Mechanism of Papain Catalysis: Studies of Active-Site Acylation and Deacylation by the Stopped-Flow Technique†

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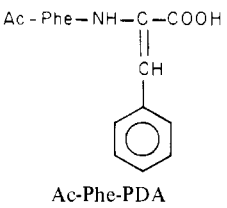
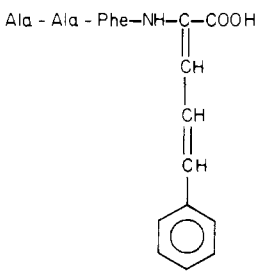
ABSTRACT: The acylation of papain by substrates and competitive inhibitors with a free α-carboxyl adjacent to the point of catalysis (position P_1) and the deacylation of acyl–papain complexes were studied by the stopped-flow technique. The substrates and inhibitors contained the amino acids β-phenyldehydroalanine (PDA) or β-styryldehydroalanine (SDA) at position P_1 as spectroscopic probes of acyl–papain formation. The rate constants of the acylation of papain by the inhibitors (k_2 = 0.23–4.6 s^{-1}) were found to be much greater than the deacylation rate constants (k_{-2} = 0.11–0.16 s^{-1}) at pH 4.3. The ratios $K_2 = k_2/k_{-2}$ indicate that when the studied competitive inhibitors, with a free α-carboxyl at P_1, bind to the active site of papain at pH 4.3, most of the enzyme-bound inhibitor molecules are at equilibrium in the form of a covalent acyl–enzyme complex. The rate constants of the acylation of papain by esters and amides were compared to the acylation rate constants of inhibitors at pH 4.3. In these reactions the enzyme was in excess to the substrates and inhibitors. The results show that the inhibitors acylate papain at pH 4.3 with rate constants that are not smaller than those of the corresponding methyl and ethyl esters. On the other hand, the acylation rate constants of the amides were 10–50-fold smaller. The acylation rate constants of papain by inhibitors are dependent on the amino acid composition and on the length of the peptides. The results show that the energy required for acylation comes from the noncovalent binding. When the "*productivity*" of the binding is increased, more energy is required for acylation and the total free energy of binding is lowered. It results in a lower binding constant.

In the hydrolysis of amides and esters by papain an acyl–enzyme intermediate is formed (Stockell & Smith, 1957; Kirsch & Katchalski, 1965; Lowe & Williams, 1965a; Brubacher & Bender, 1966; Sluyterman, 1968; Hinkle & Kirsch, 1970). I have recently shown that an acyl–enzyme complex is also formed when competitive inhibitors with a free α-carboxyl at position P_1[1] interact with papain (Smolarsky, 1978). These competitive inhibitors were synthesized according to the specificity of the active site of the enzyme (Berger & Schechter, 1970; Berger et al., 1971) and contained the amino acids β-phenyldehydroalanine (PDA) and β-styryldehydroalanine (SDA) in position P_1 as spectroscopic probes



Ac-Phe-PDA



Ala-Ala-Phe-SDA

of acyl–papain complex formation. The light absorption spectra of these α,β-unsaturated aromatic amino acids are greatly affected by chemical substitutions at their carboxyl group. In this respect, PDA and SDA are similar to β-arylacrylic acids which have been used to study the chemical nature of catalytically active nucleophiles in the active sites of various enzymes (Bender et al., 1961, 1962; Bender & Kaiser, 1962; Bernhard et al., 1965; Brubacher & Bender, 1966; Oliver et al., 1967; Malhotra & Bernhard, 1968, 1973; Hinkle & Kirsch, 1970). This property of PDA and SDA made it possible to observe the formation of the acyl–enzyme complex directly by following the characteristic changes in the absorption spectra of the chromophores. The spectral changes are large red shifts of the wavelength of the maximal absorption (λ_{max} is shifted from 277 to 326 nm in the case of PDA and from 318 to 377 nm in the case of SDA).

In a previous paper I showed qualitatively that when a competitive inhibitor with a free α-carboxyl at P_1 interacts with papain at pH 4–5 most of the enzyme-bound inhibitor molecules are bound to the active site covalently in a thioester bond (Smolarsky, 1978). This was concluded from the characteristic absorptions of the acyl–enzyme complexes observed when the inhibitors and papain were mixed at pH 4–5. This conclusion was confirmed quantitatively in the study presented in this paper, in which the individual rate constants of the acylation and deacylation reactions were obtained by the stopped-flow technique.

I also report here results on the comparison of the acylation rate constants of competitive inhibitors with a free α-carboxyl at P_1 with those of substrate esters and amides and on the effect of the amino acid composition of substrates and inhibitors of papain on the acylation and deacylation rate constants.

Materials and Methods

Buffer. Buffer A: 0.05 M potassium acetate and 0.01 M KCl, pH 4.3.

Peptide Syntheses. The syntheses of peptides were described in a previous publication (Smolarsky, 1978).

Peptide Solutions. Concentrated stock solutions (0.01–0.1 M) were made in freshly distilled anhydrous dimethylformamide and stored in the dark at −20 °C. The peptides stored under these conditions did not show any detectable chemical change after 1 year. Before the experiments the stock solutions were diluted in buffer A. The concentration of DMF in the

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[1] 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 papain is according to Berger & 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.

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reaction mixtures did not exceed 0.1%.

Papain. A commercial enzyme 2 times crystallized prepared by the method of Kimmel & 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 ~50% activatable enzyme as measured by active-site titrations. Prior to the experiments, the enzyme suspensions were diluted in buffer A (previously deaerated with nitrogen), the pH was adjusted to 4.3, and the solutions were clarified by filtration through a 0.45-μm Millipore filter. The enzyme was activated by DTT and EDTA just prior to the measurements.

Stopped-Flow Measurements. Reactions were carried out in buffer A at room temperature (23 °C). The acylation and deacylation reactions were followed at wavelengths characteristic of the absorptions of the acyl–papain complexes, 325 nm for peptides with PDA at P_1 and 375 nm when SDA was at this position. The mixing cell and observation cell unit of the stopped-flow spectrophotometer was a slight modification of that described by Gutfreund (1965, 1967). Various mixing ratios of reactant solutions were obtained by using the appropriate syringes.

