Thermodynamic Investigation of the Heparin−Mucus Proteinase Inhibitor Binding

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Abstract: The thermodynamics of the interaction of mucus proteinase inhibitor (MPI) with heparin has been studied. The apparent association constant K' and the enthalpy of reaction have been determined in buffered aqueous solutions (Hepes + NaCl) using flow microcalorimetry. The measurements have been performed as a function of pH, temperature, and NaCl concentration, and the standard transformed Gibbs energy, enthalpy, entropy, and heat capacity of association have been calculated. The following values have been found in 0.05 M Hepes and 0.1 M NaCl at pH 7.4 and 25 °C: $K' = (4.9 \pm 1.3) \times 10^6$; $\Delta_r G'^\circ = -38.2 \pm 0.6$ kJ mol^{-1}; $\Delta_r H'^\circ = -64.9 \pm 1.3$ kJ mol^{-1}; $T\Delta_r S'^\circ = -26.7 \pm 1.9$ kJ mol^{-1}; $\Delta_r C_p'^\circ = -180 \pm 20$ J K^{-1} mol^{-1}. The pH dependency of K' shows that one proton of the medium is transferred to the MPI−heparin complex upon association. This proton uptake is controlled by a group on the protein whose pK_a is lower than 9. NaCl strongly decreases K', an effect that indicates that heparin binds to MPI through five or six ionic interactions. NaCl also increases $\Delta_r H'^\circ$ and $\Delta_r S'^\circ$, but the association process is enthalpy-driven over the whole range of NaCl concentrations. The small effect of temperature on $\Delta_r H'^\circ$ and $\Delta_r S'^\circ$ suggests that hydrophobic interactions are not involved in the association process. Our thermodynamic data also suggest that heparin behaves as a ligand of the agonist type for the "receptor" MPI which may undergo a structural transition following the binding of the ligand.

Introduction

Heparins are naturally occurring glycosaminoglycans with variable degrees of sulfation and polymerization. Mammalian heparin mostly consists of trisulfated disaccharide units formed of L-iduronic acid 2-sulfate linked to D-glucosamine *N*,3,6-disulfate. It has been known for decades as an anticoagulant drug.

Heparin forms tight complexes with a number of proteins, whose biological properties are usually modified by the polymer.[1] For example, heparin binds serine proteinase inhibitors belonging to the serpin superfamily, namely, antithrombin, heparin cofactor, protease nexin, plasminogen activator inhibitor I, and protein C, and increases the rate of inhibition of their target proteinases. The heparin-promoted acceleration of thrombin inhibition by antithrombin provides the molecular basis for the anticoagulant effect of the sulfated glycosaminoglycan.

We have recently shown[2] that heparin also binds to mucus proteinase inhibitor (MPI), a reversible neutrophil elastase inhibitor that does not belong to the serpin superfamily, and accelerates the enzyme inhibition. MPI is an 11.7 kDa, highly basic nonglycosylated protein naturally occurring in the airways where it acts as a physiologic antielastase. The inhibitor has been crystallized in complex with chymotrypsin, and its tertiary structure has been determined at 2.5 Å resolution.[3] The molecule is organized in two homologous domains, each of which is strongly stabilized by four disulfide bridges. The majority inhibitory reactive site is located on the C-terminal domain. A second reactive site has been demonstrated but not yet localized.[4]

Using fluorescence spectroscopy, we have shown[2] that commercial low molar mass heparin with an average mass of 5 kDa forms a 1:1 complex with MPI with an equilibrium constant K' of 2×10^7 in 0.05 M Hepes and 0.1 M NaCl at pH 7.4 and 25 °C. Furthermore, from the variation of K' with ionic strength we have inferred that about seven ionic interactions are involved in heparin−inhibitor binding. A heparin-induced conformational change of the inhibitor was evidenced by a 4-fold increase in the quantum yield of tryptophan 30, a blue shift of its maximum emission wavelength, a decrease of the acrylamide quenching rate, and an increase in the mean intensity weighted lifetime.

Thermodynamics helps in understanding how subunits of multimeric proteins assemble[5−8] and how monomeric proteins are stabilized.[9] Microcalorimetry is a powerful tool for measuring the thermodynamic parameters that characterize interacting molecules. It not only measures enthalpy changes but may also help determine association constants, even in the case of weak interactions.[10,11] Literature is poorly documented on microcalorimetric studies on protein−heparin equilibria.[12,13] DeLauder *et al.*[12] investigated the thermodynamics of the binding

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of heparin to antithrombin while Thompson *et al.*[13] used calorimetry to assess the effect of site-directed mutagenesis of basic fibroblast growth factor (bFGF) on the energetics of the binding of heparin to the growth factor. Both investigations used commercial polydisperse heparins at constant pH and temperature. The present report describes the use of flow calorimetry to investigate the effect of pH, ionic strength, and temperature on the thermodynamics of MPI binding to a fairly monodisperse heparin preparation.

