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Possible Role for Water Dissociation in the Slow Binding of Phosphorus-Containing Transition-State-Analogue Inhibitors of Thermolysin†

Paul A. Bartlett* and Charles K. Marlowe
Department of Chemistry, University of California, Berkeley, California 94720
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ABSTRACT: A number of phosphonamidate and phosphonate tripeptide analogues have been studied as transition-state-analogue inhibitors of the zinc endopeptidase thermolysin. Those with the form Cbz-Gly^P(Y)Leu-X [ZG^P(Y)LX, X = NH_2 or amino acid, Y = NH or O linkage] are potent (K_i = 9–760 nM for X = NH, 9–660 μM for X = O) but otherwise ordinary in their binding behavior, with second-order rate constants for association (k_{on}) greater than 10^5 M^{-1} s^{-1}. Those with the form Cbz-X^P(Y)-Leu-Ala [ZX^P(Y)LA, X^P = α-substituted phosphorus amino acid analogue] are similarly potent (K_i for ZF^{P}LA = 68 pM) but slow binding ($k_{on} \leq 1300$ M^{-1} s^{-1}). Several kinetic mechanisms for slow binding behavior are considered, including two-step processes and those that require prior isomerization of inhibitor or enzyme to a rare form. The association rates of ZF^{P}LA and ZF^P(O)LA are first order in inhibitor concentration up to 1–2 mM, indicating that any loose complex along the binding pathway must have a dissociation constant above this value. The crystallographic investigation described in the preceding paper [Holden, H. M., Tronrud, D. E., Monzingo, A. F., Weaver, L. H., & Matthews, B. W. (1987) *Biochemistry* (preceding paper in this issue)] identifies a specific water molecule in the active site that may hinder binding of the α-substituted inhibitors. The implication of this observation for a mechanism for slow binding is discussed.

Enzyme inhibitors have been used to study the importance of metabolic pathways and the role of individual enzymes, to shed light on enzyme mechanisms, and to aid in the development of useful pharmacological and agricultural agents. In contrast to investigations of enzyme–substrate interactions, which generally focus on the *kinetic* details of the process, most studies of enzyme inhibitors have focused on the *thermodynamic* aspects of the binding phenomenon. Of interest in the latter regard are the protein–inhibitor contacts that contribute incrementally to overall affinity, the interplay between inhibitor potency and its relevance to the enzyme mechanism, or the potential of an inhibitor as a pharmaceutical agent. However, with greater refinement in the design of potent and mechanistically relevant inhibitors, there is increasing interest in the kinetic details of their binding behavior (Frieden, 1970; Cha, 1975, 1976; Williams & Morrison, 1979; Duggleby et al., 1982; Morrison & Walsh, 1987; Kurz et al., 1987). This interest stems in great measure from the increasing frequency with which "slow binding behavior" is observed, that is, instances in which formation of an enzyme–inhibitor complex takes place at a rate considerably slower than expected for a diffusion-limited process. In spite of increasing interest in this behavior, there remains limited insight into the molecular basis of the phenomenon, either in the form of direct evidence to support the varied explanations proposed or through the identification of the responsible structural elements in the inhibitors themselves. In this paper, we describe the behavior of a series of inhibitors of the zinc endopeptidase thermolysin and the identification of a specific element in their structure that produces slow binding behavior. In the preceding paper, Holden et al. (1987) present a crystallographic comparison of the structures of thermolysin complexes with representative slow- and fast-binding inhibitors and identify what may be the key element in the slow binding process.

Thermolysin, a 34.6-kDa, zinc-containing endopeptidase isolated from *Bacillus thermoproteolyticus*, is important as one of the prototypical metalloproteases and a model for the more pharmacologically important members of this class (Cushman & Ondetti, 1981; Maycock et al., 1981; Hangauer et al., 1984; Hersh & Morihara, 1986). Its substrate specificity, including its selectivity for hydrophobic amino acids at the P_1 and P_1' sites (Schechter & Berger, 1967), has been well characterized (Morihara & Tsuzuki, 1970; Morgan & Fruton, 1978; Hersh & Morihara, 1986), and it has been the focus of a number of inhibitor (Maycock et al., 1981; Bartlett & Marlowe, 1983; Shenvi & Kettner, 1985, and references cited therein) and crystallographic studies (Monzingo & Matthews, 1984; Tronrud et al., 1986, 1987, and references cited therein). The most recent proposal with respect to its mechanism is that of Hangauer, Monzingo, and Matthews (1984), who incorporated both kinetic and structural information in suggesting a sequence involving attack of the zinc-bound water on the scissile carbonyl group, with simultaneous coordination of the carbonyl oxygen to the metal. Key

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* Author to whom correspondence should be addressed.

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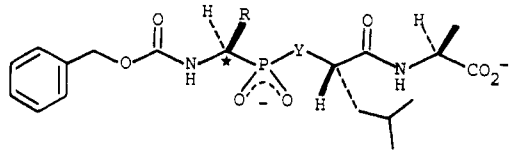
Chart I



ZG^PLA	Y = NH, R = H
ZF^PLA	Y = NH, R = $CH_2C_6H_5$
$ZG^P(O)LA$	Y = O, R = H
$ZA^P(O)LA$	Y = O, R = CH_3
$ZL^P(O)LA$	Y = O, R = $CH_2CH(CH_3)_2$
$ZF^P(O)LA$	Y = O, R = $CH_2C_6H_5$

($Z(D)F^PLA$, $Z(D)A^P(O)LA$, $Z(D)L^P(O)LA$, $Z(D)F^P(O)LA$ have the opposite configuration at the starred center)

support for this proposal came from crystallographic studies of inhibitor complexes, including that between thermolysin and the *N*-phosphoryl dipeptide phosphoramidon (Weaver et al., 1977). Interestingly, phosphoramidon and its stereoisomer talopeptin are slow-binding inhibitors of thermolysin (Kam et al., 1979; Kitagishi & Hiromi, 1984).

Our initial studies on the thermodynamic and structural aspects of the binding of phosphonate peptide analogues to thermolysin concerned derivatives of (aminomethyl)phosphonic acid (Bartlett & Marlowe, 1983, 1987) (Chart I). The focus of this paper is the behavior of peptide derivatives in which α-substituted phosphonic acids (e.g., Ala^P, Leu^P, and Phe^P)[1] are incorporated at the P_1 position of the inhibitors. These compounds were investigated to probe the limits of the correlation between K_m/k_{cat} and K_i for transition-state analogues (Westerik & Wolfenden, 1972; Thompson, 1973; Bartlett & Marlowe, 1983) as well as to use this correlation to develop more potent inhibitors. An unanticipated finding was that the inhibitors incorporating these α-substituted analogues are slow binding, in contrast to those derived from Gly^P.

