The avid binding of tissue inhibitors of metalloproteinases (TIMPs) to matrix metalloproteinases (MMPs) is crucial for the regulation of pericellular and extracellular proteolysis. The interactions of the catalytic domain (cd) of MMP-1 with the inhibitory domains of TIMP-1 and TIMP-2 (N-TIMPs) and MMP-3cd with N-TIMP-2 have been characterized by isothermal titration calorimetry and compared with published data for the N-TIMP-1/MMP-3cd interaction. All interactions are largely driven by increases in entropy but there are significant differences in the profiles for the interactions of both N-TIMPs with MMP-1cd as compared with MMP-3cd; the enthalpy change ranges from small for MMP-1cd to highly unfavorable for MMP-3cd (−0.1 ± 0.7 versus 6.0 ± 0.5 kcal mol⁻¹). The heat capacity change (ΔCₚ) of binding to MMP-1cd (temperature dependence of ΔH) is large and negative (−210 ± 20 cal K⁻¹ mol⁻¹), indicating a large hydrophobic contribution, whereas the ΔCₚ values for the binding to MMP-3cd are much smaller (−53 ± 3 cal K⁻¹ mol⁻¹), and some of the entropy increase may arise from increased conformational entropy. Apart from differences in ionization effects, it appears that the properties of the MMP may have a predominant influence in the thermodynamic profiles for these N-TIMP/MMP interactions.

The matrix metalloproteinases (MMPs) catalyze the turnover of components of the extracellular matrix and have important roles in tissue remodeling, wound healing, embryo implantation, cell migration, and shedding of cell surface proteins (1, 2). MMP-1 is a well-characterized collagenase that catalyzes the turnover of collagen fibrils in the matrix, whereas MMP-3 (stromelysin 1) cleaves multiple extracellular matrix components and functions in tissue remodeling and other processes (2). Collagenolysis is a key feature of biological processes including development, morphogenesis, and wound repair yet unregulated collagen breakdown contributes to important diseases including cancer, arthritis, emphysema, and fibrosis (1).

An understanding of the molecular basis of the regulation of MMP activities is crucial for understanding MMP-associated diseases and developing therapies for them. The tissue inhibitors of metalloproteinases (TIMP-1 to −4) are a family of four endogenous MMP inhibitors that can form high affinity 1:1 complexes with most MMPs. TIMP-3 also inhibits some members of the distantly related disintegrin metalloproteinase (ADAM) and disintegrin metalloproteinase with thrombospondin type 1 motif (ADAMTS) families (2). A loss of balance between the TIMPs and their target proteases is linked to diseases such as cancer and arthritis (2, 3).

TIMPs are slow, tight-binding inhibitors of the MMPs with Kᵢ values typically in the sub- to low nanomolar range (3). Mammalian TIMPs have two domains, a larger (−125 residue) N-terminal domain that can be expressed separately and carries the MMP inhibitory activity (2, 3) and a smaller C-terminal domain that is absent from the TIMPs of some invertebrates (3). In the crystal structures of the MMP-3-TIMP-1, MMP-1-N-TIMP-1, MT1-MMP-TIMP-2, and MMP-13-TIMP-2 complexes (4–7), most of the MMP interaction surface is located within the N-domains of the TIMPs; as shown in Fig. 1, the N-terminal region (residues 1–5) inserts into the active site of the MMP, whereas the α-amino group together with the carboxyl oxygen of Cys¹ coordinate the catalytic zinc (4–7). Modification of the α-amino group by addition of an alanine (8, 9), carbamylation (10), or acetylation (11) radically reduces the inhibitory activities of TIMPs for MMPs. In the all inhibitory TIMP-MMP complexes, the side chain of residue 2 of the TIMP (Ser or Thr in vertebrate TIMPs) sits over the mouth of the key S₁’ subsite of the MMP active site (4–7), and substitution by glycine results in large loss of affinity for most MMPs (9, 12). However, both the Ala extension and Thr² to Gly mutation in N-TIMP-3 have little effect on the inhibition of ADAM-17, ADAMTS-4, or ADAMTS-5 (9, 13). The inhibitory domain of TIMP has an OB-fold structure, a 5-stranded β-barrel structure with two small helices. Other regions in TIMPs that contact MMPs include the connector between the C and D β strands and the loops connecting the A to B, and E to F strands (4–7). In
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FIGURE 1. Structure of the N-TIMP-1-MMP-1cd complex with N-TIMP-2 superimposed on the N-TIMP-1 component. The N-TIMP-1/MMP-1cd structure used was chains A and D from PDB code 2J0T and the N-TIMP-2 structure was extracted from the structure of the TIMP-2-MT1-MMP complex (1BUV). The image was constructed using Chimera (43). The MMP-1cd Zn\(^{2+}\)-binding motif, His\(^{211}\)-Glu-Leu-Gly-His\(^{212}\) is colored yellow and Zn\(^{2+}\) ions are red; the catalytic Zn\(^{2+}\) is the most prominent. This region of MMPs, and the N terminus, A-B loop and C-D loop of TIMPs (labeled), are key components of interaction sites in TIMP-MMP complexes.

