reported for several other systems including troponin I (Olwin et al., 1982), phosphorylase kinase (Cohen et al., 1978), and *Bordetella pertussis* adenylase cyclase (Greenlee et al., 1982; Kilhoffer et al., 1983). However, all of these proteins exhibit higher affinity for CaM·Ca^{2+}_n than CaM. P-57 is the first CaM binding protein discovered that actually has higher affinity for CaM compared to CaM·Ca^{2+}_n.

We have avoided the temptation of naming P-57 more specifically until its function and relationship to other CaM binding proteins are more thoroughly examined. Thus far, we have been unable to detect kinase, phosphodiesterase, phosphatase, adenosinetriphosphatase, or adenylate cyclase activity associated with the pure protein. It is possible that P-57 is a proteolytic fragment derived from some other CaM binding protein; however, its unusual CaM binding properties make that possibility seem unlikely. The most interesting property of P-57 is its relative affinities for CaM in the presence and absence of Ca^{2+}, which suggest one possible function of P-57. This protein subunit may function to bind CaM at some local site (e.g., a multisubunit enzyme complex or the inner surface of the membrane), thereby concentrating and localizing CaM in the cell. Increases in free Ca^{2+} in response to a stimulus would result in the release of CaM from P-57. Such stimulus-induced release of CaM from membrane sites has been detected in rat brain (Gnegy et al., 1977). For example, a protein such as P-57 could be used to localize CaM near Ca^{2+} channels. The relatively large amounts of P-57 present in cerebral cortex do suggest that a significant amount of the total CaM present will be associated with P-57 when Ca^{2+} levels are low (<0.1 μM). P-57 was not detected in a variety of other tissues including bovine heart, rat heart, liver, lung, and skeletal muscle (data not shown). Definition of the function of P-57 in brain will clearly require further experimentation.

Registry No. Calcium, 7440-70-2.

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Phosphonamidates as Transition-State Analogue Inhibitors of Thermolysin†

Paul A. Bartlett* and Charles K. Marlowe

ABSTRACT: Six phosphorus-containing peptide analogues of the form Cbz-$NHCH_2PO_2^-$-L-Leu-Y (Y = D-Ala, NH_2, Gly, L-Phe, L-Ala, L-Leu) have been prepared and evaluated as inhibitors of thermolysin. The K_i values for these compounds range from 1.7 μM to 9.1 nM and correlate well with the K_m/k_{cat} values for the corresponding peptide substrates [Morihara, K., & Tsuzuki, H. (1970) *Eur. J. Biochem. 15*, 374–380] but not with the K_m values alone. The correlation noted between inhibitor K_i and substrate K_m/k_{cat} is the most extensive one of this type, providing strong evidence that the phosphonamidates are transition-state analogues and not simply multisubstrate ground-state analogues. Cbz-$NH_2CH_2PO_2^-$-L-Leu-L-Leu (K_i = 9.1 nM) is the most potent inhibitor yet reported for thermolysin.

The concept of transition-state analogues (TS analogues)[1] is a successful one for the design of potent enzyme inhibitors (Wolfenden, 1976; Lienhard, 1973; Stark & Bartlett, 1983). The rationale for this approach is the recognition that addi-

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[1] Abbreviations: TS, transition state; M-S, multisubstrate; Cbz, benzyloxycarbonyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; FAGLA, 3-(2-furylacryloyl)glycyl-L-leucinamide.

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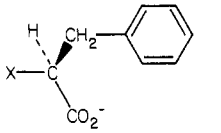
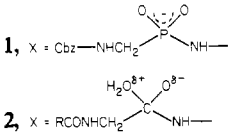
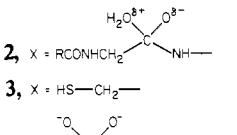
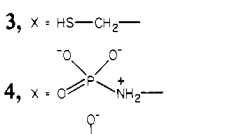
tional, favorable binding interactions develop between an enzyme and the substrate as their complex approaches its transition-state conformation. Such extra binding interactions, which are not available to the ground-state complex, assist in lowering the energy of activation of the reaction and therefore are a mechanism for enzymatic rate enhancement (Jencks, 1975, 1980, 1981). Transition-state analogue inhibitors seek to take advantage of these extra binding interactions by incorporating key structural elements of the unstable, transition-state form of the substrate in the stable structure of the inhibitor.

