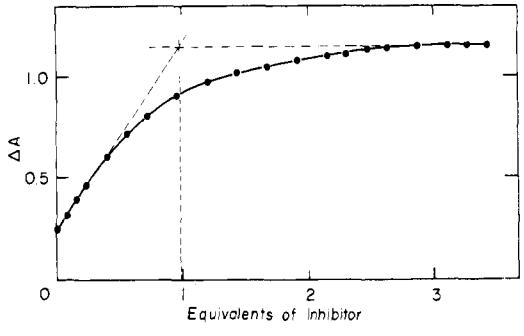


FIGURE 4: Spectrophotometric titration of papain with Ac-Phe-PDA. Enzyme concentration 10^{-4} M. The reaction was carried out in buffer A containing 7.7×10^{-4} M DTT and 8.7×10^{-4} M EDTA, at 25 °C. Optical path length 5 mm. ΔA is the light absorption at 330 nm in the sample cell, which contained papain + inhibitor, vs. the light absorption in the reference cell which contained only the inhibitor at the same concentrations.

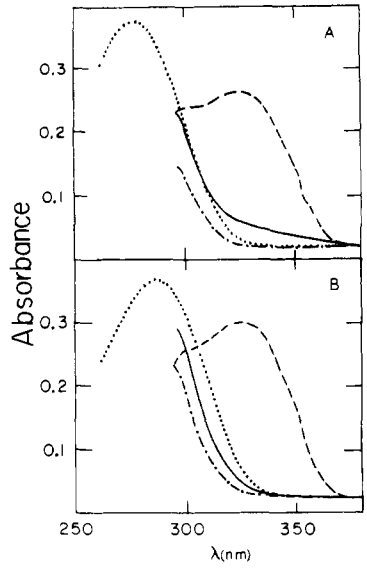


FIGURE 5: Absorption spectra representing the acylation of papain by Ac-Phe-PDA and deacylation in the presence of $HgCl_2$ (A) or Boc-PIP-Leu (B) at pH 4.3. Papain concentration 1.25×10^{-4} M, Ac-Phe-PDA concentration 10^{-4} M. The reaction was carried out in buffer A at 37 °C: (···) inhibitor in the absence of enzyme; (- • -) inhibitor with unactivated enzyme; (- - -) inhibitor with papain activated by 2×10^{-4} M DTT and 2×10^{-4} M EDTA; (—) inhibitor with activated papain subsequently poisoned with 10^{-3} M $HgCl_2$ (A) or with 4×10^{-4} M Boc-PIP-Leu (B). Optical path length 2 mm. The composition of the solution in the reference cell was as in the sample cell but without Ac-Phe-PDA.

papain is an acyl-enzyme complex. From the difference spectra, shown in Figure 8, it can be seen that the formation of the new species can be followed at 330 nm.

Observation of a New Light Absorption Band When Specific Substrates or Inhibitors, Containing SDA at P_1, Interact with Activated Papain. Z-Phe-SDA (IV), Ala-Ala-Phe-SDA (V), and Lys-Ala-OBT-SDA (VI) bind to papain in an equimolar ratio (determined by spectrophotometric titration similar to that described in Figure 4, data not shown here) and are strong competitive inhibitors. The inhibition constants are given in Table I. A solution of inhibitor V in buffer A has a light absorption spectrum with λ_{max} at 318 nm, which is caused by the chromophoric amino acid SDA. When Ala-Ala-Phe-SDA was mixed with activated papain at pH 4.3, a new absorption band appeared with a λ_{max} at 377 nm (Figure 6), indicating the formation of a new species. The data presented in this paper indicate that this new species is an acyl-enzyme complex in which SDA is bound to the active site by a thioester bond. The new species was formed only after activation of the enzyme, and it disappeared when the enzyme was poisoned by mercury

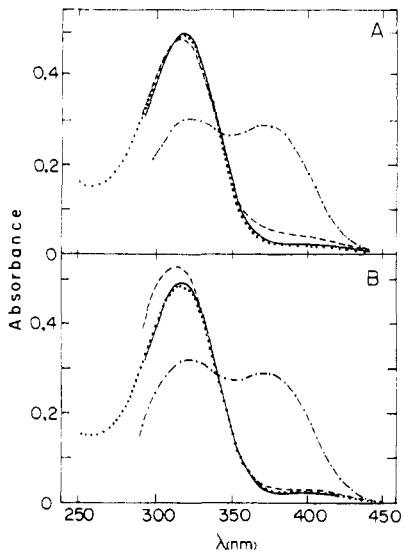


FIGURE 6: Absorption spectra representing the acylation of papain by Ala-Ala-Phe-SDA and deacylation by Boc-PIP-Leu (A) or $HgCl_2$ (B) at pH 4.3. The reaction was carried out in buffer A at 37 °C: Enzyme concentration 1.25×10^{-4} M, Ala-Ala-Phe-SDA concentration 10^{-4} M, optical path length 2 mm. (· · ·) inhibitor in the absence of enzyme; (—) inhibitor with unactivated papain; (- • -) inhibitor with papain activated by 2×10^{-4} M DTT and 2×10^{-4} M EDTA; (- - -) inhibitor with activated papain subsequently poisoned with 4×10^{-4} M Boc-PIP-Leu or 10^{-3} M $HgCl_2$. Solutions in the reference cell were as in the sample cell omitting the inhibitor.

