Stromelysin Inhibitors Designed from Weakly Bound Fragments: Effects of Linking and Cooperativity

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Abstract: In the preceding paper,[1] we reported on the discovery of potent, nonpeptide inhibitors of the matrix metalloproteinase stromelysin that were prepared by linking two ligands which bind weakly to adjacent sites on the protein. Here we describe the enthalpic and entropic contributions to the observed binding energy for both the linked and unlinked compounds using isothermal titration calorimetry. The results of the calorimetric experiments were interpreted on the basis of NMR-derived structures of stromelysin/inhibitor complexes. In addition, enzyme kinetic assays were performed to measure the cooperative binding of the untethered ligands. For the untethered compounds, the presence of acetohydroxamic acid increases the binding energy of biaryl ligands by ∼1.3 kcal/mol. This gain in energy is enthalpic in nature and can be attributed, in part, to a direct dispersion interaction between the two ligands. For the linked compounds, enthalpic contributions to the binding energy depend critically on the linker length, whereas the entropic contributions show virtually no dependence. The significant gains in enthalpy observed for a compound which linked the hydroxamate to the biaryl with a two methylene bridge was not observed for compounds with longer linkers due to a difference in the position of the biaryl moiety in the binding pocket. This difference disrupts key interactions between the ligand and the protein and highlights the importance of the linker in the design of tethered compounds.

Introduction

Recently, we described an NMR-based technique for discovering high-affinity ligands for proteins called SAR by NMR (structure−activity relationships by nuclear magnetic resonance).[2] With the use of this method, small molecules that bind to proximal subsites of a protein are identified by NMR and subsequently linked together on the basis of NMR-derived structural information. One of the central tenets of the SAR by NMR method is that high-affinity ligands can be produced by linking together two ligands that only bind weakly to the protein. This approach assumes that the binding energy of a linked compound can be approximated by the sum of the binding energies of each component plus an additional term to account for linking. Using this model, the binding energy of a linked molecule, $\Delta G(AB)$, composed of two components A and B can be represented by[3]

$$\Delta G(AB) = \Delta G(A) + \Delta G(B) + \Delta G(L) \qquad (1)$$

where $\Delta G(A)$ and $\Delta G(B)$ are the intrinsic binding energies of the unlinked components and $\Delta G(L)$ includes contributions to the binding energy due to linking.

Many factors must be considered when estimating the binding energy of a linked compound based on its component parts.[4−6]

First, in order to retain the intrinsic binding energy of each component, the linked compound must maintain the same binding orientation as the untethered ligands, which will depend on the length and geometry of the linker. In addition, the linker itself may affect the overall binding energy by making favorable or unfavorable enthalpic interactions with the protein. Furthermore, contributions from entropic changes can be large and difficult to predict. Entropic gains are expected upon linking due to the reduction in the number of components which are immobilized upon binding to the protein.[7] Although this effect might be expected to be quite large on the basis of the calculated loss of translational and rotational degrees of freedom in the gas phase,[7] the actual contribution is generally much smaller due to new vibrational modes of the protein−ligand complex and the residual motional freedom of the ligand.[5,8−11] Predicting entropic changes upon linking is also complicated by the effects of solvation and desolvation of the ligands in their tethered and untethered states.[5,12,13] These terms may favor or oppose complex formation, leading to an observed binding energy which is a delicate balance between large and counteracting effects.