Materials and Methods

Synthesis of Inhibitors

General. Unless otherwise indicated, NMR spectra were determined in $CDCl_3$ solvent. All NMR spectra were acquired on Fourier transform instruments with the following combination of frequencies, solvents, and references. ^1H NMR: 300, 250, or 200 MHz; chemical shifts are reported in ppm on the δ scale relative to tetramethylsilane ($CDCl_3$ solvent) or $(CH_3)_3SiCD_2CD_2CO_2Na$ (D_2O solvent) as 0 ppm [data are presented as chemical shift (multiplicity, number of protons, coupling constants in hertz)]. ^{13}C NMR: 50.8 or 45.3 MHz; chemical shifts are referenced to $CDCl_3$ as 77.0 ppm or to dioxane as 66.5 ppm in D_2O. ^{31}P NMR: 72.9 or 81.8 MHz; chemical shifts are reported in ppm downfield from 85% H_3PO_4 (sealed capillary). Melting points are uncorrected. Chloroform was rendered ethanol free by washing with concentrated H_2SO_4 and water, drying ($CaCl_2$), and distilling from P_2O_5. Combustion analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. Column chromatography was performed by the method of Still, Kahn, and Mitra (1978). Triethylammonium bicarbonate buffer (TBK) was prepared by saturating a rapidly stirred mixture (0 °C) of freshly distilled triethylamine and water with CO_2 until a pH of 8.6 was reached.

N-[[1-[(Phenylmethoxycarbonyl)amino]-2-phenylethyl]-methoxyphosphinyl]-L-leucyl-L-alanine Methyl Ester (ZF^PLA Dimethyl Ester). To a solution of 747 mg (2.13 mmol) of methyl hydrogen [1-[(phenylmethoxycarbonyl)amino]-2-phenylethyl]phosphonate (ZF^P-OCH_3) (Bartlett & Lamden, 1986) in 4 mL of ethanol-free $CHCl_3$ under a nitrogen atmosphere was added 171 μL (2.34 mmol) of thionyl chloride. After stirring at 23 °C for 4 h, the solution was concentrated, redissolved in $CHCl_3$, and concentrated again to remove traces of HCl and excess $SOCl_2$. The crude chloridate was then dissolved in 3 mL of $CHCl_3$ and cooled to −10 °C. After the addition of 795 μL (5.7 mmol) of dry triethylamine, a dry solution of 360 mg (1.43 mmol) of L-leucyl-L-alanine methyl ester hydrochloride[1] in 1 mL of $CHCl_3$ was added. The solution was allowed to warm to 23 °C and stir for 10 days. The phosphonamidate was isolated by chromatography on silica gel (7% ethanol/dichloromethane) to give 329 mg (42% yield) of a slightly yellow crystalline solid as an equal mixture of four diastereomers. A 158-mg sample was separated by HPLC (Altex Ultrasphere-Si, 10 mm × 25 cm) by elution with 3% ethanol/dichloromethane to give three distinct peaks (k' values in parentheses) as white solids: peak A, 22 mg (0.22); peak B, 12 mg (0.18); peak c, 52 mg as an equal mixture of two diastereomers, (0.16). The NMR spectral details are recorded in the supplementary material; λ_{max}(MeOH) 257 nm (ϵ = 226). Anal. Calcd for $C_{27}H_{38}N_3O_7P$: C, 59.22; H, 7.00; N, 7.67; P, 5.66. Found: C, 59.53; H, 6.87; N, 7.46; P, 5.48.

N-[[(L)-1-[(Phenylmethoxycarbonyl)amino]-2-phenylethyl]hydroxyphosphinyl]-L-leucyl-L-alanine Dilithium Salt (ZF^PLA). A solution of 30 mg (0.054 mmol) of ZF^PLA dimethyl ester, obtained from peak B above, in 0.11 mL of acetonitrile was treated with 0.59 mL (0.162 mmol) of 2.75 M aqueous LiOH and 0.12 mL of water at 21 °C for 24 h. After lyophilizaton, the crude salt was purified by chromatography on a 20-cm × 1-cm column of DEAE-Sephadex A-25 (HCO_3^- form) using 300 mL of a linear gradient of 0–0.5 M TBK buffer (pH 8.6). Fractions containing the desired compound (monitored by absorbance at 257 nm) were pooled, treated with 0.49 mL of 2.75 M LiOH (2.2 equiv based on ϵ_{257} = 340) and lyophilized, and finally evaporated from methanol (to remove traces of triethylammonium carbonate) to give a quantitative yield of the white dilithium salt of ZF^PLA: ^1H NMR (D_2O) δ 0.92 (d, 6, J = 6.5), 1.34 (d, 3, J = 7.2), 1.40–1.67 (m, 2), 1.67–1.83 (m, 1), 2.67 (A of ABXP, 1, J_{AB} = 12.5, J_{AX} = 13.6, J_{AP} = 12.5), 3.23 (B of ABXP, 1), 3.79 (m, 1), 3.87 (X of ABXP, 1), 4.13 (q, 1, J = 7.2), 4.85 (d, 1, J = 12.9), 5.01 (d, 1, J = 12.9), 7.23 (m, 10); ^{13}C NMR (D_2O) δ 17.6, 21.6, 22.1, 23.9, 36.4, 43.6 (J_{CP} = 3.3), 50.8, 53.2 (J_{CP} = 139), 54.3, 66.4, 127.2, 127.3, 128.0, 128.4, 128.6, 129.2, 136.4, 138.8 (J_{CP} = 4.3), 157.6 (J_{CP} = 7.9), 177.0; ^{31}P NMR (D_2O) δ 20.2 (major), 19.5 (minor).

Preparation and characterization of $Z(D)F^P$ monomethyl ester and $Z(D)F^PLA$ are described in the supplementary material.

Determination of the Absolute Configuration of the Isomers of N-[[(L,D)-1-[(Phenylmethoxycarbonyl)amino]-2-phenylethyl]hydroxyphosphinyl]-L-leucyl-L-alanine Dilithium Salt (ZF^PLA). To a solution of 150 mg (0.43 mmol) of a 2:1 mixture of the D and L isomers of methyl hydrogen [1-[(phenylmethoxycarbonyl)amino]-2-phenylethyl]phosphonate

[1] Abbreviations: G^P = Gly^P, A^P = Ala^P, L^P = Leu^P, F^P = Phe^P, the α-(aminoalkyl)phosphonic acid corresponding to the indicated amino acid; FAGLA, *N*-[3-(2-furyl)acryloyl]glycyl-L-leucinamide; TBK, triethylammonium bicarbonate; HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

in 1.7 mL of dry $CHCl_3$ was added 31 μL (0.43 mmol) of thionyl chloride. After stirring 4 h at 23 °C, the solution was concentrated in vacuo, and the residue was dissolved in 1.7 mL of $CHCl_3$ and cooled to 0 °C. A 189-mg (0.43 mmol) sample of L-leucyl-L-alanine methyl ester hydrochloride was added, followed by 180 μL (1.3 mL) of dry triethylamine. After stirring for 2 days at 23 °C, the solution was extracted with 1 N HCl and 5% $NaHCO_3$, dried ($CaSO_4$), and concentrated under reduced pressure to give the dimethyl esters of the ZF^PLA diastereomers. ^{31}P NMR analysis showed enrichment in two of four resonances: δ 31.4 and 29.7 (63%) and 30.9 and 29.1 (37%). Therefore, the resonance at 31.4, which corresponds to HPLC peak A, was shown to have the D configuration and to be the precursor to the poor inhibitor of K_i = 2 μM. The resonance at 30.9, which corresponds to HPLC peak B, is of the L configuration and is the precursor to the more potent inhibitor.