TABLE I: Wavelengths of Maximal Absorptions (λ_{max}) of Substrates and Inhibitors of Papain, Containing PDA or SDA at P_1 and the Inhibition Constants of the Competitive Inhibitors.[a]

peptide	λ_{max} of a free peptide (nm)	λ_{max} of acyl-papain (nm)	K_i (M)
inhibitors			
Ac-Phe-PDA (I)	277	326	5.3×10^{-6}
Ac-Phe-PDA (*p*-NO_2)	318	326	2.5×10^{-4}
Z-Phe-SDA (IV)	317	377	5.6×10^{-5}
Ala-Ala-Phe-SDA (V)	318	377	2.9×10^{-5}
Lys-Ala-OBT-SDA (VI)	318	377	1.1×10^{-5}
Ala-Ala-DAP-SDA (XI)	318	377	2.5×10^{-6}
substrates			
Ac-Phe-PDA-OEt (II)	283	326	
Ac-Phe-PDA-NH_2 (III)	279	326	
Ala-Ala-Phe-SDA-OMe (VII)	325	377	
Ala-Ala-Phe-SDA-NH_2 (VIII)	320	377	
Lys-Ala-OBT-SDA-OMe (IX)	325	377	
Lys-Ala-OBT-SDA-NH_2 (X)	320	377	
Ala-Ala-DAP-SDA-OMe (XII)	325	377	
Ala-Ala-DAP-SDA-NH_2 (XIII)	320	377	

[a] The experimental conditions are as described in the legends to the figures.

ions or when inhibitor V in the enzyme-inhibitor complex was displaced by an excess of another strong competitive inhibitor like Boc-PIP-Leu. The same results were obtained with the competitive inhibitors IV and VI and with the related substrates VII, VIII, IX, and X (Table I). From the different spectra that are shown in Figure 8, it can be seen that the formation of the new species can be followed by the increase in absorption at 377 nm or by the decrease at 318 nm.

A Spectroscopic Probe for the Study of the Noncovalent

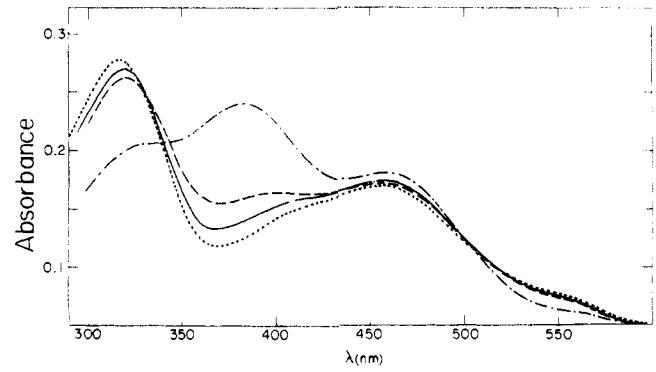


FIGURE 7: Absorption spectra representing the acylation of papain by Ala-Ala-DAP-SDA and deacylation of the acyl enzyme in the presence of Boc-PIP-Arg in buffer A at 37 °C: enzyme concentration 5×10^{-5} M; inhibitor concentration 1.67×10^{-5} M; optical pathlength 10 mm; (· · ·) inhibitor in the absence of enzyme; (—) inhibitor with unactivated papain; (- • -) inhibitor with papain activated by 8.4×10^{-4} M DTT and 4×10^{-4} M EDTA; (- - -) after displacement of inhibitor XI from the active site of the enzyme by 1.67×10^{-4} M Boc-PIP-Arg. Solutions in the reference cell were as in the sample cell but without Ala-Ala-DAP-SDA.

Binding of Substrates and Inhibitors to the Active Site of Papain. To follow the noncovalent interaction of papain with substrate or inhibitor, independently from the acyl enzyme formation, inhibitor XI and substrates XII and XIII were prepared. These peptides contain the chromophoric amino acid DAP at position P_2. This amino acid had already been used by Berger et al. (1971) to follow the binding of the inhibitor Ac-DAP-Arg to papain. Inhibitor XI is a strong competitive inhibitor with a K_i of 2.5×10^{-6} M. The changes in the absorption spectra that were observed when inhibitor XI was mixed with activated papain are shown in Figures 7 and 8. It can be seen that, in addition to the absorption changes resulting from the SDA chromophore (presumably as an acyl-enzyme complex), spectral differences at wavelengths higher than 450 nm can be observed, which result from the DAP chromophore. When papain, mixed with inhibitor XI, was activated, an increase in the absorption at 480 nm and a decrease at 550 nm were observed (Figure 7). These differences in the absorption spectra of the DAP result from the changes in the environment of this chromophore, which take place when peptides XI–XIII diffuse from the aqueous medium into the hydrophobic pocket in the active site. Some spectral changes could also be seen with the unactivated enzyme, probably due to a small amount of already active papain which existed in the preparation. The spectral changes diminished when the inhibitor was displaced by Boc-PIP-Arg. Since there are no spectral differences resulting from the SDA chromophore at wavelengths longer than 450 nm (Figure 8), the changes in the absorption of the DAP chromophore can be followed independently. On the other hand, from the difference spectrum observed when the inhibitor Ac-DAP-Arg ($K_i = 1.7 \times 10^{-6}$ M) was bound to papain, it could be seen that around 375 nm there is practically no spectral difference resulting from the DAP chromophore (Berger et al., 1971). These findings show that it is possible to follow the absorption changes resulting from the SDA chromophore and from the DAP chromophore simultaneously and independently.

