cannot rule out unequivocally any of the above possibilities. The large pressure-induced increase of T_{c0} cannot be understood in terms of current theories. It is consistent, however, with the conjectures (*2*) of interfacial superconductivity (arising from mixed phases, or interfaces between layers, or concentration fluctuations even within the K_2NiF_4 phase) and noninterfacial superconductivity due to a strong electron-pairing interaction resulting from the mixed valence state in LBCO. A *d-f* mixing due to *f*-electrons in lanthanum slightly above the Fermi level can lead to a high electron density of states and thus a high T_c. This mixing is also sensitive to pressure. This possibility can be tested in future specific heat measurements. Finally, it should be pointed out that the T_{c0} observed by us exceeds the limit previously predicted by theory (*15*) based on the conventional electron-phonon interaction.

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Evaluation of Intrinsic Binding Energy from a Hydrogen Bonding Group in an Enzyme Inhibitor

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This and two accompanying reports describe the intrinsic binding energy derived from a single hydrogen bond between an inhibitor and an enzyme. The results were obtained by comparing matched pairs of inhibitors of the zinc endopeptidase thermolysin that bind to the enzyme in an essentially identical manner but differ in the presence or absence of a specific hydrogen bond. This report describes five phosphorus-containing analogs of the peptides carbobenzoxy-Gly-Leu-X, in which the Gly-Leu peptide linkage is replaced with a phosphonate ester (-PO_2^--O-). Values for the inhibition constants of these inhibitors show a direct relation with those of the corresponding phosphonamidate analogs (-PO_2^--NH- in place of the Gly-Leu peptide moiety), which have been characterized previously as transition state analogs. However, each phosphonate ester is bound about 840 times more weakly than the analogous phosphonamidate, reflecting the loss of 4.0 ± 0.1 kilocalories per mole in binding energy. From these results and the crystallographic analysis in the next report, it can be inferred that the value of 4.0 kilocalories per mole represents the intrinsic binding energy arising from a highly specific hydrogen binding interaction.

A GOAL YET TO BE ATTAINED IN THE study of any enzyme mechanism is a full understanding of the relations among active site structure, substrate binding, and the dynamics of catalysis (*1*). While we have a qualitative understanding of the interactions between substrates and enzyme active sites, quantitative understanding of the roles played by separate substructural elements is clouded by the difficulty in distinguishing effects due to solvation and, in particular, entropy. Jencks has pointed out that the incremental change in Gibbs (*G*) free energy of binding due to the addition of a group X to a reference molecule A provides a measure of the "intrinsic binding energy" due to X (Eq. 1) (*2*). This value is free from entropic complications if A and A–X do not undergo differences in strain or in rotational and translational entropy loss on binding to the enzyme (*2*). However, these qualifications are not easily met, especially in view of the fact that, as an appendage to A, the X-moiety seldom represents an insignificant structural perturbation. Moreover, the attribution of observed intrinsic binding energies to a specific interaction is risky in the absence of corroborating structural information.

$$(\Delta G^{i}_{X} = \Delta G^{O}_{A-X} - \Delta G^{O}_{A}) \qquad (1)$$

We previously reported that phosphonamidate peptide analogs **1**, X = NH_2 or amino acid (ZG^PLX; see Table 1), are potent inhibitors of the zinc endopeptidase thermolysin, and that their free energies of interaction with the enzyme, reflected in the inhibition constants K_i, show a strong correlation with the (hypothetical) binding energies of the transition states for hydrolysis of the corresponding amide substrates, reflected in the second order rate constants for enzymatic turnover (K_m/k_{cat}) (*3*). From this correlation we concluded that the phosphonamidates mimic the transition state configurations of the enzyme-substrate complexes (*4*). We now report our findings with a series of phosphonate esters **2** [$ZG^P(O)LX$], which differ from the amidates in replacement of the NH linkage between phosphorus and the leucine moiety with an oxygen atom (*5*). The structural consequences of this substitution are minimal; however, the effect that it has on the magnitude of K_i is significant (Fig. 1 and Table 1).

