Analysis of the Binding of Hydroxamic Acid and Carboxylic Acid Inhibitors to the Stromelysin-1 (Matrix Metalloproteinase-3) Catalytic Domain by Isothermal Titration Calorimetry

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ABSTRACT: Matrix metalloproteinases (MMPs) are implicated in diseases such as arthritis and cancer. Among these enzymes, stromelysin-1 can also activate the proenzymes of other MMPs, making it an attractive target for pharmaceutical design. Isothermal titration calorimetry (ITC) was used to analyze the binding of three inhibitors to the stromelysin catalytic domain (SCD). One inhibitor (Galardin) uses a hydroxamic acid group ($pK_a \cong 8.7$) to bind the active site zinc; the others (PD180557 and PD166793) use a carboxylic acid group ($pK_a \cong 4.7$). Binding affinity increased dramatically as the pH was decreased over the range 5.5−7.5. Experiments carried out at pH 6.7 in several different buffers revealed that approximately one and two protons are transferred to the enzyme−inhibitor complexes for the hydroxamic and carboxylic acid inhibitors, respectively. This suggests that both classes of inhibitors bind in the protonated state, and that one amino acid residue of the enzyme also becomes protonated upon binding. Similar experiments carried out with the H224N mutant gave strong evidence that this residue is histidine 224. ΔG, ΔH, ΔS, and ΔC_p were determined for the three inhibitors at pH 6.7, and ΔC_p was used to obtain estimates of the solvational, translational, and conformational components of the entropy term. The results suggest that: (1) a polar group at the P1 position can contribute a large favorable enthalpy, (2) a hydrophobic group at P2′ can contribute a favorable entropy of desolvation, and (3) P1′ substituents of certain sizes may trigger an entropically unfavorable conformational change in the enzyme upon binding. These findings illustrate the value of complete thermodynamic profiles generated by ITC in discovering binding interactions that might go undetected when relying on binding affinities alone.

Matrix metalloproteinases (MMPs)[1] play essential roles in tissue remodeling and the healing of wounds (*1, 2*). However, inappropriate expression or localization of MMPs can play a role in many pathological processes, including tumor growth and metastasis (*3, 4*), multiple sclerosis (*5, 6*), ulceration (*7*), osteoporosis (*8*), periodontal disease (*9*), and psoriasis (*10*). (MMPs have been extensively reviewed in refs *11*−*15*.) Among the MMPs, stromelysin-1 (MMP-3) is directly involved in rheumatoid and osteoarthritis (*16, 17*) and can also cleave the proenzymes of several other MMPs to their active forms (*15, 16*). Stromelysin thus provides an excellent therapeutic target for a broad range of diseases and has consequently received a great deal of attention in recent years. (Over 200 papers concerning stromelysin were published in 1998 alone.) Several classes of both peptidic and nonpeptidic stromelysin inhibitors have been developed, many with K_i or IC_{50} values in the nanomolar range (*18*−*30*). Clinical trials have been initiated for several of them (*15, 27, 31*).

One of the most clinically advanced MMP inhibitors, Galardin (Glycomed GM-6001), has been shown to be effective in the treatment of corneal ulcers when applied topically (*31*). However, this compound has poor oral bioavailability, and further clinical development has not been reported. A major reason for this problem is that Galardin, like the majority of potent MMP inhibitors developed to date, uses a hydroxamic acid group to bind to the catalytic zinc atom at the active site. Hydroxamic acids undergo rapid metabolism in the liver and have limited aqueous solubility. Although hydroxamic acids are more effective than, for example, carboxylic acids at binding to the active-site zinc (*25*), inhibitors that use a different type of zinc-binding group might have longer half-lives and/or better solubility and thus be more effective in vivo. Also, like many other potent MMP inhibitors developed to date, Galardin is peptidic, making it susceptible to enzymatic hydrolysis in vivo.

X-ray and NMR structures have been published for complexes of several inhibitors with the stromelysin catalytic domain (SCD) (*20, 22, 27, 28, 32*−*35*). In addition to chelating the catalytic zinc, potent inhibitors also form at least one or two hydrogen bonds to the enzyme's peptide

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[1] Abbreviations used: MMP (matrix metalloproteinase), ITC (isothermal titration calorimetry), SCD (stromelysin catalytic domain), PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)), MES (2-(*N*-morpholino)ethane sulfonic acid), BES (*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid), HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)), DMSO (dimethyl sulfoxide).

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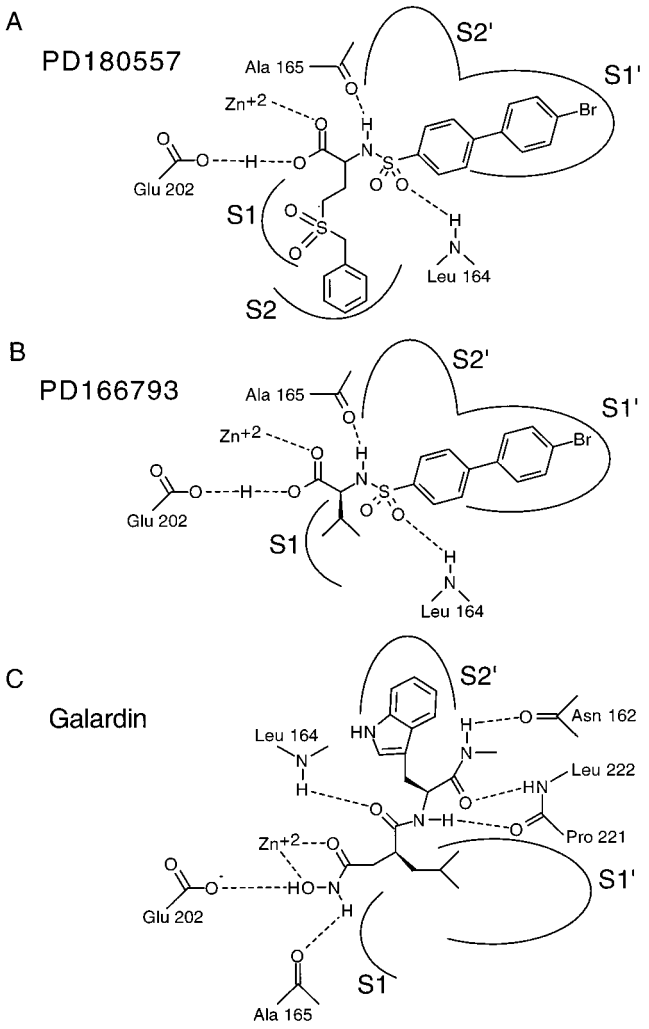

FIGURE 1: Structures of stromelysin inhibitors and their proposed interactions with the enzyme: (A) PD180557; (B) PD166793; (C) Galardin. Panels A and B are based on structures of complexes with similar inhibitors (*35*, *75*) and molecular modeling (*36*); panel C is based on Levy et al. (*25*) and Dhanaraj et al. (*34*). Hydrogen bonds and electrostatic interactions are denoted by dashed lines. In this model, the S2′ sites are unoccupied by PD180557 and PD166793, the S1 site is unoccupied by Galardin, and the S2 site is unoccupied by PD166793 and Galardin.

backbone. Of the "specificity pockets" to which substrate side chains bind, the S1′ subsite appears to be the most important for determining selectivity of inhibition vs other MMPs. The S1′ pocket (actually a "channel" that is open to solvent at the bottom) is lined with hydrophobic residues and is deep in stromelysin but shallower in many other MMPs. The other stromelysin binding sites (S1, S2, S2′, etc.) are more exposed to solvent (see Figure 2A in (*22*) and Figure 5 in (*36*)). Exploitation of hydrophobic interactions with the S1′ pocket has thus been a major goal in the design of stromelysin inhibitors.