Results

Deacylation Rates of Acyl–Papain Complexes. In a solution of activated papain and a competitive inhibitor (I) with a free α-carboxyl at P_1, the free enzyme (E) and the free inhibitor are in equilibrium with a noncovalent enzyme–inhibitor complex (EI) and with a covalent acyl–enzyme complex (EI*). This equilibrium can be presented in a simplified way (not including tetrahedral and other possible intermediates) by eq 1.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^* \qquad (1)$$

If an excess of a second inhibitor with a high affinity to papain like Hg^{2+} or Boc-PIP-Arg[2] ($K_i = 3.3 \times 10^{-6}$ M; Berger et al., 1971) is added to this solution, the free enzyme molecules will be captured by the second inhibitor and the equilibrium will be shifted toward dissociation of EI and EI*. This reaction is shown in eq 2. The mathematical analysis of the

$$EI^* \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} EI \xrightarrow{k_{-1}} \underset{+I}{E} \xrightarrow{Hg^{2+}} E\cdot Hg \qquad (2)$$

kinetics of this reaction is described in the supplementary material (for information regarding supplementary material, see paragraph at end of paper). The solution that gives the concentration of the acyl–enzyme complex (Y) as a function of time is

$$Y = Y_e\frac{p}{p-q}e^{-qt} - Y_e\frac{q}{p-q}e^{-pt} \qquad (3)$$

The complex rate constants p and q (eq 4 and 5) are functions

$$p = \frac{k_2 + k_{-1} + k_{-2} - [(k_2 + k_{-1} + k_{-2})^2 - 4k_{-1}k_{-2}]^{1/2}}{2} \qquad (4)$$

$$q = \frac{k_2 + k_{-1} + k_{-2} + [(k_2 + k_{-1} + k_{-2})^2 - 4k_{-1}k_{-2}]^{1/2}}{2} \qquad (5)$$

of the acylation rate constant (k_2), the deacylation rate constant (k_{-2}), and the rate constant of the dissociation of the noncovalent enzyme–inhibitor complex (k_{-1}). According to this mathematical solution, the experimental curves had to be analyzed as a sum of two exponential terms (eq 3). Since only one exponential term could be clearly detected within the time scale and the resolution of the stopped-flow technique used, the mathematical solution was simplified by assuming that $k_{-1} \gg k_2, k_{-2}$ (see Discussion), as described in the supplementary material. The result is

$$Y = Y_e e^{-k_{-2}t} \qquad (6)$$

and

$$p \approx k_{-2} \qquad q \approx k_{-1}$$

Examples of the experimental results obtained are shown in Figure 1. In these experiments solutions of activated papain and the inhibitors were mixed in the stopped-flow spectrophotometer with equal volumes of solutions of the deacylation reagents $HgCl_2$ or Boc-PIP-Arg, which were shown to bind to the active site of papain (Drenth et al., 1971a,b; Berger et al., 1971) and compete with the binding of inhibitors I, IV, V, and X (Smolarsky, 1978). In Figure 1 the results are expressed in terms of the voltage generated in the photomultiplier of the stopped-flow spectrophotometer that was linearly dependent on the intensity of the light coming from the reaction cell. The measurable parameters are the time (t), the time-dependent voltage (V), and the voltage after the reaction was completed (V_∞). k_{-2} is obtained from the slope of the line described by eq 7.

$$\ln \log (V_\infty/V) = -k_{-2}t + \ln \log (V_\infty/V_e) \qquad (7)$$

Figure 1 shows that the experimental curves could be linearized as single exponential functions according to eq 7.

The deacylation rate constants (k_{-2}) obtained with inhibitors I, IV, V, and X are given in Table I. It can be seen that the values of k_{-2} obtained for all four inhibitors tested are similar.

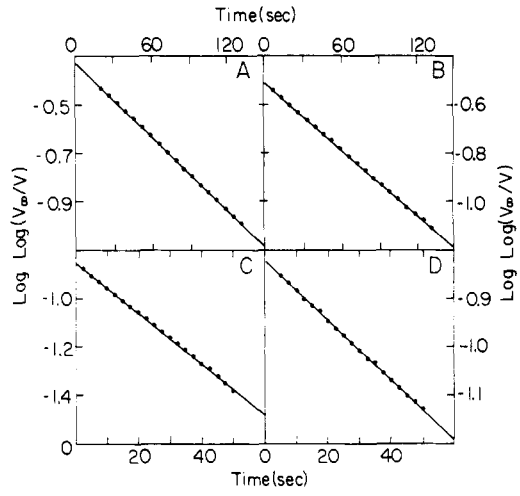

FIGURE 1: Kinetics of deacylation of acyl–papain complexes in the presence of $HgCl_2$ or Boc-PIP-Arg. The straight lines were plotted according to eq 7 by the linear least-squares technique. (A) Deacylation of Ac-Phe-PDA-papain. (B) Deacylation of Ala-Ala-Phe-SDA-papain. (C) Deacylation of Lys-Ala-OBT-SDA-papain. (D) Deacylation of Ala-Ala-DAP-SDA-papain. Concentrations of reactants were the following: (A) 5×10^{-5} M papain, 5×10^{-5} M Ac-Phe-PDA, and 5×10^{-4} M Boc-PIP-Arg or $HgCl_2$; (B) 5×10^{-5} M papain, 5×10^{-5} M Ala-Ala-Phe-SDA, and 5×10^{-4} M Boc-PIP-Arg or $HgCl_2$; (C) 2×10^{-5} M papain, 2×10^{-5} M Lys-Ala-OBT-SDA, and 10^{-3} M Boc-PIP-Arg or $HgCl_2$; (D) 1.25×10^{-5} M papain, 10^{-5} M Ala-Ala-DAP-SDA, and 5×10^{-4} M Boc-PIP-Arg or $HgCl_2$. In these reactions $HgCl_2$ was in excess to the EDTA + DTT that was used to activate the enzyme. V is the voltage as a function of time. V_∞ is the voltage after the reaction was completed.