N-[(L)-2-[[[1-[(*Phenylmethoxycarbonyl*)*amino*]*ethyl*]-*methoxyphosphinyl*]*oxy*]-4-*methylpentanoyl*]-L-*alanine Methyl Ester* (*ZA*^{P}(*O*)*LA Dimethyl Ester*). To a solution of 492 mg (1.80 mmol) of methyl hydrogen [1-[(phenylmethoxycarbonyl)amino]ethyl]phosphonate (Bartlett & Lamden, 1986) in 5 mL of ethanol-free $CHCl_3$ under a nitrogen atmosphere was added 244 μL (3.3 mmol)of thionyl chloride. After stirring at 23 °C for 4 h, the solution was concentrated, redissolved in $CHCl_3$, and concentrated again to remove traces of HCl and excess $SOCl_2$. The crude chloridate was dissolved in 4 mL of CH_2Cl_2, the solution was cooled to −10 °C, and a solution of 242 mg (1.11 mmol) of (−)-*N*-(L-2-hydroxy-4-methylpentanoyl)-L-alanine methyl ester in 1 mL of CH_2Cl_2 and 269 μL (3.3 mmol) of dry pyridine were added. After 18 h at 23 °C, the reaction mixture was diluted with 10 mL of CH_2Cl_2 and washed twice with 20-mL portions each of 1 N HCl and 5% $NaHCO_3$, dried ($CaSO_4$), and concentrated in vacuo to give 500 mg (100%) of a clear oil. Purification of this material by chromatography (3:2 EtOAc/hexane) gave 377 mg (75%) of the diester as a 6:4:1:1 mixture of diastereomers. These isomers were separated by HPLC into three peaks on a 25-cm Altex silica gel column eluted with 70% EtOAc/hexane (9.9 mL/min): A (k' = 3.0, 37%); B (k' = 3.5 and 3.8, 21 and 12% of two diastereomers); and C (k' = 5.4, 30%). The NMR spectral details are recorded in the supplementary material. Anal. (mixture of diastereomers) Calcd for $C_{21}H_{33}N_2O_8P$: C, 53.36; H, 7.04; N, 5.93; P, 6.56. Found: C, 53.03; H, 7.11; N, 5.89; P, 6.40.

N-[(L)-2-[[[(L)-1-[(*Phenylmethoxycarbonyl*)*amino*]-*ethyl*]*hydroxyphosphinyl*]*oxy*]-4-*methylpentanoyl*]-L-*alanine* (*ZA*^{P}(*O*)*LA*). To a solution of 59.6 mg (0.126 mmol) of ZA^P(O)LA methyl ester, obtained from peak C above, in 0.25 mL of hexamethylphosphoramide (HMPA) was added 0.57 mL (3.15 mmol) of a 0.55 M solution of lithium propanethiolate in HMPA at 23 °C under nitrogen atmosphere (Bartlett & Johnson, 1970). After 1 h, 2 mL of water was added and the solution was extracted 4 times with 1-mL portions of $CHCl_3$ to remove the HMPA. The aqueous layer was lyophilized and purified on a 1-cm × 25-cm column of DEAE-Sephadex (A-25), HCO_3^- form, and eluted with a 300-mL linear gradient of 0–0.3 M TBK buffer (pH 8.6). Fractions (6 mL) were monitored by absorbance at 257 nm. Combination of fractions 26–39, lyophilization, ion exchange of the resulting white solid through Dowex 50W-8X resin (H^+ form) with 1:1 water/methanol, and final lyophilization gave 42 mg (75%) of the diacid ZA^P(O)LA as a white, hygroscopic solid: ^1H NMR δ 0.85 (d, 3, J = 6.7), 0.87 (d, 3, J = 6.9), 1.34 (dd, 3, J = 17.5, 7.3), 1.43 (d, 3, J = 7.1), 1.61 (m, 2), 1.73 (m, 1), 4.20 (dq, 1, J = 7.4), 4.54 (dd, 1, J = 7.3), 4.87 (br s, 1), 5.07 (s, 2), 5.94 (br s, 1), 7.30 (s, 5), 7.97 (br s, 2); ^{13}C NMR δ 15.0, 17.1, 21.3, 23.2, 24.0, 42.3, 44.2 (J_{CP} = 159), 48.0, 67.2, 74.8, 128.1, 128.2, 128.5, 136.1, 156.4, 172.5, 175.3; ^{31}P NMr δ 25.9. Anal. (determined on a mixture of diastereomers) Calcd for $C_{19}H_{29}N_2O_8P \cdot 0.25H_2O$: C, 50.83; H, 6.62; N, 6.24; P, 6.90. Found: C, 50.92; H, 6.59; N, 6.20; P, 7.12.

Preparation and characterization of ZL^P(O)LA, ZF^P(O)LA, and the D isomers are described in the supplementary material.

Table I: Concentration Ranges Explored for Determination of On-Rates

inhibitor	[I] range (μM)	[E] range (nM)	k_{on} (M^{-1} s^{-1})	K_i[a] (nM)
ZG^PLA[b]	0.01–0.15	10	$>10^5$	16.5
ZF^PLA	0.2–12	10–20	1000	0.068[c]
ZF^PLA[d]	20–1600	200–3000	1000	0.068[c]
ZG^P(O)LA[b]	15–75	4–24	$>10^5$	13000
ZF^P(O)LA	0.1–100	0.15–40	470	45
ZF^P(O)LA[d]	50–3660	40–4000	470	45
ZL^P(O)LA	1–30	1.3–40	480	680
ZA^P(O)LA	0.5–80	2.5–100	1250	1800
Z(D)F^PLA	0.5–20	1–50	1300	480
Z(D)F^P(O)LA	5–150	10	400	30000
Z(D)L^P(O)LA	50–1000	3.3–22.5	2.8	42000
Z(D)A^P(O)LA	20–2700	2.5–50	2.1	24000

[a] Determined by steady-state kinetics, unless otherwise indicated. [b] Thermodynamic data from Bartlett and Marlowe (1983, 1987); slow binding not observed; k_{on} given as lower limit only. [c] Calculated from $K_i = k_{off}/k_{on}$. [d] Determined under stopped-flow conditions.

Enzyme Assays

Materials. Thermolysin (3× crystallized) was obtained from Calbiochem and used without further purification. Stock solutions were prepared in 0.05 M Tris, 2.5 M NaBr, and 10 mM $CaCl_2$, pH 7.0, and stored at 4 °C; enzyme concentration was determined by using $E_{1\%}^{280}$ = 17.65 and a molecular weight of 34600. *N*-[3-(2-Furyl)acryloyl]glycyl-L-leucinamide (FAGLA), obtained from Chemalog, was used as substrate; a stock solution (4.0 mM) was prepared by dissolving the substrate in dimethylformamide (DMF) and diluting it with buffer to a final concentration of 0.1 M Tris, 0.1 M NaBr, and 2.5 mM $CaCl_2$, pH 7.0 (final concentration of dimethylformamide, 2.5%).

