Binding Energetics of Phosphorus-Containing Inhibitors of Thermolysin†

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ABSTRACT: The importance of a specific hydrogen bond between thermolysin and a phosphonamidate inhibitor, Z-NHCH$_2$-PO(O$^-$)-Leu-Leu (**1**) [Bartlett, P. A., & Marlowe, C. K. (1987) *Science* (*Washington D.C.*) *235*, 569–571], has been reevaluated. We have determined the inhibition constants (binding free energies) for thermolysin of phosphonamidate *n*-hexyl-P(O)(O$^-$)-Leu-Trp-NHMe (**4**), phosphonate *n*-hexyl-P(O)(O$^-$)OCH(iBu)CO-Trp-NHMe (**5**), and phosphinates *n*-hexyl-P(O)(O$^-$)CH$_2$CH(iBu)CO-Trp-NHMe (**6**) and Z-NHCH$_2$PO(O$^-$)CH$_2$CH(iBu)CO-Leu (**3**). Replacement of the P–NH group by P–CH$_2$ (**1** → **3** and **4** → **6**) weakens the overall binding free energy by about 1.5 kcal/mol. A negligible difference in solvation energy has been measured for these pairs, and the basicity of the P–O$^-$ ligand for zinc in each pair remains nearly unchanged as determined by pH titration of their ^{31}P NMR resonances. Therefore, this value of 1.5 kcal/mol can be assigned to the specific hydrogen bond known to exist between the P–NH of **1** and thermolysin [Tronrud, D. E., Holden, H. M., & Matthews, B. W. (1987) *Science* (*Washington, D.C.*) *235*, 871–574] and inferred to exist between **4** and the enzyme. Substitution of P–O for P–NH (**1** → **2** [Bartlett, P. A., & Marlowe, C. K. (1987) *Science* (*Washington, D.C.*) *235*, 569–571] and **4** → **5**) weakens the overall binding free energy by 4.1 kcal/mol for each pair as the basicity of the P–O$^-$ ligand decreases by about 1.6 pH units. The measured solvation energy difference between **4** and **5** (and by inference between **1** and **2**) is negligible. Therefore, we postulate that the loss of 4.1 kcal/mol in binding energy for this substitution results from the loss of a specific hydrogen bond of energy 1.5 kcal/mol present in both phosphonamidates **1** and **4** and a loss of 2.6 kcal/mol in the energy of liganding of the less basic phosphonate inhibitors **2** and **5**.

Bartlett and Marlowe (1987) have compared the inhibition constants for thermolysin of a series of phosphorus-containing analogues of the peptide carbobenzoxy-Gly-Leu-X in which the Gly–Leu peptide bond was replaced with either a phosphonate ester (P–O, **2**) or a phosphonamidate (P–NH, **1**) linkage. The phosphonamidates bound approximately 1000-fold more tightly to thermolysin than their phosphonate ester isosteres. From these results and the crystallographic analysis of the structure of one isosteric P–NH → P–O pair[1] (Tronrud et al., 1987), Bartlett and Marlowe (1987) concluded that the difference in binding free energy between the phosphonamidates and their isosteric phosphonate esters is due to a hydrogen bond donated by the phosphonamidate P–NH to the carbonyl oxygen atom of Ala 113 in the enzyme, which of course is not present in the phosphonate–enzyme complex.