Replacement of the phosphonamidate NH with phosphonate O reduces the binding energy of each inhibitor by a factor of 840, almost uniformly across the two orders of magnitude in absolute binding affinity within each series. The constancy of this increment reflects an identical correlation between structural variation and binding energy within each series, and therefore suggests that the phosphonamidate and phosphonate inhibitors bind to the enzyme active site in a similar manner, and, aside from those due to the NH group itself, with similar interactions with the protein. The difference in K_i values between the phosphonamidates and phosphonates represents

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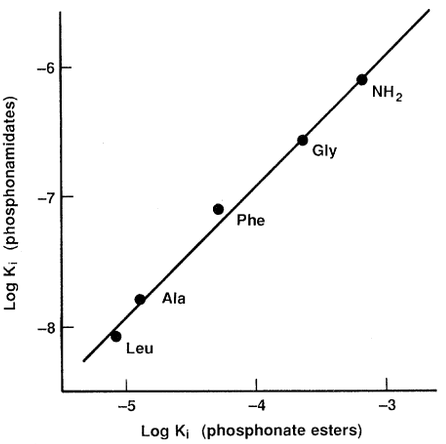
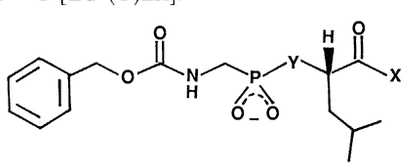


Fig. 1. Comparison of inhibition constants for phosphonamidate and ester inhibitors; the line represents the relationship:
K_i (ester) = 840 × K_i (amidate).

Table 1. Phosphonate and phosphonamidate inhibitors of thermolysin. **1**: Y = NH (ZG^PLX); **2**: Y = O [$ZG^P(O)LX$].



X*	K_i† (n*M*)	
	1‡	2
NH_2	760.0	660,000
Gly	270.0	230,000
Phe	78.0	53,000
Ala	16.5	13,000
Leu	9.1	9,000

*All chiral amino acids, as well as the α-hydroxy analog of Leu, are of the L-configuration. †Determined at 25°C, 0.1*M* tris-HCl, 0.1*M* NaBr, and 2.5 m*M* $CaCl_2$, *p*H 7.0, as described in reference (*3*) and citations therein. Typical concentrations of enzyme and substrate were 10 n*M* and 2.0 m*M*, 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 (*14*) was used to correct for inhibitor depletion. The values obtained were reproducible within ±8%. ‡Data from (*3*).

on average 4.0 ± 0.1 (SE) kcal/mol in binding energy that can be attributed to the O-for-NH substitution. The most obvious difference between the amides and esters is the possibility of a hydrogen bond between the NH of the phosphonamidate inhibitor and an acceptor group on the protein. Indeed, this hydrogen bonding interaction, as well as the absence of other significant differences in the way the amidates and esters bind, is revealed by the crystal structures of the thermolysin complexes with amidate **1** (X = L-Leu) and ester **2** (X = L-Leu), as described in the accompanying report by Tronrud *et al.* (*6*) and shown in figure 4 in that report. The value of 4.0 kcal/mol therefore represents the intrinsic binding energy that can be attributed to the phosphonamidate NH group.

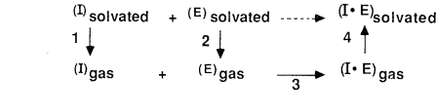
These results have several implications. Since the phosphonamidate structure presumably mimics a tetrahedral intermediate along the normal reaction path (*3, 7*), an analogous hydrogen bond may play a role in stabilizing the latter complex. However, hydrogen bond donation from the N–H moiety in the tetrahedral intermediate will be significant only for the ammonium form, suggesting that the phosphonamidates mimic the tetrahedral intermediate prior to its collapse to the trigonal products.

Evaluation of the value of 4.0 kcal/mol for a hydrogen bonding interaction is complicated by the nature of the exchange process involved (*1, 8, 9*). In previous analyses, the formalism of Eq. 2 has been used to point out that the number of hydrogen bonds is unchanged on association of hydrated inhibitor with hydrated active site and thus that the process is, to a first approximation, isoenthalpic. A key assumption in this analysis is that the hydrogen bonds made by the solvated components are similar in energy to those formed after association. Moreover, when this approach is adapted to association of an active site with a solute that lacks the hydrogen bonding capability, the accounting becomes less tidy (Eq. 3), and the rationalization of isoenergetic behavior requires that hydrophobic binding effects (primarily entropic) compensate for loss of discrete hydrogen bonds (*7*).