Experimental Section

Materials. Human recombinant MPI was obtained from Synergen (Boulder, CO) through the courtesy of Dr H. P. Schnebli, Ciba-Geigy (Basel, Switzerland). The amino acid composition of the expression product was confirmed by protein sequencing[14] and its purity checked by SDS–PAGE and reversed-phase HPLC.[14] The inhibitor was active-site-titrated with human neutrophil elastase which was isolated from purulent sputum[15] and itself active-site-titrated with acetyl-Ala_2-Aza-Ala-*p*-nitrophenyl ester[16] from Enzyme System Products (Livermore, CA). Commercial heparin with a mean molar mass of 5.1 kDa (Calbiochem, La Jolla, CA) is a depolymerization product of porcine mucosal heparin. Buffering solutions were prepared using 0.05 M Hepes and NaCl, and the pH was adjusted with NaOH. The buffer was filtered through a 0.45 μm microporous cellulose acetate membrane on a Millipore device (Molsheim, France) and degassed prior to use.

Heparin Fractionation and Characterization. A low molar mass fraction of heparin with reduced polydispersity was prepared from commercially available heparin by gel filtration chromatography on a Sephadex G-50 column and rechromatography as described elsewhere.[17] Salt-free sodium heparin was obtained by lyophilization and gel filtration through a Sephadex G-10 column followed by relyophilization. The fraction has a molar mass of 4.5 ± 0.5 kDa, as determined by ultracentrifugation, and binds MPI with a 1:1 stoichiometry.[17]

Microcalorimetry. All measurements were performed using a multichannel microcalorimeter (LKB 2277 thermal activity monitor) equipped with a flow-mix measuring vessel. Suurkuusk and Wadsö[18] have thoroughly described this twin thermopile heat-conduction calorimeter and analyzed its performance. Association of MPI with heparin was followed by measuring the heats of mixing of MPI solutions at constant concentration and heparin solutions at varying concentration. The molarities were on the order of 10^{-6}–10^{-5}. The buffered MPI and heparin solutions were injected into the mixing vessel at about the same flow rate (*ca.* 3.3×10^{-3} mL s^{-1}) using a two-channel peristaltic pump (Gilson Minipuls). The power values observed upon mixing were measured on the most sensitive scale, namely, 3 μW. There was less than 0.1 μW mean noise. The heats of dilution of both components, measured under the same conditions, were found to be totally negligible. Consequently, the experimentally determined heats were considered to be the heats of reaction. The measurements were performed at five temperatures ranging from 20 to 40 °C in the following buffer: 0.05 M Hepes, 0.1 M NaCl, pH 7.4. Hepes has a pK_a of 7.55 which undergoes little variation with temperature ($\Delta pK_a/\Delta T$ of -0.014 K^{-1}).[19] The effects of pH and NaCl concentration were both studied at 25 °C. Nonlinear regression analysis of the data was done using the Enzfitter program (Elsevier-BIOSOFT).

Results

Theoretical Background. The binding of heparin to MPI may be described by the biochemical reaction

$$\text{MPI} + \text{heparin} \rightleftarrows \text{MPI–heparin}$$

which, following the IUPAC last recommendations concerning biochemical reactions,[20] is characterized by an apparent association constant K' in a medium of specified temperature, pressure, pH, pNa, and ionic strength I. The K' constant is made up of the equilibrium concentrations of the reactants relative to the standard state concentration $c^\circ = 1$ M:

$$K' = \frac{[\text{MPI–heparin}]/c^\circ}{([\text{MPI}]/c^\circ)([\text{heparin}]/c^\circ)} \quad (1)$$

pNa can obviously be considered as constant since the reactant concentrations are very low as compared with the buffer and NaCl concentrations. On the other hand, the ionic strength can be considered as being equal to the NaCl concentration, since the contribution of the zwitterionic buffer Hepes to the ionic strength can be neglected.