Another factor which must be considered when analyzing the interactions of multiple ligands with different protein binding sites is that the presence of one of the ligands may affect the binding energy of the other.[14−16] This is shown schematically in Figure 1 for two ligands A and B with dissociation constants

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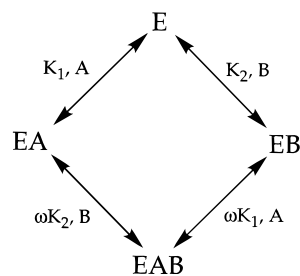
Figure 1. Diagram of the multiple equilibria involved in the binding of two ligands, A and B, to different sites on a protein, E. For the Discussion and Tables, ligand A represents acetohydroxamic acid (**1**), with dissociation constants K_1 and ωK_1, while ligand B represents the biaryls, with dissociation constants K_2 and ωK_2.

of K_1 and K_2, respectively. The cooperativity factor, ω, is a measure of the change in binding affinity of one ligand due to the presence of the other. In this scheme, the cooperativity factor can be less than 1.0 (cooperative), equal to 1.0 (non-cooperative), or greater than 1.0 (anticooperative). A cooperative (or anticooperative) effect can be due to direct interactions between the ligands or to conformational changes that occur upon binding.[16] These additional enthalpic interactions or conformational alterations must be considered when comparing the measured binding energies of the untethered compounds with that of linked compounds.

In the preceding paper,[1] we described the discovery of potent, nonpeptide inhibitors of the matrix metalloproteinase stromelysin that were prepared by linking acetohydroxamic acid (**1**) to biaryls. The binding affinities of the biaryls were found to be strongly dependent on the presence of **1**, suggesting that these two ligands bind cooperatively. In this paper, we present a detailed analysis of the cooperative binding of acetohydroxamic acid and several biaryl ligands to stromelysin by measuring the inhibition of stromelysin using different concentrations of both inhibitors. The enthalpic and entropic contributions to the cooperativity were determined by calorimetry and interpreted on the basis of NMR-derived structural information of stromelysin/inhibitor complexes. Calorimetric studies have also been performed on several tethered ligands to elucidate the enthalpic and entropic contributions to the binding energy due to linking. The structural basis for the observed differences in binding energy for compounds with different linker lengths was also determined. In addition to furthering our understanding of ligand binding to stromelysin, these studies reveal some of the important factors that should be considered when applying the SAR by NMR method.

Results and Discussion

Cooperative Binding of Untethered Ligands. To investigate the cooperative binding of acetohydroxamic acid (**1**) and the biaryl ligands, the inhibition of stromelysin cleavage of a fluorogenic substrate was measured using a matrix of concentrations of **1** and various biaryl ligands. Figure 2 shows the relative inhibitory activity of acetohydroxamic acid in the presence of increasing amounts of **3**. The increased inhibition of stromelysin activity observed for **1** in the presence of **3** is indicative of cooperative binding. We have analyzed these data and those for other biaryl compounds in the context of the multiple simultaneous equilibria involved in the binding of two ligands to a protein (Figure 1). Dissociation constants and cooperativity
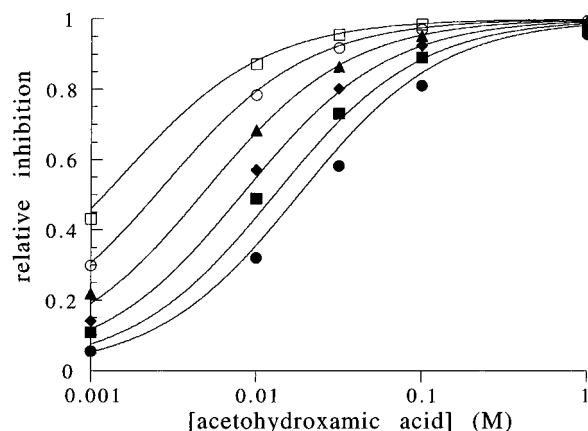
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Figure 2. Inhibitory data from the modified enzyme inhibition assay (data points) and fitted simulations of the data (solid lines) as described in methods. Data points (shown as the mean of duplicate assays) are for 0.0 (●), 0.125 (■), 0.25 (◆), 0.5 (▲), 1.0 (○), and 2.0 (□) mM **3**. Errors in the data points are small and are not shown for clarity.