[2] Abbreviations of amino acids and peptides are according to the IUPAC–IUB Commission on Biochemical Nomenclature (1972) recommendations. Further abbreviations used: DAP, *p*-(*p*′-dimethylaminophenylazo)phenylalanine; DTT, dithiothreitol; OBT, *o*-benzyltyrosine; PDA, β-phenyldehydroalanine; PIP, *p*-iodophenylalanine; SDA, β-styryldehydroalanine.

Table I: Rate and Equilibrium Constants[a] of Acylation of Papain by Inhibitors (k_2) and Deacylation of Acyl-Enzyme Complexes (k_{-2}) and Equilibrium Constants of Formation of Noncovalent Papain-Inhibitor Complexes (K_{-1})

inhibitor	k_2 (s^{-1})	k_{-2} (s^{-1})	k_2/k_{-2}	$K_{-1} = k_{-1}/k_1$ (M)
Ac-Phe-PDA (I)	0.23 ± 0.1	(1.1 ± 0.1) × 10^{-2}	21	(1.5 ± 1) × 10^{-4}
Ala-Ala-Phe-SDA (IV)	0.8 ± 0.1	(1.0 ± 0.1) × 10^{-2}	80	(7 ± 0.6) × 10^{-4}
Lys-Ala-OBT-SDA (V)	4.6 ± 0.8	(1.6 ± 0.15) × 10^{-2}	288	(1.4 ± 0.4) × 10^{-3}
Ala-Ala-DAP-SDA (X)	2.8 ± 0.7	(1.3 ± 0.1) × 10^{-2}	215	(1.2 ± 0.3) × 10^{-4}

[a] Values of k_2 and K_{-1} were obtained by nonlinear analysis of the curves shown in Figure 3 according to eq 14. Values of k_{-2} were obtained by linear analysis of the straight lines shown in Figure 1 according to eq 6. Concentrations of reactants are given in Figures 1 and 3.

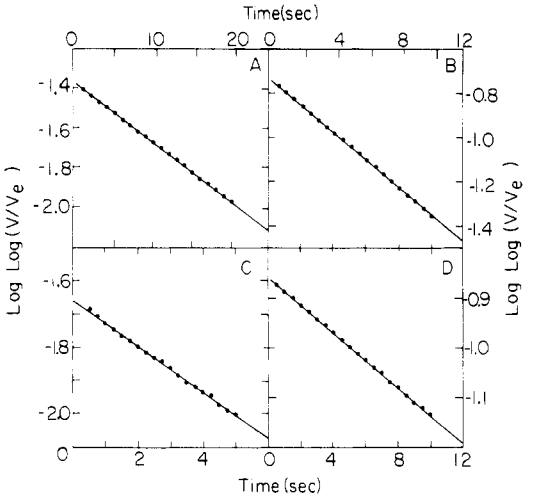

FIGURE 2: Kinetics of acylation of papain by (A) 5 × 10^{-5} M Ac-Phe-PDA, (B) 7 × 10^{-5} M Lys-Ala-OBT-SDA, (C) 6 × 10^{-6} M Ala-Ala-DAP-SDA, and (D) 5 × 10^{-5} M Ala-Ala-Phe-SDA. The straight lines were plotted according to eq 15 by the linear least-squares technique. Reactions were carried out in buffer A at room temperature. Enzyme concentrations are given in Figure 3. V is the voltage on the oscilloscope screen as a function of time. V_e is the voltage after equilibrium was reached.

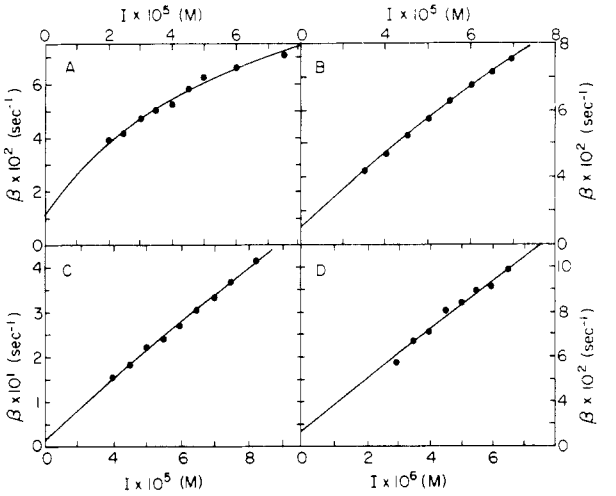

FIGURE 3: Rate constants (β) of the acylation of papain by competitive inhibitors as a function of the inhibitor concentration. (A) Ac-Phe-PDA; (B) Ala-Ala-Phe-SDA; (C) Lys-Ala-OBT-SDA; (D) Ala-Ala-DAP-SDA. The curves were calculated by a nonlinear analysis according to eq 14, from the values of β obtained with the various concentrations of the inhibitors used. Papain concentrations were (A) 5.5 × 10^{-6}, (B) 10^{-5}, (C) 10^{-5}, and (D) 10^{-6} M.

It means that the amino acid composition of the peptides has only a minor effect on the deacylation reaction. A possible explanation is that the covalent bond between the α-carboxyl at P_1 and the catalytic SH group of Cys-25, together with the hydrogen bonds between the peptide backbone and the enzyme in subsites S_1 and S_2 of the active site, is enough to fix the thioester bond in the appropriate orientation for hydrolysis.