all structurally characterized complexes of TIMP-1 and TIMP-2 with MMPs, the core of the interaction site in the TIMP is a surface ridge formed by the N-terminal five residues, Cys\(^1\)—Thr—Cys—Val—Pro\(^5\) (TIMP-1 sequence and numbering) and the connector between \(\beta\)-strands C and D, residues Met\(^{66}\)—Glu—Ser—Val—Cys\(^{70}\) in TIMP-1, which are linked by the Cys\(^1\) to Cys\(^{70}\) disulfide bond (3). Other regions that make variable contributions to MMP binding are the loops connecting \(\beta\)-strands A and B and strands E and F and the C-terminal end of \(\beta\)-strand D (3). The A-B loop of N-TIMP-2, which is longer than that of N-TIMP-1 by 7 residues (Fig. 1), makes multiple contacts with MT1-MMP in the TIMP-2-MT1-MMP and TIMP-2-MMP-13 complexes (6, 7). Although no structure is available for the N-TIMP-2-MMPcd complexes investigated here, NMR studies have shown that MMP-3cd binding reduces the internal motions of the large TIMP-2 A-B loop, suggesting that it interacts with the protease (14).

The truncated N-terminal domains of TIMPs (N-TIMPs) and isolated MMP catalytic domains (MMPcd) have been extensively used in studies of the interactions between TIMPs and MMPs (12–19) and the majority of the intermolecular interactions in structurally characterized inhibitory TIMP-MMP complexes involve residues in these domains. Some interactions involving the C-domains of TIMPs have been observed also and it has been suggested that these might affect the relative orientations of inhibitor and MMP in their complexes (6, 7). Although it is possible that interactions involving the TIMP C-domain might modulate the affinity for some MMPs, they are not necessary for binding because N-TIMPs are fully active as MMP inhibitors (12–19). The one clear example of selectivity in TIMP/MMP interactions is the weak affinity of TIMP-1 for the membrane-type MMPs, including MT1-MMP and MMP-19, whereas TIMP-2 is a potent inhibitor of these enzymes (3). This inhibitory selectivity resides in the TIMP N-terminal domains (19, 20). However, the C-domain appears to have a role in TIMP interactions with other metalloproteinases; for example, TIMP-1 and TIMP-3 are potent inhibitors of ADAM-10 but their truncated N-domains are ineffective (21).

A previous ITC study of the thermodynamics of N-TIMP-1 binding to the catalytic domain of MMP-3cd (22) revealed that the interaction has a positive (unfavorable) enthalpy change (\(\Delta H\)) that is compensated by a large increase in entropy (\(\Delta S\)). The relatively small negative heat capacity change (\(\Delta C_p\)) for the interaction implied that the hydrophobic effect accounts for only a fraction of the favorable entropy change (22). Increased conformational dynamics could be a major source of the entropy increase because NMR-based backbone dynamics results suggested that binding to MMP-3cd enhanced the mobility of the backbone of the core of N-TIMP-1 as reflected in fluctuations on the picosecond to nanosecond scale, and more widely on the time scale of microsecond to millisecond (22).

We report here an investigation by ITC of the interactions of N-TIMP-1 with the catalytic domain of MMP-1 (MMP-1cd) and N-TIMP-2 with the catalytic domains of both MMP-1 and MMP-3. Together with the previous study of the N-TIMP-1/ MMP-3cd interaction (22), this provides thermodynamic profiles of four TIMP/MMP interactions that may help to illuminate the physical basis of functional specialization among different TIMPs (3). Based on the magnitude of the heat capacity change for binding (\(\Delta C_p\)), it appears that increases in solvent entropy (hydrophobic effect) make a major contribution to the binding of both N-TIMPs to MMP-1cd, but apparently less to the binding to MMP-3cd where the “missing” entropy might arise from enhanced conformational dynamics in the inhibitor. The thermodynamic profiles are discussed in the light of the structural information on the various TIMP-MMP complexes.

**EXPERIMENTAL PROCEDURES**

**Materials**—N-TIMP-1, N-TIMP-2, and MMP-3cd were expressed as previously described (20, 23). MMP-1cd was expressed using the vector pET-3a-Mmp-1cd encoding MMP-1cd, kindly provided by Dr. H. Nagase, Kennedy Institute of Rheumatology, Imperial College, UK. Superdex-75 was purchased from Amersham Biosciences and Centriplus YM-3 centrifugal filter devices were from Millipore. Isothermal titration calorimetry studies were carried out using a MicroCal VP-ITC Microcalorimeter. All other equipment, reagents, and cells were from the same sources as in previous studies (12, 16, 23–25).
Expression, Purification, and In Vitro Folding of N-TIMP-1, N-TIMP-2, MMP-1cd, and MMP-3cd—N-TIMP-1 and N-TIMP-2 were expressed in Escherichia coli BL21 (DE3) cells (20, 23). The proteins were partially purified and folded as described for N-TIMP-1 by Wei et al. (16) and for N-TIMP-2 by Hamze et al. (20).