All steady-state enzyme assays were performed at 25 °C by the spectrophotometric method of Feder and Schuck (1970) in 0.1 M Tris, 2.5 M NaBr, 10 mM $CaCl_2$, and 2.5% DMF (pH 7.0). Typical concentrations of enzyme and substrate were 10 nM and 2.0 mM, respectively, with inhibitor concentrations ranging from 0.5 to 10 K_i. Initial velocities were determined for ≤10% reaction and were repeated twice for each inhibitor concentration, interspersing controls without inhibitor after every two runs with inhibitor. For determination of inhibition constants for the more potent derivatives, which had to be evaluated at concentrations on the order of that of the enzyme, the method of Henderson (Henderson, 1972; Segel, 1972) was employed to correct for inhibitor depletion. The values obtained were reproducible within ±8% and are listed in Table I.

Stopped-flow experiments were performed on a Union Giken Model RA-401 stopped-flow spectrophotometer interfaced with an On-Line Instruments (OLIS) 3820 stopped-flow operating system (Version 5.02). Data were collected at 345 nm by using a 14-nm slit. Temperature control was maintained at 25 ± 0.1 °C with an Endocal refrigerated cooling bath.

Determination of Slow Binding Kinetics. All assays, except for the stopped-flow experiments, were performed with a Cary 219 spectrophotometer equipped with the OLIS data collection

system. The sample chamber was maintained at 25 °C with a constant temperature bath. The buffer system consisted of 0.1 M Tris, 2.5 M NaBr, 10 mM $CaCl_2$, and 2.5% DMF (pH 7.0). A buffer salt concentration of 2.5 M NaBr was employed, since thermolysin is significantly more stable at this concentration than at 0.1 M NaBr. Inhibitor concentrations were varied from ca. K_i to the highest concentration at which the on-rate was still readily determined ($t_{1/2}$ ca. 1 min), typically up to 50–100 μM. A substrate concentration of 2.0 mM ($K_m \approx 30$ mM; Kam et al., 1979) was used to monitor the change in absorbance at 345 nm, at which wavelength absorbance changes up to 0.1 Å were shown to be linear. Enzyme concentrations were high enough to give an absorbance change of ca. 0.1 Å during the approach to steady-state conditions but sufficiently below the inhibitor concentration to maintain pseudo-first-order behavior. K_i values were determined from these reaction progress curves according to the equation $V_0/V_i = 1 + [I]/K_i$, where V_0 and V_i are the observed steady-state velocities in the absence and presence of inhibitor, respectively ($[S] < K_m$).

For the association of each inhibitor with thermolysin, the reaction progress curves conformed to eq 1, where P, t, v_0, and v_s represent product concentration, time, and the initial and final steady-state velocities, respectively. The steady-state

$$P = v_s t + (v_0 - v_s)(1 - e^{-k_{app}t})/k_{app} \quad (1)$$

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI \quad (2)$$

$$k_{app} = k_{off} + k_{on}[I] \quad (3)$$

rate was subtracted from the reaction progress curve and the residual exponential component was fitted with a single-exponential, nonlinear regression program (Cleland, 1963; Millar, 1981) to obtain k_{app}, the apparent first-order rate constant for approach to steady state. The on-rates (k_{on}) were then derived from a plot of k_{app} versus [I] (eq 2 and 3). For none of the inhibitors was a deviation from second-order behavior seen at concentrations below 0.1 mM. The concentration ranges investigated for each inhibitor and the k_{on} values are shown in Table I.

Stopped-Flow Experiments. A solution of 4.0 mM FAGLA and different amounts of the inhibitor was prepared in 2.5 M NaBr, 0.1 M Tris, 10 mM $CaCl_2$, and 2.5% DMF, pH 7.0, and placed in one chamber of the stopped-flow instrument. The other chamber was filled with enzyme solution (diluted from a 255-μM stock solution) in the same buffer. Equal amounts from each chamber were mixed to give a final substrate concentration of 2.0 mM. Pseudo-first-order rate constants for inhibitor association were determined by fitting the decaying rate of substrate hydrolysis to a single exponential. Three to five runs at each inhibitor concentration were used.

Determination of k_{off} for N-[[(L,D)-1-[(Phenylmethoxycarbonyl)amino]-2-phenylethyl]hydroxyphosphinyl]-L-leucyl-L-alanine Dilithium Salt (ZF^PLA). A 10.4-μL sample of a 9.6 μM solution of thermolysin was incubated overnight at 4 °C with 25 μL of a 0.02 mM solution of the inhibitor in 2.5 M NaBr, 0.1 M Tris, 10 mM $CaCl_2$, pH 7.0, and 2.5% DMF buffer. Elution through a 8-mm × 16-cm column of Sephadex G-25 (1-mL fractions) gave the purified EI complex in fractions 2 and 3. Dilution to 5 mL produced the stock solution from which 20-mL volumes of 1.0, 0.5, 0.1, and 0.05 nM EI were prepared. These solutions were kept at 25 °C and assayed periodically for return of enzyme activity by combining a 2-mL sample with 5 μL of a 60 mM stock solution in DMF of the fluorogenic substrate (2-aminobenzoyl)-Ala-Gly-Leu-Ala-(4-nitrobenzyl)amide and monitoring activity according to the method of Nishino and Powers (1980) on an SLM Instruments Model 4048S spectrofluorometer. As a control of enzyme activity, the above solutions were prepared without inhibitor and shown not to lose any activity over the 2-week period of the experiment.

The return of activity was followed over a 12-day period, and the data were fit to a single exponential by using a nonlinear regression program (Cleland, 1963; Millar, 1981); the average value for the rate constant over the four different concentrations was found to be $(5.95 \pm 0.18) \times 10^{-3}$ day^{-1}.

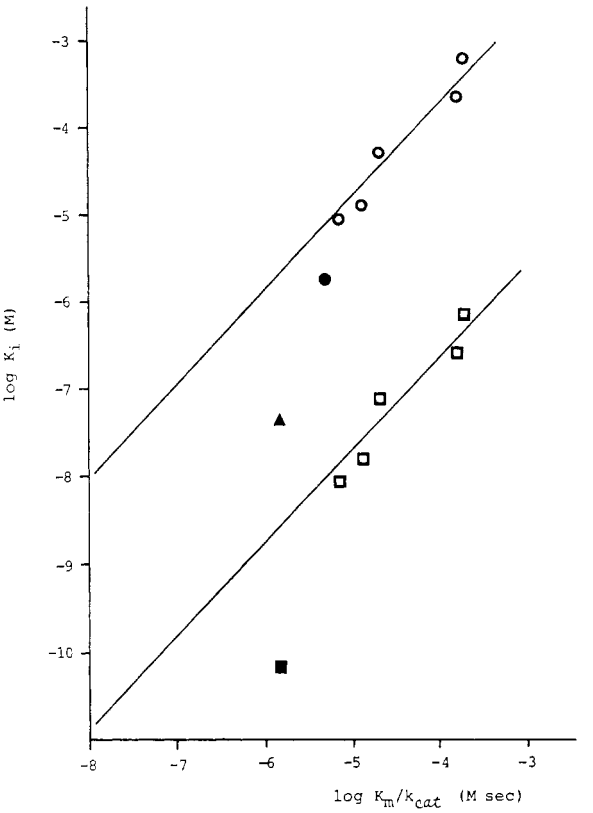


FIGURE 1: Comparison of inhibitor K_i values with K_m/k_{cat} values for the analogous peptide substrates: (open circles) $ZG^P(O)LX$; (open squares) ZG^PLX; (filled circle) $ZA^P(O)LA$; (filled triangle) $ZF^P(O)LA$; (filled square) ZF^PLA. Substrate data taken from Morihara and Tsuzuki (1970); data on ZG^PLX analogues from Bartlett and Marlowe (1983); data on $ZG^P(O)LX$ analogues from Bartlett and Marlowe (1987).