In most cases, quantitative comparisons of the potencies of enzyme inhibitors rely solely upon dissociation constants (K_d or K_i) or IC_{50} values obtained from enzyme kinetics or other methods. While these data are very useful, in some cases, an enthalpically favorable interaction, such as a hydrogen bond, can be masked by an unfavorable entropy change if the conformational flexibility of the inhibitor and/or enzyme is greatly decreased when this interaction occurs. This can result in small or undetectable differences in the

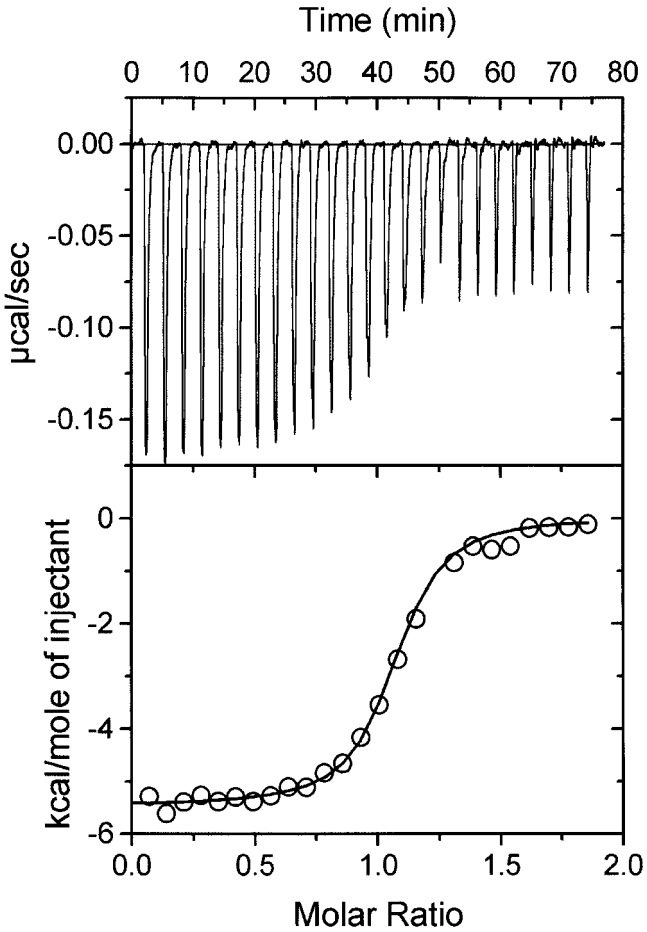

FIGURE 2: Representative data from an ITC experiment. The cell (1.34 mL) contained 10.0 μM stromelysin catalytic domain in 50 mM PIPES, 10 mM $CaCl_2$, 1% (v/v) DMSO, pH 6.7 at 22 °C. The syringe contained 100 μM PD166793 in the same buffer. Twenty-five injections of 9.5 μL apiece were made at 180 s intervals. Top panel: raw data with integration baseline shown. Bottom panel: data after peak integration, subtraction of blank titration data (not shown), and concentration normalization. The solid line is the fit to a single binding site model (*49*, *50*). Results of the curve fitting are shown in Table 1.

dissociation constants of different inhibitors, even though the thermodynamics of binding may be very different. Isothermal titration calorimetry (ITC), however, is ideally suited for this type of analysis (*37*). Under the appropriate conditions, both the dissociation constant (and hence ΔG) and ΔH can be determined directly, and ΔS can be calculated from these values. Additionally, the stoichiometry of binding (N) is determined. This can be useful in determining, for instance, the number of competent active sites in a dimeric enzyme, or for detecting allosteric or cooperative binding sites.

Many binding interactions involve the transfer of protons to or from the complex. This can have enormous effects on the binding affinity, often resulting in very steep pH-dependence (*38*). The degree of proton transfer can be determined by ITC by performing experiments in buffers with different enthalpies of protonation (*38*−*40*). This can give clues as to the chemical mechanism of binding that are difficult to obtain in other ways. For example, the protonation state of a chemical group cannot be determined with certainty from X-ray crystal structures (due to the small size of protons), and only rarely in NMR solution structures (because of exchange with solvent). pK_a values for these groups can

Table 1: Binding Data for Stromelysin Inhibitors Obtained by Isothermal Titration Calorimetry

inhibitor	pH	T (°C)	buffer	ΔG^a (kcal/mol)	$\Delta H_{apparent}{}^a$ (kcal/mol)	K_d (nM)[a]	$N^{a,b}$
PD180557	5.5	22	MES	−11.6 (0.2)	−9.32 (0.06)	2.7 (1.0)	0.972 (0.003)
PD180557	6.0	22	MES	−11.2 (0.2)	−11.0 (0.1)	4.5 (1.5)	0.964 (0.004)
PD180557	6.7	22	MES	−10.1 (0.1)	−10.0 (0.1)	35.5 (7.3)	0.989 (0.007)
PD180557	6.7	22	PIPES	−10.1 (0.1)	−12.8 (0.2)	31.3 (7.1)	0.991 (0.007)
PD180557	6.7	22	HEPES	−10.1 (0.1)	−7.84 (0.11)	30.4 (7.7)	0.985 (0.008)
PD180557	6.7	22	BES	−10.1 (0.1)	−6.06 (0.08)	34.7 (8.3)	1.04 (0.01)
PD180557	7.5	22	BES	−8.65 (0.10)	−5.61 (0.14)	394 (70)	1.06 (0.02)
PD180557	6.7	17	PIPES	−9.95 (0.12)	−12.2 (0.1)	32.2 (6.9)	1.06 (0.01)
PD180557	6.7	12	PIPES	−10.1 (0.2)	−11.2 (0.2)	18.1 (5.3)	1.01 (0.01)
PD180557	6.7	7	PIPES	−10.1 (0.2)	−11.3 (0.1)	14.0 (4.3)	1.09 (0.01)
PD166793	5.5	22	MES	−12.7 (1.2)	−3.27 (0.06)	0.40 (0.84)	1.01 (0.01)
PD166793	6.0	22	MES	−11.3 (0.3)	−4.43 (0.05)	4.4 (2.2)	1.04 (0.01)
PD166793	6.7	22	MES	−9.74 (0.17)	−3.60 (0.07)	61.7 (17.6)	1.07 (0.01)
PD166793	6.7	22	PIPES	−9.48 (0.10)	−5.46 (0.07)	96.1 (16.9)	1.04 (0.01)
PD166793	6.7	22	HEPES	−9.65 (0.22)	−1.54 (0.04)	71.9 (27.3)	1.12 (0.02)
PD166793	7.5	22	PIPES	−7.74 (0.20)	−4.88 (0.59)	1860 (650)	0.825 (0.069)
PD166793	6.7	17	PIPES	−9.61 (0.14)	−5.30 (0.08)	57.3 (13.7)	0.998 (0.010)
PD166793	6.7	12	PIPES	−9.61 (0.25)	−4.44 (0.11)	43.3 (19.0)	1.04 (0.02)
PD166793	6.7	7	PIPES	−9.69 (0.25)	−4.64 (0.11)	27.6 (12.6)	0.994 (0.013)
Galardin	5.5	22	MES	−12.8 (1.2)	−6.24 (0.09)	0.32 (0.68)	0.881 (0.005)
Galardin	6.0	22	MES	−12.4 (0.9)	−5.85 (0.12)	0.60 (0.88)	1.02 (0.01)
Galardin	6.7	22	MES	−11.2 (0.3)	−5.79 (0.09)	5.2 (2.7)	1.02 (0.01)
Galardin	6.7	22	PIPES	−11.0 (0.2)	−6.94 (0.06)	6.7 (2.1)	1.05 (0.004)
Galardin	6.7	22	HEPES	−11.2(0.2)	−5.43 (0.04)	5.1 (1.5)	1.03 (0.003)
Galardin	6.7	22	BES	−11.2 (0.4)	−4.38 (0.07)	5.2 (3.3)	1.04 (0.01)
Galardin	7.5	22	BES	−9.96 (0.22)	−3.58 (0.10)	41.8 (15.5)	1.11 (0.02)
Galardin	6.7	17	PIPES	−10.9 (0.2)	−6.43 (0.08)	6.0 (2.4)	1.00 (0.01)
Galardin	6.7	12	PIPES	−11.1 (0.2)	−5.63 (0.05)	3.4 (1.3)	0.998 (0.004)
Galardin	6.7	7	PIPES	−11.0 (0.5)	−5.27 (0.08)	2.8 (2.6)	0.968 (0.007)