Active N-TIMP-1 was separated from N-acetylated inactive protein in some preparations by medium pressure cation exchange chromatography as described previously (11) but, as previously noted (22), the presence of the inactive form does not interfere with the results and the material from the CM-52 separation was used in most experiments. The concentrations of active N-TIMP-1 and N-TIMP-2 (about 44% of the total) were determined by activity titration and ITC. MMP-1cd and MMP-3cd were expressed and folded as previously described (12) and were purified by ion exchange followed by gel filtration with a column (2.5 × 35 cm) of Superdex-75 pre-equilibrated and eluted with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 20 mM CaCl₂. The eluate was collected in 4.5-ml fractions at a flow rate of 1.5 ml/min. Fractions containing folded N-TIMPs were pooled and concentrated using Centricon YM-3 centrifugal filter devices (Millipore).

Fluorescence Assays for N-TIMP-1 and N-TIMP-2 Activity—The inhibition of MMPs by N-TIMP-1 and N-TIMP-2 was measured by assaying MMP activities for hydrolysis of fluorogenic substrates using a PerkinElmer LS50B luminescence spectrometer. TNC buffer (50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 10 mM CaCl₂, and 0.02% Brij-35) was used for both dilution of MMP and TIMP samples and all assays.

To determine the \( K_{p}^{pp} \) of N-TIMP-1 and N-TIMP-2 for MMP-1cd, and of N-TIMP-2 for MMP-3cd at 25 °C, various concentrations of inhibitor were incubated with 5 nM enzyme at 30 °C for 3 h before addition of the Knight substrate (for MMP-1cd) or, for MMP-3cd, the NFF-3 substrate (Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH₂) to a final concentration of 3 μM and the fluorescence intensity was measured as described above. Reaction velocities were measured as the slope of the linear portion of the fluorescence curve. The percentage of residual MMP activity was calculated by dividing the velocities measured with inhibitor by the velocities measured without inhibitor \( (v/v_o) \).

\[ \frac{v}{v_o} = \frac{E - I - K + ((E - I - K)^2 + 4EK)^{0.5}}{2E} \]  
\( (Eq. 1) \)

where \( v \) is the experimentally determined reaction velocity, \( v_o \) is the activity in the absence of inhibitor, \( E \) is enzyme concentration, \( I \) is inhibitor concentration, and \( K \) is the apparent inhibition constant \( (K_{p}^{pp}) (9) \).

For stoichiometric titration of N-TIMP-1 and N-TIMP-2 various concentrations of the inhibitor were incubated with MMP-3cd (300 nM) for 4 h at 37 °C, diluted 300-fold with TNC buffer, and immediately assayed with 1.5 μM NFF-3 substrate, as described above. Residual MMP activity (%) was calculated as described above and plotted against the molar ratio of TIMP/MMP (0–4 in this case). The stoichiometry was determined by linear regression analysis of the appropriate data points.

Isothermal Titration Calorimetry of N-TIMP/MMPcd Interactions—Protein solutions were dialyzed extensively against various buffers at 20 mM concentrations containing 250 mM NaCl, 10 mM CaCl₂, and 50 μM ZnCl₂ and degassed prior to use. N-TIMPs (12–30 μM) were titrated with the MMPcd (120–300 μM) at different temperatures using a MicroCal VP-ITC microcalorimeter. Titrations of N-TIMP-1 were conducted at pH 6.8 as in the previous study of the N-TIMP-1/MMP-3cd interaction but, because this pH is close to the pl of N-TIMP-2 (6.98 cf. 8.58 for N-TIMP-1), the studies with N-TIMP-2 were conducted at pH 7.4 to avoid solubility problems at the protein concentrations needed for ITC. The instrument was programmed to carry out 16 injections of 10–20 μl each over 16 s, spaced at 300-s intervals. The stirring speed was 200 rpm. Heats of binding were determined by integrating the signal from the calorimeter, and binding isotherms were generated by plotting the heats of binding against the ratio of enzyme to inhibitor. The data were corrected for heats of dilution of the MMPs and the Origin 5.0 from Microcal Inc. was used to calculate the enthalpy changes (\( \Delta H \)) and stoichiometry (\( N \)). To determine the heat capacity, the following relationship was used: \( \Delta H = \text{measured at a series of temperatures and the data were fitted to Equation 2.} \)

\[ \Delta C_p = \frac{(d\Delta H^o/dT)_p}{(dT)} \]  
\( (Eq. 2) \)

\( \Delta H \) was measured at a series of temperatures and \( \Delta C_p \) was determined by linear regression analysis.

Correlation of Thermodynamics with Structure—It has been proposed that the \( \Delta C_p^a \) of protein interactions is related to changes in non-polar and polar accessible surface areas (26, 27), \( \Delta ASA_{np} \) and \( \Delta ASA_{pol} \) (where surface burial has a negative sign).

\[ \Delta C_p = a \times \Delta ASA_{np} + b \times \Delta ASA_{pol} \]  
\( (Eq. 3) \)

This expression has been routinely used for analyzing interactions (27, 28). Structures are not available for N-TIMP-2 or TIMP-2 complexes with either MMP-1 or MMP-3 but the structure of the N-TIMP-1/MMP-1cd complex has been determined (5) (PDB code 2JOT). The parameterizations of the coefficients for changes in nonpolar and polar surface used here were a = 0.28 ± 0.12 cal mol⁻¹ K⁻¹ Å⁻² and b = -0.09 ± 0.30 cal mol⁻¹ K⁻¹ Å⁻² (29).