Acylation of Papain by Competitive Inhibitors with a Free α-Carboxyl at P_1. Solutions of activated papain were mixed in the stopped-flow spectrophotometer with equal volumes of solutions of inhibitors I, IV, V, and X (see Table I) which have a free α-carboxyl at position P_1 and are competitive inhibitors of papain (Smolarsky, 1978). The concentrations of reactants used are given in Figure 3. In these experiments the concentrations of the inhibitors were much higher than the concentrations of the enzyme. The mathematical analysis of the acylation reaction (eq 1) under these conditions ($I_0 \gg E_0$) is described in the supplementary material. The solution that gives the concentration of the acyl–enzyme (Y) as a function of time is

$$Y = \frac{b}{a-b}Y_e e^{-at} - \frac{a}{a-b}Y_e e^{-bt} + Y_e \qquad (8)$$

where Y_e is the acyl–enzyme concentration at equilibrium ($t \rightarrow \infty$) and

$$a = [k_1I + k_{-1} + k_2 + k_{-2} + [(k_1I + k_{-1} + k_2 + k_{-2})^2 - 4(k_1k_{-2}I + k_1k_2I + k_{-1}k_2)]^{1/2}]/2 \qquad (9)$$

$$b = [k_1I + k_{-1} + k_2 + k_{-2} - [(k_1I + k_{-1} + k_2 + k_{-2})^2 - 4(k_1k_{-2}I + k_1k_2I + k_{-1}k_2)]^{1/2}]/2 \qquad (10)$$

The concentration of the noncovalent inhibitor–enzyme complex as a function of time is

$$X = Y_e\frac{b(k_{-2}-a)}{k_2(a-b)}e^{-at} + Y_e\frac{a(b-k_{-2})}{k_{-2}(a-b)}e^{-bt} + \frac{k_{-2}}{k_2}Y_e \qquad (11)$$

In practice, the mathematical solution obtained could not be used without simplifying it because of the following. (1) The analysis of an experimental curve as a sum of two exponents, according to eq 8, could not be done since only one exponential term could be found in the time scale of the stopped-flow technique with a resolution good enough for such analysis. (2) Obtaining the values of the parameters a and b is not enough to calculate the acylation rate constant (k_2) if the other rate constants (k_1, k_{-1}, and k_{-2}) are unknown. Substitution of a and b in eq 9 and 10 gives two equations and four unknowns.

The solution was therefore simplified by assuming k_1I, $k_{-1} \gg k_2$, k_{-2} (see Discussion), as is described in the supplementary material. The result is

$$Y = -Y_e\frac{\alpha}{\alpha-\beta}e^{-\beta t} + Y_e \qquad (12)$$

$$a \approx \alpha = k_1I + k_{-1} \qquad (13)$$

$$b \approx \beta = \frac{k_1k_{-2}I + k_1k_2I + k_{-1}k_{-2}}{k_1I + k_{-1}} \qquad (14)$$

Figure 2 shows that the experimental curves could be linearized as a single exponential function according to eq 15, derived from eq 12. The parameters in eq 15 are V_0 = the

$$\ln \log \frac{V}{V_e} = -\beta t + \ln\left(\frac{\alpha}{\alpha-\beta}\log\frac{V_0}{V_e}\right) \qquad (15)$$

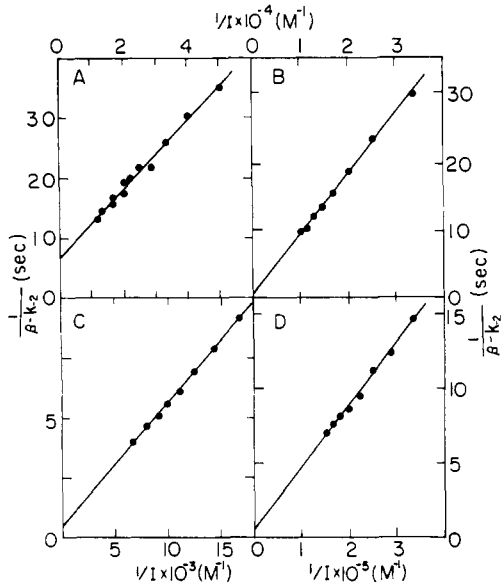

FIGURE 4: Rate constants (β) of the acylation of papain by competitive inhibitors as a function of the inhibitor concentrations (I). (A) Ac-Phe-PDA; (B) Ala-Ala-Phe-SDA; (C) Lys-Ala-OBT-SDA; (D) Ala-Ala-DAP-SDA. The curves were calculated by a linear analysis according to eq 16, from the values of β obtained with the various concentrations of the inhibitors used. The concentrations of reactants are given in Figure 3.

voltage generated in the photomultiplier when the complete reaction mixture is in the observation cell, but without the acyl–enzyme (at $t = 0$ for acylation and at $t \rightarrow \infty$ for deacylation), V = the voltage at time t, and V_e = the voltage at equilibrium ($t \rightarrow \infty$). The acylation reactions were carried out with various concentrations of inhibitors, I, IV, V, and X. The corresponding values of β (eq 15) were calculated from the slopes of the straight lines like those shown in Figure 2. The acylation rate constants (k_2) and the equilibrium constants ($K_{-1} = k_{-1}/k_1$) for the noncovalent enzyme–inhibitor complex (EI) formation were calculated from the values of β by nonlinear analysis according to eq 14. Figure 4 shows that the reciprocals of the experimental values of β are linearly dependent on $1/I$ according to

$$\frac{1}{\beta - k_{-2}} = \frac{1}{k_2} + \frac{k_{-1}}{k_1 k_2}\frac{1}{I} \qquad (16)$$

The values of k_2 and K_{-1} obtained are shown in Table I. It can be seen from the values of k_2/k_{-2} that all the inhibitors acylate papain when they bind to the active site at pH 4.3. At equilibrium most of the inhibitor molecules are bound to the active site in a thioester bond. It can also be seen that both k_2 and K_1 are affected by the amino acid composition of the peptides. In contrast to what might have been expected, a stronger noncovalent binding (lower K_{-1}) does not result in a higher degree of acylation. Thus, inhibitor IV has a higher K_{-1} than inhibitor I but it acylates papain more efficiently than inhibitor I. Its binding is therefore more productive. A possible reason for this is the difference in the length of the two peptides. The two additional alanyl residues of inhibitor IV in positions P_3 and P_4 may increase the productivity of the binding by interacting with subsites S_3 and S_4 in the active site and thus "forcing" the α-carboxyl at P_1 into a position more suitable for the formation of the thioester bond with Cys-25. A second possible reason is the difference between PDA and SDA. The additional double bond in SDA removes the bulky phenyl ring away from the α-carboxyl, as compared to PDA, and thus may affect the steric interactions between the side chain and the active site. The additional double bond may also affect the chemical properties of the amino acids and the acylation reaction.

