Thermodynamics of Charged Oligopeptide−Heparin Interactions†

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Received August 15, 1994; Revised Manuscript Received December 1, 1994⊗

ABSTRACT: To better understand the electrostatic component of the interaction between proteins and the polyanion heparin, we have investigated the thermodynamics of heparin binding to positively charged oligopeptides containing lysine or arginine and tryptophan (KWK-CO_2 and RWR-CO_2). The binding of these peptides to heparin is accompanied by an enhancement of the peptide tryptophan fluorescence, and we have used this to determine equilibrium binding constants. The extent of fluorescence enhancement is similar for both peptides, suggesting that the tryptophan interaction is similar for both. Titrations of these peptides with a series of simple salts suggest that this fluorescence enhancement is due to the interaction of tryptophan with sulfate moieties on the heparin. Equilibrium association constants, K_{obs} (M^{-1}), for each peptide binding to heparin were measured as a function of temperature and monovalent salt concentration in the limit of low peptide binding density. At pH 6.0, 25 °C, 20 mM KCH_3CO_2, K_{obs} = 3.2 (±0.3) × 10^3 M^{-1} for KWK-CO_2 binding, whereas K_{obs} = 4.5 (±0.5) × 10^3 M^{-1} for RWR-CO_2. However, the dependence of K_{obs} on KCH_3CO_2 concentration is the same for both oligopeptides, each of which possesses a net charge of +2 at pH 6.0. The logarithm of K_{obs} is a linear function of the logarithm of [KCH_3CO_2] over the range from 12 mM ≤ KCH_3CO_2 ≤ 30 mM (pH 6.0, 25 °C), with ($\partial \log K_{obs}/\partial \log [KCH_3CO_2]$) = −2.0 ± 0.3, indicating that ~2 ions are released per bound peptide upon formation of the complex. The van't Hoff $\Delta H°_{obs}$ for each peptide−heparin interaction is independent of [KCH_3CO_2], with $\Delta H°_{obs}$ = −1 ± 1.5 kcal/mol for KWK-CO_2, and $\Delta H°_{obs}$ = −3.5 ± 1.5 kcal/mol for RWR-CO_2. Comparison of these results with similar studies of the binding of these same peptides to single-stranded polynucleotides indicates that binding of these peptides to heparin at low salt concentrations is largely driven by the favorable increase in entropy resulting from the release of ions, presumably K^+ from the heparin. The results from these model peptide studies are compared with similar studies of protein−heparin interactions.

The sulfated polysaccharide heparin interacts with a number of proteins involved in a variety of biological processes including cell growth and differentiation (Hassel et al., 1986) and control of blood coagulation (Bjork et al., 1989). Among these proteins are fibroblast growth factors (FGF) (Rapraeger et al., 1991; Yayon et al., 1991; Thompson et al., 1994), thrombospondin (Dixit et al., 1984; Guo et al., 1992), thrombin (Olson et al., 1991), and antithrombin (Olson & Bjork, 1991). Since heparin is a polyanion, one expects that some component of its free energy of interaction with proteins will be electrostatic. In fact, since heparin is a highly charged linear polyelectrolyte, counterions (e.g., Na^+, K^+, Mg^{2+}) will bind to heparin in order to reduce its net charge density (Oosawa, 1971; Manning, 1969), and this has been observed experimentally (Delville & Laszlo, 1983). As a result, when a positively charged ligand or a protein with a positively charged heparin binding site binds to heparin, some fraction of the heparin charge will be neutralized resulting in the displacement of some of the heparin-bound counterions in a manner similar to what has been observed for the binding of charged ligands to linear nucleic acids (Record et al., 1976b, 1978, 1991; Mascotti & Lohman, 1990, 1992, 1993; Lohman & Mascotti, 1992a). Of course, for proteins binding to heparin, the salt dependence of the equilibrium constant will generally also be influenced by contributions from ion release or uptake by the protein.