Table 1. Dissociation Constants and Cooperativity Data for Ligand Binding to Stromelysin

No.	Compound	$\omega K_2{}^a$	$\omega{}^b$	$\Delta G(\omega){}^c$
2		7.4	0.088	-1.4
3		0.32	0.090	-1.4
4		0.11	0.095	-1.4
5		0.70	0.132	-1.2
6		1.5	0.154	-1.2

^{a} ωK_2 is the dissociation constant of the biaryls for stromelysin in the presence of saturating amounts of acetohydroxamic acid (**1**) as described in Figure 1 and as determined by a modified enzyme inhibition assay. ^{b} ω is the cooperativity factor as described in Figure 1. ^{c} $\Delta G(\omega) = RT \ln \omega$.

factors for each ligand were obtained and are given in Table 1. The results indicate that the cooperativity factors for all of the biaryl ligands are similar and positive ($\omega < 1.0$), despite significant differences in the structures of the biaryl compounds. The enhancement in binding energy of one compound due to the presence of the other is on average −1.3 kcal/mol.

Nature of the Cooperative Binding. In order to determine the relative enthalpic and entropic contributions to the binding of the untethered ligands and the observed cooperativity, the binding of several biaryls to stromelysin was examined by calorimetry in the presence and absence of **1**. The heats of binding were measured (e.g., Figure 3), and the thermodynamic parameters were obtained (Table 2). Although it might be expected that the biaryls would exhibit classical "hydrophobic" binding[6,17] (exemplified by large entropic contributions), the binding of the biaryls to stromelysin is characterized by a large net increase in enthalpy. Thus, the entropic gains expected from the release of ordered waters from the binding pocket and the ligand upon complexation are offset by other entropic changes that occur.

In the presence of acetohydroxamic acid, there is a large increase in the binding energy of the biaryls which is due to enthalpic contributions. Biaryl **4** gains 3.2 kcal/mol in enthalpy in the presence of **1** (Table 2), while **3** gains 2.9 kcal/mol. This gain in enthalpy suggests that additional interactions are available to the biaryls when **1** is present. Indeed, in isotope-

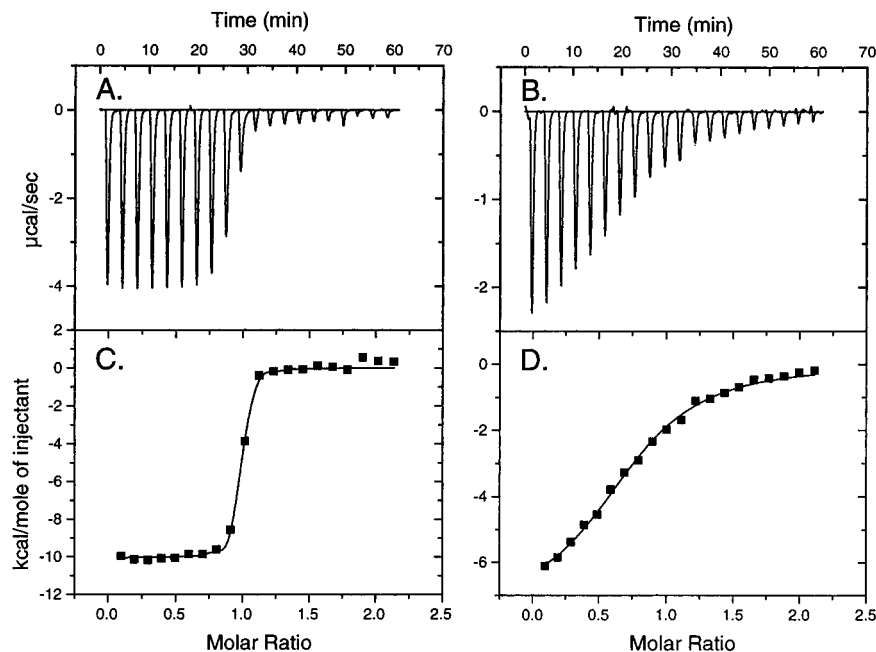(17) Kauzmann, W. *Adv. Protein Chem.* **1959**, *14*, 1−63.