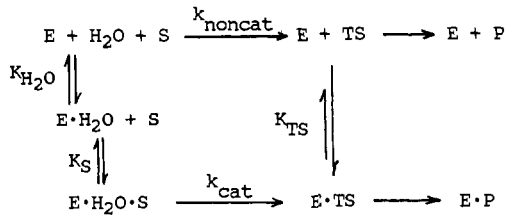
Results and Discussion

Correlation of Inhibitor K_i with Substrate K_m/k_{cat} Values. The peptide analogues that incorporate an α-substituted phosphonic acid at the P_1 site are excellent inhibitors of thermolysin. Indeed, as the results in Figure 1 indicate, they are *more* potent than expected according to the correlation previously suggested as diagnostic of the binding of transition-state analogues (Westerik & Wolfenden, 1972; Thompson, 1973; Bartlett & Marlowe, 1983). Although appropriate data for the peptide substrate counterparts are available only for the Phe and Ala derivatives (Morihara & Tsuzuki, 1970), the deviation from the theoretical line is greater for the bulkier of the two substituents. Rather than implying that the α-substituted inhibitors are not transition-state analogues, this deviation may reveal some of the limitations of the assumptions made in deriving the correlation of K_i for the inhibitors and K_m/k_{cat} for the substrates (Scheme I, eq 4 and 5). One

$$K_{TS} = (k_{noncat}/k_{cat})K_S K_{H_2O} \quad (4)$$

$$K_i(\text{TS analogue}) \propto K_{TS} \propto K_m/k_{cat} \quad (5)$$

Scheme I

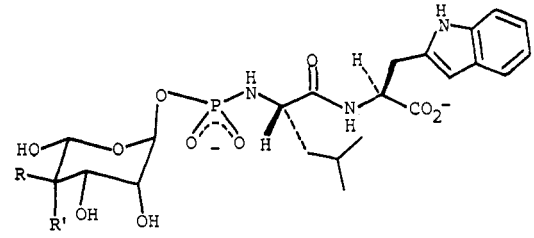


assumption made in deriving eq 5 from eq 4 is that the readily measured Michaelis constant, K_m, corresponds to the actual substrate dissociation constant, K_S. By determining the on- and off-rates by stopped-flow techniques, Morgan and Fruton (1978) were able to show that K_m indeed approximates K_S for the fluorescent thermolysin substrate Mns-FLA. It would be surprising if this correspondence did not extend to ZFLA; thus, an alternative interpretation should be sought for the observed deviation from the correlation. An implicit assumption in the correlation is that the K_m/k_{cat} values reflect the same rate-determining step throughout the series. Consideration of the information in Figure 1 from a different perspective suggests that this may not be the case, at least for ZFLA. The observed deviation may be viewed either as unexpectedly tight binding of the α-substituted inhibitors *or* as unexpectedly slow hydrolysis of ZFLA as a substrate. The binding affinities of the Phe^P-containing inhibitors suggest that the enzyme stabilizes the tetrahedral transition state or intermediate enough to catalyze hydrolysis of ZFLA at a rate greater than 10^7 M^{-1} s^{-1}. However, the measured on-rate for the related mansyl substrate, Mns-FLA, is an order of magnitude slower than this value (Morgan & Fruton, 1978), suggesting that turnover of ZFLA may be limited by the rate at which it binds to the enzyme. Under such circumstances, the phosphorus-containing inhibitors could no longer be considered analogues of the transition state for the overall enzymatic process, although they may remain good mimics of the transition state for the specific chemical steps involved.

Another explicit assumption is made in going from eq 4 to eq 5, namely, that k_{noncat} is constant across the series of comparison substrates (Bartlett & Marlowe, 1983).[2] While it is not unreasonable to assume that the ease with which the scissile carbonyl is converted to a tetrahedral adduct in the hypothetical noncatalyzed reaction is insensitive to structural variation in the P_2' position, such is not likely to be the case for substitution adjacent to the carbonyl (Jarboe et al., 1971; Levene et al., 1932). If attack is slowed by steric bulk at this position, k_{noncat} and therefore K_{TS} will be overestimated.

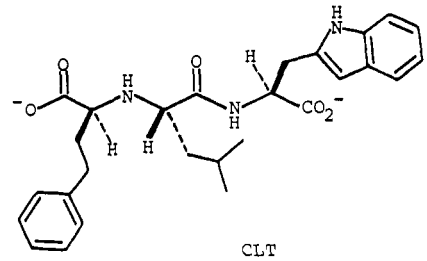
Structural Dependence of Slow Binding Behavior. The α-substituted phosphonic acid inhibitors are slow binding, in contrast to those incorporating Gly^P at the P_1 position (Table I). A lower limit of $k_{on} \geq 10^5$ M^{-1} s^{-1} is given for ZG^{P}LL, since an association rate as slow as this would have been noted in our earlier experiments (Bartlett & Marlowe, 1983). All of the P_1-substituted analogues, however, bind with on-rates of 1300 M^{-1} s^{-1} or less. The slow binding behavior does not depend upon the size of the substituent or its stereochemical configuration, whether it is in the amide or ester series, or whether the inhibitor shows strong or weak thermodynamic affinity.

[2] In our original discussion, we made a distinction between the water molecule coordinated to the zinc ion and that which is the cosubstrate of the reaction (Bartlett & Marlowe, 1983, footnote 2). This distinction can be discarded, since it appears that these water molecules are the same (Hangauer et al., 1984, and Scheme I).



R = OH, R' = H: Phosphoramidon

R = H, R' = OH: Talopeptin



CLT

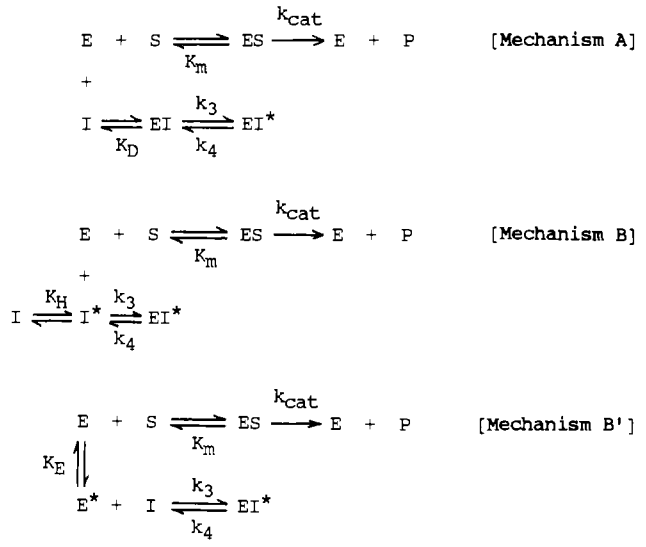
FIGURE 2

Kam et al. (1979) and Kitagishi and Hiromi (1983, 1984) have reported slow binding behavior of phosphoramidon and talopeptin (Figure 2) and of the aglycone *N*-phosphoryl-Leu-Trp. For phosphoramidon, the rate constant for formation of the inhibited complex (k_{on}) is on the order of 2×10^4 M^{-1} s^{-1} at pH 7.0. In contrast, other potent inhibitors of thermolysin, such as the *N*-carboxyalkyl dipeptide CLT, which share common structural elements at the P_1' and P_2' positions as well as a zinc-coordinating ligand, bind normally (Maycock et al., 1981). The molecular events that result in slow binding are potentially different between congeners of phosphoramidon and the α-substituted phosphonic acid derivatives, since the trigger for this behavior in the latter compounds is found at a position that is not even part of the structure of the former.