$$I\text{–}H\cdots OH_2 + HOH\cdots :E \rightleftharpoons I - H\cdots :E + HOH\cdots OH_2 \quad (2)$$

$$I + HOH\cdots :E \rightleftharpoons I :E + HOH \quad (3)$$

An alternative approach is to consider the thermodynamic cycle shown in Fig. 2, in which desolvation of the components and their association and hydrogen bonding are separated hypothetically. The important point is the incremental change in these quantities as a result of the O-for-NH substitution. Step 1, desolvation of the inhibitor, will be more difficult for the amidate than for the ester, largely because of the hydrogen bonding capability of the former, both as a donor and as an acceptor. Step 3, desolvation of the enzyme active site, is the same for both amidates and esters. If the van der Waals forces due to the ester oxygen are similar to those of the amidate nitrogen, the only difference in step 3, association of inhibitor and enzyme, is the energy of the amidate-to-ester hydrogen bond and the increased ligand strength of the amidate over the less basic ester. Finally, step 4, resolvation of the enzyme-inhibitor complex, is similar in both series, since water is



Step	Amide versus ester	Difference
1	Desolvation	$\Delta G_{solvation}$
2	(Identical)	0
3	(a) Phosphonate coordination	ΔG_{ligand}
	(b) Amidate H-bonding	$\Delta G_{H\text{-}bond}$
4	(Similar)	~0

Fig. 2. Hypothetical comparison of solvation and association steps.

excluded from the region where the structural modification is made. In this mode of analysis (Eq. 4), the difference in binding energies arises from steps 1 and 3: the contribution from the favorable energy of the amidate-enzyme hydrogen bond is, on the one hand, augmented by the increased ligand strength of the amidate and, on the other, partially offset by the greater free energy loss on its desolvation.

$$\Delta G = \Delta G_{solv} + \Delta G_{ligand} + \Delta G_{H\text{-}bond} \quad (4)$$

How much better should the phosphonamidates be over the esters as ligands for the zinc (and the other polar residues with which they interact); that is, what magnitude is expected for ΔG_{ligand} in Eq. 4? The first ionization constants, pK_{a1}, for phosphoramidates are generally 1 to 1.5 units higher than those for related alkyl phosphate esters (*10*). Although parallel substituent effects are seen for phosphonic acids, the difference in basicity of phosphonate anions is not directly reflected in the formation constants of their metal complexes. For example, for a series of alkyl phosphonic acids, complexation of copper(II) is only moderately sensitive to the second ionization constant, pK_{a2}, of the phosphonate (log K_{eq} = 0.23 + 0.434 pK_{a2}) (*11*).

How significant is the difference in solvation between the phosphonamidates and the esters, that is, what magnitude is expected for ΔG_{solv} in Eq. 4? Experimental comparison of phosphorus esters with amides is limited, and available only for the fully esterified, neutral derivatives (*12*). However, this evidence does suggest that neutral phosphoramidates are hydrated only moderately more than similar esters. In contrast, as pointed out by Bash *et al.*, molecular dynamics simulations imply that a much greater difference in solvation exists for the ionized derivatives (*13*). This difference is likely to be due to the basicity of the phosphonamidate nitrogen, that is, to its greater hydrogen-bond accepting ability, in comparison with the corresponding oxygen of a phosphonate ester. The foregoing analysis suggests that the favorable contribution to step 4 of the association process made by the

H-bond itself ($\Delta G_{H\text{-}bond}$) is significantly higher than the observed ΔG of 4.0 kcal/mol.

The "intrinsic binding energy" of the amide N–H in **1** is considerably greater than the values reported by Fersht *et al.* (*9*) as representative of hydrogen bonds between uncharged components in the active site of tyrosyl-tRNA synthetase. Although a formal charge does not reside on the nitrogen, it may be argued that the phosphonamidate is not an uncharged species. However, the hydrogen bond formed between the phosphonamidate-NH and the active site hydrogen bond acceptor must be a highly favorable one from the point of view of entropy: both components are held in the same orientation in the absence of the interaction, hence no further entropy is lost on establishment of the hydrogen bond. This system therefore comes closest to approximating the situation envisaged by Jencks for determination of a true intrinsic binding energy (*2*). Were the interaction between flexible side chains, or between groups that are free to rotate before the hydrogen bond is established, a less favorable incremental binding energy would be seen. Thus, the value observed for a hydrogen bonding interaction may reflect the degree with which the interacting components are fixed as much as it does their charged or uncharged nature.