In order to provide the criterion for equilibrium at specified pH and pNa, a transformed Gibbs energy G' must be defined in terms of the Gibbs energy G by using the Legendre transform[21,22]

$$G' = G - n'(H^+)\,\mu(H^+) - n'(Na^+)\,\mu(Na^+) \quad (2)$$

where $n'(H^+)$ and $n'(Na^+)$ are the total amounts of free and bound H^+ and Na^+, respectively, and $\mu(H^+)$ and $\mu(Na^+)$ are the chemical potentials of H^+ and Na^+, respectively. This new way of describing biochemical reactions in terms of transformed thermodynamic properties has been thoroughly presented by Alberty in a recent review.[23] The standard transformed Gibbs energy of reaction $\Delta_r G'^\circ$ is related to the apparent association constant by

$$\Delta_r G'^\circ = -RT \ln K' \quad (3)$$

It is made up of the standard transformed enthalpy of reaction $\Delta_r H'^\circ$, which is given by

$$\Delta_r H'^\circ = RT^2\left(\frac{\partial \ln K'}{\partial T}\right)_{P,\text{pH},\text{pNa},I} \quad (4)$$

and the standard transformed entropy of reaction $\Delta_r S'^\circ$:

$$\Delta_r S'^\circ = \frac{\Delta_r H'^\circ - \Delta_r G'^\circ}{T} \quad (5)$$

In the present case, calorimetric titration was used to measure K' as described above. The power value P is related to K' and to the MPI and heparin concentrations through the following equation:

$$P = P_{sat}[(K'[\text{MPI}]_o + K'[\text{heparin}]_o + 1 - \{(K'[\text{MPI}]_o + K'[\text{heparin}]_o + 1)^2 - 4K'^2[\text{MPI}]_o[\text{heparin}]_o\}^{1/2})/(2K'[\text{MPI}]_o)] \quad (6)$$

where P_{sat} is the asymptotic value of the power at infinite heparin concentration, and $[\text{MPI}]_o$ and $[\text{heparin}]_o$ are the analytical MPI and heparin concentrations after mixing, respectively. The expression in square brackets corresponds to the fraction of complexed MPI. The regression analysis of the data yielded the best estimates of K' and P_{sat}, and their standard errors. It can be noted that the tangent drawn to the titration curve of

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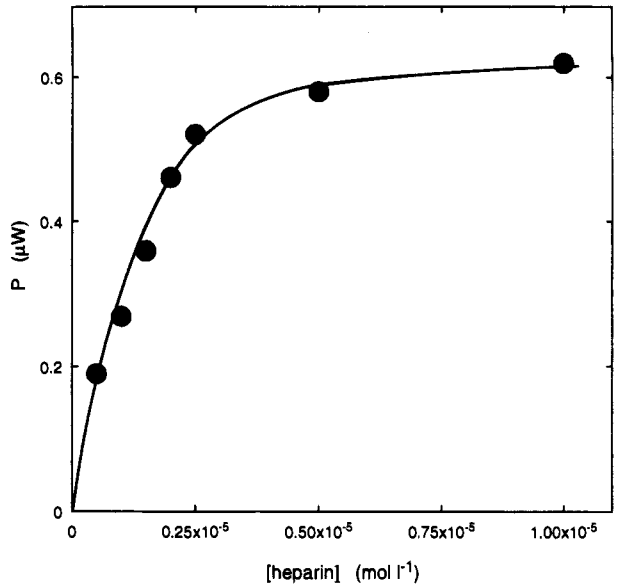

Figure 1. Calorimetrically measured power generated by mixing MPI solutions at constant concentrations with heparin solutions at increasing concentrations in 0.05 M Hepes and 0.1 M NaCl at pH 7.4 and 25 °C.

Table 1. Effect of pH on the Association of Heparin with MPI in 0.05 M Hepes and 0.1 M NaCl at 25 °C

pH	K'	Δ_rH(cal) (kJ mol^{-1})	$\Delta_rG'^\circ$ (kJ mol^{-1})	$\Delta_rH'^\circ$ (kJ mol^{-1})	$T\Delta_rS'^\circ$ (kJ mol^{-1})
6.4	$(1.5 \pm 0.7) \times 10^7$	-60.4 ± 1.4	−41.0	−60.4[a]	−19.4
6.9	$(1.5 \pm 0.7) \times 10^7$	-47.0 ± 1.2	−41.0	−66.7	−25.7
7.4	$(4.9 \pm 1.3) \times 10^6$	-45.2 ± 1.3	−38.2	−64.9	−26.7
7.9	$(1.5 \pm 0.2) \times 10^6$	-41.2 ± 1.2	−35.3	−60.9	−25.6
8.4	$(6.6 \pm 0.2) \times 10^5$	-38.7 ± 0.8	−33.2	−58.4	−25.2

[a] Uncorrected result (see text).