The enthalpy of binding (\( \Delta H^o \)) at 60 °C (the mean melting temperature of a group of proteins used in the analysis (27)) was calculated using the relationship,

\[ \Delta H^o(60 ^\circ C) = c \times \Delta ASA_{np} + d \times \Delta ASA_{pol} \]  
\( (Eq. 4) \)

where \( c \) is -7.27 cal mol⁻¹ Å⁻² and \( d \) is 29.16 cal mol⁻¹ Å⁻² (26). \( \Delta H^o \) at 25 °C is then calculated using the calculated value for \( \Delta C_p^a \). Changes in polar and apolar surface areas were measured from atomic coordinates using NACCESS⁴ and ProFace (30).

⁴ S. J. Hubbard and J. M. Thornton, Department of Biochemistry and Molecular Biology, University College London, London.
RESULTS AND DISCUSSION

Isothermal Titration of N-TIMP-2 by MMP-1cd and MMP-3cd Both Show an Enthalpy Increase Compensated by Favorable Entropy—The isothermal titration of N-TIMP-2 with MMP-1cd at 291 K exhibits heat uptake until the protease is saturated with the inhibitor; analysis of the titration data indicate that the interaction has a positive enthalpy change of about 3.2 kcal/mol under these conditions (Fig. 2B). Although uncommon among protein-protein interactions, this result is similar to previous calorimetric observations for the binding of N-TIMP-1 to MMP-3cd although the enthalpy increase is less. A similar result was obtained for the titration of N-TIMP-2 with MMP-3cd. However, the integrated value for $\Delta H_{\text{obs}}$ is smaller (2.22 versus 3.21 kcal/mol in Hepes buffer at 291 K). The stoichiometry at 288–303 K ranged from 0.36 to 0.42, which is consistent with the fraction of active N-TIMP-2 (~40%), determined by titration with MMP-3cd and MMP-1cd.

To determine the contribution to $\Delta H_{\text{obs}}$ of enthalpy changes arising from protonation or deprotonation on complex formation, the enthalpies of binding ($\Delta H_{\text{int}}$) for the interactions of N-TIMP-2 with both MMPs were measured at 291 K in buffers of different enthalpies of ionization, Pipes, Hepes, Bes, and Aces (Table 1 and Fig. 3). These were analyzed by linear regression based on the relationship: $\Delta H_{\text{obs}} = \Delta H_{\text{int}} + N_{\text{H+}} \times \Delta H_{\text{ion}}$, where $N_{\text{H+}}$ is the number of protons taken up (positive values) or released to the buffer and $\Delta H_{\text{int}}$ is the enthalpy change independent of buffer (31). Fig. 4 shows graphical plots of these data that show a small fractional uptake of protons (0.14) for the interaction with MMP-1 but the release of about 0.7 protons on binding to MMP-3cd.

| Buffer | $\Delta H_{\text{int}}$ | $\Delta H_{\text{obs}}$ |
|--------|----------------|----------------|
| MMP    | 2.726           | 1.97 ± 0.03    |
| Pipes  | 5.195           | 3.85 ± 0.07    |
| Hepes  | 5.993           | 5.47 ± 0.04    |
| Aces   | 7.479           | 6.63 ± 0.06    |
|        | -0.86 ± 0.56    | 6.26 ± 0.14    |
| $N_{\text{H+}}$ | 0.96 ± 0.10 | 0.058 ± 0.002 |

| Buffer | $\Delta H_{\text{int}}$ | $\Delta H_{\text{obs}}$ |
|--------|----------------|----------------|
| MMP    | 2.705           | 2.92 ± 0.01    |
| Pipes  | 5.190           | 3.21 ± 0.01    |
| Hepes  | 6.012           | 3.38 ± 0.02    |
| Aces   | 7.552           | 3.60 ± 0.01    |
|        | 2.53 ± 0.03     | 5.78 ± 0.24    |
| $N_{\text{H+}}$ | 0.14 ± 0.01 | -0.68 ± 0.04 |

* Data from Ref. 22 at 298 K.

The $K_i$ values for the N-TIMP-2 interactions are too large (~10$^7$) to be reliably determined by ITC and were calculated from $K_i$ values determined by inhibition kinetics under the same conditions; these were corrected by multiplying by the fraction of active N-TIMP-2 to give values of 0.036 ± 0.003 and 4.3 ± 1.0 nM for the interactions with MMP-1cd and MMP-3cd, respectively, at 298 K. Using the relationship $\Delta G = RT \ln K_i$, $\Delta G$ for the interactions of N-TIMP-2 with MMP-1cd and MMP-3cd were calculated to be −14.2 and −11.1 kcal/mol,
respectively. Together with the measured $\Delta H_{\text{int}}$ values these generated $T\Delta S^o$ values of $-14.8$ and $-16.6 \text{ kcal/mol}$, respectively, for the interactions with MMP-1cd and MMP-3cd (Table 2).