Figure 3. Calorimetric titration data of stromelysin with (A) compound **7** and (B) compound **4** in the presence of 450 mM acetohydroxamic acid (**1**). Integrated calorimetric heats (data points) and best fit curves using a single binding site model (solid lines) are shown for **7** and **4** in panels C and D, respectively.

Table 2. Thermodynamic Parameters for Ligand Binding to Stromelysin[a]

No.	Compound	$[1]$[b]	ΔG (cal.)[c]	ΔG (NMR)[d]	ΔG (assay)[e]	$<\Delta G>$[f]	ΔH[g]	$-T\Delta S$[i]
1		0	n.d.	-2.4	-2.3	-2.4 ± 0.1	-3.0± 0.6[h]	0.6 ± 0.6
3		0	n.d.	n.d.	-3.4	-3.4	-2.4± 0.6[h]	-1.0 ± 0.6
		450	-4.9	-5.2	-4.8	-5.0 ± 0.2	-5.3± 0.4	0.3 ± 0.4
4		0	-5.5	n.d.	-4.0	-4.8 ± 1.0	-4.7± 0.5	-0.1 ± 1.1
		450	-6.9	-6.4	-5.4	-6.2 ± 0.8	-7.9± 0.5	1.7 ± 0.9
7		0	-9.6	n.d.	-10.0	-9.8 ± 0.3	-9.3± 0.4	-0.5 ± 0.5
8		0	-6.8	n.d.	-6.6	-6.7 ± 0.1	-6.1± 0.5	-0.6 ± 0.5

[a] All thermodynamic parameters are given in units of kcal/mol and are calculated at 303 K. n.d. means that the value was not determined. [b] Denotes the concentration (in mM) of acetohydroxamic acid (**1**) under which the experiment was performed. [c] Values for ΔG derived from dissociation constants obtained calorimetrically (cal.). [d] Values for ΔG derived from dissociation constants obtained from NMR titrations. [e] Values for ΔG derived from dissociation constants obtained from an enzyme inhibition assay. [f] Value for ΔG averaged over all available data. Errors are the standard deviations of these averages, where available. [g] Errors are given as the standard deviation in the average of two measurements. [h] Enthalpy measurements for these equilibria were determined using a single injection of ligand into protein as described in methods. [i] The value for $-T\Delta S$ was determined from the relation $\Delta G = \Delta H - T\Delta S$. Errors in $-T\Delta S$ were obtained using standard statistical methods for propagation of errors. For **3** in the absence of **1**, the error in $-T\Delta S$ is given as that for the enthalpy.

filtered NOE studies of ternary complexes comprised of stromelysin, acetohydroxamic acid, and various biaryl ligands, NOEs were observed between the biaryls and the methyl group of **1**.[1] These results suggest that at least part of the additional enthalpic contribution is due to a direct dispersion interaction between the ligands. In contrast to the favorable enthalpic effects, there is an unfavorable change in entropy for the binding of the biaryls in the presence of **1**. This loss in entropy suggests that the biaryl ligands and the protein are more ordered under these conditions. The observed cooperativity between the two ligands is a factor that should be considered when optimizing compounds for binding to nearby sites, since a portion of the binding energy is due to the cooperativity rather than interactions between the ligands and the protein.