Figure 1 intercepts the asymptote to this curve at $[\text{heparin}]_o = [\text{MPI}]_o$, thus confirming the 1:1 stoichiometry.

The calorimetrically measured enthalpy change Δ_rH(cal) was calculated from P_{sat} using

$$\Delta_rH(\text{cal}) = -\frac{P_{sat}}{2f_{MPI}[\text{MPI}]_o} \quad (7)$$

where f_{MPI} is the flow rate of the inhibitor solution. Δ_rH(cal) theoretically includes the enthalpies of reaction of H^+ and Na^+ (consumed or produced) with the buffer. In the present case, there is no reaction between Na^+ and the buffer used (Hepes). The standard transformed enthalpy of association $\Delta_rH'^\circ$ can thus be calculated from Δ_rH(cal), provided correction is made for the enthalpy of reaction caused by the change in the binding of H^+. This change corresponds to the difference between the number of H^+ ions bound by the products and the number of H^+ ions bound by the reactants at specified T, P, pH, and pNa. Thus, following IUPAC recommendations,[20,23] $\Delta_rH'^\circ$ can be calculated using

$$\Delta_rH'^\circ = \Delta_rH(\text{cal}) - \Delta_rN(H^+)\,\Delta_aH^\circ(\text{Hepes}) \quad (8)$$

where $\Delta_rN(H^+)$ is the change in binding of H^+ and Δ_aH°(Hepes) is the standard enthalpy for the acid dissociation of the buffer. At 25 °C, the enthalpy for the deprotonation of 0.02 M Hepes in 0.1 M NaCl is equal to 21.41 kJ mol^{-1}.[24]

Effect of pH. The MPI−heparin association was studied as a function of pH in 0.1 M NaCl at 25 °C. The K' and Δ_rH(cal) values calorimetrically determined in the pH range of 6.4−8.4 are reported in Table 1 together with their standard errors. The

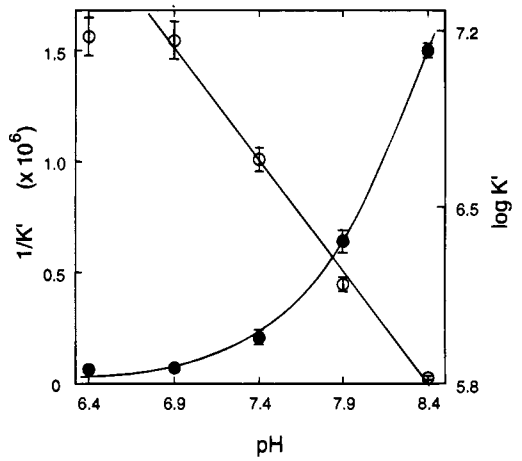

Figure 2. Effect of pH on the apparent constant K' for the heparin−MPI complex in 0.05 M Hepes and 0.1 M NaCl at 25 °C: $1/K'$ *vs* pH (●) and log K' *vs* pH (○).

Table 2. Effect of NaCl Concentration on the Association of Heparin with MPI in 0.05 M Hepes at pH 7.4 and 25 °C[a]

[NaCl] (M)	K'	Δ_rH(cal) (kJ mol^{-1})	$\Delta_rG'^\circ$ (kJ mol^{-1})	$\Delta_rH'^\circ$ (kJ mol^{-1})	$T\Delta_rS'^\circ$ (kJ mol^{-1})
0.05	$(1.9 \pm 0.8) \times 10^7$	-64.9 ± 1.3	−41.6	−84.4	−42.8
0.10	$(4.9 \pm 1.3) \times 10^6$	-45.2 ± 1.3	−38.2	−64.9	−26.7
0.15	$(6.0 \pm 2.2) \times 10^5$	-33.4 ± 1.7	−33.0	−53.2	−20.2
0.20	$(6.1 \pm 0.8) \times 10^4$	-23.6 ± 1.0	−27.3	−43.5	−16.2
0.25	$(2.8 \pm 0.6) \times 10^4$	-9.1 ± 0.8	−25.4	−29.2	−3.8

[a] Assumed $\Delta_rN(H^+) = 0.92$.

buffering range of Hepes did not allow further measurements in the alkaline zone. It can be noticed in Figure 2 that, between pH 6.9 and pH 8.4, log K' varies linearly with pH; the point at pH 6.4 is clearly outside this straight line. The slope of the straight line (−0.92), which is equal to $-\Delta_rN(H^+)$,[25] indicates that one proton of the medium is transferred to the MPI−heparin complex in this pH range. It follows from eq 8 that

$$\Delta_rH'^\circ = \Delta_rH(\text{cal}) - 0.92 \times 21.41 \text{ kJ mol}^{-1} = \Delta_rH(\text{cal}) - 19.7 \text{ kJ mol}^{-1} \quad (9)$$