The Association of N-TIMP-2 with MMP-1cd Has a Larger $C_p$ Than the Interaction with MMP-3cd—The heat capacity changes ($\Delta C_p$) for the N-TIMP-2/MMP-1cd and N-TIMP-2/MMP-3cd interactions were calculated from the temperature dependence of $\Delta H_{\text{obs}}$ measured under standard experimental conditions (Table 2). For both interactions $\Delta H_{\text{obs}}$ decreases linearly with temperature (Fig. 5) but the slopes of the two lines differ; regression analysis of two sets of data gave values for $\Delta C_p$ of $-278 \pm 10$ and $-54.7 \pm 3.7 \text{ cal K}^{-1} \text{ mol}^{-1}$ for MMP-1 and MMP-3, respectively. As indicated by the experimental data shown in Fig. 2, $\Delta H_{\text{obs}}$ for the interaction with MMP-1cd changes from positive to negative over this temperature range. A titration at 30 °C (303 K) gave a negligible signal, in keeping with the plot shown in Fig. 5. It should be noted that because the N-TIMP-2/MMP-3 interaction (and also the N-TIMP-1/MMP-3 interaction discussed below) is associated with significant ionization changes and the experimentally determined

| Temperature (K) | MMP-1cd | MMP-3cd* |
|----------------|---------|---------|
| N-TIMP-1       |         |         |
| 288            | 4.42 ± 0.07 | 2.222 ± 0.015 |
| 293            | 3.42 ± 0.05 | 1.838 ± 0.011 |
| 298            | 2.37 ± 0.03 | 1.639 ± 0.030 |
| 303            | 1.51 ± 0.02 | 1.162 ± 0.037 |
| $\Delta C_p$ (cal/mol K) | $-189 \pm 12 (-194)^b$ | $-50.4 \pm 6.2$ |
| $\Delta S_{\text{solv}}$ (cal/mol) | 49 | 13 |
| N-TIMP-2       |         |         |
| 291            | 3.207 ± 0.011 | 2.222 ± 0.015 |
| 298            | 1.390 ± 0.08 | 1.838 ± 0.011 |
| 303            | (0)      | 1.639 ± 0.030 |
| 305            | $-0.815 \pm 0.06$ | ND$^c$ |
| 310            | $-1.918 \pm 0.028$ | 1.162 ± 0.037 |
| $\Delta C_p$ (cal/mol K) | $-278 \pm 10$ | $-54.8 \pm 3.7 (-47.2)^b$ |
| $\Delta S_{\text{solv}}$ (cal/mol) | 71 | 12 |

$^a$ From Ref. 22.
$^b$ Corrected for buffer ionization dependence (32) as described in the text.
$^c$ ND, not determined.

FIGURE 3. Effect of buffers with different enthalpies of ionization on calorimetric titration curves of N-TIMP-2 with MMP-3cd at 291 K. A, aliquots (25 μl) of MMP-3 (158 μM) were injected into N-TIMP-2 (14.3 μM) in 20 mM Pipes buffer, pH 7.4. B, aliquots (25 μl) of MMP-3 (180 μM) were injected into N-TIMP-2 (16.8 μM) in 20 mM Aces buffer, pH 7.4.

FIGURE 4. Relationship of buffer enthalpy of ionization ($\Delta H_{\text{buf}}$) on $\Delta H_{\text{obs}}$ for different N-TIMP/MMPcd interactions. Symbols for the different interactions are: N-TIMP-1/MMP-1cd, open circles; N-TIMP-2/MMP-1cd, closed circles; and N-TIMP-2/MMP-3, closed squares.

### Table 2

| Temperature (K) | MMP-1cd | MMP-3cd* |
|----------------|---------|---------|
| N-TIMP-1       |         |         |
| 288            | 4.42 ± 0.07 | 2.222 ± 0.015 |
| 293            | 3.42 ± 0.05 | 1.838 ± 0.011 |
| 298            | 2.37 ± 0.03 | 1.639 ± 0.030 |
| 303            | 1.51 ± 0.02 | 1.162 ± 0.037 |
| $\Delta C_p$ (cal/mol K) | $-189 \pm 12 (-194)^b$ | $-50.4 \pm 6.2$ |
| $\Delta S_{\text{solv}}$ (cal/mol) | 49 | 13 |
| N-TIMP-2       |         |         |
| 291            | 3.207 ± 0.011 | 2.222 ± 0.015 |
| 298            | 1.390 ± 0.08 | 1.838 ± 0.011 |
| 303            | (0)      | 1.639 ± 0.030 |
| 305            | $-0.815 \pm 0.06$ | ND$^c$ |
| 310            | $-1.918 \pm 0.028$ | 1.162 ± 0.037 |
| $\Delta C_p$ (cal/mol K) | $-278 \pm 10$ | $-54.8 \pm 3.7 (-47.2)^b$ |
| $\Delta S_{\text{solv}}$ (cal/mol) | 71 | 12 |

$^a$ From Ref. 22.
$^b$ Corrected for buffer ionization dependence (32) as described in the text.
$^c$ ND, not determined.
ΔCp values require correction for the contribution of the buffer (32). However, these corrections are relatively small (+7.6 and −5 cal/mol K, respectively, Table 2).