The origins of slow binding behavior are poorly understood. The rate of diffusion-limited association of a small molecule with an enzyme active site is typically on the order of 10^7–10^9 M^{-1} s^{-1} (Brouwer & Kirsch, 1982; Hardy & Kirsch, 1984). For the thermolysin substrate Mns-FLA, Morgan and Fruton (1978) have measured an on-rate of 3×10^6 M^{-1} s^{-1}. Rates slower than this, and typically slower than 10^6 M^{-1} s^{-1}, must therefore reflect an unusual barrier to binding of the small molecule to the enzyme. Slow-binding inhibition is manifested by a lag in the establishment of steady-state turnover on combination of enzyme, inhibitor, and substrate. It appears as an initial exponential component in a plot of the concentration of product against time; the form of this curve is given by the integrated eq 1 (Morrison & Walsh, 1987). Slow binding behavior has been observed in many contexts, but it is commonly noted in conjunction with high-affinity inhibitors since for such compounds it is readily detected (Cha, 1975; Williams & Morrison, 1979). However, there does not appear to be a specific correlation between slow binding and high-affinity inhibitors, as demonstrated by the results in Table I and those reported by others (Kurz & Frieden, 1983).

A priori, there are two limiting mechanisms that could lead to slow binding behavior. One involves a staged binding process in which an initial, "loose" complex is formed at the rate of diffusion, followed by a slower isomerization to the ultimate "tight" complex (mechanism A) (Scheme II). Alternatively, slow binding may arise from an association process that requires either a rare form of the inhibitor or a rare form of the enzyme (mechanisms B and B′) (Scheme II). Although

Scheme II



the actual binding steps in mechanisms B and B′ may occur at the rate of diffusion, the observed on-rates would be reduced by the fraction of enzyme or inhibitor in the rare form (see eq 8 and 10). In each instance, some sort of isomerization is envisioned, usually a conformational change, and the distincton between the two modes depends upon whether this isomerization takes place after or before, respectively, the inhibitor and enzyme encounter each other.

The rate constant k_{app} (eq 1) depends on the concentration of inhibitor in a way that is characteristic of the kinetic mechanism of the particular slow binding process, as indicated in eq 6, 8, and 10 (Frieden, 1970; Cha, 1975; Duggleby et al., 1982; Morrison & Walsh, 1987).

$$k_{app} = k_4 + k_3\left(\frac{[I]/K_D}{1 + [S]/K_m + [I]/K_D}\right) \quad (6)$$

$$k_{on} = \frac{k_3/K_D}{1 + [S]/K_m + [I]/K_D} \quad (7)$$

$$k_{app} = k_4 + k_3\left(\frac{K_H[I]}{1 + [S]/K_m}\right) \qquad K_H = \frac{[I^*]}{[I]} \ll 1 \quad (8)$$

$$k_{on} = k_3\left(\frac{K_H}{1 + [S]/K_m}\right) \quad (9)$$

$$k_{app} = k_4 + k_3\left(\frac{K_E[I]}{1 + K_E + [S]/K_m}\right) \qquad K_E = \frac{[E^*]}{[E]} \quad (10)$$

$$k_{on} = k_3\left(\frac{K_E}{1 + K_E + [S]/K_m}\right) \quad (11)$$

Mechanism A, invoking postassociation isomerization of a loose to a tight complex, has been the most frequently observed kinetic mechanism for slow binding behavior (Morrison & Walsh, 1987). The slow isomerization step is usually attributed to a conformational change on the part of the protein (Morrison & Walsh, 1987; Kurz & Frieden, 1983). Even if such a conformational change is not involved, a stepwise mechanism must be involved in any slow binding process in which the initial species that combine are the major forms of both inhibitor and enzyme. Indeed, a stepwise mechanism, perhaps involving a number of intermediates, is likely to be involved in the association of fast- as well as slow-binding inhibitors or substrates (eq 12). Many, if not all, of these

$$E + I \rightleftharpoons (EI)_1 \rightleftharpoons (EI)_2 \rightleftharpoons (EI)_{n-1} \rightleftharpoons EI^* \quad (12)$$

intermediates are kinetically invisible, and the distinction between slow and fast binders arises only when the conversion of one to the next is observably slow.

The simplest staged binding process involves a single intermediate (mechanism A). Whether this intermediate can be observed depends on the interplay of its dissociation constant and rate of isomerization to the tightened complex. The rate of onset of inhibition, k_{app}, shows a hyperbolic dependence on inhibitor concentration, much like the Michaelis–Menton relationship. Only if this rate can be determined at inhibitor concentrations approaching K_D, the dissociation constant of the loose complex, can saturation of the preequilibrium be observed and kinetic evidence for its existence be obtained; otherwise, the behavior observed is simple second order (first order in [I]). For example, a two-step process has been observed for talopeptin (K_D ca. 0.3 mM) but not phosphoramidon, although these two inhibitors show similar second-order on-rates (k_{on} ca. 2×10^4 M^{-1} s^{-1} at pH 7.0) (Kitagishi & Hiromi, 1983, 1984).

The most potent of our inhibitors, ZF^{P}LA, has a thermodynamic inhibition constant K_i = 68 pM and a second-order association rate constant k_{on} = 1000 M^{-1} s^{-1} at 25 °C. We determined this rate constant at a range of inhibitor concentrations in an attempt to discern a change in reaction order consistent with a more loosely bound intermediate. However, second-order behavior was observed for combinations of inhibitor and enzyme ranging from [E] = 10 nM and [I] = 200 nM ($t_{1/2}$ = 1 h) to [E] = 3 μM and [I] = 1.6 mM ($t_{1/2}$ = 0.43 s). Similar behavior was observed for phosphonate ester ZF^P(O)LA at concentrations up to 3.7 mM. Reciprocal plots of $1/k_{app}$ against $1/[I]$ indicate that any intermediate complexes along the binding path must have dissociation constants $K_D > 4$ mM. This lower limit is an order of magnitude higher than K_m for the substrate ZFLA, which is 0.58 mM (Morihara & Tsuzuki, 1970), and 8 orders of magnitude higher than K_i in the case of the phosphonamidate ZF^{P}LA.