The Chemical Nature of the Species Identified by the New Absorption Bands of the PDA and SDA Chromophores. The light absorption maxima of several derivatives of PDA and SDA, dissolved in various solvents, are shown in Table II. It is seen that neither the difference in polarity of the environment (H_2O vs. ethanol) nor the protonation of the terminal carboxyl

TABLE II: Wavelength (nm) of Maximal Absorption (λ_{max}) for Various RC(=O)X Derivatives.

		RC(=O)			
-X	solvent	Ac-Phe-PDA	Ala-Ala-Phe-SDA	Ac-SDA	Z-Phe-SDA
$-O^-$	pH 8[a]	273	317		
-OH	pH 2.1[b]	284	325		
-OH	ethanol	280	322		
-OH + O^-	buffer A	277	318	318[c]	317[d]
$-NH_2$	buffer A	279	320		319[d]
-OEt	buffer A	283			326[d]
-OMe	buffer A		325		
azlactone	buffer A	312		366[c]	362[e]
$-S-CH_2COOH$	buffer A			342[c]	
-papain (denatured)	pH ~ 3		340-350[f]		
-papain (native)	buffer A	326	377		377

[a] Buffer composition: 0.01 M KH_2PO_4, 0.01 M $Na_2B_4O_7$, 0.01 M citric acid, 0.005 M EDTA. [b] In 0.2 M citric acid. [c] In buffer A that contained 20% IPOH. [d] In buffer A that contained 30% CH_3CN. [e] In H_2O-CH_3CN (1:1). [f] Acyl-papain complex was formed and denatured as described in the legend to Figure 10.

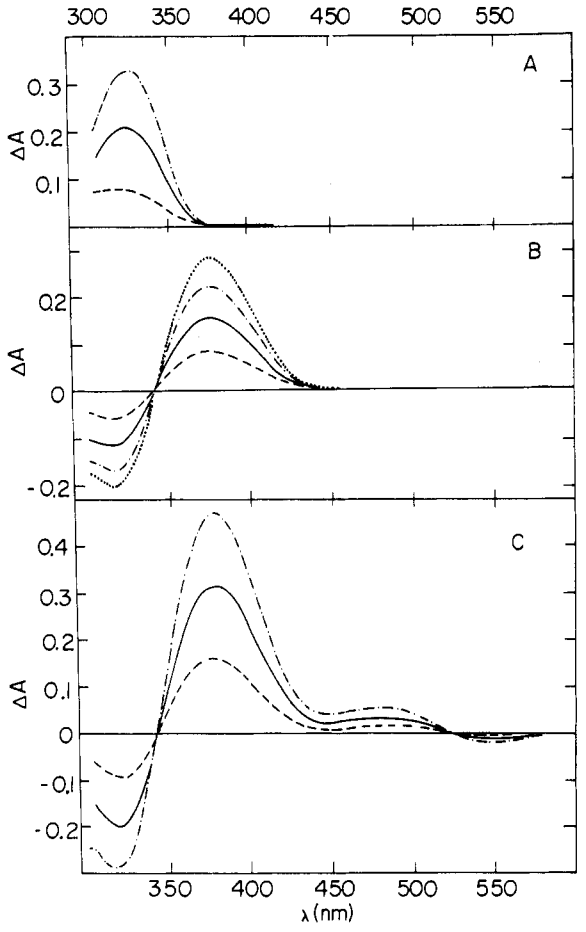


FIGURE 8: Difference spectra. Absorption of light by solutions of activated papain and Ac-Phe-PDA (A), Ala-Ala-Phe-SDA (B), and Ala-Ala-DAP-SDA (C) vs. the absorption by solutions of the same inhibitors but with unactivated papain, at pH 4.3. The reactions were carried out in buffer A at 37 °C. Enzyme concentrations were 10^{-4} M in A and B and 4.9×10^{-5} M in C. Concentrations of inhibitor I in A are: (- - -) 7.7×10^{-6} M; (—) 23.1×10^{-6} M; (- • -) 38.5×10^{-6} M. Concentrations of inhibitor V in B are: (- - -) 1.6×10^{-5} M; (—) 3.2×10^{-5} M; (- • -) 4.8×10^{-5} M; (• • •) 6.4×10^{-5} M. Concentrations of inhibitor XI in C are: (- - -) 0.785×10^{-5} M; (—) 1.57×10^{-5} M; (- • -) 2.36×10^{-5} M. DTT and EDTA respective concentrations are: (A) 19.3×10^{-4} and 8.7×10^{-4} M; (B) 2×10^{-3} and 1.25×10^{-3} M; (C) 10^{-3} and 4.8×10^{-4} M: optical path length 5 mm in A and B and 10 mm in C.