[a] Values in parentheses are the standard deviations. These were obtained directly from the fitting routine for ΔH, N, and K_{assoc}. Standard deviations for the other parameters were calculated as described in Casella and Berger (*78*). [b] Stoichiometry of the enzyme−inhibitor complex.

sometimes be determined as well, by analyzing the pH-dependence of proton transfer by ITC (*38*, *41*).

We have used ITC to examine the binding of three inhibitors (Figure 1) to the stromelysin catalytic domain. One inhibitor (Galardin) uses a hydroxamic acid group and two (PD180557 and PD166793) use carboxylic acid groups to chelate the active-site zinc. We report here on the relative contributions of enthalpic and entropic forces to the binding affinities and describe insights into the mechanism of binding obtained from this analysis.

MATERIALS AND METHODS

Enzyme Preparation. Recombinant SCD, consisting of residues 83−256, was expressed and purified in *E. coli* as described previously (*42*).[2] The enzyme was stored at a concentration of 120−130 μM in 10 mM PIPES, 10 mM $CaCl_2$, pH 7.5 at 4 °C. Purity was ≥99% as determined by loading 20, 2, and 0.2 μg of enzyme onto a denaturing gel and staining with Coomassie Blue. Protein concentrations were measured using an extinction coefficient at 280 nm of 28 790 (±760) M^{-1} cm^{-1} (average of five determinations), determined by the method of Gill and von Hippel (*43*). This is in excellent agreement with the value of 28 460 M^{-1} cm^{-1} determined previously (*44*) using the same method.

Construction, expression, and purification of the H224N mutant has been described (*45*).

Sources of Inhibitors. Galardin (*46*) was purchased from AMS Scientific (Concord, CA). PD180557 and PD166793 were synthesized as described previously (*36*, *47*, *48*).

Isothermal Titration Calorimetry. Titrations were performed with a VP−ITC microcalorimeter (MicroCal, Inc., Northampton, MA). The stirred cell contained enzyme at 5.00 or 10.0 μM and the syringe contained 50.0 or 100 μM inhibitor. After an initial injection of 0.5 μL (which was not used in data fitting), 25 injections of 9.5 μL each were performed at 180-second intervals. Blank titrations of inhibitor into buffer were also performed in order to correct for heats of dilution and mixing. Data were fit to a single binding site model (*49*, *50*) using Origin (version 5.0, MicroCal, Inc.).[3] Experiments were performed in 50 mM MES, (pH 5.5, 6.0, and 6.7), BES (pH 6.7 and 7.5), PIPES (pH 6.7 and 7.5), or HEPES (pH 6.7), 10 mM $CaCl_2$, at 7, 12, 17, or 22 °C (experimental temperatures were within 0.02° of the nominal values). All solutions contained 1% (v/v) DMSO. Control experiments indicated that DMSO concentrations as high as 3% had no effect on the thermodynamic parameters obtained (data not shown).

RESULTS

Binding of Inhibitors is Strongly pH-Dependent. The pH optimum for stromelysin activity is approximately 6.0, an unusually low value for an MMP (*42*, *51*). To investigate the pH-dependence of inhibitor binding, inhibitors were titrated into solutions of the SCD at pH 5.5, 6.0, 6.7, and 7.5. (A representative ITC curve is shown in Figure 2, and the thermodynamic parameters for all of the wild-type experiments are listed in Table 1.) For all three inhibitors,

[2] The numbering used in ref *42* included the 17-residue signal sequence and was, therefore, listed as 100−273. Since most publications currently assign residue numbers to the protein with the signal sequence cleaved away, we will use the latter nomenclature.

[3] Some older curve-fitting programs, including earlier versions of Origin, report the "asymptotic" standard errors for the fitted parameters. This can result in underestimates of these values (*76*). Version 5.0 of Origin uses an improved method in which elements of the variance−covariance matrix are incorporated into these calculations.