In the absence of evidence for a multistep binding process for the phosphonate inhibtors, we considered the other explanations of slow binding, namely, isomerization of either the inhibitor or enzyme before association (mechanisms B and B′). These kinetic mechanisms would lead to second-order behavior provided that the preassociation interconversions of I ⇌ I* or E ⇌ E*, governed by K_H and K_I, respectively, are rapid in comparison with the association step. It has already been shown that kinetic mechanisms in which slow binding reflects slow interconversions of the inhibitor or enzyme would not lead to the second-order behavior (first order in [I]) observed for ZF^{P}LA and ZF^P(O)LA (Duggleby et al., 1982).

Evidence against the involvement of a significant overall conformational change of the enzyme is provided by the crystallographic results described in detail in the accompanying paper by Holden et al. (1987), which demonstrate that the structure of the protein within the thermolysin–ZF^{P}LA complex is virtually superimposable with that of either the native, unliganded enzyme or the complex with the fast binder ZG^{P}LL. In contrast, the bound conformation of ZF^{P}LA is significantly different from that of ZG^{P}LL, with the Cbz moiety of the slow-binding analogue penetrating more deeply into the S_2 site. The conformation that ZP^{P}LA adopts in the active site is not an unfavorable one for the peptide analogue, however, and it is an unlikely candidate for the rare conformer of mechanism B.

Thus, the most straightforward explanations of slow binding behavior appear to be excluded for the α-substituted phos-

phonate inhibitors; neither an overall protein conformational change nor an unfavorable conformational equilibrium on the part of the inhibitor appears to be responsible. The necessity of a transient conformational change to admit the inhibitor into the active site could be invoked; such a process would correspond to mechanism B′, in which E and E* represent native enzyme with a "closed" active site and the transient conformer with an "open" site, respectively. Although such a mechanism has not been ruled out, it does not appear from close inspection of the structure of $ZF^{P}LA$–thermolysin complex that the binding regions which accept the carbamate moiety or the α-substituent are particularly constricted. It is in the vicinity of the zinc atom and in the S_1' pocket that the inhibitor is embraced most tightly by the protein, yet these cavities are occupied almost identically by both fast- and slow-binding inhibitors.

An intriguing implication of the crystallographic results is that displacement of a specific water molecule from the active site might constitute the barrier that the α-substituted inhibitors must surmount on binding. A similar proposal has been made by Rich (1985) in rationalizing the behavior of pepstatin derivatives that bind slowly to pepsin. The process whereby a flexible inhibitor or substrate enters a binding site and displaces loosely and tightly bound solvent molecules is poorly understood. What certainly does *not* occur in such a sequence is initial dissociation of all of the waters from the active site, followed by association of the small molecule (eq 13). Such

$$E(H_2O)_n + I \rightleftharpoons E(\ ) + nH_2O + I \rightleftharpoons E(I) + nH_2O \quad (13)$$

$$E(H_2O)_n + I \rightleftharpoons E(H_2O)_nI \rightleftharpoons E(H_2O_{n-1}I) + H_2O \rightleftharpoons E(H_2O_{n-2}I) + 2H_2O \rightleftharpoons \rightleftharpoons E(I) + nH_2O \quad (14)$$

a pathway would involve a very large enthalpic as well as entropic barrier, while a pathway with stepwise dissociation of water molecules and the involvement of multiple intermediate binding states would not (eq 14). According to this model, from one intermediate complex to the next, dissociation of a water molecule occurs and part of the inhibitor takes its place in the active site. A flexible molecule would settle into the active site as the water molecules slip out, and the high barrier would be reduced to a succession of smaller ones. Under most circumstances, the equilibria for these steps must be established very rapidly. Each succeeding step in the sequence, the replacement of a vacating water molecule by the inhibitor, is an intramolecular process, and the rate at which the enzyme–inhibitor complex proceeds through these steps could be as fast as the rate at which the water molecules dissociate. Most waters associated with a protein surface undergo exchange with bulk solvent on a nanosecond time scale (Edsall & McKenzie, 1983; Koenig et al., 1975), although the existence of specific water molecules bound more tightly or whose exit could be blocked by the inhibitor cannot be ruled out (Kossiakoff, 1982; Rich, 1985).

Two pathways for association that lie between the impossibility of eq 13 and the likelihood of eq 14 could result in slow binding behavior. In the first, hindered escape of one of the water molecules, perhaps impeded by the presence of the inhibitor, could slow the binding sequence. Such a process would be kinetically indistinguishable from mechanism A. Whether or not the complex preceding dissociation of the critical water molecule is observable kinetically depends upon K_D. Although only a single-step process is found for $ZF^{P}LA$ and $ZF^{P}(O)LA$, K_D may simply be too high (>4 mM). One can imagine a situation in which the water molecule dissociates from an intermediate complex more slowly than the inhibitor itself does, either because this complex is very weakly bound or because exit of the water molecule is very hindered. Under such circumstances, a pathway involving dissociation of the water molecule before inhibitor association may be more rapid (eq 15).

$$E(H_2O)_n + I \underset{K_{H_2O}}{\rightleftharpoons} E(H_2O)_{n-1} + H_2O + I \overset{k_3}{\underset{k_4}{\rightleftharpoons}} E(H_2O_{n-1}I) + H_2O \overset{\text{(fast)}}{\rightleftharpoons} E(I) + nH_2O \quad (15)$$

The requirement that the inhibitor must encounter the enzyme when it is in a rare form, namely, the one missing the water molecule, could lead to slow binding behavior. This would be formally equivalent to mechanism B′, where E would be $E(H_2O)_n$ and E* would be $E(H_2O)_{n-1}$, the forms of the enzyme in which the specific water molecule is present or absent, respectively, and $K_E = [E(H_2O)_{n-1}]/[E(H_2O)_n] = K_{H_2O}[H_2O]$. This slow binding process does not involve any events that are particularly slow at the molecular level. Rather, it is the restriction of the binding pathway to a sequence that is merely improbable which results in the macroscopically observed phenomenon of slow binding. Reduction of the second-order rate constant for binding by a factor of 10^4 from the diffusion limit (e.g., from 10^7 to 10^3 M^{-1} s^{-1}) would require only that for this molecule of water K_{H_2O} = 5 mM. These numbers are compatible with our observations and indicate that the water molecule need not be bound excessively tightly. Moreover, if the binding to $E(H_2O)_{n-1}$ is diffusion limited, this mechanism predicts that inhibitors which bind by this pathway will show similar on-rates, as is indeed observed for all of the L-configured, α-substituted compounds.

Relationship between Slow Binding, Tight Binding, and Transition-State Analogy. Slow binding behavior is associated experimentally with tight-binding inhibitors, because for these compounds it is simply more difficult to overlook. However, there is no a priori reason why a relationship should exist between the kinetic property of slow binding and the thermodynamic property of tight binding. The ultimate affinity between enzyme and inhibitor is solely a function of the difference between the solvation energies of free inhibitor and native enzyme and the interaction energies between the two molecules in the final complex. The pathway whereby this final complex is attained and, therefore, the magnitude of its rate of formation have no relevance to the binding constant. Although this may appear difficult to reconcile intuitively with the obvious relationship between K_i, k_{off}, and k_{on}, the principle of microscopic reversibility must not be overlooked: if there is an unusually high barrier to association of a reversible inhibitor, the same barrier must be crossed in the dissociative process.