The nature of the amino acid in position P_2 affects both the noncovalent binding and the acylation rate constant. This can be seen when comparing k_2/k_{-2} and K_{-1} of inhibitors IV and X. Substitution of the phenylalanine by DAP in position P_2 was shown to increase the binding energy of competitive inhibitors of papain by ~2.3 kcal/mol (Berger et al., 1971). Table I shows that substitution of phenylalanine by DAP in position P_2 affects both the noncovalent binding and the acylation. Inhibitor V has the highest productivity of acylation although it has the highest K_{-1}.

Acylation of Papain by Substrates. The acylation of papain by substrates can be presented in a reduced form (not including tetrahedral and other possible intermediates) by

$$E + S \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} ES \xrightarrow{k_3} \underset{+P}{EI^*} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} EI \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + I \qquad (17)$$

According to this scheme the enzyme (E) and the substrate (S) associate first to form a noncovalent complex (ES). This complex is then converted to an acyl–enzyme (EI*) while releasing the alcoholic or the amine portion of the substrate (P). The acyl–enzyme complex is in equilibrium with the noncovalent complex EI, which is in equilibrium with E + I. I is the acylic product of the reaction and is a competitive inhibitor. The kinetics of this reaction is much more complex than that of the acylation by competitive inhibitors, and its complete mathematical analysis might be very complicated, if possible at all. Therefore, the following assumptions were made to simplify it. (a) $E_0 \gg K_{-1} = k_{-1}/k_1$, $E_0 \gg K_{-1}' = k_{-1}'/k_1'$. (b) The noncovalent association rates are much faster than the acylation and deacylation reactions (see Discussion). (c) The initial concentration of the enzyme is much larger than the initial concentration of the substrate ($E_0 \gg S_0$). When these conditions hold, most of the substrate molecules will be bound to the enzyme a short time after the mixing of the substrates with the enzyme, as compared to the time scale of the acylation reaction. In addition, since the enzyme is in large excess to the substrate, its concentration can be considered constant during the reaction. Under these conditions the steps $E + S \rightleftharpoons ES$ and $E + I \rightleftharpoons EI$ can practically be neglected a short time after mixing of the reactants.

Equation 17 can then be simplified to eq 18. The mathematical solution of this equation is described in the supplementary material. The solution that gives the concentration of the acyl–enzyme complex (Y) as a function of time is given in eq 19. After expressing Y in terms of the voltage generated

$$ES \xrightarrow{k_3} EI^* \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} EI \qquad (18)$$

$$Y = \left(\frac{k_3 - k_2}{-k_3 + k_2 + k_{-2}}\frac{k_2 + k_{-2}}{k_2}Y_e e^{-k_3 t}\right) - \left(\frac{k_3 - k_2}{-k_3 + k_2 + k_{-2}}\frac{k_2 + k_{-2}}{k_2} + 1\right)Y_e e^{-(k_2 + k_{-2})t} + Y_e \qquad (19)$$

in the photomultiplier of the stopped-flow spectrophotometer, eq 19 was rewritten as eq 20. All the parameters in eq 20

$$\frac{\log (V_0/V)}{\log (V_0/V_e)} - 1 = \left(\frac{k_3 - k_2}{k_2 + k_{-2} - k_3}\frac{k_2 + k_{-2}}{k_2}e^{-k_3 t}\right) - \left(\frac{k_3 - k_2}{k_2 + k_{-2} - k_3}\frac{k_2 + k_{-2}}{k_2} + 1\right)e^{-(k_2 + k_{-2})t} \qquad (20)$$

except k_3 are known. The rate constants k_2 and k_{-2} are ob-

Table II: Rate Constants[a] of the Acylation of Papain by Substrates and Inhibitors

substrates (or inhibitors[b])	k_3 (s^{-1})
Ac-Phe-PDA (I)	0.14 ± 0.02
Ac-Phe-PDA-OEt (II)	0.073 ± 0.003
Ac-Phe-PDA-NH_2 (III)	0.004 ± 0.0001
Ala-Ala-Phe-SDA (IV)	0.18 ± 0.01
Ala-Ala-Phe-SDA-OMe (VI)	0.18 ± 0.01
Ala-Ala-Phe-SDA-NH_2 (VII)	0.014 ± 0.001
Lys-Ala-OBT-SDA (V)	0.56 ± 0.04
Lys-Ala-OBT-SDA-OMe (VIII)	0.65 ± 0.03
Lys-Ala-OBT-SDA-NH_2 (IX)	0.03 ± 0.002
Ala-Ala-DAP-SDA (X)	2.0 ± 0.3
Ala-Ala-DAP-SDA-OMe (XI)	2.3 ± 0.2
Ala-Ala-DAP-SDA-NH_2 (XII)	0.14 ± 0.01

[a] Measured under the conditions $E \gg S$ according to eq 20, with the assumption $E \gg K_{-1}$. Enzyme concentration was 1.4×10^{-4} M. Concentrations of peptides were the following: I–IX, 1.4×10^{-5} M; X, 2.8×10^{-6} M; XI and XII, 5.5×10^{-6} M. [b] For inhibitors $k_3 = k_2$.

tained from the kinetics of the acylation and deacylation reactions with the appropriate inhibitors (Table I). V, V_0, V_e, and t are measurable parameters.

For the purpose of matching the above assumptions as much as possible, the concentration of papain in this set of experiments was 1.4×10^{-4} M, which is close to the maximal solubility of the enzyme in buffer A at room temperature (not including the unactivatable part of the commercial enzyme). The concentration of peptides I–IX was 1.4×10^{-5} M. Peptide X was used at a concentration of 2.8×10^{-6} M, and peptides XI and XII were used at 5.5×10^{-6} M. For minimization of the dilution of the enzyme when mixed with the peptide solutions in the stopped-flow spectrophotometer, the volume of the enzyme solution injected into the mixing cell was 5 times the volume of the peptide solutions.