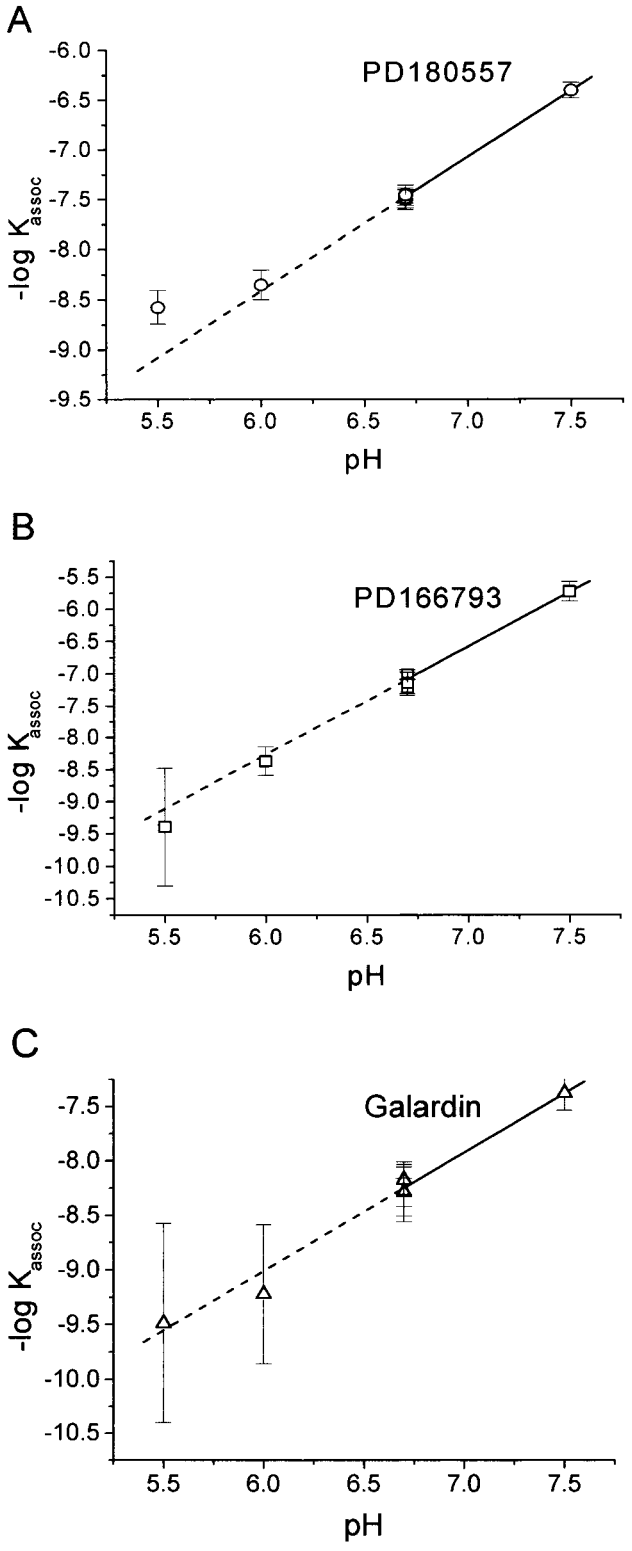

FIGURE 3: Binding affinity of carboxylic and hydroxamic acid inhibitors for the SCD increases as the pH is lowered between 7.5 and 5.5. Values of $-\log K_{assoc}$ are plotted vs pH; linear regressions for the pH range 6.0−7.5 are shown with a solid line and are extrapolated to the low-pH range with a dashed line. Titrations were carried out at 22 °C in MES buffer at pH 5.5, 6.0, and 6.7, in BES, PIPES, and HEPES at pH 6.7, and in BES or PIPES at pH 7.5 as described in the text: (A) PD180557; (B) PD166793; (C) Galardin.

binding affinities increased as the pH was lowered over the range 7.5−5.5. The values of $-\log K_{assoc}$ are plotted vs pH in Figure 3. Slopes of the plots of $-\log K_{assoc}$ vs pH over the pH range 6.7−7.5 were 1.34 (±0.03), 1.69 (±0.20), and

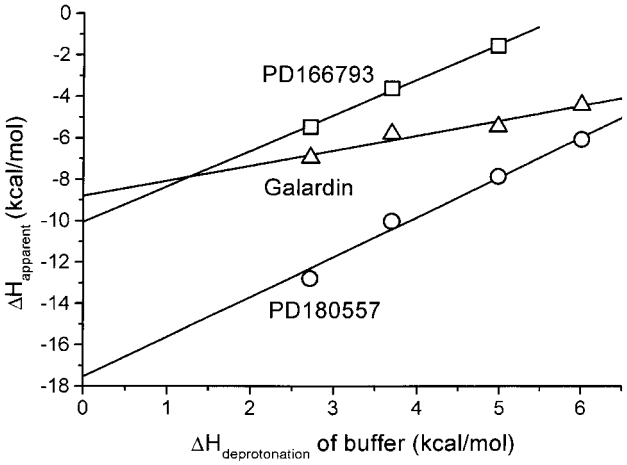

FIGURE 4: Protons are transferred to the enzyme−inhibitor complex during the binding of carboxylic and hydroxamic acid inhibitors to the stromelysin catalytic domain. Titrations with PD180557 (○), PD166793 (□), and Galardin (△) were performed at 22 °C, pH 6.7, in PIPES, MES, HEPES, and BES buffers as described in the text. Symbols are larger than the error bars for the measurements. Values for the ΔH and ΔC_p of deprotonation of the buffers at 25 °C were obtained from Fukada et al. (*77*) and used to calculate the $\Delta H_{deprotonation}$ values at 22°: PIPES: +2.72 kcal/mol; MES: +3.70 kcal/mol; HEPES: +4.99 kcal/mol; BES: +6.02 kcal/mol. The *y*-intercept for each plot of $\Delta H_{apparent}$ vs $\Delta H_{deprotonation}$ gives the buffer-corrected enthalpy change upon binding (Table 2). The sign and magnitude of the slope give the direction (from the buffer to the enzyme−inhibitor complex in these cases) and average number of protons transferred, respectively. The slopes were: 1.91 (±0.15) for PD180557, 1.71 (±0.07) for PD166793, and 0.71 (±0.11) for Galardin.

1.08 (±0.09) for PD180557, PD166793, and Galardin, respectively. (These slopes did not incorporate data obtained at lower pH values due to the large standard error in these points and the apparent curvature observed in the PD180557 plot.) This suggests that one to two protons are transferred to the enzyme−inhibitor complexes during binding (*40*).

Protons are Transferred to the Enzyme−Inhibitor Complexes during Binding. The rather dramatic pH-dependence of the binding affinities suggested that protons are transferred to or from the enzyme−inhibitor complex during binding. The extent of proton transfer during a binding event can be determined by measuring the observed ΔH of binding in buffers that have different enthalpies of protonation (*38*−*40*):

$$\Delta H_{apparent} = \Delta H_{corrected} + n\Delta H_{ionization}$$

where $\Delta H_{corrected}$ is the actual heat of binding at the pH employed, independent of buffer ionization effects, and the magnitude and sign of n give the net number of protons transferred during binding and the direction of transfer, respectively. All of the inhibitors tested, as well as the stromelysin active site, have ionizable groups that could undergo changes in protonation state upon binding. Therefore, we measured the degree of proton transfer during the binding of each inhibitor. Titrations were carried out at 22 °C, pH 6.7, in four different buffers for PD180557 and Galardin and in three buffers for PD166793 (Table 1). (The apparent ΔH for PD166793 was too small to be measured accurately in BES buffer.) The results are plotted in Figure 4 and the thermodynamic parameters are given in Table 2.

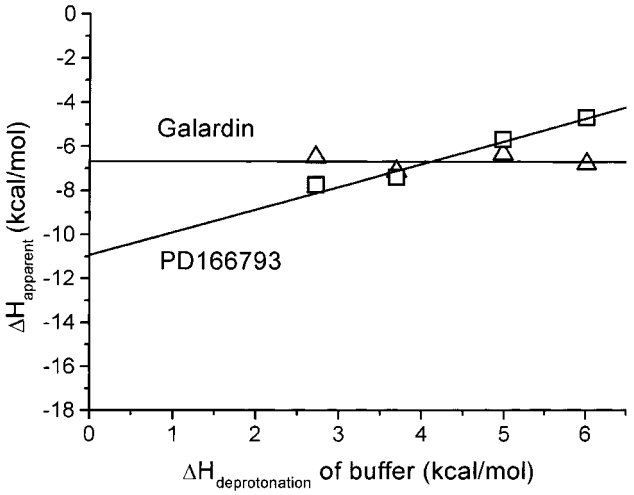

FIGURE 5: Protonation of histidine 224 accounts for one of the protons transferred during binding of stromelysin inhibitors. Titrations were carried out at pH 6.7, 22 °C with the H224N mutant in each of the four buffers described in Figure 4. Symbols are larger than the error bars for the measurements. For comparison, data is plotted at the same scale as used in Figure 4. The plots of $\Delta H_{apparent}$ vs $\Delta H_{deprotonation}$ of the buffers gave slopes of 1.03 (±0.15) for PD166793 (□) and −0.006 (±0.155) for Galardin (△).