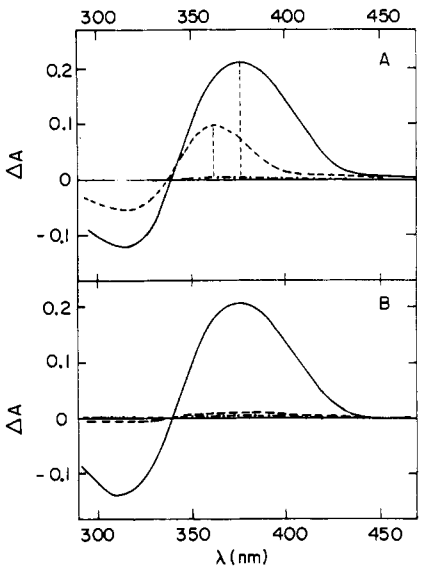


FIGURE 9: Difference spectra representing the effect of denaturation on the light absorption by the complex Ala-Ala-Phe-SDA-papain: (- • -) solution of 1.25×10^{-4} M *unactivated* papain and 10^{-4} M inhibitor in 0.04 M sodium acetate buffer (pH 4.3) and 0.01 M NaCl in *both* the sample and the reference cells; (—) after the addition of 2×10^{-3} M DTT and 8×10^{-4} M EDTA to the sample cell; (- - -) in A, 0.04 volume of 5 M HCOOH solution that contained 25% $NaDodSO_4$ was added to *both* the sample and the reference cells; in B, 4×10^{-4} M Boc-PIP-Arg was added to the sample and the reference cells and, subsequently, 0.04 volume of the 5 M HCOOH + 25% $NaDodSO_4$ solution was added to the sample and the reference cells. The pH after denaturation was ~3.

group can account for the large red shifts observed when peptide-PDA-X or peptide-SDA-X are mixed with activated papain (49 and 59 nm, respectively). Similar results with other arylacrylic derivatives have been described by Bernhard et al. (1965) and by Bender et al. (1962). On the other hand, certain chemical substitutions at the carboxyl of PDA and SDA (Table II) and of other arylacrylic acids (Bernhard et al., 1965; Bender et al., 1962; Oliver et al., 1967; Hinkle and Kirsch, 1970; Bender and Brubacher, 1964; Malhotra and Bernhard, 1968, 1973) *do* cause large red shifts of the light absorption maxima of these compounds. To test whether a covalent bond is formed when inhibitors like I, IV, V, VI, and XI interact with activated papain, the experiment described in Figure 9 was carried out. Here inhibitor V was mixed with papain at pH 4.3. When the enzyme was activated, the typical absorption band with λ_{max} at 377 nm appeared, indicating the formation of a papain-inhibitor complex. When this complex was denatured (with a simultaneous acidification to pH ~3 to inhibit the deacylation reaction), a large blue shift of λ_{max} was observed. If, on the other hand, inhibitor V in the papain-inhibitor complex was displaced prior to denaturation by the strong competitive

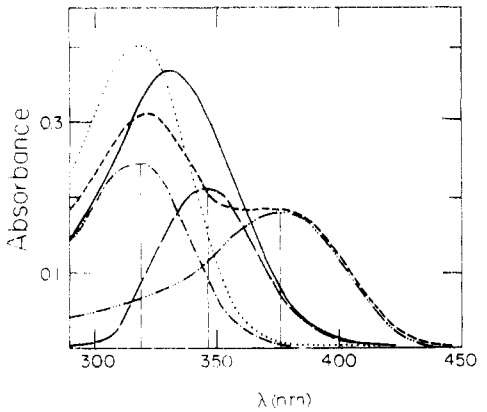


FIGURE 10: Absorption spectra of the complex Ala-Ala-Phe-SDA–papain at pH 4.3 and after denaturation by 0.2 M HCOOH + 1% $NaDodSO_4$. Buffer composition is as described in Figure 9: (···) 10^{-4} M inhibitor V and 1.25×10^{-4} M unactivated papain in buffer, pH 4.3; (- - -) after activation of the enzyme by 2×10^{-3} M DTT and 8×10^{-4} M EDTA; (—) after denaturation by 0.2 M HCOOH +1% $NaDodSO_4$ (final pH ~3). The solutions in the reference cell were as in the sample cell omitting the inhibitor. Denaturation was carried out by adding 0.04 volume of a 5 M HCOOH solution containing 25% $NaDodSO_4$. The absorption spectrum of the solution before denaturation (- - -) can be *approximately* described as the sum of two absorption curves: the absorption by the free inhibitor (- • -) and the absorption by the enzyme–inhibitor complex (- • • • -). The absorption spectrum of the solution after denaturation (solid line) can also be *approximately* described as the sum of two absorption curves: the absorption by the free inhibitor (- • -) and the absorption by the denatured enzyme–inhibitor complex (– –). The resolutions of the absorption curves were done according to assumptions described in the text.