In order to reevaluate the magnitude of the energy of this specific hydrogen bond, we have synthesized phosphinate **3** in which the P–NH of phosphonamidate **1** is replaced by the P–CH$_2$ linkage. We have also prepared a second series of isosteres that are analogues of the protected peptide *n*-hexanoyl-Leu-L-Trp-NHMe in which the peptide bond to the leucine is replaced by P–NH (**4**), P–O (**5**), and P–CH$_2$ (**6**). This series is thus analogous to the series **1**, **2**, and **3**. The substitution of P–O for P–NH in **4** to give **5** decreases the basicity of the oxygen anion that ligands zinc by about 1.6 pH units, in agreement with observations in model compounds. The substitution of P–CH$_2$ for P–O in **5** to give **6** increases the basicity of the oxygen ligand by about 1.6 pH units, also in agreement with model compounds. Thus phosphonate **5** is expected to be a poorer ligand for the active site zinc atom than either phosphonamidate **4** or phosphinate **6**, which are similar in basicity. All three isosteres have a very similar free energy of solvation, measured as the partition coefficient between 1-octanol and pH 8.0 buffer. We show here that both the liganding energy of the oxygen anion and the hydrogen-bonding energy of the P–NH hydrogen, but not the solvation energy, must contribute to the binding energy difference of 4.1 kcal/mol observed between phosphonamidate **1** and phosphonate **2** by Bartlett and Marlowe (1987). The structures and K_i's for these inhibitors of thermolysin are given in Table I. The consistent relative potencies of the three classes of thermolysin inhibitors (phosphonamidates, phosphonates, and phosphinates) are clearly illustrated by comparing the K_i's of the two sets of isosteres **1**–**3** and **4**–**6**.

Experimental Procedures

Enzyme Kinetics. Thermolysin was from Calbiochem, San Diego, CA, and was assayed with the substrate furanacryloyl-Gly-Leu-NH$_2$ from Sigma, St. Louis, MO, according to Bartlett and Marlowe (1983). The final concentration of substrate was 2 mM in 2.0 mL of 0.1 M Tris buffer, 0.5 M in sodium bromide, 2.5 mM in calcium chloride, and 2.5% in dimethylformamide. The absorbance change at 345 nm was recorded on a Varian 2200 spectrophotometer in a 1-cm path-length cell at 25 °C. The enzyme concentration was 10–20 nM. For inhibitors with K_i's greater than 100 nM, K_i was calculated from the formula uninhibited velocity/inhibited velocity = $1 + I/K_i$, where I, the inhibitor concentration, was varied over a range of at least a factor of 10 (six or more different concentrations in the range of the K_i). For more potent inhibitors, K_i was calculated from a Henderson plot (Henderson, 1972). For each inhibitor, K_i was determined at

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[1] Abbreviation: P–NH → P–O (or **4** → **5**), P–NH has been replaced by P–O in the inhibitor.

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least twice in independent experiments, and the values were averaged. The average range of the individual K_i's was within ±10% of the mean value.

The reaction with substrate was always started by the addition of substrate to a mixture of enzyme and inhibitor **3**, **4**, or **6** that had been incubated for 15 min at 25 °C. Progress of inhibition curves (initial velocity as a function of time of incubation of enzyme and inhibitor) showed that this time was sufficient to achieve steady-state velocities with the lowest inhibitor concentrations where the approach to equilibrium is slowest. Inhibitors **5** and **7** exhibited steady-state velocities without preincubation of enzyme and inhibitor.

Partition Coefficients. The partition coefficients of **4**, **5**, and **6** between 1-octanol and 0.1 M tricine buffer at pH 8.0, 0.5 M in sodium bromide, were measured three times in independent experiments. The two phases were equilibrated at 25 °C and then separated, and inhibitor was dissolved in the aqueous phase at a concentration of about 0.1 mM. The two phases were recombined in a ratio by volume of 1:9 octanol to buffer and shaken at 25 °C for 5 min and centrifuged. The concentration of inhibitor in each phase was determined spectrophotometrically with extinction coefficients previously determined at 280 nm for inhibitor in each phase saturated with its opposing phase. The mean partition coefficient for each inhibitor had a standard deviation of about 20% of its value.

Determination of the pK_a's of **4**, **5**, *and* **6**. The pK_a's of **4**, **5**, and **6** were determined by pH titration of their ^{31}P resonances with the Varian VXRS 500-MHz NMR spectrometer in the Department of Biochemistry at the University of Kentucky. Phosphorus-31 spectra were acquired at 202.334 MHz at a pulse width of 18 μs (pulse angle 80°) with an acquisition time of 0.6 s at 25 °C. Samples were at concentrations of approximately 0.05–10 mM in 5-mm tubes with an external reference of 45 mM phosphoric acid (0 ppm) in a concentric capillary tube. Initial spectra were taken at near-neutral pH in unbuffered 10% deuterium oxide in water, and the pH was adjusted down with a small volume of an appropriate concentration of hydrochloric acid. pH was measured with an Ingold microelectrode calibrated at pH 7.0 and at pH 0.1 [1 N hydrochloric acid (Weast, 1981)]. Low solubility at low pH required increasing numbers of transients to be acquired.