Effects of Linking. When the hydroxamate was linked to the biaryl **4** using a two-methylene linker (producing **7**), a gain of 2.6 kcal/mol in binding energy was obtained over the sum

of the individual binding energies of **1** and **4** (Table 2).[18] As shown in Table 2, the increase in binding energy of **7** over its component ligands is due to a 1.6 kcal/mol increase in enthalpy and a 1.0 kcal/mol increase in entropy. Part of this enthalpic gain may be attributed to interactions between the protein and the additional methylene unit in the linker. However, interpreting the energetic differences is complicated by possible changes in the binding modes of the compounds. In the untethered ligands, a large number of conformational states may be accessible which are, on average, less favorable enthalpically but more favorable entropically. In the linked compounds, on

(18) In the case of stromelysin, the intrinsic binding energy of the biaryl ligands is that measured in the absence of acetohydroxamic acid. The additional enthalpic interactions between the biaryl and acetohydroxamic acid in the ternary complexes are irrelevant in the linked compounds. Therefore, the additional gains in binding observed for the untethered compounds due to cooperative effects were not included in the intrinsic binding energies, $\Delta G(A)$ and $\Delta G(B)$.
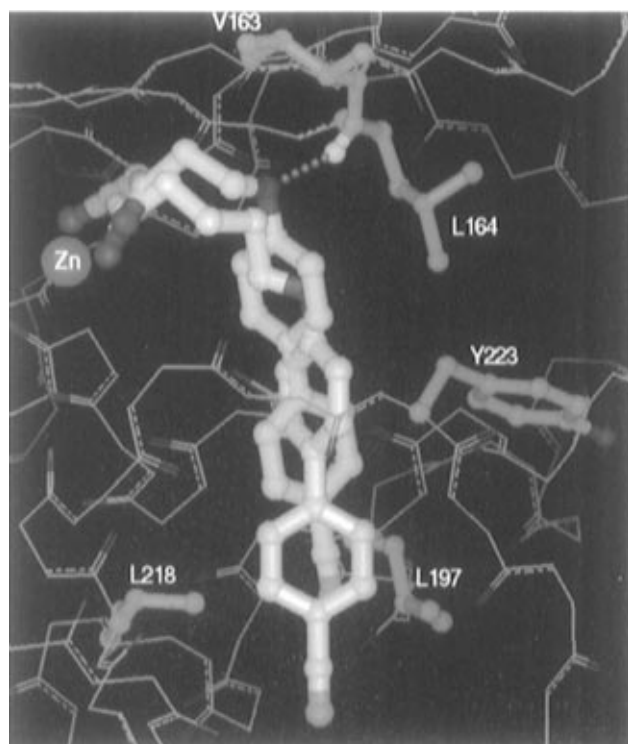
Figure 4. Superposition of compound **7** (yellow carbon atoms) and compound **8** (white carbon atoms) when bound to stromelysin as determined by NMR. The catalytic zinc is shown in magenta. Side chains of stromelysin which make NOE contacts with the ligands are also shown (green carbon atoms). The magenta dotted line indicates the presence of a hydrogen bond between the phenolic oxygen of compound **7** and the backbone amide of Leu[164], which is not present with compound **8**.

the other hand, the number of possible binding conformations are restricted and may be dominated by enthalpically favored conformations.

Compounds containing shorter or longer methylene linkers did not inhibit stromelysin as well as the two methylene-linked compound.[1] When a four methylene linker was incorporated to produce **8**, the free energy of binding is only −6.7 kcal/mol, which is a 0.5 kcal/mol loss over the sum of energies for **1** and **4** (Table 2).[18] On the basis of calorimetric measurements, this loss in binding energy can be attributed primarily to a 1.6 kcal/mol loss in enthalpy compared to its components (or a 3.2 kcal/mol decrease in binding enthalpy compared to compound **7**). As with **7**, a small but favorable gain in entropy (−1.1 kcal/mol) was observed upon linking with a four methylene linker.