The rate constants of the acylation of papain by substrates (k_3) were calculated from the experimental curves in a numerical way according to eq 20. The acylations by the related inhibitors I, IV, V, and X were also studied under the same conditions. The resulting values of the acylation rate constants obtained with the inhibitors in this way were compared to the correct values (for inhibitors $k_2 = k_3$) already known from the other experiments (Table I). This comparison enabled me to test whether the assumptions made were valid for the inhibitors and to some extent also for the related substrates. The results are shown in Table II. It can be seen that with inhibitors I and X the values of k_3 are close to the values of k_2 shown in Table I. On the other hand, for inhibitors IV and V k_3 is much smaller than k_2. These differences result from the high values of K_{-1} of inhibitors IV and V as compared to the enzyme concentration ($E = 1.4 \times 10^{-4}$ M). According to K_{-1}, 48% of inhibitor I and 54% of inhibitor X were bound to the enzyme at the beginning of the acylation reaction, 74 and 77% were bound after half of the inhibitors have reacted, and 96.4% was bound at equilibrium. On the other hand, only 17% of IV and 9.1% of V were bound to the enzyme at the beginning of the acylation reaction. About 55% of IV and V was bound after half of the inhibitors had reacted, and >96% was bound at equilibrium. It is clear therefore that the assumptions made in the derivation of eq 19 are not valid for inhibitors IV and V.

If the affinities of the substrates to the active site of papain are not less than those of the corresponding inhibitors, then the results presented in Table II show that at pH 4.3 the rate constants of the acylation of the enzyme by the inhibitors are not smaller than those of the esters. On the other hand, amides acylate papain with a much lower rate constant.

Discussion

The spontaneous formation of a thioester from a free carboxyl and a thiol group can be considered thermodynamically unfavored because of the high energy of hydrolysis of thioesters [6–8 kcal/mol at pH 7; see tables in Jencks (1968)]. The acylation of thiols by amides can also be considered thermodynamically unfavored because of the low energy of hydrolysis of amides. However, it was shown that a thioester acyl–enzyme complex is an intermediate in the hydrolysis of substrates by papain (Lowe & Williams, 1965a; Brubacher & Bender, 1966; Sluyterman, 1968; Hinkle & Kirsch, 1970). Recently, I have shown also that papain is acylated by competitive inhibitors with a free α-carboxyl group at P_1 (Smolarsky, 1978). This was done by direct spectrophotometric measurements using the amino acids PDA and SDA as spectroscopic probes at position P_1 of the substrates and inhibitors. The unusually high chemical reactivity of the catalytic thiol group of papain (Sluyterman, 1968; Brocklehurst & Little, 1972) can only partly account for this phenomenon, especially in view of the unique spectral properties of acyl–papain complexes that may be indicative of a high energy of hydrolysis (Smolarsky, 1978). A rough calculation, based on the molar absorption coefficients of the thioesters of PDA and SDA, showed that when competitive inhibitors bind to papain at pH 3.8–4.5, most of the enzyme-bound inhibitor molecules are at equilibrium in the form of an acyl–enzyme complex. This result was confirmed quantitatively in this study by direct measurement of the acylation and deacylation rate constants (k_2 and k_{-2}, see Table I). Moreover, the results show that at pH 4.3 the inhibitors acylate papain with rate constants that are not smaller than those of the corresponding methyl or ethyl esters. It should be noted that the pH dependence of the acylation of papain by inhibitors with a free α-carboxyl at P_1 is different from that of uncharged substrates. The optimal pH for the acylation of papain by inhibitors is ~4.1 (Smolarsky, 1978), and for substrates it is in the range of 5–7 (Glazer & Smith, 1971). It was found that it is the protonation of the enzyme at low pH that provides the free energy required to shift the equilibrium toward acylation when the inhibitors interact with papain. This energy, however, is still not equivalent to the energy of hydrolysis of the thioester bond in the acyl–enzyme. The rest probably comes from the noncovalent binding of the inhibitors or substrates to the active site. This energy can be used for acylation by (a) formation of a local high concentration of the α-carboxyl at S_1, close to the catalytic thiol group in the right orientation and location, (b) induction of conformational changes in the peptide and in the enzyme [the bonds to be hydrolyzed (–CO–N– or –CO–O–) can be distorted and polarized], and (c) desolvation of both the substrate and the active site of the enzyme. The results of these effects would be a decrease in the free energy of activation and in the total free energy of the acylation reaction. It has been shown that both the length of the peptide substrates (or inhibitors) and the chemical properties of the amino acids determine the energy and the specificity of the binding to the extended active site of papain (Berger & Schechter, 1970; Berger et al., 1971). Substrates which are long enough to occupy the seven subsites in the active site (four toward the amino terminus and three toward the carboxy terminus of the substrate) are usually better substrates than short peptides which can bind to only part of the subsites. It was also shown that the binding energy of competitive inhibitors to papain is dependent on the properties of the amino acid in position P_2. Substitution of alanine in this position by

phenylalanine increases the energy of binding by 3 kcal/mol, and substitution of alanine by DAP increases the binding energy by 5.3 kcal/mol. Therefore, it may be expected that the acylation rate constants of papain by substrates and inhibitors will be affected by the amino acid composition. The data shown in Table I show that this is indeed the case. Inhibitor I binds to papain with a higher noncovalent binding constant (K_I) than inhibitors IV and V, but the binding is much less productive. This result may indicate that the noncovalent binding energy of inhibitor I is not utilized for the acylation of the thiol group, as it is with inhibitors IV and V. Thus, a more productive binding, in which a larger part of the noncovalent binding energy is utilized for acylation, results in a *lower* binding constant. It was indeed found that inhibitors with a free α-carboxyl at P_1 that can acylate papain have much lower binding constants than inhibitors that can bind to the active site with little or no investment of energy in acylating it. Thus, Ala-Ala-Phe-Gly is a weak competitive inhibitor of papain with $K_d = 2.2 \times 10^{-3}$ M (corresponding to a free energy of binding of 3.7 kcal/mol). By contrast, Ala-Ala-Phe-glycinenitrile is a strong competitive inhibitor with $K_d = 3.5 \times 10^{-6}$ M (corresponding to a free energy of binding of 7.6 kcal/mol; unpublished result). Similarly, Ac-Phe-Gly binds to papain with a free energy of only 4.1 kcal/mol (H. Benderly, personal communication) while Ac-Phe-$NHCH_2CHO$ is a very strong competitive inhibitor with $K_d = 2.2 \times 10^{-8}$ M (10.3 kcal/mol; Westerik & Wolfenden, 1972). The lower productivity of binding of inhibitor I, as compared with IV, V, and X, may result from the fact that it is made of only two amino acids. Therefore, its interaction with the active site is not enough to "force" the α-carboxyl at P_1 into the position and orientation which is optimal for acylation. The binding of inhibitors IV, V, and X is more productive because they are made of four amino acids, according to the specificity of papain (Berger & Schechter, 1970; Berger et al., 1971), and they fully interact with the four subsites of the active site S_1, S_2, S_3, and S_4. Substitution of phenylalanine at position P_2 with DAP increases both the productivity of the binding and the noncovalent binding energy. The case of inhibitor V is another example where the noncovalent binding energy is utilized for acylation. Inhibitor V is a stronger competitive inhibitor of papain ($K_i = 1.1 \times 10^{-5}$ M) than inhibitor IV ($K_i = 2.9 \times 10^{-5}$ M). It was also shown that substitution of phenylalanine at position P_2 with OBT increases the energy of binding by 1.5 kcal/mol (Berger et al., 1971). Therefore, inhibitor V has a higher noncovalent binding energy than IV, but since its binding is more productive a larger part of this energy is utilized for acylation. The result is a higher K_{-1} and a higher $K_2 = k_2/k_{-2}$.