Hydrolysis of **4** at low pH to *n*-hexylphosphonic acid (see the supplementary material) prevented observation of the inhibitor resonance at complete protonation. Only the resonance of *n*-hexylphosphonic acid (measured pK_a = 2.5, data not shown [lit. pK_a = 2.6; p 308 of Fasman (1976)]) was observed at very low pH's. The resonance of *n*-hexylphosphonic acid nearly superimposed with the resonance of **5** at pH's between 1.0 and 2.5. However, no hydrolysis of **5** was observed at low pH. The pK_a's of **4**, **5**, and **6** were calculated at each measured chemical shift with the modified Henderson–Hasselbalch equation $pH = pK_a + \log [(\delta_{max} - \delta)/(\delta - \delta_{min})]$ and with the assumption that the highest chemical shift observed represented complete protonation. Since complete protonation of phosphonamidate **4** was not reached, the pK_a reported here for **4** could be several tenths of a pH unit too high.

Synthesis of the Inhibitors. The synthesis and characterization of inhibitors **3**, **4**, **5**, **6**, and **7** is described in the supplementary material for this paper (see paragraph at end of paper for information regarding the supplementary material).

Results and Discussion

The structures and K_i's for all of the inhibitors against thermolysin are given in Table I. Phosphinates **3** and **6** are mixtures of the two possible diastereomers. The relationship between the K_i's of isosteric phosphonate, phosphinate, and phosphonamidate inhibitors of thermolysin is described by eq 1 (compounds **2**, **3**, and **1**) and eq 2 (compounds **5**, **6**, and **4**).

Table I: K_i's of Phosphonamidate, Phosphonate, and Phosphinate Inhibitors of Thermolysin

inhibitor	K_i (nM)
Z-NHCH$_2$-PO$_2$-Leu-Leu (**1**)	9.1[a]
Z-NHCH$_2$-PO$_2$-OCH(iBu)CO-Leu (**2**)	9000[a]
Z-NHCH$_2$-PO$_2$-CH$_2$CH(iBu)CO-Leu (**3**)	180[b]
n-hexyl-PO$_2$-Leu-Trp-NHMe (**4**)	1.5
n-hexyl-PO$_2$-OCH(iBu)CO-Trp-NHMe (**5**)	1450
n-hexyl-PO$_2$-CH$_2$CH(iBu)CO-Trp-NHMe (**6**)	53[b]
EtO-PO$_2$-CH$_2$CH(iBu)CO-Trp-NHMe (**7**)	1600[b]

[a] Data from Bartlett and Marlowe (1987). [b] Mixture of two diastereomers.

$$K_i(P\text{–}O) \simeq 100K_i(P\text{–}CH_2) \simeq 1000K_i(P\text{–}NH) \quad (1)$$

$$K_i(P\text{–}O) \simeq 55K_i(P\text{–}CH_2) \simeq 1000K_i(P\text{–}NH) \quad (2)$$

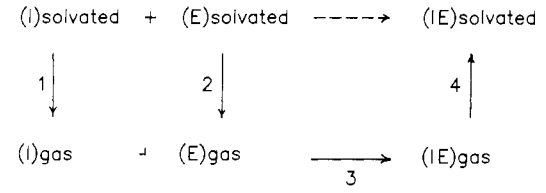
The K_i's for **3** and **6** from Table I have been divided by 2 to correct for the presence of a weakly inhibiting stereoisomer in the diastereomeric mixture (Bartlett & Marlowe, 1983). The phosphinates are thus about 1 order of magnitude and the phosphonates 3 orders of magnitude less potent than their phosphonamidate isosteres. In terms of relative energy changes, the substitution of an oxygen atom for the NH group in **1** or **4** decreases the overall binding energy $\Delta\Delta G$ by 4.1 kcal/mol. However, the CH_2 for NH substitution in **1** (**1** → **3**) and **4** (**4** → **6**) causes a significantly smaller change in $\Delta\Delta G$ (~1.7 and ~1.4 kcal/mol, respectively).