ΔCp in is generally regarded as a measure of the extent of solvent release on binding; the entropy of desolvation, ΔSsolv, at any temperature T, can be estimated from the relationship ΔSsolv = ΔCp,0 × ln(T/Ts), where Ts is the reference temperature (385 K) at which the hydrophobic contribution to ΔS is zero (33, 34). Based on this interpretation, it can be estimated that the hydrophobic effect (−TΔSsolv) contributes ~21 kcal/mol to the free energy of interaction of N-TIMP-2 with MMP-1cd but only ~3.6 kcal/mol to the N-TIMP-2/MMP-3cd interaction (Table 3).

Thermodynamic Profile for the Interaction of N-TIMP-1 with MMP-1cd—Previous studies of the interaction of N-TIMP-1 with MMP-3cd showed a similar pattern to the N-TIMP-2/MMP-3cd interaction: a positive ΔH compensated by a large favorable ΔS of which only a small fraction appears to arise from the hydrophobic effect (22). This suggests that the properties of the MMP may predominate in determining the thermodynamic profiles for the N-TIMP/MMPcd interactions. This view is supported by ITC data for the N-TIMP-1/MMP-1cd interaction under the conditions previously used for calorimetric studies of the N-TIMP-1/MMP-3cd interaction (22). The results (Tables 1–3) show that the N-TIMP-1/MMP-1cd interaction is characterized by the uptake of about 1 proton and that the extrapolated value for the ionization-independent enthalpy of interaction, ΔHint, is small and favorable (−0.9 kcal/mol at 25 °C). ΔG° calculated from the Ki value is comparable with those of the other interactions, being slightly more negative than for the N-TIMP-1/MMP-3cd interaction. ΔHobs for the N-TIMP-1/MMP-1cd interaction shows a strong negative dependence on temperature (Fig. 5), giving a value for ΔCp° of −189 cal/mol (corrected to −194 for buffer contribution). Using this heat capacity change, −TΔSsolv is estimated at ~13 kcal/mol, having a much larger (negative) magnitude than for the N-TIMP-1/MMP-3cd interaction (~3.8 kcal/mol).

Sources of Binding Energy for the Different N-TIMP/MMPcd Interactions—There is an entropic cost to molecular associations that arise from a loss of translational and rotational freedom T(ΔStrans + ΔSrot). This was estimated to be about 3 (±2.4) kcal/mol for associations in general (35, 36), including N-TIMP interactions with MMP catalytic domains. Based on this approximation and the −TΔSint values for the interactions given in Table 3, it can be estimated that the hydrophobic contribution (−TΔSsolv) for the interaction of N-TIMP-1 with MMP-1cd accounts for most of the entropic contribution to the free energy of binding. In the case of the N-TIMP-2/MMP-1cd interaction, it more than accounts for −TΔSint. In contrast to the interactions with MMP-1cd, the magnitude of the hydrophobic effect and association-derived entropy loss suggested by ΔCp do not account for −17.4 and −16 kcal/mol of −TΔSint for the interactions of MMP-3cd with N-TIMP-1 and N-TIMP-2, respectively.

Structural Correlations with Thermodynamics—Crystallographic structures are currently available for the TIMP-1/MMP-3cd and N-TIMP-1/MMP-1cd complexes. Although the crystallographic structures of the corresponding complexes of N-TIMP-2 are not available, a NMR study of the effects of MMP-3cd binding on the conformational dynamics of N-TIMP-2 has been reported (14) and crystallographic structures are available for complexes of TIMP-2 with MMP-13 and MT1-MMP (6, 7).

**TABLE 3**

| Parameter | N-TIMP-1 | N-TIMP-2 |
|-----------|----------|----------|
| ΔHobs (kcal/mol) | 0.58 ± 0.02 | 5.46 ± 0.24 |
| ΔScal (cal/mol) | 49.7 | 55.7 |
| K (µM) | 0.036 ± 0.003 | 4.3 ± 1.0 |
| ΔG° (kcal/mol) | −14.2 ± 1.3 | −11.1 ± 0.2 |
| −TΔSsolv (kcal/mol) | −14.82 | −16.6 |
| −TΔSrot (kcal/mol) | −21.2 | −3.6 |
| Possible −TΔStrans (kcal/mol) | 3.4 | 16.0 |

* The thermodynamic parameters for N-TIMP-1 binding to MMP-3cd at pH 6.8 are from Ref. 22.
* Calculated from ΔHint at 291 K using ΔCp,0 value.
* Values estimated from ΔCp,0.
* Estimated by assuming a cost of 3 kcal/mol for decrease in entropy of translational and rotational degrees of freedom arising from the protein-protein interaction.
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TABLE 4
Character of interaction interfaces and calculated and experimentally determined $\Delta C_p$ and $\Delta H$ for the association of MMP-1cd and MMP-3cd with N-TIMP-1

| MMP1/N-TIMP-1 | ProFace | NACCESS (complex) | NACCESS (free vs bound) | Experimental |
|---------------|---------|-------------------|-------------------------|--------------|
| Buried surface areas Å² | | | | |
| Polar | 655 (41%) | 611 (40%) | 591 (36%) | |
| Non-polar | 816 (59%) | 919 (60%) | 1072 (64%) | |
| Total | 1472 | 1530 | 1663 | |
| Parameters | | | | |
| $\Delta H$ (kcal/mol) | $-7.2$ | $-4.1$ | $-1.2$ | $-0.86$ |
| $\Delta C_p$ (cal/mol/K) | $-169$ | $-202$ | $-247$ | $-194$ |