For all three inhibitors, the observed ΔH values changed linearly as the $\Delta H_{deprotonation}$ of the buffer was varied. For the carboxylic acid inhibitors, PD180557 and PD166793, the slopes of the lines were 1.91 (±0.15) and 1.71 (±0.07), respectively, indicating that a net average of approximately two protons are transferred to the enzyme−inhibitor complexes during binding at pH 6.7. For the hydroxamic acid inhibitor Galardin, a net average of 0.71 (±0.11) protons were transferred, indicating that approximately one proton is transferred to the complex with Galardin at this pH. The simplest interpretation of these results is that the net uptake of one proton (in the case of the hydroxamic acid inhibitor) reflects a change in the protonation state of a single functional group and that the net uptake of two protons (for the carboxylic acid inhibitors) reflects the protonation of the same functional group plus one additional group.

For reasons discussed below, we reasoned that protonation of histidine 224 might account for one of these proton transfers. Therefore, we performed titrations of the H224N mutant stromelysin catalytic domain with Galardin and PD166793 in each of the four buffers at pH 6.7. The results are plotted in Figure 5 and listed in Table 3. For the carboxylic acid inhibitor PD166793, a net of 1.03 (±0.15) protons were transferred to the enzyme−inhibitor complex during binding at pH 6.7. For the hydroxamic acid inhibitor Galardin, −0.006 (±0.155) protons were transferred. Carboxylic and hydroxamic acids have very different acidities and exist in different protonation states in solution at neutral pH. Therefore, the transfer of a single proton to the PD166793−H224N complex most likely reflects protonation of the carboxylic acid group during binding (see Discussion). Comparison of the H224N results to those obtained with the wild-type enzyme strongly suggests that histidine 224 becomes protonated during binding at pH 6.7.

To determine whether histidine 224 contributes to the pH-dependence of inhibitor binding, the dissociation constants for binding of PD166793 to the H224N mutant were measured at 22 °C in PIPES buffer at pH 6.7 and 7.5. For H224N, the K_d values were 48.1 (±3.6) and 117 (±30) nM at pH 6.7 and 7.5, respectively, a difference of 2.4-fold. For the wild-type enzyme, the corresponding values were 76.6 (±17.7) and 1860 (±650) nM, respectively, a 24-fold difference. Protonation of histidine 224 thus appears to be a major source of the pH-dependence of inhibitor binding.

Table 2: Thermodynamic Parameters for Binding of Inhibitors to the Wild-Type Stromelysin Catalytic Domain at pH 6.7, 22 °C

inhibitor	PD180557	PD166793	Galardin
K_d (nM)[a]	33.0 (2.7)	76.6 (17.7)	5.5 (2.1)
ΔG (kcal/mol)[a]	−10.1 (0.1)	−9.62 (0.13)	−11.6 (0.2)
ΔH (kcal/mol)[b]	−17.4 (0.8)	−10.1 (0.3)	−8.82 (0.52)
$-T\Delta S$ (kcal/mol)	+7.34 (0.77)	+0.43 (0.33)	−2.34 (0.54)
protons transferred[c]	1.91 (0.15)	1.71 (0.07)	0.71 (0.11)
ΔC_p (cal/mol K)	−106 (30)	−66.1 (23.4)	−118 (11)
ΔS_{total} (cal/mol K)	−24.9 (2.6)	−1.46 (1.12)	+7.93 (1.83)
ΔS_{solv} (cal/mol K)[d]	+28.2 (8.0)	+17.6 (6.2)	+31.4 (2.9)
ΔS_{other} (cal/mol K)[e]	−42.9 (8.4)	−8.86 (6.30)	−13.3 (3.4)

[a] Average values from titrations carried out in three (PD166793) or four (PD180557, Galardin) buffers. Standard deviations were calculated as described in Casella and Berger (*78*). [b] Buffer-corrected values obtained from the *y*-intercepts of data in Figure 4. [c] Net number of protons transferred to the enzyme−inhibitor complex upon binding, obtained from the slopes of data in Figure 4. [d] Estimated contribution to ΔS from changes in solvation (*39*, *55*). [e] Estimated contribution to ΔS from changes in conformational rigidity (see text).

Table 3: Thermodynamic Parameters for Binding of Inhibitors to the H224N Mutant Stromelysin Catalytic Domain at pH 6.7, 22 °C

Inhibitor	PD166793	Galardin
K_d (nM)[a]	48.1 (3.6)	1.21 (5.26)
ΔG (kcal/mol)[a]	−9.89 (0.10)	−12.2 (1.6)
ΔH (kcal/mol)[b]	−10.9 (0.7)	−6.67 (0.76)
$-T\Delta S$ (kcal/mol)	−1.05 (0.67)	−5.54 (1.82)
protons transferred[c]	1.03 (0.15)	−0.006 (0.155)

[a] Average values from titrations carried out in four buffers. Standard deviations were calculated as described in Casella and Berger (*78*). [b] Buffer-corrected values obtained from the y-intercepts of data in Figure 5. [c] Net number of protons transferred to the enzyme−inhibitor complex upon binding, obtained from the slopes of data in Figure 5.

Heat Capacity Changes upon Binding. The change in heat capacity (ΔC_p; i.e., $d\Delta H/dT$) provides a measure of changes in the hydration of a system (*52*, *53*), and thus can provide information about the degree of solvent expulsion that occurs during binding. ΔC_p values were determined in PIPES buffer at pH 6.7 by measuring the ΔH of binding for each inhibitor at four temperatures (Table 1; Figure 6) and assuming that ΔC_p does not vary with temperature within this range. Differential scanning calorimetry experiments indicated that the isolated stromelysin catalytic domain undergoes a complex, non two-state unfolding transition between about 30 and 70 °C at pH 6.7 (data not shown). Therefore, we restricted our experiments to temperatures between 7 and 22 °C.

ΔC_p values of −106 (±30), −66.1 (±23.4), and −118 (±11) cal/mol K were obtained for PD180557, PD166793, and Galardin, respectively. The negative signs of the ΔC_p values suggest that hydrophobic interactions make significant contributions to the binding of these inhibitors to the SCD (*54*). The ΔC_p values were used to obtain estimates of the solvational and other components of the ΔS terms (see Discussion).