Bartlett and Marlowe (1987) have proposed that the difference in binding free energy to thermolysin of 4.1 kcal/mol between phosphonamidate **1** and isosteric phosphonate **2** is due entirely to a hydrogen bond donated from the NH of **1** to the enzyme. We show from measurement of K_i's of isosteric P–NH **4**, P–O **5**, and P–CH_2 **3** and **6** inhibitors that this hydrogen bond can only have a free energy of about 1.5 kcal/mol. Furthermore, we show that the increase in binding energy of phosphonamidate **1** compared to that of phosphonate **2** could be partially due to the increased ligand strength of the more basic phosphonamidate PO_2^- group for the zinc atom, a possibility discounted by the above authors. Finally, we show that a negligible difference in solvation free energy is observed among the three isosteric inhibitors **4**, **5**, and **6**, in contrast to the large difference in solvation free energy calculated by Bash et al. (1987) for the isosteres **1** and **2**.

Let us use Bartlett and Marlowe's thermodynamic cycle shown in Figure 1 [Figure 2 in Bartlett and Marlowe (1987)] for analysis of the observed differences in binding energies of P–NH, P–O, and P–CH_2 inhibitors. The difference in binding energies between the phosphonate, phosphinate, and phosphonamidate inhibitors arises from steps 1 and 3 (eq 3). In

$$\Delta\Delta G = \Delta G(\text{solvation}) + \Delta G(\text{ligand}) + \Delta G(\text{H-bond}) \quad (3)$$

previous analyses (Bartlett & Marlowe, 1987) it was assumed that ΔG(solvation) and ΔG(ligand) contribute little to the overall $\Delta\Delta G$ of 4.1 kcal/mol, and it was concluded that this value represents the intrinsic binding energy arising from a specific hydrogen bond between the P–NH of **1** and thermolysin [$\Delta\Delta G \simeq \Delta G$(H-bond)] which cannot and does not exist for the P–O inhibitor **2**. However, if the assignment of the difference in intrinsic binding energy to this hydrogen bond is correct, then we should observe K_i values for the phosphinate inhibitors (P–CH_2) **3** and **6** (which also must lack this hydrogen bond) approximately equal to the K_i's for their re-



Step in cycle	Difference in free energy change between isosteres
1. desolvation	ΔG(salvation)
2. (identical)	0
3. (a) PO_2^- coordination	ΔG(ligand)
(b) NH H-bonding	ΔG(H-bond)
4. (similar)	0

FIGURE 1: Hypothetical comparison of solvation and association steps for the thermodynamic cycle of enzyme–inhibitor complex formation. This figure is essentially identical with Figure 2 in Bartlett and Marlowe (1987).

spective isosteric phosphonates **2** and **5**. In fact, we find that the substitution of CH_2 for NH increases the K_i by only a factor of 10 for **1** → **3** and by a factor of 18 for **4** → **6** compared to factors of 1000 for the P–O for P–NH substitution.

Evaluation of the Liganding Free Energy. We assume that replacement of the phosphonamidate NH with the phosphinate CH_2 has minimal effect on the overall structure of the inhibitor and that the mode of inhibition of thermolysin by all three compounds is similar (as shown by crystallography for the NH and O substitution; Tronrud et al., 1987). However, we propose that the pK_a differences between the P–NH compounds and their respective P–O and P–CH_2 isosteres result in different free energies of liganding to zinc [ΔG(ligand)] and therefore do contribute to the observed differences in binding energy ($\Delta\Delta G$) between the isosteres.

A quantitative assessment of the pK_a's of phosphonamidates is difficult because of their low stability under acidic conditions (Poncz et al., 1984). However, as recognized by Bartlett and Marlowe (1987), the pK_a's of phosphonamidates are expected to be about 1.5 pH units higher than those for analogous phosphonates. For example, the pK_a of MeOP(O)OHNH-cyclohexyl is 3.1 (Hamer, 1965) while the pK_a of the corresponding MeOP(OH)$_2$ is 1.54 (Kumler & Eiler, 1943). The pK_a's of phosphinic acids are also about 1 pH unit higher than those of their corresponding phosphonate monoesters. For example, the pK_a of $Et_2P(O)OH$ is 3.29 (Crofts & Kosolapoff, 1953) while the pK_a of EtP(O)OH(OEt) is 2.27 (Razumov & Kehn, 1956). We have measured the pK_a's of **4**, **5**, and **6** to be 2.9, 1.4, and 3.1, respectively, in good agreement with the above model compounds. The pH titrations of the ^{31}P resonances of **4**, **5**, and **6** by NMR spectroscopy are shown in Figure 2.