Structural Basis for Differences in Binding Energy of Compounds 7 and 8. In order to understand why the four methylene-linked compound (**8**) did not bind to stromelysin as well as **7**, intermolecular NOEs between stromelysin and **8** were obtained from isotope-edited/filtered NMR experiments and compared to those for the complex of stromelysin and **7**. The most striking difference in the NOE data for the two ligands is the increase in the number of NOEs between the biaryl moiety of **8** and residues of stromelysin which lie at the bottom of the S1' subsite (Leu218 and Leu197). A decrease in the number of contacts between the biaryl of **8** and Val163 (which sits above the S1′ pocket) was also observed. These data suggest that the biaryl moiety of **8** sits deeper into the S1′ pocket than the biaryl moiety of **7**. Energy minimizations of a complex of stromelysin and **8** result in more than a 2 Å shift of the biaryl moiety deeper into the pocket than the biaryl moiety of **7** (see Figure 4). This difference in the position of the biaryl alters many of the key interactions with the protein, including the loss of a hydrogen bond between the backbone amide of Leu164 and the phenolic oxygen of **7** that was previously suggested on the basis of the

reduced amide exchange rate of Leu164 in this complex.[1] The loss of this hydrogen bond is supported by the rapid exchange of the backbone amide of Leu164 when stromelysin is complexed to **8**. Both the change in the position of the biaryl and the loss of the hydrogen bond between the phenolic oxygen of the ligand and Leu164 contribute to the more than 3 kcal/mol loss in the enthalpy of binding for compound **8** relative to compound **7**.

In addition to the large enthalpic gains realized upon linking, entropic gains (1.0 kcal/mol) also contribute to the increased binding energy of **7** relative to its component parts. While favorable, however, the increase is much smaller than the potential theoretical gains expected in going from a ternary to a binary complex. Thus, other changes (i.e., in the conformational and vibrational entropy for this class of compounds) must significantly reduce this effect. Furthermore, the two methylene-linked compound (**7**) and the four methylene-linked compound (**8**) have similar entropic contributions to binding. The expected entropic gains from reducing the number of rotatable bonds in **7** vs **8** is difficult to separate from changes in solvation or conformational entropy that are a result of the different binding orientations of these two compounds.

Conclusions

On the basis of enzyme inhibition and calorimetric measurements, we have been able to determine the enthalpic and entropic contributions to the binding energy of several ligands for stromelysin. In addition, NMR studies on stromelysin/inhibitor complexes have provided a structural basis for many of the thermodynamic results. In the case of stromelysin, the observed cooperativity between acetohydroxamic acid (**1**) and the biaryl compounds may be due in part to enthalpic interactions between the two ligands.

Linking acetohydroxamic acid and biaryl **4** with methylene linkers of varying length produced compounds which exhibit a significant increase in binding energy relative to either of the fragment molecules (**1** and **4**). Furthermore, the introduction of a two methylene linker between the two fragments produced a compound whose binding energy was greater than the sum of the binding energies of its component parts. However, compounds with longer linkers were less potent enzyme inhibitors. On the basis of NMR-derived structural information on stromelysin/inhibitor complexes, this loss in potency was attributed to a shift in the position of the biaryl moiety. This result highlights the importance of the length and geometry of the linker in the design of high affinity ligands using SAR by NMR.

The gain in binding energy for the two methylene-linked compound above a simple summing of the binding energies for the untethered compounds was due to both enthalpic and entropic contributions. Although the enthalpic contributions for the different linked compounds was highly dependent on linker length, the differences in entropy were negligible. These results suggest a strategy for linker design in which relatively flexible linkers (e.g., methylene bridges) are first incorporated into the linked ligands. This can be followed by incorporating other linkers with potentially improved properties (e.g., rigid linkers or those with additional functionality) designed on the basis of the three-dimensional structure of the protein complexed with the initial linked compound.

Methods

Samples. All experiments were performed using the catalytic domain of stromelysin (residues 81−256) cloned from human skin fibroblast mRNA, expressed in *Escherichia coli*, and purified as previously described.[19] The experiments were conducted in a buffer solution consisting of 20mM $CaCl_2$, 50mM Tris, and 0.05% sodium azide at pH 7.0.