Recently we reported that the phosphonamidate dipeptide analogue **1**, designed to be a TS analogue for carboxypeptidase A, is a potent inhibitor of the enzyme (K_i = 89 nM) (Jacobsen & Bartlett, 1981). Appropriate phosphorus peptide analogues have been shown to be effective inhibitors for a variety of other zinc peptidases as well (Holmquist, 1977; Weaver et al., 1977; Kam et al., 1979; Nishino & Powers, 1979; Thorsett et al., 1982; Galardy, 1982; Fukuhara et al., 1982; Petrillo & Ondetti, 1982; Galardy et al., 1983). In spite of the resemblance between the tetrahedral phosphorus atom of inhibitor **1** and the tetrahedral intermediate **2**, involved in one of the postulated mechanisms of the zinc peptidases (Breslow & Wernick, 1977; Rees & Lipscomb, 1981; Weaver et al., 1977), there is no rigorous evidence that the high-binding affinity of **1** in fact results from its mimicry of this tetrahedral intermediate. The phosphorus moiety may simply be a good, chelating ligand for the zinc ion, for example. The most potent inhibitor yet reported for carboxypeptidase A, thiol **3** (K_i = 11 nM) (Ondetti et al., 1979), bears no resemblance to **2**. Moreover, the simpler phosphoramidate **4** (5000 nM) (Kam et al., 1979), even though tetrahedral, is a weaker inhibitor than the trigonal carboxylate **5** (450 nM) (Byers & Wolfenden, 1973).



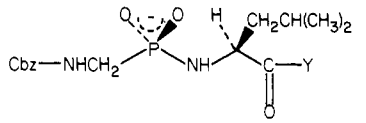






In spite of the importance of the TS analogue strategy for generation of potent enzyme inhibitors, there are few demonstrations that such inhibitors in fact take advantage of the special binding interactions available only in the transition-state complex, i.e., that they actually *are* TS analogues. We now provide evidence that a series of phosphonamidate peptide derivatives, **6–11**, are indeed TS analogues for the zinc peptidase thermolysin. We chose thermolysin because extensive information is available both on substrate selectivity (Morihara & Tsuzuki, 1970; Morgan & Fruton, 1978) and on the three-dimensional structure of a variety of enzyme–inhibitor complexes (Weaver et al., 1977; Kester & Matthews, 1977; Bolognesi & Matthews, 1979; Holmes & Matthews, 1981; Monzingo & Matthews, 1982; Holmes et al., 1983).

The project was also undertaken with the intention of demonstrating that a general, rigorous criterion exists for



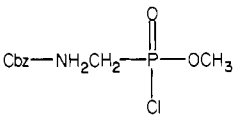
6, Y = D-Ala
7, Y = NH_2
8, Y = Gly
9, Y = L-Phe
10, Y = L-Ala
11, Y = L-Leu

distinguishing TS analogues from other types of inhibitors. It has already been noted that the inhibition constant K_i for a TS analogue should be related to the K_m/k_{cat} value of the corresponding substrate; however, previous correlations of this kind have been limited (Thompson, 1973; Westerik & Wolfenden, 1972; Wolfenden et al., 1977; Thompson & Bauer, 1979). We now report an extensive correlation of this type, supporting both the TS analogue designation for phosphonamidate inhibitors of the zinc peptidases and an enzyme mechanism involving the tetrahedral intermediate **2**.

Materials and Methods

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 $E^{280}_{1\%}$ = 17.65 (Ohta et al., 1966) and a molecular weight of 34 600 (Titani et al., 1972). 3-(2-Furylacryloyl)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 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%).