Structure−Activity Relationships Determined by ITC. The thermodynamic parameters for binding of the three inhibitors

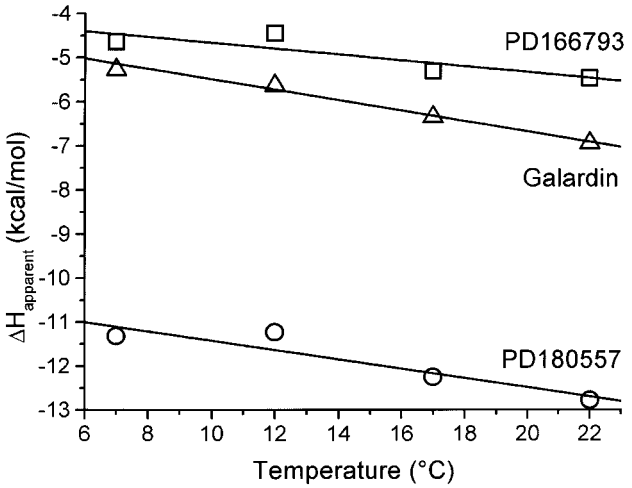

FIGURE 6: Heat capacity changes (ΔC_p) for binding of three inhibitors to the stromelysin catalytic domain. Titrations were carried out at 7, 12, 17, and 22 °C in PIPES buffer at pH 6.7 as described in the text. Symbols are larger than the error bars for the measurements. The slope of the plot of observed ΔH vs temperature provides the ΔC_p (Table 2). ○: PD180557; □: PD166793; △: Galardin.

at pH 6.7 and 22 °C are listed in Table 2. While Galardin had the highest affinity, the relative contributions of enthalpic and entropic factors to the total free energies of binding were very different for the three inhibitors (see below).

DISCUSSION

The binding of three inhibitors (Figure 1) to the stromelysin catalytic domain was analyzed by ITC. One inhibitor (Galardin) contains a hydroxamic acid group for chelating the active-site zinc atom, while the others (PD180557 and PD166793) have carboxylic acid groups. The binding affinities of all three inhibitors changed as the pH was varied (Table 1, Figure 3), with higher observed affinities at lower pH values. In light of this observation, we note that the potencies of stromelysin inhibitors should be compared at the same pH, as the dissociation constants can vary by more than an order of magnitude per pH unit (Table 1).

NMR and X-ray crystal structures of complexes between the SCD and hydroxamic and carboxylic acid inhibitors provide insight into the effects of pH upon binding and the likely sites of protonation. For example, it has been suggested that a hydrogen bond forms between the hydroxyl proton of hydroxamic acid inhibitors and the carboxylate of glutamic acid 202 of the enzyme (*34, 35*). The position of the proton is consistent with the higher pK_as of hydroxamic acids compared to carboxylic acids. The pK_a of acetohydroxamic acid is 8.7. In contrast, the pK_a of acetic acid is 4.7, and structural data suggest that a proton is either shared between the carboxylate of a carboxylic acid inhibitor and glutamic acid 202 or may even reside completely on the enzyme (*36, 75*). In the absence of strong inductive effects that might change the pK_as, hydroxamic acid inhibitors are essentially completely protonated in solution near neutral pH, while carboxylic acids are essentially deprotonated. Subsequent titration experiments were, therefore, carried out at pH 6.7, as this is midway between the pK_as of these two classes of inhibitors. If the proposed structural models are correct, hydroxamic acid inhibitors should not undergo protonation upon binding at pH 6.7, while the carboxylic acid inhibitors are expected to acquire one proton at this pH.

To address this question, we conducted ITC experiments in buffers with various heats of ionization. The apparent ΔH of binding for each of the inhibitors changed in a linear fashion with the $\Delta H_{deprotonation}$ of the buffers (Figure 4), indicating that proton transfer occurs during binding (*38*−*40*). The sign and magnitudes of the slopes of these plots indicated that, at pH 6.7, approximately two protons are transferred to the enzyme−inhibitor complex when a carboxylic acid is used as the zinc-chelating group. However, approximately one proton was transferred to the complex with the hydroxamic acid inhibitor (Table 2). The nonintegral values obtained for the number of protons transferred suggest that one of these groups has a pK_a in the vicinity of 6.7.

For the carboxylic acid inhibitors, an explanation that would account for the transfer of one of the two protons is that the inhibitor's carboxylate (or glutamic acid 202) becomes protonated during binding, as suggested by the structural data discussed above. Protonation of this group might be driven by the potential to form an additional hydrogen bond between the inhibitor and glutamic acid 202 as well as by the need to reduce electrostatic repulsion between the two ionized carboxylates. (Glutamic acid 202 is almost certainly ionized in the apoenzyme, as this residue is believed to polarize the zinc-bound hydrolytic water molecule for nucleophilic attack on the substrate (*33*).) The hydroxamic acid groups of inhibitors such as Galardin appear to form hydrogen bonds to the backbone carbonyl of alanine 165 and the carboxylate group of glutamic acid 202 (Figure 1).

Since these inhibitors do not contain other titratable groups, at least one amino acid of the enzyme must undergo protonation as well. One possibility is histidine 224, located near the bottom of the deep S1′ subsite. Holman et al. (*51*) have shown, through mutagenesis of this residue to glutamine, that histidine 224 is responsible for the unusually low pH optimum (5.75−6.25) observed for stromelysin activity. They propose that protonation of histidine 224 allows it to form a hydrogen bond during the binding of substrates and thus enhances activity.

To test this, we measured the ΔH for binding of PD166793 and Galardin to the histidine 224 to asparagine (H224N) mutant stromelysin catalytic domain (*45*) in four different buffers (Figure 5). The results clearly indicated that mutation of histidine 224 to a nonionizable amino acid decreased the number of protons transferred to either class of inhibitors by approximately one, resulting in a net transfer of zero protons for the hydroxamic acid inhibitor and one proton for the carboxylic acid (Tables 2 and 3). Therefore, we conclude that histidine 224 is the amino acid residue of the enzyme that becomes protonated during binding.

Since the observed ΔH of binding varied according to the $\Delta H_{deprotonation}$ of the buffer (Figure 4), it was necessary to correct the observed ΔH values for buffer ionization effects. This was done by extrapolating the plots in Figures 4 and 5 to the *y*-intercepts. The corrected thermodynamic parameters for binding of the three inhibitors to the wild-type enzyme at pH 6.7, 22 °C are listed in Table 2. Results for the H224N mutant are listed in Table 3.

Of the three inhibitors, Galardin had the lowest K_d for the wild-type SCD, followed by PD180557 and PD166793.

However, examination of the enthalpic and entropic components of the binding energy reveals significant differences in the modes of binding. While 76% of the binding energy for Galardin was contributed by a favorable enthalpy term [−8.82 (±0.52) kcal/mol], the enthalpy terms for the other inhibitors were more negative (i.e., more favorable): −10.1 (±0.3) and −17.4 (±0.8) kcal/mol for PD166793 and PD180557, respectively. Furthermore, the entropic contributions to binding were unfavorable for the latter two inhibitors.