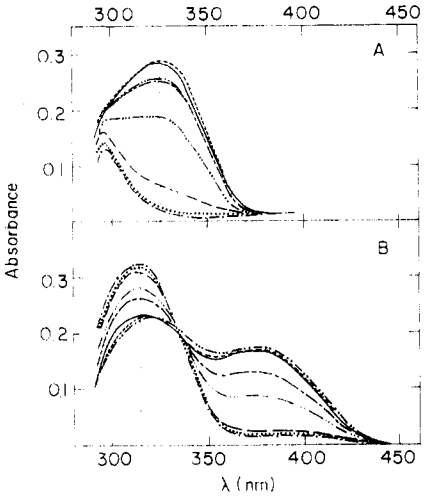


FIGURE 11: Absorption spectra representing the pH dependence of acylation of papain by Ac-Phe-PDA (in A) and by Ala-Ala-Phe-SDA (in B): enzyme concentration 1.25×10^{-4} M; inhibitor concentration 10^{-4} M; optical pathlength 2 mm; temperature 37 °C; (- • • -) pH 3.4; (- - -) pH 3.8; (—) pH 4.3; (– – –) pH 4.8; (- • • • -) pH 5.2; (– – –) pH 6.2; (• • •) pH 7.1; (- • - • -) pH 7.7. Buffers contained 0.01 M KH_2PO_4, 0.01 M $Na_2B_4O_7$, 0.01 M citric acid, and 0.004 M EDTA. pH was adjusted by KOH or by HCl. The enzyme was activated by 3×10^{-3} M DTT. Solutions in the reference cell were as in the sample cell omitting the inhibitor.

inhibitor Boc-PIP-Arg, the absorption band disappeared (Figure 9B). To determine λ_{max} for the denatured papain–inhibitor V complex, the experiment described in Figure 10 was carried out. In this experiment, the complex Ala-Ala-Phe-SDA–papain was formed by mixing activated papain with inhibitor V at pH 4.3. After tracing the absorption spectrum, the enzyme–inhibitor complex was denatured by adding 0.04 volume of 5 N HCOOH solution that contained 25% $NaDodSO_4$. The absorption spectra of the reaction mixture prior to and after denaturation can be *approximately* described as a sum of the absorption spectra of the free inhibitor and of the inhibitor–papain complex. The resolution of the absorption curves was done by the aid of a computer according to the following assumptions: (a) The absorption spectrum of the free inhibitor is not changed significantly during the denaturation process. This assumption is based on the observation that there is little change in the spectrum of the SDA chromophore in different environments as compared with the large spectral changes that result from chemical substitutions (Figure 10). (b) The denaturation process is fast compared to the deacylation reaction, which is also inhibited by the simultaneous acidification. Under these conditions, those molecules of inhibitor that were in the form of an acyl–enzyme complex prior to denaturation remain bound covalently to the enzyme after denaturation. As can be seen in Figure 10, the maximal light absorption of the denatured papain–inhibitor V complex is at 340–350 nm and is very similar to λ_{max} of the synthetic thioester Ac-SDA-S-CH_2COOH (342 nm, Table II). The similarity of the absorption spectra of denatured arylacryloyl–papain complexes and the related synthetic low-molecular-weight thioesters has also been described by other investigators (Bender and Brubacher, 1964; Hinkle and Kirsch, 1970).

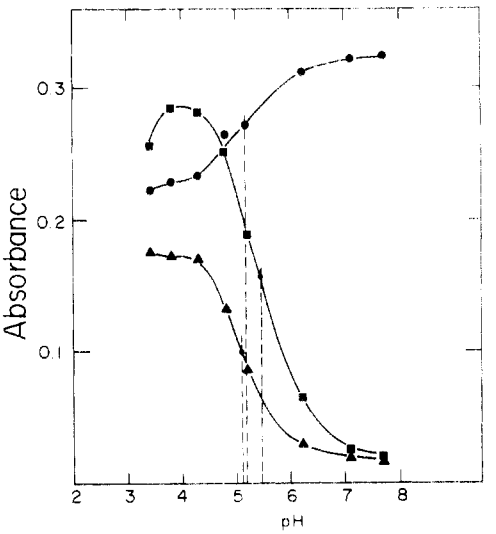


FIGURE 12: pH dependence of acylation of papain by Ac-Phe-PDA and by Ala-Ala-Phe-SDA. Data are taken from Figure 11: (▲) absorption of Ala-Ala-Phe-SDA at 380 nm; (●) absorption of Ala-Ala-Phe-SDA at 315 nm; (■) absorption of Ac-Phe-PDA at 325 nm.