Synthesis of Inhibitors. The phosphonamidate peptide analogues **6** and **8–11** were prepared by coupling the acid chloride **12** (Jacobsen & Bartlett, 1981) with the appropriate



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N-leucyl amino acid methyl ester, followed by alkaline hydrolysis. The dipeptide analogue **7** was obtained from a coupling reaction with leucine methyl ester, followed by ammonolysis of the carboxyl ester prior to phosphorus ester hydrolysis. In each case purification was accomplished by chromatography on DEAE-Sephadex A-25 (HCO_3^- form) using a linear gradient of 0–0.5 M triethylammonium bicarbonate (pH 8.6); an excess of LiOH was added prior to lyophilization to prevent acidification and hydrolysis of the phosphonamide linkage (Jacobsen & Bartlett, 1981). The inhibitors were obtained as white powders after lyophilization. All intermediates and products were completely characterized by NMR spectroscopy (^1H, ^{13}C, and ^{31}P NMR); satisfactory combustion analyses were obtained for the ester precursors prior to hydrolysis. The dilithium salts of the inhibitors themselves were shown to be free of organic contaminants by NMR spectroscopy; an average purity of 92 ± 6% was verified for compounds **8**, **10**, and **11** by amino acid analysis with an internal standard. These compounds had similar absorbances; hence, ϵ_{257} = 200 (20 mM Tris, pH 8.5) was used for **6** and **7** as well. ϵ_{257} = 372 was determined for the dimethyl ester

Table I: Inhibition of Thermolysin by Phosphonamidate Peptide Analogues

inhibitor	K_i (nM)	corresponding substrate data[a] K_m (mM)	K_m/k_{cat} (μM s)
6	1700[b]	16.6	3200
7	760	20.6	196
8	270	10.8	165
9	78	2.4	20
10	16.5	10.6	13.6
11	9.1	2.6	7.0

[a] Kinetic data for the corresponding peptide substrates (i.e., Cbz-Gly-L-Leu-D-Ala, etc.) from Morihara & Tsuzuki (1970). [b] Observed K_i for material contaminated with $\simeq$0.9% of the L-Ala isomer **10**.

of **9** and assumed to be unaffected by hydrolysis (Jacobsen & Bartlett, 1981). Complete experimental and spectral details may be found in the supplementary material (see paragraph at end of paper regarding supplementary material). Stock solutions of the inhibitors (ca. 2 mM) prepared in 20 mM Tris, pH 8.5, were stable for months at 4 °C.

Determination of the Degree of Contamination of D-*Alanine Derivative* **6** *with* L-*Alanine Diastereomer* **10**. A solution of 5 μL (0.068 mmol) of thionyl chloride in 0.5 mL of methanol was kept at −10 °C for 10 min, and 20 mg (0.045 mmol) of **6** was added. After 1 h at −10 °C, followed by 4 h at 50 °C, the solution was concentrated at reduced pressure. The residue was dissolved in 0.5 mL of CH_2Cl_2 and 11 μL (0.95 mmol) of *N*-methylmorpholine and treated with 7 μL (0.07 mmol) of trifluoroacetic anhydride overnight at room temperature. Washing the mixture with 1 N HCl and 5% $NaHCO_3$, drying ($MgSO_4$), and concentration afforded *N*-[*N*-(trifluoroacetyl)leucyl]alanine methyl ester. The L,D and L,L diastereomers were cleanly resolved on GC analysis on a 12-m fused silica-cross-linked methyl silicone capillary column on a Hewlett-Packard Series 5790A gas chromatograph with temperature programming. Under the conditions used, the L,D isomer had a retention time of 12.27 ± 0.06 min and the L,L isomer a retention time of 12.13 ± 0.05 min. The preparation of **6** was shown to be contaminated with 0.89 ± 0.37% of the L-leucyl-L-alanine isomer **10**.

Enzyme Assay. All enzyme assays were performed at 25 °C by the spectrophotometric method of Feder et al. (1974). Typical concentrations of enzyme and substrate (FAGLA) were 1×10^{-8} M 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 inhibitors. 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 (1972) was employed to correct for inhibitor depletion. The values obtained were reproducible within ±8% and are displayed in Table I.