NMR Spectroscopy. NMR data were collected on Bruker AMX-500, AMX-600, or DMX-500 MHz spectrometers. All NMR data were collected at 32 °C and processed using software written in-house. In all NMR experiments, pulsed field gradients were applied where appropriate as described[20] to afford the suppression of the solvent signal and spectral artifacts. Quadrature detection in the indirectly detected dimensions was accomplished by using the States-TPPI method.[21]

A ^{13}C-separated 3D NOESY-HMQC spectrum[22,23] was recorded on the complex of stromelysin and **8** using a mixing time of 80 ms. Side-chain ^{1}H and ^{13}C assignments for the stromelysin/**8** complex were obtained by comparison of this data set to known assignments for stromelysin/inhibitor complexes. To identify amides that exchanged slowly with solvent, a series of ^{15}N-HSQC spectra were recorded at 32 °C at 2 h intervals after the protein was exchanged into D$_2$O. The first ^{15}N-HSQC spectrum was recorded 2 h after the addition of D$_2$O.

NOEs between the ligand and the protein were obtained from a 3D ^{12}C-filtered, ^{13}C-edited NOESY spectrum with a mixing time of 80 ms. The pulse scheme consisted of a double ^{13}C-filter[24] concatenated with a NOESY-HMQC sequence.[22]

Dissociation constants were obtained by following the changes in chemical shifts of selected residues in the ^{15}N-HSQC spectra as a function of ligand concentration. Data were fit using a single binding site model. A least-squares grid search was performed by varying the values of K_D and the chemical shift of the fully saturated protein. ΔG values were derived from the calculated dissociation constants using the relation: $\Delta G = RT \ln K_D$.

Model of the Stromelysin/8 Complex. A total of 49 intermolecular distance restraints between stromelysin and **8** were derived from the NMR data and given lower and upper bounds of 1.8 and 5.0 Å, respectively. Center-average corrections were used where appropriate. The structure was obtained by manually docking the ligand to the structure of stromelysin in a ternary complex as previously described[1] and performing successive minimizations with the X-PLOR 3.1 program[25] on Silicon Graphics computers. Structures of the complex of stromelysin and **7** and the ternary complexes of stromelysin with acetohydroxamic acid (**1**) and various biaryl ligands were previously described.[1]

Calorimetry. All experiments were performed at 30 °C on a Microcal Omega titration microcalorimeter. Details of the instrument design and data analysis software are given elsewhere.[26] The experiments were conducted in a buffer solution consisting of 20 mM CaCl$_2$, 50 mM Tris, 0.5% DMSO, and 0.05% sodium azide at pH 7.0. DMSO was included in all solutions to solubilize the biaryl ligands. The solutions of titrant and titrate were matched in DMSO concentration. A good match was indicated experimentally by the lack of additional heat being evolved after an excess of titrant had been added. Some measurements were performed with 450 mM acetohydroxamic acid in both the protein and ligand solution.

Isothermal calorimetric titrations of protein (0.7−1.5 mM) into a solution containing the ligands (0.025−0.1 mM) were used to measure the enthalpy and dissociation constants for compounds **3** (in the presence of **1**), **4**, **7**, and **8**. Values for ΔG were obtained from the K_D values derived from these calorimetric titrations. Single injections were used to get estimates of the enthalpic contributions to binding of compounds **1** and **3** (in the absence of **1**) due to their low binding affinities (and their resultant small heats of binding). In these experiments, a total of 200 μL of titrant was added to 1.4 mL of protein in the cell. Initial concentration of protein was 0.2 mM. The concentration of the two titrants, **1** and **3**, was 450 and 5 mM, respectively. The values of K_D determined from our enzymatic assay were used to calculate the fraction of complex formed by the injection of ligand. This value was used to

calculate the reported ΔH and $-T\Delta S$. Errors due to dilution effects were corrected by measuring the heat evolved by separately injecting ligand and protein into buffer solutions.