In the mathematical analysis of the kinetics of the acylation and deacylation reactions described in this work, I assumed that the noncovalent association (k_1I) and dissociation (k_{-1}) are much faster than the acylation (k_2 and k_3) and deacylation (k_{-2}) steps. This assumption is based on a large body of experimental data described in the literature. The noncovalent association rate constants of many enzymes with their substrates in solutions have been found to be in the range of 10^6–10^9 M^{-1} s^{-1} (Eigen & Hammes, 1963; Gutfreund, 1971). The dissociation rate constants of noncovalent enzyme–substrate complexes are also high, although they are usually lower than the diffusion-controlled rates (10^9–10^{11} s^{-1}) and in most cases are in the range of $(5–50) \times 10^4$ s^{-1} (Eigen & Hammes, 1963). Because of the high rates of the noncovalent association and dissociation reactions, it is usually impossible to observe these steps with the stopped-flow technique under the conditions $E_0 \gg S_0$ or $S_0 \gg E_0$ and when the reactant concentrations are high enough to get significant detectable signals. These are the conditions under which the measurements described in this paper were performed. In our laboratory Nurith Kurn studied the association of the competitive inhibitor Ac-DAP-Arg with papain. She found that in this case $k_1 = 2.3 \times 10^6$ M^{-1} s^{-1} (Berger et al., 1971). N. Kurn, H. Bosshard, and I. Pecht studied the association and dissociation of Z-Ala-Ala-(β-naphthyl)alanine with chymotrypsin by the T-jump technique (personal communication). They found the values $k_1 = 2.7 \times 10^8$ M^{-1} s^{-1} and $k_{-1} = 3.4 \times 10^4$ s^{-1}. Gutfreund & Sturtevant (1956) studied the hydrolysis of *p*-nitrophenyl acetate by chymotrypsin with the stopped-flow technique. They found that under the conditions $S_0 \gg E_0$ the noncovalent association was too fast to be detected. The acylation and deacylation rates were found to be much slower (3.15 and 0.025 s^{-1}, respectively). Similar results were found by other investigators (Bender et al., 1962; Spencer & Sturtevant, 1959; Gutfreund, 1955; Hollaway et al., 1969; Brubacher & Bender, 1966; Lucas & Williams, 1969; Lowe & Williams, 1965b).

The acylation and deacylation rate constants found in this study are 0.23–4.6 and 0.011–0.016 s^{-1}, respectively. The numerical calculations that are described in the supplementary material (Figures 5 and 6) show that the assumption made ($k_1I, k_{-1} \gg k_2, k_{-2}, k_3$) is justified when $k_1I, k_{-1} > 10k_2, 10k_{-2}, 10k_3$. Comparison of k_2, k_{-2}, and k_3 found in this study with k_1 for the binding of Ac-DAP-Arg to papain (see above), and with the rate constants of the noncovalent association and dissociation of enzymes and substrates (or ligands) that were described in the literature, shows that the assumptions made in this study are probably correct.

Supplementary Material Available

Mathematical analysis of the kinetics of the acylation of papain by inhibitors and substrates and the deacylation of acyl–papain complex, theoretical curves representing kinetic rate constants in the acylation of papain by inhibitors (Figure 5), and theoretical curves representing kinetic rate constants in the deacylation of acyl–papain complex (Figure 6) (15 pages). Ordering information is given on any current masthead page.

References

Bender, M. L., & Kaiser, E. T. (1962) *J. Am. Chem. Soc. 84*, 2556.

Bender, M. L., Schonbaum, G. R., & Zerner, B. (1961) *J. Biol. Chem. 236*, 2930.

Bender, M. L., Schonbaum, G. R., & Zerner, B. (1962) *J. Am. Chem. Soc. 84*, 2540.

Berger, A., & Schechter, I. (1970) *Philos. Trans. R. Soc. London, Ser. B 257*, 249.

Berger, A., Schechter, I., Benderly, H., & Kurn, N. (1971) *Pept., Proc. Eur. Pept. Symp., 10th*, 290.

Bernhard, S. A., Lau, S. J., & Noller, H. (1965) *Biochemistry 4*, 1108.

Brocklehurst, K., & Little, G. (1972) *Biochem. J. 128*, 471.

Brubacher, L. J., & Bender, M. L. (1966) *J. Am. Chem. Soc. 88*, 5871.

Drenth, J., Jansonius, J. N., Koekoek, R., & Wolthers, B. G. (1971a) *Adv. Protein Chem. 25*, 79.

Drenth, J., Jansonius, J. N., Koekoek, R., & Wolthers, B. G. (1971b) *Enzymes, 3rd Ed. 3*, 485.

Eigen, M., & Hammes, G. G. (1963) *Adv. Enzymol. Relat. Subj. Biochem. 25*, 1.