This relation was used to calculate $\Delta_rH'^\circ$ from Δ_rH(cal) for the pH values ranging from 6.9 to 8.4 (Table 1). Since K' does not vary between pH 6.4 and pH 6.9, it was considered that $\Delta_rH'^\circ = \Delta_rH$(cal) at pH 6.4. It must be noted that the uncorrected value at pH 6.4 is consistent with the corrected ones calculated at the other pH values. A direct plot of $1/K'$ *vs* pH yielded the acidic tail of a titration curve constructed using $pK_a = 8.7$. The calculated standard transformed Gibbs energies and entropies of association are also reported in Table 1.

Effect of NaCl Concentration. The concentration of NaCl was varied from 0.05 to 0.25 M in order to study the effect of the Na^+ counterion on the thermodynamic properties of association at 25 °C and pH 7.4. Because of the paucity of the reactants, it was not possible to study the pH effect at each ionic strength. We therefore assumed that $\Delta_rN(H^+)$ was equal to 0.92 at any [NaCl]. Olofsson and co-workers[24] determined the standard enthalpy for the acid dissociation of Hepes at varying ionic strength: they gave Δ_aH°(Hepes)(kJ mol^{-1}) = $20.7 + 2.2I^{1/2}$ at 25 °C. This variation does not significantly affect the results but was nevertheless used to estimate the second term on the right-hand side of eq 8 at each NaCl concentration. The calorimetrically determined association constants and enthalpy changes are reported in Table 2 together with the calculated standard transformed thermodynamic parameters of association.

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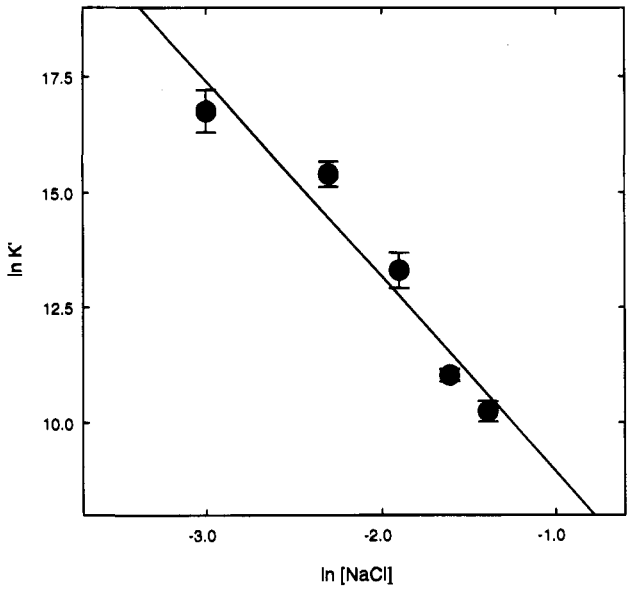

Figure 3. Effect of $[Na^+]$ on the apparent constant K' for the heparin–MPI complex in 0.05 M Hepes at pH 7.4 and 25 °C: ln K' *vs* ln $[Na^+]$.

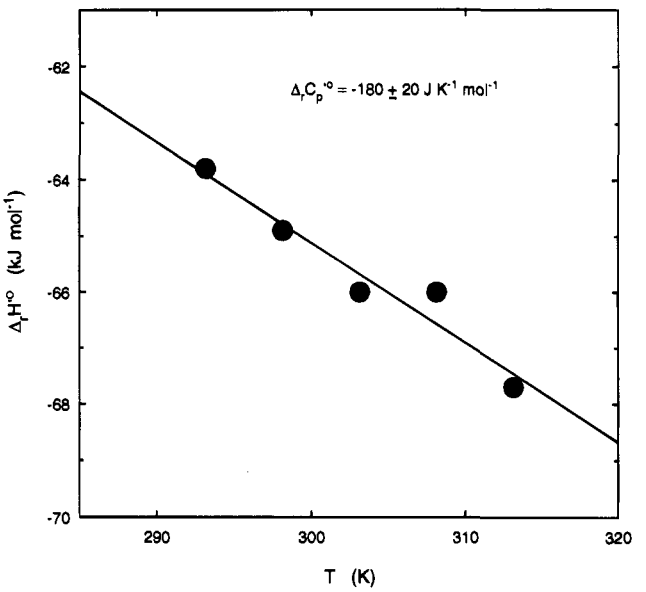

Figure 4. Effect of temperature on the standard transformed enthalpy of association of heparin with MPI in 0.05 M Hepes and 0.1 M NaCl at pH 7.4 and 25 °C. A linear least-squares analysis of the data yielded a slope of -180 ± 20 J K^{-1} mol^{-1} (see Table 3).