In general, negative contributions to ΔH reflect the formation of hydrogen bonds, van der Waals forces, and/or electrostatic interactions. A number of factors contribute to the ΔS term:

$$\Delta S_{total} = \Delta S_{solv} + \Delta S_{trans} + \Delta S_{other}$$

where ΔS_{solv} refers to changes in the degree of hydration of the enzyme and inhibitor upon binding, ΔS_{trans} reflects the loss of translational and rotational degrees of freedom upon formation of the complex (*39, 55, 56*), and ΔS_{other} includes changes in rotational and vibrational entropy due to the loss of conformational flexibility in the enzyme and inhibitor as well as any entropic effects which might result from changes in ionization state. Favorable (positive) ΔS values are usually dominated by the release of ordered water molecules into bulk solvent and are reflected in the ΔS_{solv} term. Negative contributions to ΔS come from losses of translational, rotational, and vibrational degrees of freedom that reduce the conformational flexibility of the ligand and/or the receptor upon binding and are reflected in the ΔS_{trans} and ΔS_{other} terms.

The signs and magnitudes of ΔS_{total} varied considerably among these inhibitors: Galardin had a positive value, PD166793 had a slightly negative value, and PD180557 had a large negative value (Table 2). The major contribution to the difference between the ΔS values for the PD inhibitors probably arises from loss of conformational flexibility of the P1−P2 moiety of PD180557 upon binding. Comparing PD166793 and Galardin, the data suggests that there is a much smaller loss of entropy for the binding of Galardin, which we propose arises in part from a conformational change in the S1′ site of the enzyme upon binding of the PD inhibitors (see below).

To analyze the binding in terms of the various components of the entropy term, we determined the change in heat capacity (ΔC_p) for the binding of each inhibitor over the temperature range 7−22 °C (Figure 5, Table 2). The ΔC_p for binding of Galardin was similar to that of PD180557, and more negative than for PD166793, even though both PD compounds have larger hydrophobic substituents at P1′. Since Galardin has an indole group at P2′ that is not present in the other two inhibitors, this suggests that the P2′ substituent forms a hydrophobic interaction with the S2′ subsite. This type of interaction would contribute negatively to the ΔC_p value for the binding of Galardin (*54, 57*).[4]

ΔC_p values can also be used to obtain an estimate of the contribution to the ΔS value made by changes in solvation. It has been estimated that at a temperature T^* [385 (±1) K] the contribution of changes in solvation to ΔS is zero (*56, 58, 59*), so at a given temperature T, ΔS_{solv} can be approximated by

$$\Delta S_{solv} = \Delta C_p \ln(T/T^*).$$

The proper method for calculating ΔS_{trans} has been a subject of much debate. A "cratic" or mixing entropy term (*60*) has been proposed as providing a reasonable approximation of experimental data (*39*). This approach has received both criticism (*61*−*63*) and support (*64, 65*). We favor the treatment proposed by Amzel (*64*), in which, for a binding interaction with 1:1 stoichiometry,

$$\Delta S_{trans} \cong -[R\ln 55 + R + (0-0.6 \text{ cal/mol K})] = -(9.95-10.55) \text{ cal/mol K}$$

wherein the third term depends on the partial specific volume in the occupied binding site. We, therefore, used an estimate of −10.2 (±0.1) cal/mol K for ΔS_{trans} where the term in parentheses ≡ one standard deviation.

Estimates of ΔS_{solv} and ΔS_{other} at 22 °C, pH 6.7, are listed in Table 2 for each of the three inhibitors. PD180557 and Galardin had very similar values for ΔS_{solv}, while the value for PD166793 was somewhat less positive. In contrast, PD166793 and Galardin had similar values for ΔS_{other}, while the value for PD180557 was much more negative.

Since PD180557 and PD166793 differ only in their P1−P2 substituents, comparison of their ΔS_{solv} values suggests that the P1−P2 moiety of PD180557 forms additional interactions with the enzyme that result in the release of bound water molecules. Molecular modeling of the PD180557−SCD complex suggests that the phenyl ring of the inhibitor may bind in a hydrophobic region of the shallow S2 subsite containing phenylalanine 86 and tyrosines 155 and 168 (*36*). Although structural data is not available for this complex, the modeling results suggest that both hydrophobic and pi-stacking interactions might occur at this site. The hydrophobic interaction would lead to a positive contribution to ΔS_{solv}. A phenyl−phenyl pi-stack with ideal geometry could contribute a maximum of −6 to −7 kcal/mol of enthalpy at 22 °C (*66*). The observed enthalpy difference between PD180557 and PD166793 was 7.3 kcal/mol, although the modeling data suggests that potential pi-stacking interactions would probably have less than ideal geometry and thus contribute only a fraction of the 6−7 kcal/mol maximum value.

Using values for the dehydration of benzene determined at 25 °C (*67, 68*), and assuming that about half of the phenyl ring at P2 becomes buried upon binding, we estimate that the ΔH and ΔS for desolvation of this moiety would be +2.6 kcal/mol and +5.7 cal/mol K, respectively. The net contribu-

[4] A possible complication in the determination of the ΔC_p values might arise from the ΔC_p of ionization of PIPES buffer, histidine 224, and the carboxylate groups. The ΔC_p for deprotonation of PIPES is +4.5 cal/mol K (*77*) and thus does not make a significant contribution to the ΔC_p of complex formation. For histidine 224 and the carboxylates, it is difficult to obtain precise estimates of their contributions due to the effects of their local environments in the active site. The ΔC_p for protonation of imidazole has been reported to be +3.8 cal/mol K in solution (*77*). The ΔC_p for protonation of acetate buffer is +31.6 cal/mol K (*77*), so protonation of the carboxylate moieties of the PD inhibitors could make a significant contribution to their observed ΔC_p values. Incorporation of these corrections (which, we emphasize, may not be accurate within the local environment of an enzyme's active site), resulted in ΔC_p values of −146, −106, and −126 cal/mol K for PD180557, PD166793, and Galardin, respectively. The trend in ΔC_p values among the three inhibitors is similar to that calculated without the use of this correction (Table 2). Estimates of ΔS_{solv} and ΔS_{other} calculated using these ΔC_p values display similar trends and values to those reported in Table 2 (data not shown).

tion to ΔG would be +0.9 kcal/mol at 25 °C, so desolvation of the P2 phenyl group probably makes a slightly unfavorable overall contribution to the binding affinity. Similar estimates are not available for sulfone groups. However, Cabani et al. (*69*) have estimated the ΔH for dehydration of a sulfoxide group to be approximately +16 kcal/mol at 25 °C. The estimated $\Delta H_{dehydration}$ for a carbonyl group was estimated to be +6.3 kcal/mol at this temperature. It may be possible to decrease the enthalpic desolvation penalty by replacing the P1 sulfone with a carbonyl, although this might also affect the strength of the hydrogen bond that the sulfone appears to form with the enzyme (see below).

The entropic penalty associated with loss of flexibility in this moiety and/or the S1 and S2 subsite residues upon binding (ΔS_{other}) was much greater than the favorable ΔS_{solv} value for interactions in this site. This suggests that any entropic advantage gained by PD180557 from release of bound solvent in the S1 and S2 subsites is more than offset by the unfavorable entropy change caused by the concomitant loss of flexibility. This loss of conformational entropy is large enough to almost completely offset the favorable enthalpic interactions as well.

Like the other inhibitors, Galardin had a negative value for ΔS_{other}, most likely due to its highly flexible peptidic structure and the large number of hydrogen bonds it forms upon binding. However, this was more than compensated for by the ΔS_{solv} term, a large part of which is probably contributed by hydrophobic interactions in the S2′ site (see below).