The differences in pK_a's between phosphonamidate inhibitors and their isosteric phosphonates are likely to result in a difference in the liganding free energy to the zinc atom of the oxygen atom bound to phosphorus. In general, the stability constants (β) of complexes of a metal with a series of structurally similar ligands are approximately linearly dependent on the basicity of the ligands as in eq 4 (Perrin, 1964). For

$$\log \beta = b + \alpha pK_a \quad (4)$$

example, a linear relationship is obtained between the stability and acidity constants of a series of phosphonic acids and their complexes with copper(II), where $\log \beta = 0.23 + 0.434(pK_{a2})$ (Wozniak & Nowogrocki, 1978). In this example an increase in pK_{a2} by 1 pH unit stabilizes the complex by ~0.6 kcal/mol.

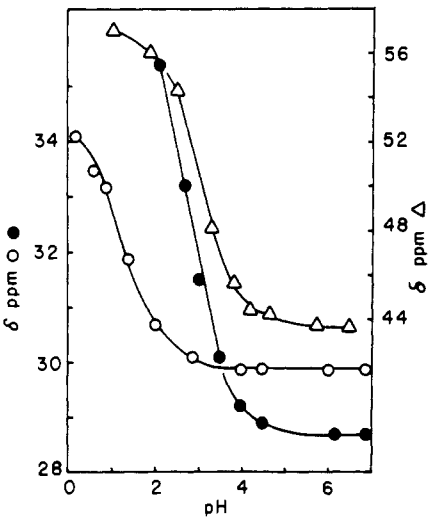


FIGURE 2: pH titration of the ^{31}P NMR resonance of the P–NH **4** (●), P–O **5** (○), and P–CH_2 **6** (△) inhibitors at 25 °C. Note that the chemical shift scale is different for the P–CH_2 inhibitor.

This increasing stabilization with increasing basicity is even more pronounced in the case of berylium complexes with alkylphosphonic acids. An increase of 1 pH unit in the second pK_a of the ligand stabilizes the complex by about 1.4 kcal/mol (Dyatlova et al., 1968). The effect of ligand basicity on the ligand binding energy, ΔG(ligand), in the enzyme–inhibitor complex should be stronger than that in solution due to the exclusion of water from the enzyme active site. Electrostatic interactions are no longer shielded by solvent molecules, and there is a better approximation of the interacting species. The small but significant differences in the coordination of isosteric phosphonamidate **1** and phosphonate **2** inhibitors to thermolysin observed by Tronrud et al. (1987) might therefore result from the differences in basicity of the PO_2^- ligands in these isosteres. Thus, it is very likely that the basicity of the PO_2^- ligand for a zinc atom might be an important factor in the overall binding energy ($\Delta\Delta G$) and that the value of ΔG(H-bond) of the amide NH of **1** and **4** is considerably smaller than 4.1 kcal/mol proposed by Bartlett and Marlowe (1987).

Evaluation of the Solvation Free Energy. Another factor that must be reevaluated is the relative free energy loss of desolvation of P–NH compared to P–O and P–CH_2 inhibitors. There are few experimental data that would allow the evaluation of solvation energies of these isosteres. Neutral phosphoramidates are hydrated only moderately more than similar esters (Wolfenden & Williams, 1983). In contrast, the theoretical estimate of the ΔG(solv) between anionic phosphonamidate **1** and its phosphonate **2** isostere is unexpectedly high (Bash et al., 1987). Bash calculated the relative change in binding free energy of phosphonamidate **1** versus phosphonate **2** to thermolysin using standard computational methods. According to this analysis, the difference in binding energy for the P–NH and P–O pair in solution $\Delta\Delta G$ is equal to ΔG(bind) – ΔG(solv), where ΔG(bind) is the *calculated net free energy difference in the two enzyme–inhibitor complexes* and ΔG(solv) is the net difference in solvation free energy between the two inhibitors. Bash's calculation gave 7.6 kcal/mol for ΔG(bind) and 3.4 kcal/mol for ΔG(solv).