Table 3. Effect of Temperature on the Association of Heparin with MPI in 0.05 M Hepes and 0.1 M NaCl at pH 7.4[a]

Θ (°C)	K'	$\Delta_r H$(cal) (kJ mol^{-1})	$\Delta_r G'^\circ$ (kJ mol^{-1})	$\Delta_r H'^\circ$ [a] (kJ mol^{-1})	$T\Delta_r S'^\circ$ (kJ mol^{-1})
20	$(4.1 \pm 0.9) \times 10^6$	-44.4 ± 1.1	−37.1	−63.8	−26.7
25	$(4.9 \pm 1.3) \times 10^6$	-45.2 ± 1.3	−38.2	−64.9	−26.7
30	$(3.4 \pm 1.0) \times 10^6$	-46.0 ± 1.6	−37.9	−66.0	−28.1
35	$(2.6 \pm 0.6) \times 10^6$	-45.7 ± 1.5	−37.8	−66.0	−28.2
40	$(8.5 \pm 1.7) \times 10^5$	-47.1 ± 2.4	−35.5	−67.7	−32.2

[a] Assumed $\Delta_r N(H^+) = 0.92$.

The apparent association constant decreases by nearly 3 orders of magnitude over this ionic strength range (Figure 3), whereas $\Delta_r H'^\circ$ and $\Delta_r S'^\circ$ become less negative.

Effect of Temperature. The thermodynamic properties of association at pH 7.4 and 0.1 M NaCl were also determined at five temperatures ranging from 20 to 40 °C. Again, for the reason given above, it was not possible to study the effect of pH and ionic strength at each temperature. We therefore assumed that $\Delta_r N(H^+) = 0.92$ at all the temperatures studied. $\Delta_a H^\circ$(Hepes) was calculated at each temperature using $\Delta_a H^\circ(\text{Hepes})_{T_2} = \Delta_a H^\circ(\text{Hepes})_{T_1} + 65.8 \times 10^{-3}(T_2 - T_1)$.[24] Although this relation was established for Hepes in 0.15 M NaCl, we used it for 0.1 M NaCl because the variation of $\Delta_a H^\circ$(Hepes) with I is very weak. The $\Delta_a H^\circ$(Hepes) values calculated at each temperature were then introduced into eq 8 to correct the calorimetrically determined enthalpies of association (Table 3). From the slight decrease of $\Delta_r H'^\circ$ with temperature (Figure 4), it was possible to estimate the standard transformed heat capacity of association: $\Delta_r C_p'^\circ = -180 \pm 20$ J K^{-1} mol^{-1}.

Discussion

Studies on heparin–protein interactions usually focus more on the functional role of these interactions (*e.g.*, proteinase or growth factor activity) than on their thermodynamic characterization. Only two calorimetric investigations have been reported to date.[12,13] Both of them used commercial polydisperse heparin at constant pH and temperature. In one study the ionic strength was varied.[13] Here we describe a thorough thermodynamic characterization of the heparin–MPI interaction using a homogeneous heparin preparation and studying the binding energetics at variable pH, ionic strength, and temperature.

The heparin–MPI affinity is insensitive to pH between pH 6.4 and pH 6.9 but decreases by a factor of 23 between pH 6.9 and pH 8.4. The slope of the log K' *vs* pH plot (Figure 2) indicates that one proton of the medium is transferred to the MPI–heparin complex. An identical transfer was observed with the antithrombin–heparin complex.[12] A number of lysine and arginine residues have been shown to be involved in the binding of heparin to MPI.[26] The normal pK_a values of lysine and arginine residues in proteins at 25 °C are 10.0 ± 0.6 and 12.0 ± 0.4, respectively. The 23-fold decrease in K' between pH 6.9 and pH 8.4 can therefore not be due to the titration of a lysine or an arginine residue with a normal pK_a. It more likely involves a residue with a lower pK_a as suggested by the plot of $1/K'$ *vs* pH (Figure 2) which yields the acidic tail of a titration curve fitted to a pK_a of 8.7. Lys 87 of MPI is a good candidate for such a titration because it participates in heparin binding,[26] and is distant by 3.8 Å from Phe 5, which might decrease its pK_a.