Slow-binding and tight-binding inhibitors have often been considered together (Morrison & Walsh, 1987, and references cited therein) because under normal assay conditions both lead to lag times in the approach to equilibrium. These lag times have different origins, however. In the case of slow-binding inhibitors, the lag time arises from characteristics of the association process itself, i.e., the fact that it takes place at a rate demonstrably slower than that allowed by diffusion. In contrast, the lag time observed in a typical assay of a tight-binding inhibitor is an experimental artifact arising from the low concentration of inhibitor that must be used to avoid saturation of the enzyme (Morrison & Walsh, 1987). For example, with an inhibitor of $K_i = 10^{-11}$ M, for 10% of the enzyme activity to be preserved at steady state ($[I] = 10K_i$, $[I] \gg [E]_t$), the half-time for approach to equilibrium will be more than 10 min, even if $k_{on} = 10^7$ M^{-1} s^{-1}. This lag time is ultimately traced to the low rate of *dissociation* of the inhibitor from the enzyme, not the rate at which binding takes

place; hence, it would be incongruous to consider such inhibitors "slow binding". We suggest that the term slow-binding be reserved for noncovalently bound inhibitors for which $k_{on} \leq 10^6$ M^{-1} s^{-1}, irrespective of the K_i values.

If ZF^PLA is an accurate model for the transition state, the same water molecule must be displaced in the course of turnover of ZFLA. It is thus disconcerting to find that the inhibitor binds to thermolysin more slowly than the corresponding substrate turns over (k_{on} for $ZF^PLA = 10^3$ M^{-1} s^{-1}; k_{cat}/K_m for ZFLA = 6.7×10^5 M^{-1} s^{-1}). However, it should be emphasized that the transition-state complex and the inhibitor complex are reached by different pathways and that it is the specific pathway to which we attribute the slow binding behavior. Thus, there is no reason to expect a relationship between the rates of inhibitor binding and substrate hydrolysis.

Conclusion. Studies of slow-binding inhibition, particularly for transition-state analogues, have been stimulated by the possibility that this behavior may reflect a protein conformational change which is relevant to the catalytic event. For the phosphonamidate and phosphonate inhibitors of thermolysin, however, the slow-binding phenomenon per se does not appear to involve such a conformational change nor to offer any insight into the catalytic sequence. However, the possibility that a specific water molecule may slow the binding of an inhibitor to an enzyme active site may provide insight on the molecular level into the pathways whereby small molecules associate with macromolecules.

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SUPPLEMENTARY MATERIAL AVAILABLE

Details for the synthesis and characterization of inhibitors and intermediates not described above (11 pages). Ordering information is given on any current masthead page.

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Purification and Characterization of Hen Oviduct Microsomal Signal Peptidase†

R. Keith Baker and Mark O. Lively*
Department of Biochemistry, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27103

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ABSTRACT: Hen oviduct signal peptidase requires only two proteins for proteolysis of fully synthesized secretory precursor proteins in vitro: one with a molecular mass of 19 kilodaltons (kDa) and one which is a glycoprotein whose mass varies from 22 to 24 kDa depending on the extent of glycosylation. Purified signal peptidase has been analyzed both as part of an active catalytic unit and after electroelution of the individual proteins out of a preparative polyacrylamide gel. The multiple forms of the glycoprotein component of signal peptidase bind to concanavalin A and are shown to be derived from the same polypeptide backbone. Removal of their oligosaccharides by digestion with *N*-glycanase converts these proteins to a single 19.5-kDa polypeptide. The glycoproteins all exhibit very similar profiles following individual digestion with trypsin and separation of the resulting peptides by reverse-phase high-performance liquid chromatography. In addition, sequence analysis of selected peptides from corresponding regions in chromatograms representing each form of the glycoprotein reveals the same amino acid sequences. The 19-kDa signal peptidase protein does not bind concanavalin A, has a distinct tryptic peptide map from that of the glycoprotein, and appears to share no amino acid sequences in common with the glycoprotein. Its copurification on a concanavalin A–Sepharose column indicates that it must interact directly with the glycoprotein subunit.

The current model for translocation of secretory proteins across the lipid bilayer of the endoplasmic reticulum is founded on a sequence of recognition events which are initiated by an amino-terminal extension peptide of the nascent protein (Blobel & Dobberstein, 1975a; Walter et al., 1984; Hortsch & Meyer, 1986; Walter & Lingappa, 1986). The role of this signal peptide is transient, and its removal is catalyzed by a highly specific endoproteinase located on the lumenal side of the endoplasmic reticulum (Blobel & Dobberstein, 1975b). Hen oviduct signal peptidase (HOSP)[1] belongs to this unique class of enzymes which are integral membrane proteins (Jackson & Blobel, 1977; Lively & Walsh, 1983; Mollay et al., 1982; Fujimoto et al., 1984) requiring a phospholipid environment for activity in vitro (Jackson & White, 1981; Baker et al., 1986).

Two distinct signal peptidases have been identified in *Escherichia coli* (Wolfe et al., 1983; Innis et al., 1984). Each of these enzymes is a single-chain molecule which requires detergent for solubilization, yet, unlike the eukaryotic enzyme, neither appears to require phospholipid for activity. In contrast to the apparently simple prokaryotic enzymes, purified microsomal signal peptidase from canine pancreas is associated with a glycosylated complex of from four to six polypeptides (Evans et al., 1986a). It has not yet been determined which of the proteins of this complex are absolutely required for signal peptide cleavage.

It is not yet known to which proteolytic enzyme family, if any, the signal peptidases may belong, and information regarding their mechanisms of action is very limited. No clear pattern of inhibition of these enzymes has emerged, and additional studies of the purified proteases are required to delineate the nature of their reaction mechanisms. In an effort to further our understanding of the enzymology of signal peptidase and its role in the translocation process, we have purified the enzyme from tubular gland cells from the magnum region of hen oviduct using affinity chromatography on concanavalin A–Sepharose. The purified enzyme is composed of polypeptides of 24, 23, 22, and 19 kDa. The 24-, 23-, and 22-kDa proteins are differentially glycosylated forms of a single 19.5-kDa polypeptide. The 19-kDa protein is not a glycoprotein and is distinct from the glycosylated polypeptides. We demonstrate that HOSP requires no more than two proteins for cleavage of full-length secretory precursor proteins in vitro.

Experimental Procedures

Materials. DEAE-cellulose and CM-cellulose were obtained from Whatman (Clifton, NJ). Phosphatidylcholine (egg, L-α-lecithin) was purchased from Avanti Polar-Lipids (Birmingham, AL). Hydroxylapatite (Bio-Gel HTP) and Affi-Gel 15 are products of Bio-Rad (Richmond, CA). Con A–Se-

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[1] Abbreviations: HOSP, hen oviduct signal peptidase; DEAE, diethylaminoethyl; CM, carboxymethyl; HA, hydroxylapatite; NP-40, Nonidet P-40; Con A, concanavalin A; HRP, horseradish peroxidase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, phosphatidylcholine; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; endo H, endo-β-*N*-acetylglucosaminidase H; TPCK–trypsin, L-1-(*p*-tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin; HPLC, high-performance liquid chromatography; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane; PTH, phenylthiohydantoin; ER, endoplasmic reticulum.

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