Results

The synthesis of the phosphonamidate peptide analogues was accomplished in a straightforward manner with a sequence analogous to the one we reported previously for the phosphonamidate analogue of Cbz-Gly-Phe (**1**) (Jacobsen & Bartlett, 1981). The last step in the synthesis of these inhibitors, alkaline hydrolysis of the methyl esters, leads to a small amount of epimerization of the C-terminal amino acid. For compound **6**, mild acid hydrolysis of the phosphonamide linkage and derivatization and capillary GC analysis of the leucylalanine released indicated that epimerization had occurred only to the extent of 0.89 ± 0.37%. Use of the most obvious alternative ester protecting groups was precluded by the sensitivity of the Cbz moiety to hydrogenolysis and the acid lability of the phosphonamide linkage. We were not successful in finding chromatographic conditions that would resolve **6** and **11**. Such epimerization is of no importance for the L,L isomers **9**–**11** since slight contamination by the more weakly bound L,D diastereomers has an immeasurable effect on the observed K_i. However, for the D-alanine derivative **6**, this epimerization has a profound effect because the contaminating L,L isomer **10** is bound over 100 times as tightly (see Table I). The K_i value reported for **6** is therefore a lower limit for that of the pure diastereomer.

Not unexpectedly, the phosphonamidates proved to be very potent inhibitors of thermolysin, with the observed inhibition constants ranging from 1.7×10^{-6} M for the D-alanine derivative **6** to 9×10^{-9} M for the L-leucyl compound **11** (Table I). The latter is in fact the most potent inhibitor yet reported for thermolysin (Nishino & Powers, 1978, 1979; Maycock et al., 1981; Komiyama et al., 1975; Weaver et al., 1977). The Michaelis constant of FAGLA, the substrate used in the assay, is relatively high (K_m ca. 30 mM; Kam et al., 1979); hence it was not possible to determine the mode of inhibition of the phosphonamidates. In view of their similarity to related inhibitors of thermolysin and other zinc peptidases, however, it is reasonable to assume that they act competitively.

Discussion

As the multitude of potent TS analogues demonstrates, this approach to enzyme inhibition has become popular as well as successful. With few exceptions, however, the designation "transition state analogue" in truth is a revelation of the underlying *design* concept, which led to the inhibitor, or an a posteriori rationalization of its tight binding. Evidence to show that an inhibitor is actually mimicking the transition state in the way it is bound is elusive and frequently open to differing interpretation (Schray & Klinman, 1974). The simple magnitude of the inhibition constant, or its relationship to the substrate dissociation constant, has at times been used to support the TS analogue designation. However, as Wolfenden (1976) has pointed out, there are clearly cases in which tightly bound inhibitors are simply opportunistic, taking advantage of binding interactions that have no relevance to an enzyme's complex with the transition state vis à vis that with the substrate.

In some cases, the rate of formation of an enzyme–inhibitor complex can furnish evidence for or against TS analogous binding. For example, for a series of adenosine deaminase inhibitors, Frieden et al. (1980) have noted that inhibitors considered for structural reasons to be TS analogues exhibit second-order rate constants for formation of tightly bound E·I complexes that are significantly slower than for inhibitors considered to be ground-state analogues. Slow-binding behavior of this sort suggests that tight binding of TS analogue inhibitors may require a conformational change on the part of the enzyme, related to that which the normal E·S complex undergoes during the catalytic event. In recent years, an increasing number of enzyme–TS analogue interactions have been shown to be slow-binding ones (Pierce et al., 1980; Schloss et al., 1980; Rich & Sun, 1980; Schmidt et al., 1982; Schloss & Cleland, 1982; Schloss & Lorimer, 1982). However, slow-binding inhibitors are known for which other evidence rules out the possibility that they are TS analogues. The inhibition of dihydrofolate reductase by methotrexate is a case