Glazer, A. N., & Smith, E. L. (1971) *Enzymes, 3rd Ed. 3*, 581.

Gutfreund, H. (1955) *Discuss. Faraday Soc. 20*, 167.
Gutfreund, H. (1965) *An Introduction to the Study of Enzymes*, Blackwell Scientific Publications, Oxford.
Gutfreund, H. (1967) *Fast React. Primary Processes Chem. Kinet., Proc. Nobel Symp., 5th*, 429.
Gutfreund, H. (1971) *Annu. Rev. Biochem. 40*, 315.
Gutfreund, H., & Sturtevant, J. M. (1956) *Biochem. J. 63*, 656.
Hinkle, P. M., & Kirsch, J. F. (1970) *Biochemistry 9*, 4633.
Hollaway, M. R., Antonini, E., & Brunori, M. (1969) *FEBS Lett. 4*, 299.
IUPAC-IUB Commission on Biochemical Nomenclature (1972) *J. Biol. Chem. 247*, 977.
Jencks, W. P. (1968) *CRC Handbook of Biochemistry* (Sober, H. A., Ed.) pp J-146-147, Chemical Rubber Co., Cleveland, OH.
Kimmel, J. R., & Smith, E. (1954) *J. Biol. Chem. 207*, 515.
Kirsch, J. F., & Katchalski, E. (1965) *Biochemistry 4*, 884.
Lowe, G., & Williams, A. (1965a) *Biochem. J. 96*, 189.
Lowe, G., & Williams, A. (1965b) *Biochem. J. 96*, 199.
Lucas, E. C., & Williams, A. (1969) *Biochemistry 8*, 5125-5135.
Malhotra, O. P., & Bernhard, S. A. (1968) *J. Biol. Chem. 243*, 1243.
Malhotra, O. P., & Bernhard, S. A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2077.
Oliver, R. W. A., Viswanatha, T., & Whish, W. J. D. (1967) *Biochem. Biophys. Res. Commun. 27*, 107.
Sluyterman, L. A. AE. (1968) *Biochim. Biophys. Acta 151*, 178.
Smolarsky, M. (1978) *Biochemistry 17*, 4606.
Spencer, T., & Sturtevant, J. M. (1959) *J. Am. Chem. Soc. 81*, 1874.
Stockell, A., & Smith, E. L. (1957) *J. Biol. Chem. 227*, 1.
Westerik, J. O., & Wolfenden, R. (1972) *J. Biol. Chem. 247*, 8195.

Pyridoxal Phosphate as a Probe of Reovirus Transcriptase†

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ABSTRACT: The ribonucleoprotein core of reovirus is a multienzyme complex that transcribes messenger ribonucleic acid (mRNA) from double-stranded RNA templates. So far, the core has resisted attempts to disassemble it and identify the polypeptide species responsible for RNA polymerase activity. As an alternative approach, we tested pyridoxal 5-phosphate (PLP) as a potential affinity labeling reagent for reovirus transcriptase in vitro; PLP has been used as an affinity reagent for cellular and viral nucleic acid polymerases. We found that PLP inhibited reovirus transcriptase reversibly (apparent K_i = 0.2 mM), but the inhibition was noncompetitive with respect to each of the four ribonucleoside triphosphates. This interaction required both the aldehyde and phosphate moieties in PLP, since pyridoxamine and pyridoxal were relatively inactive. To identify the polypeptides involved, we labeled the PLP–core complex by reductive alkylation with [^{3}H]borohydride. At PLP concentrations close to the apparent K_i, labeling was selective for the two largest virion polypeptides, λ_1 and λ_2. At saturation, there were only 10 high-affinity PLP binding sites per core in each of the λ polypeptide species. These findings implicate either or both λ polypeptide species in viral transcription and they indicate that a special population, representing no more than 10% of the total λ molecules in each core, participates in RNA synthesis.

Several types of RNA viruses carry a complete mRNA-synthesizing apparatus from cell to cell as required by the structure of their genomes (Raghow & Kingsbury, 1976). Although these viruses possess few genes, they contain a transcriptive apparatus that has many of the capabilities of the cell nucleus, including, besides a transcriptase, four or five additional enzymatic activities that cap, methylate, and polyadenylate the transcripts (Shatkin, 1976; Raghow & Kingsbury, 1976). The question arises of how these activities are apportioned among so few viral gene products. Little progress has been made toward an answer for any of these viruses, because it is difficult to obtain adequate amounts of viral proteins for study or to separate the proteins from the virus particles in an enzymatically active form.

Reovirus, which contains 10 genes in the form of separate segments of double-stranded RNA, is a case in point. Only four protein species have been identified in virus cores that display a full spectrum of RNA synthesizing and modifying activities (Joklik, 1974; Shatkin & Both, 1976), and only denaturing and inactivating treatments have, as yet, been able to dislodge any of these proteins from the core (White & Zweerink, 1976). This is unfortunate, because in other respects reovirus is an attractive subject for studies of virus transcriptase, being easy to grow and possessing a prodigious RNA synthetic capacity (Joklik, 1974).

For these reasons, we chose reovirus as the subject of this study to evaluate affinity labeling as a means of identifying the enzymatically relevant polypeptides in an intact and functioning viral transcriptive complex. In affinity labeling of enzymes, the aim is to form a covalent bond between the enzyme and a labeled analogue of a substrate or allosteric effector, identifying the site on the enzyme where the labeled compound binds (Singer, 1967). At another level of analysis, as in the present work, the method can identify the enzyme in a mixture of proteins, such as a multienzyme complex. We chose to investigate pyridoxal 5-phosphate (PLP) as a probe of reovirus transcriptase, because it is a compound with a record of application to affinity labeling of several nucleic acid polymerases (Bull et al., 1975; Martial et al., 1975; Modak, 1976; Papas et al., 1977; Venegas et al., 1973), among other types of enzymes (Colombo & Marcus, 1974; Marie, 1976; Piszikiewicz et al., 1977; Rippa et al., 1967; Schnackerz & Noltmann, 1971; Shapiro et al., 1968). PLP has a structural

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