| MMP3/N-TIMP-1 | | | | |
| Buried surface areas Å² | | | | |
| Polar | 623 (31%) | 538 (27%) | 1141 (60%) | |
| Non-polar | 1366 (69%) | 1452 (73%) | 767 (40%) | |
| Total | 1989 | 1989 | 1908 | |
| Parameters | | | | |
| $\Delta H$ (kcal/mol) | $+3.14$ | $+7.4$ | $-23.8^b$ | $+6.5^c$ |
| $\Delta C_p$ | $-325$ | $-358$ | $-112^b$ | $-50^c$ |

$^a$ Calculated on the basis that there are no structural changes during complex formation.

$^b$ Calculated using the structures of free vs bound forms, accounting for binding linked conformational change as described (22). In the MMP-1cd/N-TIMP-1 analysis the free form of only N-TIMP-1 was used in the analysis.

Analyzes of the chemical nature of the contact surfaces and atoms in the interfaces of the N-TIMP-1-MMP-1cd and N-TIMP-1-MMP-3cd complexes have been previously reported (5, 22). Analyses of the interaction sites indicate that the interface in the MMP-1 complex is significantly less hydrophobic than in the MMP-3 complex (60 versus 70% non-polar) (Table 4). This difference largely reflects differences in the active sites of the two MMPs because the structure of the N-TIMP-1 component is similar in the two MMPcd complexes and the core of the TIMP reactive sites in the two complexes are the same, although there are some variations in contacts at the periphery of the binding site.

A comparison of the structures of the free form of MMP-1cd (8) (PDB 1CGE) and the structure in its complex with N-TIMP-1 (9) (PDB 2JOT) indicates that the interaction with N-TIMP-1 induces only minor conformational changes. If the interactions result in insignificant structural changes in both of the proteins, the interaction interfaces will correspond to the accessible surface areas in the free proteins that are buried on complex formation ($\Delta$ASA). This appears to be the situation for the N-TIMP-1-MMP-1cd interaction because when the non-polar and polar surface areas for the complex determined using NACCESS or ProFace are used in Equation 3 with the parameters from Ref. 29, values for $\Delta C_p$ of $-202$ and $-169$ cal mol$^{-1}$ K$^{-1}$ are calculated (Table 4), in good qualitative agreement with the corrected $\Delta C_p$ of $-194$ cal mol$^{-1}$ K$^{-1}$ measured by ITC (Table 1). When the values were employed to calculate $\Delta H^o$ (60 °C) (Equation 4), and then adjusted to 25 °C, values of $-4.1$ and $-7.2$ kcal/mol were obtained, which agree less well with the experimentally determined value of $-0.9$ kcal/mol (Table 4). When the analysis is conducted using the solution NMR structure of free N-TIMP-1 and the bound form of MMP-1cd, the calculated $\Delta H^o$ is $-1.2$ kcal/mol, in good agreement with the experimental value. In contrast, the calculated values of both $\Delta C_p$ and $\Delta H^o$ for the N-TIMP-1/MMP-3 interaction differ substantially from those determined experimentally. It was previously proposed that the positive enthalpy change for the N-TIMP-1/MMP-3cd interaction must include a strongly unfavorable enthalpic contribution, which might arise from conformational changes, particularly in MMP-3cd (22). Differences between the structure of free MMP-3cd and the structure in the complex include a rearrangement of the N-terminal region of the MMP-3 that includes disruption of a salt bridge between the $\alpha$-amino group of Phe$^{84}$ and the side chain carboxyl of Asp$^{237}$ and a 14 Å movement of residues 83 to 90 to interact with Met$^{66}$ of TIMP-1. Also, as with the binding of other inhibitors to MMP-3 (37), the $S_1'$ loop (Leu$^{222}$ to Arg$^{231}$) moves along with the side chain of Tyr$^{223}$, which covers the $S_1'$ pocket in the uninhibited state. Although the solvation or desolvation of non-polar surfaces has been useful for correlating structural changes associated with protein interactions and unfolding/folding processes with heat capacity changes, $\Delta C_p$, (27, 28, 34, 36), there have been a number of previous reports of anomalous heat capacity effects (see Refs. 38 and 39). In the present case, $\Delta C_p^o$ for the N-TIMP-1/MMP-3cd interaction is much less negative than the structure-based prediction suggesting that there is a source of positive change in $\Delta C_p$. There are two potential explanations. One is the conformational change in MMP3cd induced by the binding of N-TIMP-1 that exposes 600 Å² of surface outside of the interface (22). This results in the exposure of non-polar surface, thereby reducing the net hydrophobic effect arising from the interaction and diminishing $\Delta C_p^o$. This brings the predicted $\Delta C_p^o$ of $-112$ cal/mol/K into better agreement with the experimentally measured $\Delta C_p^o$ of $-50$ cal/mol/K for the association (Table 4). A second explanation, suggested by previous NMR studies (22), is that a large component of the entropy increase driving the interaction of N-TIMP-1 and MMP-3cd could arise from an increase in conformational entropy in the core of the TIMP $\beta$-barrel; this could result from the disruption of cooperative non-covalent interactions, a phenomenon that has been linked to “unconventional” positive heat capacity changes, (39). A modest disruption of non-covalent packing interactions in N-TIMP-1 could also contribute to the positive $\Delta H$ of binding.