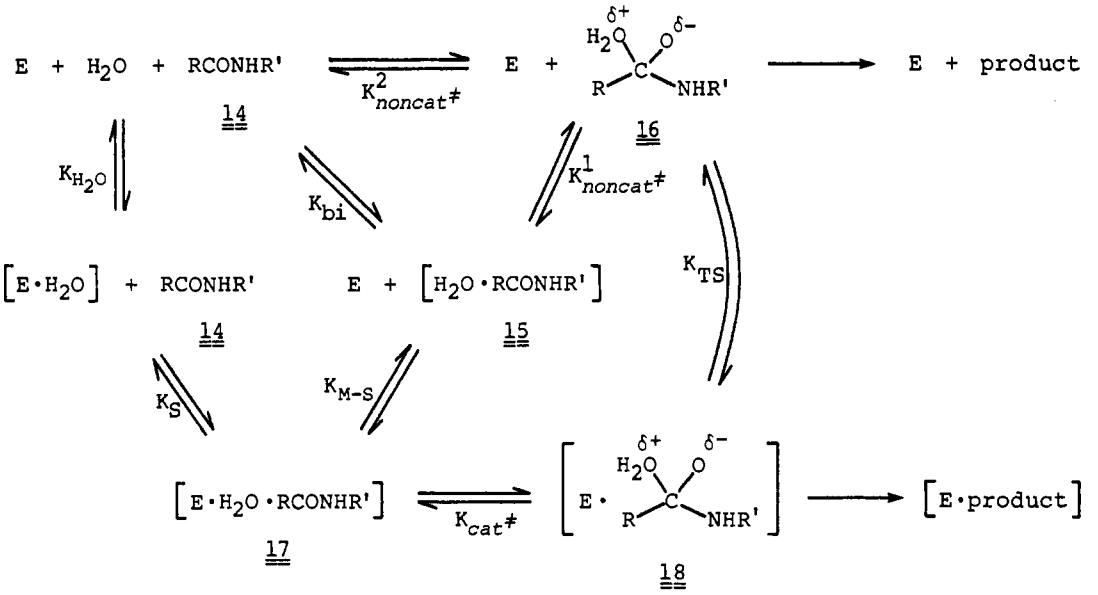
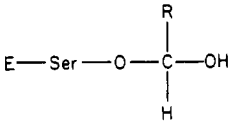


FIGURE 1: Mechanistic scheme for peptide–water–enzyme interaction. All equilibria are defined as dissociation constants.

in point. Methotrexate is a slow-binding, extremely potent inhibitor of the enzyme (K_i = 58 pM; Williams et al., 1979), yet crystallographic studies indicate that it binds to the enzyme with an orientation that is inverted with respect to dihydrofolate itself (Matthews et al., 1978; Charlton et al., 1979).

In seeking to show that a pair of peptide aldehydes are TS analogue inhibitors of the serine peptidase elastase, Thompson (1973) found that a structural modification which produces a 105-fold decrease in substrate K_m/k_{cat} gives a 78-fold decrease in aldehyde K_i. The fact that this modification has no significant effect on substrate K_m indicates that the enzyme-bound form of the inhibitor, hemiacetal **13**, takes substantial



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advantage of the binding forces that are experienced by the enzyme–substrate transition state but not the ground-state complex. A similar relationship was independently noted by Westerik & Wolfenden (1972) for three peptide aldehyde inhibitors of papain and by Thompson & Bauer (1979) with a series of serine peptidases.

In contrast to the peptide aldehydes, most TS analogues are not synthesized within the enzyme active site and do not become covalently bound. Equilibria different from those discussed previously must therefore be considered in the general case. For inhibitors of the zinc peptidases, the scheme in Figure 1 is necessary.

According to the mechanism depicted, these enzymes catalyze a bimolecular reaction: attack of a water molecule on the scissile carbonyl group, with formation of the tetrahedral transition state **18** in the rate-limiting step (Breslow & Wernick, 1977; Rees & Lipscomb, 1981; Weaver et al., 1977). There are three limiting types of substrate–enzyme interaction that can be emulated by an inhibitor. The inhibitor can try to take advantage of the ground-state interaction of either a single substrate or both substrates or the transition-state interaction of the tetrahedral species. In the first instance, the inhibitor would be considered simply a ground-state analogue of the peptide substrate **14**, emulating its binding with the "hydrated"[2] form of the enzyme to give the Michaelis complex **17**. β-Phenylpropionyl-L-phenylalanine and carbobenzoxy-L-phenylalanine are examples of this type of inhibitor for thermolysin (Kester & Matthews, 1977). An inhibitor of the second kind is of greater interest, in that it substitutes for **15**, a hypothetical collision complex between the peptide substrate and the attacking water molecule, binding to the "unhydrated" form of the enzyme. Such an inhibitor would be a ground-state multisubstrate (M-S) analogue (Wolfenden, 1972). The third type of inhibitor, a TS analogue, would interact with the "unhydrated" enzyme in a manner similar to the unstable tetrahedral species **16**.