The reported enthalpies are averages for two different experimental measurements. Error bars correspond to the standard deviation of the measurements. In all of the titrations, the apparent ligand to protein stoichiometry was close to one (range from 0.64 to 1.03).

Enzyme Inhibition Assay. Kinetic determination of stromelysin activity was performed using a fluorescent substrate of the following sequence: Gly-Glu(EDANS)-Gly-Pro-Leu-Gly-Leu-Tyr-Ala-Lys(D-ABCYL)-Gly. The proximity of the DABCYL group quenches the fluorescence of the naphthalene sulfonate moiety (EDANS). Upon stromelysin cleavage of the Gly-Leu bond of the substrate, a 30-fold enhancement of signal intensity is observed (excitation 335 nm, emission 485 nm). A substrate containing these groups has been previously used in the assay of HIV protease activity.[27] Assays were conducted in a total volume of 150 μL in a 96 well microtiter plate at ambient temperatures using a Fluoroskan II plate reader (ICN). All experiments were carried out in 50 mM Tris, 150 mM NaCl, 10 mM CaCl$_2$, pH 7.5 with 0.2% Pluronic F-68, and 4% DMSO. While these conditions differ slightly from the conditions used for NMR or calorimetry, the experimental ΔG values agree well between the various techniques. The concentration of the enzyme used (~5 nM) resulted in about 10% cleavage of the substrate (10 μM) in 1 h in the control wells. The linear rate of enzymatic reaction (<10% substrate cleavage) was calculated using the DeltaSoft software (Biometallics, Inc.). In carrying out experiments designed to test the cooperativity of binding of acetohydroxamic acid and biaryl compounds, the hydroxamic acid concentration was varied from 10^{-3} to 1 M. The usable concentration of the various biaryl compounds was limited by the solubility of the compounds in the assay buffer upon dilution from DMSO stocks.

Analysis of the multiple equilibria in the ternary complex (Figure 1) is greatly simplified because of the low concentration of enzyme, [E], in comparison to the ligands, [A] and [B]. Since a constant, subsaturating level of substrate is used in these experiments, the concentration of the enzyme−substrate complex ([ES]) will be a constant fraction of the enzyme unbound by either ligand. Using these assumptions and applying material balance to the enzyme concentration results in an expression which relates the concentration of the enzyme/substrate complex, the concentrations of the ligands, and the dissociation constants:

$$[ES] = ([E]_0 [S])/([S] + K_m(1 + [A]_0/K_1 + [B]_0/K_2 +$$
$$[A]_0[B]_0/\omega K_1 K_2)) \quad (2)$$

where $[E]_0$ is the initial enzyme concentration, [S] is the substrate concentration, K_m is the equilibrium dissociation constant for the enzyme and the substrate, $[A]_0$ and $[B]_0$ are the concentrations of ligands A and B, ω is the cooperativity factor for the binding of the ligands, and K_1 and K_2 are the dissociation constants for A and B, respectively (see Figure 1). Thus, the experimentally determined rate of peptide cleavage, $V([A],[B]) = k_{cat}[ES]$, measured as a function of the concentrations of the two ligands can be used to determine the equilibrium constants. The enzymatic rates were fit using a grid search over possible values of the equilibrium constants. This yields a set of equilibrium constants that is the best fit of the values of V measured as a function of both ligand concentrations.

For compounds **1**, **3**, and **4**, values for ΔG were obtained from the dissociation constants given in eq 2 and Figure 1 using the relations $\Delta G = RT \ln K_{1,2}$ and $\Delta G = RT \ln (\omega K_2)$, corresponding to the absence and presence of acetohydroxamic acid, respectively. The inhibition of stromelysin by compounds **7** and **8** were determined using the same substrate and buffer conditions as described above, and the calculated IC$_{50}$ value was assumed to be equal to the K_I. The values for ΔG were derived from the relation $\Delta G = RT \ln K_I$.

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