From the limited data that are currently available it appears that the MMPcd component may determine the general fea-
tures of the thermodynamic profiles of each MMPcd/N-TIMP interaction. Based on this, it would be predicted that the interaction of N-TIMP-2 with MMP-3cd, but not with MMP-1cd, is accompanied by structural rearrangements and increased dynamics. Currently, there are no structural data to support this hypothesis but NMR studies of the effects of MMP-3cd binding to N-TIMP-2 indicate that it enhances the mobility of some residues of the inhibitor remote from the interface in two of the segments that, in N-TIMP-1, show the most pronounced increase in backbone dynamics arising from MMP-3cd binding. Specifically, MMP-3cd binding caused the $^{15}$N T$_2$ values of Ile$_{19}$–Thr$_{21}$, Leu$_{84}$, and Ala$_{86}$ of N-TIMP-2 to fall below the average T$_2$, suggesting microsecond-millisecond exchange broadening (14). Although not conclusive, these results are consistent with a possible contribution of conformational dynamics to the entropy increase that drives the interaction of MMP-3cd with N-TIMP-2 as well as N-TIMP-1.

Ionization Changes on Complex Formation—The differences in the ionization effects associated with the different complexes (Table 1) could arise from sequence differences in the interaction sites, roles of different residues in stabilizing different complexes, and changes associated with interaction-induced conformational transitions. Which groups undergo ionization state changes upon association is a matter of speculation. The interactions of both N-TIMPs with MMP-3cd have N$_{14}$ of 0.8 to 0.9 less than their interactions with MMP-1cd. The association of N-TIMP-2 with MMP-3cd is accompanied by release of a proton, suggesting the presence of a group in the MMP-3cd active site, but not in MMP-1cd, which can release a proton. This could conceivably result from the partial deprotonation of the α-amino group (pK 7.7 ± 0.5) (40) of the N-terminal residue, Phe$_{83}$ (4), of MMP-3 at pH 7.4 when its interaction with Asp$_{237}$ is disrupted.

The residue that becomes protonated at pH 6.8 on the association of N-TIMP-1 with MMP-1cd could be Glu$_{219}$, the general base in the active site. Its pK appears to be around 6 in free MMP-3 (see Holman et al. (41)) but it seems unlikely that this would increase sufficiently on binding N-TIMP-1, to account for this change. Alternatively, Glu$_{67}$ of TIMP-1 (Ser in TIMP-2) might take up a proton upon association at pH 6.8. Glu$_{67}$ makes 12 contacts with Ser$_{227}$ and His$_{228}$ in the MMP-1cd complex but only 2 with His$_{211}$ in the MMP-3cd complex.

Conclusions—Protein-protein interactions have key roles in numerous biological processes and understanding the structural and biophysical bases of high affinity binding and specificity is of fundamental interest in structural biology. The goal of the present study was to determine the thermodynamic and structural bases of high affinity binding and affinity differences in TIMP/MMP interactions. These are of particular interest because of their relevance to engineering TIMPs to be targeted inhibitors of MMPs for possible clinical application in diseases such as cancer and arthritis (3). Structural studies of the complexes of TIMP-1 with MMP-1 and MMP-3 and TIMP-2 with MMP-13 and MMP-14 have shown that the same binding surface of the inhibitor can make different interactions with different MMPs involving both shared and distinct chemical groups in the active site; also, shifts in orientation of TIMP and changes in interactions with loops at the periphery of the active site have been noted (4–7). However, a major interaction-induced structural transition has only been observed in MMP-3cd in its complex with TIMP-1. The thermodynamic profiles reported here show that the interactions of both N-TIMP-1 and N-TIMP-2 with MMP-3cd have nearly 4–5-fold smaller (negative) heat capacity changes, greater (positive) entropy changes, and less favorable enthalpy changes than those for the corresponding interactions with MMP-1cd. These may reflect conformational changes in MMP-3cd in its complexes with both N-TIMPs but only minor changes in MMP-1cd in corresponding complexes (4, 5); a redistribution of dynamics when N-TIMPs bind to MMP-3cd may also underlie the “anomalous” profiles. Disorder to order structural transitions in protein interactions have been linked to negative AC$_p$ values of greater magnitude than predicted from the composition of buried surfaces (42); it appears that the converse might apply to the interactions of the two N-TIMPs with MMP-3cd where the interacting N-TIMPs may be more ordered than their complexes. Based on these considerations we would hypothesize that the N-TIMP-2:MMP-3cd complex will show similar conformational changes to those in the TIMP-1:MMP-3cd complex but the N-TIMP-2:MMP-1cd complex will not. Because large structural adjustments have not been observed in other TIMP-MMP complexes (5–7), it is possible that they are unique to those involving MMP-3, but structural studies of additional TIMP-MMP complexes are needed to clarify this.

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