Of primary concern for a phosphonamidate inhibitor of the zinc peptidases is to distinguish between the latter two cases: M-S analogue and TS analogue inhibition. To do this, it is necessary to compare the inhibition constant K_i with respectively the hypothetical dissociation constant of the Michaelis complex **17** (to give **15**, $K_{M\text{-}S}$) or that of the enzyme–transition state complex **18** (K_{TS}). As shown in eq 1, $K_{M\text{-}S}$ can be es-

$$K_{M\text{-}S} = (K_S/K_{bi})K_{H_2O} \quad (1)$$

timated by the left half of the thermodynamic cycle of Figure 1, in which K_{bi} represents the equilibrium constant for dissociation of the collision complex **15** into the peptide substrate **14** and water (Jencks, 1975). As Jencks has pointed out, K_{bi} can approach 10^8 M, reflecting the unfavorable entropy of constraining two molecules to move as one. An M-S analogue inhibitor therefore has the potential of binding up to 10^8 M times as tightly as the product of the substrate binding constants, depending upon how rigidly the two substrates are fixed

[2] There are two water molecules in the active site of thermolysin that are important to consider in connection with this scheme: one that attacks the peptide carbonyl and that is the cosubstrate and another that is coordinated to the zinc cation and appears to be displaced by both substrates and inhibitors (Weaver et al., 1977; Kester & Matthews, 1977; Bolognesi & Matthews, 1979; Holmes & Matthews, 1981; Monzingo & Matthews, 1982; Holmes et al., 1983). The former is consumed and replaced in the course of the catalytic cycle. For the purpose of discussion, enzyme that lacks this water molecule is referred to a "unhydrated", although it is recognized that in the free form of the enzyme (in turn called "hydrated") it is present. The second water molecule is not represented in the equilibria in Figure 1, since it affects all of them identically. Although binding of M-S and TS complexes or analogues is depicted as displacing only one water molecule (eq 2 and 7), it is understood that the second one is ejected by these inhibitors as well.

relative to each other in the Michaelis complex and upon how accurately the M-S analogue mimics this relationship. In the case at hand, prediction of the *observable* dissociation constant $K'_{M\text{-}S}$ of the M-S complex (or analogue) must take into account displacement of the substrate water from the native enzyme;[2] $K'_{M\text{-}S}$ will therefore be independent of K_{H_2O} (eq 2 and 3a).

$$E\cdot H_2O + H_2O\cdot RCONHR' \overset{K_{H_2O}}{\rightleftharpoons} E + H_2O + H_2O\cdot RCONHR' \underset{K_{M\text{-}S}}{\rightleftharpoons} E\cdot H_2O\cdot RCONHR' + H_2O \quad (2)$$

$$\frac{[E\cdot H_2O][H_2O\cdot RCONHR']}{[H_2O][E\cdot H_2O\cdot RCONHR']} = \frac{K_{M\text{-}S}}{K_{H_2O}} = \frac{K_S}{K_{bi}} = \frac{K'_{M\text{-}S}}{[H_2O]} \quad (3a)$$

Prediction of the hypothetical dissociation constant of the transition-state complex **18** employs the thermodynamic cycle around the perimeter of Figure 1 and leads to eq 4. Equation 5, relating the rate constants k and the transition-state equilibrium constants $K_\ddagger$, is derived from absolute rate theory (Eyring, 1935). Together, these lead to the expression for K_{TS} shown in eq 6.

$$K_{cat,\ddagger}K_S K_{H_2O} = K_{TS}K^2_{noncat,\ddagger} \quad (4)$$

$$K^2_{noncat,\ddagger} = \frac{h}{kT}\left(\frac{1}{k^2_{noncat,\ddagger}}\right) \qquad K_{cat,\ddagger} = \frac{h}{kT}\left(\frac{1}{k_{cat}}\right) \quad (5)$$

$$K_{TS} = \left(\frac{k^2_{noncat,\ddagger}}{k_{cat}}\right)K_S K_{H_2O} \quad (6)$$