K' decreases by a factor of 680 when [NaCl] increases from 0.05 to 0.25 M (Table 2). Theoretically, NaCl may affect K' in several ways: (i) the Na^+ cations from the medium may compete with Na^+ counterions of heparin released during the heparin–protein association, thus disfavoring this association, (ii) the ionic strength of the medium may have a deleterious screening effect on the charged species, and (iii) there may be a salt effect on the hydration of the species. Assuming that the latter effect is small for salt concentrations lower than 1 M, the two other effects may be accounted for by the theory of Record and co-workers:[27,28]

$$\ln K'(T,P,\text{pH},\text{pNa}) = \ln K'(T,P,\text{pH},[\text{NaCl}] = 1\text{M},\text{id}) + z\zeta^{-1}\ln(y_\pm\delta) - z\psi \ln [Na^+] \quad (10)$$

where $K'(T,P,\text{pH},\text{pNa})$ is the apparent association constant measured at given T, P, pH, and [NaCl], K'(T,P,pH,[NaCl] = 1M,id) is the theoretical constant for NaCl in the standard state (ideal, 1 M), z is the number of charges involved in the electrostatic interaction, ζ is the reduced charge density parameter, $y_\pm$ is the mean activity coefficient of NaCl, δ is one-

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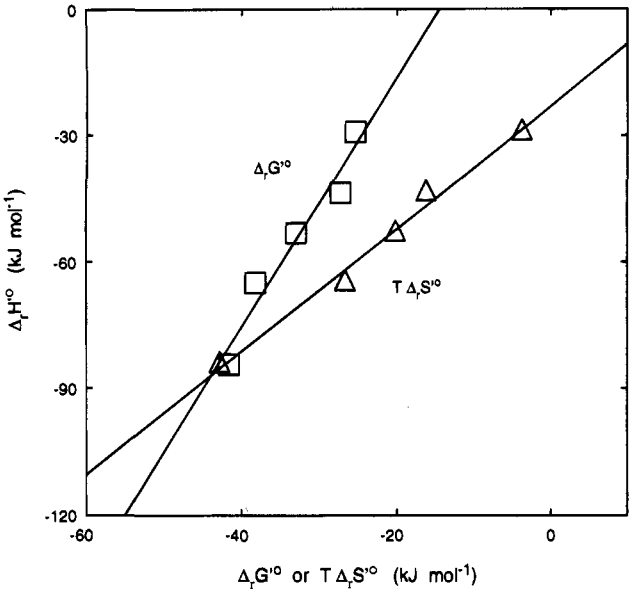

Figure 5. $\Delta_r H'^\circ$ *vs* $\Delta_r G'^\circ$ or $T\Delta_r S'^\circ$ for the association of heparin with MPI in 0.05 M Hepes at pH 7.4 and 25 °C in the presence of NaCl at various concentrations.

third of the mean distance between the charges of the polyanion, and ψ is the fraction of Na^+ bound to heparin per unit charge. $\zeta = 2.5$ and $\psi = 0.8$ were taken from the literature,[29] δ was calculated from ζ using a known relationship,[27] and $y_\pm$ was assumed to be 0.75 within the working NaCl concentration range. Hence, the term $z\zeta^{-1}$ $\ln(y_\pm\delta)$, which reflects the deviation from ideality due to Coulombic interactions, was found to be −0.81. The plot in accordance with eq 10 yielded a straight line (Figure 3) whose slope gave $z = 5.5 \pm 0.8$. Thus five to six ionic interactions are involved in the binding of our homogeneous 4.5 kDa heparin to MPI. With the same heparin preparation, four ionic interactions were detected using chemical modification procedures.[26] With a different heparin, we previously found $z = 7.2$.[2] The thrombin−heparin interaction also involves five to six interactions.[29] In contrast, only two to three Na^+ cations are displaced during the binding of heparin to basic fibroblast growth factor.[13]

On the other hand, $K'(T,P,\text{pH},[\text{NaCl}] = 1\text{M},\text{id})$ was found to be 2×10^2 which corresponds to $\Delta_r G'^\circ = -13$ kJ mol^{-1}, a value that represents 34% of $\Delta_r G'^\circ$ measured in the presence of 0.1 M NaCl (Table 2). In contrast, this percentage is much higher (70%) in the case of the interaction of heparin with basic fibroblast growth factor or antithrombin.[13,30] Table 2 shows that $\Delta_r H'^\circ$ also increases with [NaCl] although the heparin−MPI association is enthalpy-driven over the whole range of [NaCl] investigated. By differentiating eq 10 with respect to temperature, we obtained an equation (not shown) that predicted a linear variation of $\Delta H'^\circ$ with ln [Na^+] with a slope of *ca.* −1 kJ mol^{-1}. The experimental slope was found to be 30 kJ mol^{-1} when the electrolyte concentration was varied from 0.05 to 0.25 M. This increase in $\Delta_r H'^\circ$ may be due to the salt effect on the hydration of the charged species, a phenomenon that the theory of Record *et al.*[27] does not account for. It is likely that the enthalpy changes accompanying these solvation effects account for most of the increase in $\Delta_r H'^\circ$.