The thermodynamic data listed in Table 2 allowed us to determine some structure−activity relationships among these inhibitors. Our analysis focuses on three binding sites: the S1−S2, S2′, and S1′ pockets. Binding of PD180557 had a very large favorable ΔH, at least 7 kcal/mol more negative than for the other two inhibitors. Since PD180557 and PD166793 differ only in the substituent at the P1−P2 position (Figure 1), we propose that the sulfone group of PD180557 forms at least one hydrogen bond or electrostatic interaction at this site, perhaps including a hydrogen bond with the backbone nitrogen of alanine 167 in the S1 subsite. As noted above, pi-stacking interactions with the phenyl moiety of PD180557 might contribute to the favorable enthalpy value as well.

Many other investigators have observed that a polar group in the P1 position can increase affinity (see (*19*) for examples). However, in the case of PD180557, this strong favorable contribution to the binding enthalpy was almost completely compensated for by an unfavorable change in the entropy term. Formation of an enthalpically favorable interaction at S1−S2 will necessarily result in some loss of conformational entropy upon binding. We propose, however, that it would be desirable to retain a polar P1 moiety, but to also remove some of the other parts of this substituent that appear to contribute little binding energy and probably undergo a significant loss of conformational entropy upon binding (i.e., the methylene groups and probably the phenyl group). An optimal strategy might be to place the polar group into a more constrained P1 substituent, such as a ring (*30*, *70*, *71*), so that the loss of conformational flexibility upon binding would be minimized further.

While we do not have enough structural information to predict the gain in binding affinity this approach might yield, we can make some very rough estimates. It has been observed that the removal of each rotational degree of freedom in an unbound ligand results in a decrease in the free energy of binding of between 0.7 and 1.6 kcal/mol (*72* and references within). Removal of the P1−P2 moiety of PD180557 or incorporation into a cyclic structure would remove four rotational degrees of freedom. (Rotation of the phenyl group does not need to be considered in this context (*73*).) If we consider only rotational degrees of freedom, then constraining this moiety in the unbound inhibitor could yield as much as 2.8−6.4 kcal/mol of free energy. Khan et al. (*72*) covalently connected two freely rotating groups in a penicillopepsin inhibitor and obtained a 420-fold increase in binding affinity, corresponding to a free energy gain of 3.6 kcal/mol.

Galardin had the highest binding affinity of the three inhibitors studied, but its ΔH was less favorable than for the others. This was somewhat surprising in light of the large number of hydrogen bonds that peptidic inhibitors such as Galardin make with the enzyme (Figure 1). However, Galardin bound with a favorable entropy term that is large enough to account for its higher binding affinity. Galardin contains an indole group that is positioned to bind at the S2′ position, while the other two inhibitors have no P2′ substituent. The estimated ΔS_{solv} for Galardin was more positive than for PD166793, even though PD166793 has a larger hydrophobic P1′ group. We propose that hydrophobic interactions within the S2′ site contribute a significant portion of Galardin's binding energy. Addition of nonpolar P2′ groups such as aromatic rings to the other inhibitors might, therefore, increase their potency, as has been suggested previously (*25*, *28*, *74*).

Finally, the sizes of the P1′ substituents differ between the two classes of inhibitors studied. Both PD180557 and PD166793 have *p*-bromo-*p*-biphenyl groups here, while Galardin has an isobutyl group. These substituents were designed to bind in the S1′ subsite via hydrophobic interactions. The estimated ΔS_{other} terms for binding of Galardin and PD166793 were similar, despite Galardin's much more flexible peptidic structure in solution. If there were little difference between these two inhibitors in terms of the loss of conformational flexibility suffered by the *enzyme* upon binding, then the ΔS_{other} term for binding of Galardin would be much more negative than for PD166793, since Galardin itself undergoes a greater loss of flexibility than does PD166793 upon binding. (For example, compare the ΔS_{other} terms for PD166793 and PD180557). Since this is not the case, this suggests that binding of PD166793 (or PD180557) induces a significant reduction in the conformational flexibility of the enzyme, probably at the S1′ site, while in the case of Galardin the loss of flexibility in the enzyme is not as severe.

A loop of about eleven residues (221−231) can shift position in order to form tighter contacts with inhibitors in the S1′ pocket (*20*, *22*, *35*). This probably causes a decrease in the conformational entropy of the system, as this region of the enzyme is somewhat flexible in the unbound form (*22*). We propose that P1′ groups of different sizes may induce different degrees of conformational rigidification in the S1′ site, resulting in different entropic "penalties". Recent structural data tends to support this (*75*). As pointed out by Li et al. (*35*), this suggests that inhibitor design based solely

on "docking" potential inhibitors with the structure of the stromelysin catalytic domain may not be reliable with regard to the S1′ site.

Interestingly, replacement of histidine 224 with asparagine did not adversely affect the binding affinity for PD166793 at pH 6.7 (Tables 2 and 3). The ΔH for binding of PD166793 was not significantly affected either. While the experimental error in the determination of K_d for Galardin−H224N binding was too large to draw any conclusions about overall binding affinity, the ΔH for Galardin was made approximately 2 kcal/mol less favorable when histidine 224 was mutated to asparagine. This may be due to the loss of a hydrogen bond between (protonated) histidine 224 and the backbone carbonyl of alanine 217, which has been observed in the complex between the SCD and an inhibitor that, like Galardin, has an isobutyl group at P1′ (*75*). Structural data for complexes between the SCD and biphenyl inhibitors such as PD166793 and PD180557 are not yet available, so we do not know what role the protonation of histidine 224 plays in the binding of these inhibitors. One possibility is that asparagine 224 in the mutant can form an alternative hydrogen bond when PD166793 binds, replacing a hydrogen bond formed by the protonated form of histidine 224.

In conclusion, we have used ITC to obtain complete thermodynamic profiles of the binding of three inhibitors to the stromelysin catalytic domain. The binding of both hydroxamic and carboxylic acid-containing inhibitors was found to be highly pH-dependent, with tighter binding occurring at lower pH within the range 5.5−7.5. It was concluded that histidine 224 becomes protonated during binding at pH 6.7 and that the zinc-binding groups of the inhibitors also bind in the protonated form (or share a proton with glutamic acid 202) by measuring the degree of proton transfer that occurs upon binding of both classes of inhibitors to the wild-type enzyme and the H224N mutant.

This study also revealed that a polar group in the P1 position can contribute a large favorable enthalpy term to the free energy of binding. Additional favorable interactions with the inhibitor PD180557 may be contributed by hydrophobic interactions and pi-stacking with a phenyl group at the end of the extended P1−P2 moiety. However, these favorable interactions are masked by an unfavorable entropy term that appears to be due to a loss of conformational flexibility in the P1−P2 moiety upon binding. This suggests that designing conformationally-rigidified P1 and/or P2 substituents into stromelysin inhibitors might increase their binding affinities.

An aromatic group at P2′ appears to increase binding affinity through a hydrophobic interaction. Additionally, we propose that the deep S1′ subsite might be exploited to maximal advantage if the size of the inhibitor moiety is optimized so that an apparent entropic penalty caused by a conformational change in the protein is minimized. This paper demonstrates that ITC can be of great value in determining structure−activity relationships in pharmaceutical design.

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