As before, observable binding of the transition state or a TS analogue to the native enzyme, expressed by K'_{TS}, will be independent of K_{H_2O} (eq 7 and 8a).

$$E\cdot H_2O + \mathbf{16} \overset{K_{H_2O}}{\rightleftharpoons} E + H_2O + \mathbf{16} \underset{K_{TS}}{\rightleftharpoons} E\cdot\mathbf{16} + H_2O \quad (7)$$

$$\frac{[E\cdot H_2O][\mathbf{16}]}{[H_2O][E\cdot\mathbf{16}]} = \frac{K_{TS}}{K_{H_2O}} = \left(\frac{k^2_{noncat}}{k_{cat}}\right)K_S = \frac{K'_{TS}}{[H_2O]} \quad (8a)$$

The relationship between $K'_{M\text{-}S}$ and K'_{TS} can be derived if the noncatalyzed, second-order rate constant k^2_{noncat} is factored into two components, K_{bi} and a first-order rate constant k^1_{noncat} (Figure 1 and eq 9 and 10). The implication of this relationship is reasonable: the transition-state complex binds more tightly than the multisubstrate complex to the extent that the bond-forming steps occur more rapidly in the enzyme active site (**17** → **18**) than from the hypothetical collision complex (**15** → **16**).

$$K_{TS}K^1_{noncat,\ddagger} = K_{cat,\ddagger}K_{M\text{-}S} \quad (9)$$

$$K'_{TS} = \left(\frac{k^1_{noncat}}{k_{cat}}\right)K'_{M\text{-}S} \quad (10)$$

However, the derivation of eq 3a and 8a (rearranged in eq 3b and 8b) and 10 does not immediately allow one to distinguish an M-S analogue from a TS analogue inhibitor. In both cases, a substantial binding enhancement is predicted in comparison to a single substrate ground-state analogue, but

$$K'_{M\text{-}S} = (K_S/K_{bi})[H_2O] \quad (3b)$$

$$K'_{TS} = \left(\frac{k^2_{noncat}}{k_{cat}}\right)K_S[H_2O] \quad (8b)$$

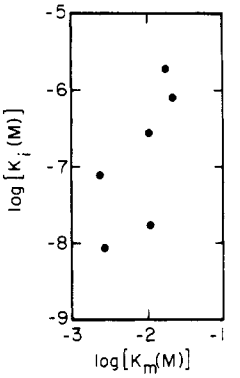

FIGURE 2: Comparison of phosphonamidate inhibitor K_i with substrate K_m.

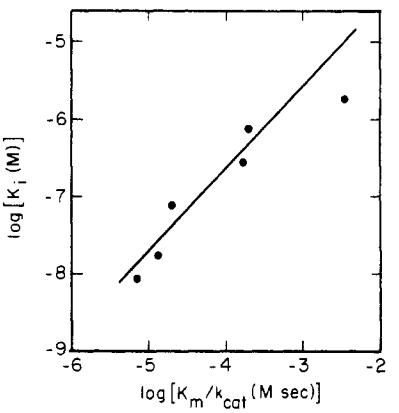

FIGURE 3: Comparison of phosphonamidate inhibitor K_i with substrate K_m/k_{cat}.

the uncertainties in estimating K_{bi}, k^2_{noncat}, and k^1_{noncat} are too great to allow accurate prediction of $K'_{M\text{-}S}$ or K'_{TS}, or their absolute relationship. Too, no inhibitor will be a perfect mimic of either the collision complex **15** or the transition state **16**, and numerical comparison of K_i with $K'_{M\text{-}S}$ vis à vis K'_{TS} would be meaningless.