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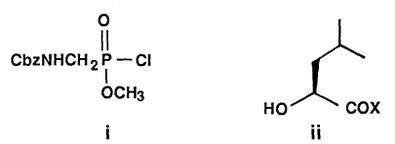

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Structures of Two Thermolysin-Inhibitor Complexes That Differ by a Single Hydrogen Bond

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The mode of binding to thermolysin of the ester analog Cbz-Gly^{P}-(O)-Leu-Leu has been determined by x-ray crystallography and shown to be virtually identical (maximum difference 0.2 angstrom) with the corresponding peptide analog Cbz-Gly^{P}-(NH)-Leu-Leu. The two inhibitors provide a matched pair of enzyme-inhibitor complexes that differ by 4.1 kilocalories per mole in intrinsic binding energy but are essentially identical except for the presence or absence of a specific hydrogen bond.

CRYSTALLOGRAPHIC ANALYSES OF the thermostable endopeptidase thermolysin have revealed the modes of binding of a broad spectrum of inhibitors (*1–5*) and have led to a detailed proposal for the stereochemical mechanism of action of the enzyme (*6*). In brief, Glu 143, in concert with the zinc, is presumed to promote the attack of a water molecule on the carbonyl carbon of the scissile bond. The proton accepted by Glu 143 is then shuttled to the leaving nitrogen. An analogous mechanism was also proposed for carboxypeptidase A (*5*) and is supported by the recent finding that Tyr 248 of carboxypeptidase A is not required for catalysis (*7*).

Here we describe the mode of binding to thermolysin of Cbz-Gly^{P}-(O)-Leu-Leu [hereafter ZG^{P}(O)LL], one of a series of phosphorus-containing inhibitors of thermolysin (*4, 8–10*) developed by Bartlett and Marlowe (*10, 11*) as putative transition-state analogs of ester substrates. [As in the preceding report (*11*) the superscript P indicates that the inhibitor has a tetrahedral phosphonamide or phosphonate ester linkage.] We found that the mode of binding of this ester analog is very similar to the corresponding peptide analog Cbz-Gly^{P}-(NH)-Leu-Leu (hereafter ZG^{P}LL). This allows the difference in affinity of the two inhibitors for thermolysin to be ascribed directly to differences in solvation and hydrogen

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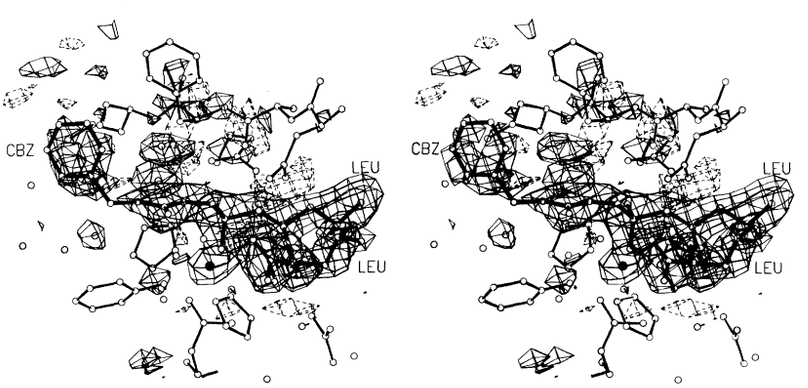

Fig. 1. Difference electron density for ZG^{P}(O)LL bound to crystalline thermolysin. Coefficients for the map are of the form ($F_{complex} - F_{native}$) where the native amplitudes and phases are calculated for the refined structure with active-site solvent atoms removed. Resolution 1.9 Å. Contours are drawn at height $+2\sigma$ (solid) and -2σ (broken) where σ is the root-mean-square density throughout the unit cell. The difference electron density at the phosphorus position is 16σ. The bound inhibitor is drawn with bonds thicker than the protein. The zinc ion is drawn solid.