The pH Dependence of the Acylation of Papain by Inhibitor with a Free α-Carboxyl at P_1. It has been reported that the inhibition constants (K_i) of various competitive inhibitors of papain, containing a free α-carboxyl group at P_1, are pH dependent (Berger and Schechter, 1970; Sluyterman, 1964). It was found that K_i increases markedly when the pH is raised above 4.5. It was therefore of interest to find out if the acylation of papain by the competitive inhibitors that were prepared in this study is also pH dependent. Inhibitors I and V were mixed with activated papain in buffers of various pH, and the light absorption spectra were traced. The results are shown in Figure 11. The absorption at the wavelength at which acylation can be measured (325 nm in the case of PDA and 380 nm in the case of SDA; see the difference spectra in Figure 8) was drawn as a function of pH (Figure 12). It can be seen that the extent of acylpapain formation by inhibitors I and V is maximal at pH 3.8–4.3, and it decreases in a sigmoidal way, with a tran-

sition point at pH 5–5.5. The pK_a of the carboxyls of *N*-acyl-PDA and *N*-acyl-SDA is 3.4–3.5 (found by spectrophotometric and potentiometric titrations). Therefore, it is the protonation of the enzyme which is required for its acylation by the inhibitors. Proton concentration may affect the noncovalent binding of the inhibitors to the active site, since above pH 5.5 the active site of papain is negatively charged because of the aspartyl residues at positions 64 and 158 which are 10 and 6.7 Å, respectively, from the catalytic thiol group of Cys-25 (Drenth et al., 1971a,b). The electrostatic repulsion between the inhibitor and the active site is eliminated when the latter is protonated. Once the inhibitor is in the active site, its free α-carboxyl may also be protonated because of the difference in the environment and the proximity of proton donors such as the imidazole of His-159, which may be protonated at a pH lower than 5.5. This may increase the acylation rate and stabilize the acyl-enzyme complex. It is also possible that the introduction of a carboxyl into the active site affects the pK_a of the ionizable groups there. This may explain why the pH dependence shown in Figure 12 is different from that found for the acylation rate constants in the hydrolysis of various substrates by papain (pK_1 = 3.9–4.3; pK_2 = 8.0–8.5; Glazer and Smith, 1971).

Discussion

The α,β-unsaturated aromatic amino acids β-phenyldehydroalanine (PDA) and β-styryldehydroalanine (SDA) have light absorption spectra which are affected by chemical substitutions at the α-carboxyl group (Table II). With respect to this property, PDA and SDA are similar to the analogous β-arylacrylic acids that have been used to study the properties of acyl-enzyme complexes of the proteolytic enzymes papain, chymotrypsin, trypsin, and subtilisin, as well as those of glyceraldehyde-3-phosphate dehydrogenase (Bender and Brubacher, 1964; Bernhard et al., 1965; Oliver et al., 1967; Bender and Kaiser, 1962; Bernhard et al., 1965; Malhodra and Bernhard, 1968). Since PDA and SDA are α-amino acids, they could be incorporated into position P_1 of oligopeptides, which are substrates or competitive inhibitors of papain. The peptides were synthesized according to the specificity of the extended active site of the enzyme and bind to it with a high noncovalent binding energy (Berger and Schechter, 1970; Berger et al., 1971). With these peptides, I was able to show that papain is acylated by peptide inhibitors which have a free α-carboxyl at P_1. The spectra of the acyl-enzyme complexes indicate that the inhibitors are bound to the active site in a thioester bond. These results explain the ability of papain to catalyze the exchange of ^{18}O between water and the free α-carboxyl of benzyloxycarbonyl amino acids (Grisaro and Sharon, 1964) and to catalyze the synthesis of peptide bonds between the free α-carboxyl of N-blocked amino acids and various amines like aniline and phenylhydrazine (Bergman and Fraenkel-Conrat, 1937; Greenstein, 1954). Acylation of papain by inhibitors with a free α-carboxyl at P_1 was suggested also by other investigators (Drenth et al., 1971a; Wolthers and Kalk, 1970; Bernhard and Lau, 1971).

What fraction of the total enzyme-bound inhibitor (both in covalent or noncovalent bonds) is at equilibrium in the form of an acyl-enzyme complex? Studies of the rate of acylation of papain by the inhibitors I, V, VI, and XI and of the rate of deacylation of the acyl-enzyme complexes by the stopped-flow technique (manuscript in preparation) showed that most of the inhibitor molecules, which are bound to the active site of the enzyme, are at equilibrium in the form of an acyl-enzyme complex. The same conclusion can be derived from the following rough calculation. It can be assumed that the molar absorption coefficient of thioesters of PDA and SDA is about $2.5 \times 10^4\ M^{-1}\ cm^{-1}$ [values of molar absorption coefficients of arylacrylic acid derivatives that were reported in the literature are in the range $1.5\text{–}3 \times 10^4\ M^{-1}\ cm^{-1}$ (Bernhard et al., 1965; Charney and Bernhard, 1967; Hinkle and Kirsch, 1970)]. According to this assumption, the calculated concentration of acyl-papain complexes formed by inhibitors I and V, in the experiments described in Figures 5 and 6, is about 6×10^{-5} M. From the total concentration of the inhibitors in these experiments (10^{-4} M), the enzyme concentration (1.25×10^{-4} M), and the inhibition constants of I and V (Table I), it can be estimated that most of the enzyme-bound inhibitors were at equilibrium in the form of an acyl-enzyme complex.