Equations 3b and 8b are not without application, however. Within a series of related substrates, in which structural variation is remote from the reactive center, it is reasonable to assume that both K_{bi} and k^2_{noncat} (and therefore k^1_{noncat}) will be essentially constant. It is improbable that differences in amino acid residue at the P'_2 site in an oligopeptide substrate, for example, would significantly affect the ease of fixing a water molecule in the proximity of the scissile carbonyl group or its rate of attack on this linkage in the noncatalyzed process. For a series of related substrates, therefore, eq 3b and 8b become proportionalities, indicating that $K'_{M\text{-}S}$ depends linearly only on K_S, while K'_{TS} depends on both K_S and k_{cat}. It is therefore possible to predict the structural variation of $K'_{M\text{-}S}$ and K'_{TS}, in spite of the fact that their absolute values cannot be determined. Comparison of the K_i values of a series of related inhibitors with the kinetic parameters of the parallel series of related substrates thus provides a means for distinguishing between M-S analogue and TS analogue inhibitors.

Table I displays both the binding constants K_i for the phosphonamidates **6**–**11** as inhibitors of thermolysin and the kinetic parameters reported for the corresponding substrates (Morihara & Tsuzuki, 1970). The same data are displayed graphically in Figures 2 and 3. From Figure 2 it is clear that there is no correlation between the K_i values for the inhibitors and the K_m values of the corresponding substrates. For thermolysin, Morgan & Fruton (1978) have shown that the Michaelis constant K_m is equal to the substrate dissociation

constant K_S. The fact that substitution at the remote amino acid site affects phosphonamide binding in a dramatically different way than it does K_m indicates that the inhibitors are not ground-state M-S analogues.

On the other hand, Figure 3 shows that over several orders of magnitude there is a reasonably good correlation between K_i for the inhibitors and K_m/k_{cat} for the corresponding substrates. The fact that remote structural changes produce similar effects on the actual binding of phosphonamidates and the predicted binding of substrate transition states is consistent with the former being a close model of the latter. The point that deviates significantly from this correlation is that for the D-alanine derivative **6**. However, as noted above this material is contaminated with $\simeq$0.9% of the more potent L isomer, enough to account for the observed K_i of **6** regardless of its actual value. The binding behavior of **6** is therefore not inconsistent with the overall correlation.

In one sense, it is surprising that the correlation of Figure 3 is so good, in view of the fact that the transition state for addition to a carbonyl group is not a fully developed tetrahedral intermediate (inter alia, Bilkadi et al., 1975; do Amaral et al., 1979; Kovach et al., 1980). The tetrahedral phosphonamidates could therefore be mimicking a species further along the reaction coordinate than the actual transition state and be "reaction-intermediate" rather than TS analogues (Schloss & Cleland, 1982). Two arguments suggest that this may not be the case. First, phosphorus–oxygen bonds are some 10–15% longer than comparable carbon–oxygen single bonds (Sutton, 1958; Sheldrick & Morr, 1981); therefore, phosphonamidates may not model the tetrahedral intermediates as well as they do the transition states, in which only partial bonding has developed between the central carbon atom and the incoming water molecule. Second, addition of water to an amide is expected to have a late transition state, with only small geometric and electronic differences from the tetrahedral intermediate (Guthrie, 1974; Kirsch, 1977; Modro et al., 1977). TS and reaction-intermediate analogues may therefore be indistinguishable in a practical sense for the zinc peptidases.

Through the relationship $\Delta G = -RT \ln K$, the graph of Figure 3 is in effect a direct comparison of the free energies of binding for the inhibitors and transition states. The slope of the line drawn in Figure 3 is 1.05, indicating that within experimental error a structural modification produces the same incremental change in the binding energy of a phosphonamidate inhibitor that it does in the binding energy of a transition state. This correlation is thus significantly stronger than related ones reported previously, in which structural effects on binding were attenuated in the inhibitors relative to the transition states (Thompson, 1973; Westerik & Wolfenden, 1972; Wolfenden et al., 1977). We therefore feel that the phosphonamidates can justifiably be labeled transition-state analogues. The fact that the transition states must in turn resemble these inhibitors provides further support for an enzyme mechanism involving direct attack of water on the peptide linkage.

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Supplementary Material Available

Experimental details for the synthesis and characterization of phosphonamidates **6**–**11** (6 pages). Ordering information is given on any current masthead page.

Registry No. **6**, 86835-12-3; **7**, 86835-13-4; **8**, 86835-14-5; **9**, 86835-15-6; **10**, 86835-16-7; **11**, 86835-17-8; thermolysin, 9073-78-3.

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