Both $\Delta_r H'^\circ$ and $T\Delta_r S'^\circ$ increase with the ionic strength and are linearly related through $\Delta_r H'^\circ = \alpha + \beta T\Delta_r S'^\circ$ where $\alpha = -22$ and $\beta = 1.5$ (Figure 5). It is noteworthy that the present data points, which were obtained with different [NaCl] in the medium, fit well on the enthalpy−entropy correlation band drawn by Gilli *et al.*[31] for 136 different ligands binding to 10 biological receptors. This confirms that the enthalpy−entropy compensation is a widespread phenomenon. Moreover, the magnitudes of $\Delta_r H'^\circ$ and $\Delta_r S'^\circ$ at pH 7.4, 25 °C, and [NaCl] = 0.1 M (Table 2) suggest that heparin may be viewed as an agonist-type ligand for the MPI "receptor".[32] An agonist is able to induce a conformational change of the receptor, a prerequisite for the modification of the receptor's activity. This is consistent with the previously demonstrated heparin-induced conformational change of MPI[2] which might be responsible for the increased activity of the inhibitor. Figure 5 also shows that $\Delta_r H'^\circ$ is linearly related to $\Delta_r G'^\circ$ with a slope $\gamma = 2.9$. This observation helps demonstrating the internal consistency of our data since, if K' and $\Delta_r H'^\circ$ are not independently determined, as in the present case, the only unbiased estimate of chemical compensation is the one which correlates $\Delta_r H'^\circ$ and $\Delta_r G'^\circ$.[33] Also it needs to be pointed out that β and γ, the slopes of the two compensation plots, are perfectly consistent since classical thermodynamics dictates that $\gamma = 1/(1 - 1/\beta)$.[33]

The negative standard transformed heat capacity of association (Figure 4) as well as the decrease of $\Delta_r S'^\circ$ with T (Table 3) at first sight suggests that hydrophobic interactions play some role in the binding of heparin to MPI. This hypothesis may, however, safely be rejected because of the small magnitudes of these two changes ($\Delta_r C_p'^\circ = -180$ J K^{-1} mol^{-1} and $\Delta\Delta_r S'^\circ = -12$ J K^{-1} mol^{-1} between 20 and 40 °C) which could as well be due to modifications in the low-frequency vibration modes of the reactants following their association.[6] Between 20 and 40 °C, there is a 5-fold decrease in K' in spite of a favorable decrease in $\Delta_r H'^\circ$. The drop in heparin−MPI affinity is in fact due to the decrease in $\Delta_r S'^\circ$ which might itself be indicative of a thermal transconformation of one or both of the reactants accompanied by an increase of the degrees of freedom. A van't Hoff plot (eq 4) gave $\Delta_r H'^\circ = -69 \pm 24$ kJ mol^{-1}, a value that favorably compares with the calorimetrically determined $\Delta_r H'^\circ$ of −65 kJ mol^{-1} (Table 3). This again emphasizes the internal consistency of our data.

Conclusion

The present work shows that heparin binds to MPI in the reference aqueous medium (0.05 M Hepes, 0.1 M NaCl, pH 7.4, 25 °C) through ionic interactions of weak energy: since five to six sites participate in the binding, the enthalpy involved per site is on the order of −10 kJ mol^{-1}. Nevertheless, the total enthalpy is rather large and controls the association process. Accordingly, heparin can be classified as a ligand of the agonist type when it binds to the MPI receptor, which might explain why it changes the activity of the inhibitor. The pH effect shows that one proton of the medium is transferred to the MPI−heparin complex upon association. On the other hand, NaCl significantly increases $\Delta_r S'^\circ$, mainly because of the dilution of the Na^+ counterions released by heparin. The ionic strength also strongly increases the enthalpy of interaction, which might be due to a salt effect on the solvation of the ionic groups combined with a screening effect. The temperature dependence of the thermodynamic properties of reaction indicates that hydrophobic interactions probably do not play a significant role in the association.

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