Can the spectral properties of acyl-papain complexes be correlated with their chemical nature? From the spectra of model compounds (Bender et al., 1962; Bernhard et al., 1965; Oliver et al., 1967; Bender and Brubacher, 1964; Hinkle and Kirsch, 1970), it was suggested that the unique spectra of native acyl enzymes indicate that the carbonyls of the ester (or thioester) bonds are more polarized than in the denatured complexes or in simple small-molecular-weight model compounds (Bernhard and Lau, 1971). This may result from a distortion of the acyl-enzyme bond and protonation of the ester's oxygen or sulfur atoms by the active site (Bernhard and Lau, 1971). From the three-dimensional model (CPK type) of acyl-papain complexes with Ac-Phe-PDA and Ala-Ala-Phe-SDA, built according to the three-dimensional structure of papain (Drenth et al., 1971a,b), and taking into account hydrogen-bond formation and hydrophobic interactions according to the specificity of the enzyme (Berger and Schechter, 1970; Berger et al., 1971), it seems that PDA and SDA are in the more stable "S-trans" configuration, but the thioester bond is distorted. The β-carbon of the catalytic cysteine residue is forced by the structure of the active site to be outside the otherwise planar thioester structure because of rotation along the thioester bond. A similar kind of distortion was found in the indoleacryloyl-α-chymotrypsin complex by X-ray analysis (Henderson 1970). The effect of this distortion is to interfere with the overlap of the p orbitals of the sulfur with the π orbital of the adjacent carbonyl. As a result, the carbonyl carbon in the native acyl enzyme is more electropositive than in usual thioesters and is more susceptible to attack by nucleophiles. This may result in a higher rate and a higher free energy of hydrolysis. It was therefore of interest to determine if a correlation exists between the free energies and rates of hydrolysis of esters and amides of arylacrylic acids (ArCH=CHCOX) and their λ_{max}. Since the free energies of hydrolysis of esters and amides of arylacrylic acids have not yet been measured, I used data available for the corresponding derivatives of acetic acid (CH_3COX) and Z-Gly-X. I assumed that the effects of -X on the free energies and rates of hydrolysis of these compounds and of the corresponding arylacrylic acid derivatives are the same. The results (Figure 13) indicate that a correlation does exist between λ_{max} of cinnamoyl-X and (a) the free energy of the conversion of acetyl-X to acetic acid anion and XH at pH 7, (b) the pK_a of the ionization of XH, and (c) the rate of the alkaline hydrolysis of Z-Gly-X. Cinnamoylimidazole and *S*-cinnamoylcysteine deviate from the rest of the compounds and have an exceptionally red-shifted λ_{max}. Since most of the other derivatives tested are oxygen esters (except cinnamoyl amide), it is possible that amides and thioesters may have a different correlation than oxygen esters. Bernhard and Lau (1971) suggested that the λ_{max} of thiol esters of arylacrylic acids are red-shifted relative to the corresponding oxygen esters, aldehydes, and ketones due to the 3d-orbital overlap from

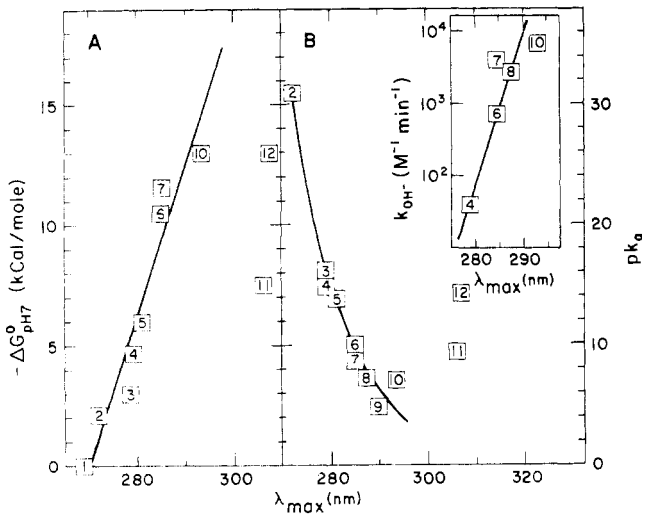


FIGURE 13: Correlation between λ_{max} of esters and amides of cinnamic acid derivatives ($C_6H_5CH{=}CHCOX$) and the chemical properties of the corresponding alcohols and amines (X): (A) The free energy of the conversion of CH_3COX to CH_3COO^- and XH in water at pH 7 vs. λ_{max} of cinnamoyl-X. (B) pK_a of alcohols and amines (XH) vs. λ_{max} of the corresponding esters and amides of cinnamic acid. Inset: Rate constants of alkaline hydrolysis of Z-Gly-X vs. λ_{max} of cinnamoyl-X. The X are: 1, O^-; 2, NH_2; 3, OH; 4, OCH_3; 5, $OCH_2CH_2N^+(CH_3)_3$; 6, OC_6H_5; 7, OC_6H_5(*m*-NO_2); 8, OC_6H_5(*o*-NO_2); 9, OCOR (anhydride); 10, OC_6H_5(*p*-NO_2); 11, S of cysteine; 12, imidazole. λ_{max} data values were from Bernhard et al. (1965), Bender et al. (1962), and Bender and Brubacher (1964). Other data are from the CRC Handbook of Biochemistry (Sober, 1966) and from Kirsch and Igelström (1966).

the sulfur. They also suggested that the spectrum of the native acyl enzyme indicates that the carbonyl of the thioester bond is more polarized than in usual thioesters. This may mean that the thioester bond in native acylpapain also has a higher energy of hydrolysis.