It was concluded that the high value of ΔG(bind) resulted from favorable NH and repulsive O interactions with the carbonyl oxygen atom of Ala 113 (Bash et al., 1987). However, the crystallographic data do not support a repulsive interaction for the P–O inhibitor **2** (Tronrud et al., 1987). In addition, the indistinguishable K_i's of **5** and **7** (which must have nearly identical pK_a's) show that the oxygen atom in **5** is

unlikely to participate in a specific repulsive interaction with Ala 113. Because the P–O and P–CH_2 pair are similar sterically and must lack the H-bond found in the P–NH inhibitor, one might assume that ΔG(bind) for the P–NH → P–CH_2 pair should be approximately equal to that calculated for the P–NH → P–O pair, 7.6 kcal/mol. By subtracting the experimental $\Delta\Delta G$ values measured by us (1.7 and 1.4 kcal/mol) from the ΔG(bind) value for each pair (7.6 kcal/mol), one might calculate ΔG(solv) to be as high as 5.9 and 6.2 kcal/mol for the **1** → **3** and **4** → **6** pairs, respectively.

In order to check the credibility of the calculated solvation free energy differences for the anions of each isostere (at a pH well above their pK_a's), we have measured the partition coefficients of phosphonamidate **4**, phosphonate **5**, and phosphinate **6** between buffer at pH 8 and 1-octanol in solute concentration ranges around 0.1 mM at 25 °C. Although octanol seems to be a very crude model for a binding site, partition into octanol has been widely used to predict the binding of ligands to proteins (Leo et al., 1971). The partition coefficients of the P–NH **4**, P–O **5**, and P–CH_2 **6** solutes from buffer into 1-octanol are equal to 11.3, 23.4, and 10.2, respectively. Thus, only a small difference in solvation energy is observed for these isosteres. If the theoretical values of ΔG(solv) calculated by Bash were correct, we should have observed differences in distribution coefficients as large as a factor of 320 for the P–NH → P–O and 25 000 for the P–NH → P–CH_2 pairs. In fact, the small experimental difference in solvation free energy between phosphonamidate **4** and phosphonate **5** (~0.4 kcal/mol) is in good agreement with the solvation energy difference observed between neutral phosphoramidate–phosphonate pairs (0–1 kcal/mol; Wolfenden & Williams, 1983). Note that no difference in solvation energy was observed between the phosphonamidate **4** and the phosphinate **6**. Thus, the ΔG(solv) has little effect on the difference in binding energy $\Delta\Delta G$ among any of the three isosteres.

In summary, by comparing the binding to thermolysin of isosteric pairs of P–NH, P–O, and P–CH_2 inhibitors, we have reevaluated the proposed free energy (Bartlett & Marlowe, 1987) of a specific hydrogen bond between the enzyme and the P–NH inhibitor. We find that the substitution of CH_2 for NH decreases the overall binding energy $\Delta\Delta G$ by ~1.5 kcal/mol while the basicity of the PO_2^- ligand remains unchanged. The substitution of O for NH decreases $\Delta\Delta G$ by 4.1 kcal/mol while the pK_a of the inhibitor is decreased by about 1.6 pH units. The value of 1.5 kcal/mol is similar to the values reported by Fersht et al. (1985) and Fersht (1987) as representative of hydrogen bonds between uncharged species in the active site of tyrosyl-tRNA synthetase. The difference of 4.1 kcal/mol must result from both the decreased basicity of the P–O compound (2.6 kcal/mol) and the loss of the specific hydrogen bond of the P–NH compound (1.5 kcal/mol). The differences in overall binding free energy among the three isosteres cannot result from differences in solvation energy since we have demonstrated these to be negligible.

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Supplementary Material Available

Syntheses and characterizations of the phosphorus-containing inhibitors **3**–**7** (9 pages). Ordering information is given on any current masthead page.

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