How is it possible to explain the spontaneous acylation of papain by the terminal free α-carboxyl of competitive inhibitors? The same question may be posed concerning the acylation of papain by amides, which are the natural substrates of the enzyme and have a low energy of hydrolysis. Protonation of the enzyme may provide 3 kcal/mol; the rest probably comes from the noncovalent binding of the inhibitors or substrates to the active site. If this is the case, then competitive inhibitors which can bind to the active site with little or no investment of energy in acylating it will have very high binding constants. This, in fact, was found to be the case. Ala-Ala-Phe-glycinenitrile is a strong competitive inhibitor of papain, with $K_d = 3.5 \times 10^{-6}$ M (corresponding to a free energy of binding of 7.6 kcal/mol). By contrast, Ala-Ala-Phe-Gly is a weak competitive inhibitor with $K_d = 2.2 \times 10^{-3}$ M (corresponding to a free energy of binding of 3.7 kcal/mol). Similarly, Ac-Phe-$NHCH_2CHO$ is a very strong inhibitor of papain with $K_d = 4.5 \times 10^7$ M^{-1} (Westerik and Wolfenden, 1972). This binding constant corresponds to a binding energy of 10.3 kcal/mol. On the other hand, Ac-Phe-Gly binds to papain with a free energy of only 4.1 kcal/mol (Benderly, H., personal communication). The results of this study show that the noncovalent binding energy is insufficient to cause acylation by inhibitors with a free carboxyl at pH >7. Protonation of the enzyme provides the additional energy required for almost quantitative acylation. At pH 4–5 the acyl–papain complex is more stable than ordinary low-molecular-weight thioesters, although the energy of hydrolysis of the thioester bond may be higher. This conclusion agrees with the suggestion of Hinkle and Kirsch (1971) that the structure of native papain contributes approximately 8 kcal/mol toward stabilizing the acyl–papain complex.

The pH dependence of the acylation of papain by competitive inhibitors with a free α-carboxyl at P_1 (Figure 12) is illustrative of a case where the energy of protonation is used to form an energy-rich chemical bond. A membranous enzyme with similar properties could use a transmembrane proton gradient to actively transport peptides from the acidic to the basic side of the membrane.

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Supplementary Material Available

Syntheses of amino acids and peptides and the chemical analysis of the products are described (20 pages). Ordering information is given on any current masthead page.

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Malate Dehydrogenase. Kinetic Studies of Substrate Activation of Supernatant Enzyme by L-Malate†

Paul A. Mueggler‡ and Raymond G. Wolfe*

ABSTRACT: At pH 8.0 in 0.05 M Tris-acetate buffer at 25 °C, homogeneous supernatant malate dehydrogenase exhibits substrate activation by L-malate. The turnover number, Michaelis constant for L-malate, and Michaelis constant for NAD are: 0.46×10^4 min^{-1}, 0.036 mM, and 0.14 mM, respectively, for nonactivated enzyme and 1.1×10^4 min^{-1}, 0.2 mM, and 0.047 mM for the same series of constants in activated enzyme. Nonactivating behavior is observed at concentrations between 0.02 and 0.15 mM L-malate and activating behavior is observed between 0.15 and 0.5 mM L-malate. L-Malate activation is compared with similar activation of mitochondrial malate dehydrogenase. While it is not possible to exclude unequivocally all mechanisms, the data seem to be consistent with the occurrence of a fundamentally ordered bi bi mechanism, possibly involving activation through the allosteric binding of L-malate. It is concluded that the data are consistent with a form of the "reciprocating compulsory order mechanism" in which nonactivated enzyme reflects catalysis by one subunit and activated catalysis expresses the coordinated activity of two subunits. The allosteric interaction and the "reciprocating mechanism" are not mutually exclusive.

This study deals with two rather general problems in enzymology, the significance of identical subunits, and the role of multiple enzyme forms. Pig heart malate dehydrogenase is of interest in relationship to the above because it occurs in two forms, mitochondrial and extramitochondrial (supernatant), each of which is a dimer of structurally identical or very similar subunits. The experimental approach involves a detailed study of the non-Michaelian kinetic behavior known as substrate activation, in this case by L-malate, with the purpose of ascertaining the mechanism correctly explaining the observed behavior. This is a continuation of previously published studies attempting to explain anomalous kinetic behavior in the structure-function framework (Harada & Wolfe, 1968), and

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