Recent studies probing the effects of salt concentration on the equilibrium binding of several proteins to heparin have demonstrated these polyelectrolyte effects. Olson et al. (1991) have measured the equilibrium constant for thrombin binding to heparin as a function of NaCl concentration and temperature and by analogy with ligand−polynucleotide interactions conclude that this interaction is stabilized significantly by nonspecific electrostatic interactions and that a major component of the favorable binding free energy change results from the displacement of cations (Na^+) from heparin. Similar effects of salt concentration have been demonstrated for the equilibrium binding of heparin to antithrombin III (Olson & Bjork, 1991), mucus proteinase inhibitor (Faller et al., 1992), and basic fibroblast growth factor (bFGF) (Thompson et al., 1994), although the magnitudes of these salt effects differ for each interaction indicating different extents of ion release.

Studies of the equilibrium binding of simple positively charged peptides to DNA and RNA have proven to be very useful in probing the contributions of electrostatic interactions and the role of counterion release in stabilizing protein−

† This work was supported in part by NIH Grants GM39062 and GM30498.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1995.

0006-2960/95/0434-2908$09.00/0

nucleic acid interactions (Latt & Sober, 1967; Record et al., 1976b; Mascotti & Lohman, 1990, 1992, 1993). Such studies have provided information on the magnitude of the salt effects that can be expected for a simple positively charged ligand due solely to the release of counterions that accompanies neutralization of charges on the nucleic acid (Record et al., 1976b; Mascotti & Lohman, 1990, 1992, 1993). However, similar studies of simple charged peptides binding to heparin have not been reported, thus such comparisons can not be made for protein−heparin interactions. We have therefore undertaken a study of the effects of salt concentration and temperature on the equilibrium binding to heparin of two positively charged peptides containing tryptophan and lysine or arginine in order to facilitate the interpretation of thermodynamic studies of more complex protein−heparin interactions.

MATERIALS AND METHODS

Reagents and Buffers. All chemicals were reagent grade and buffers were made with prefiltered, distilled H_2O that was subsequently passed through a Milli-Q system (Milli-Q H_2O) (Continental, Woburn, MA). Buffer CB is 10 mM cacodylic acid and 0.2 mM trisodium ethylenediaminetetraacetic acid (EDTA), titrated to pH 6.0 with 1 M KOH. The high [salt] buffer, which was used for the "salt-back" titrations is buffer CB + 2 M KCH_3CO_2, titrated to pH 6.4 to maintain constant pH upon dilution into standard buffer (Lohman & Mascotti, 1992b).

Peptides. The synthetic peptide L-lysyl-L-tryptophanyl-L-lysine carboxylate (KWK-CO_2) was purchased from Serva Fine Chemicals (Westbury, NY), and the peptide L-arginyl-L-tryptophanyl-L-arginine carboxylate (RWR-CO_2) was synthesized by the Texas Agricultural Experiment Station support facility (Texas A&M University). Both peptides were purified by ion-paired HPLC, and their composition was verified by FAB mass spectrometry as described (Mascotti & Lohman, 1990). Peptide concentrations were determined spectrophotometrically in buffer CB + 1.0 mM KCH_3CO_2, based on an extinction coefficient of 5.7×10^3 M^{-1} cm^{-1} (Mascotti & Lohman, 1990; Lohman & Mascotti, 1992b).

Heparin. High molecular weight sodium heparinate from porcine intestinal mucosa (170 USP units/mg) was purchased from Sigma Chemicals (St. Louis, MO) (cat. no. H3125, lot no. 029f0314) and used without further purification. The average polymer size (according to Sigma) was 16 500 (±1500) g/mol. However, considerable length heterogeneity was apparent as determined by polyacrylamide gel electrophoresis (data not shown). On the basis of a molecular weight of 312 g/mol (saccharide) (sodium salt) (Silva & Dietrich, 1975), the average heparin polymer is 53 ± 5 saccharides long. Heparin of this length could theoretically bind a maximum of ~25 tripeptides, assuming a site size of two saccharides occluded per peptide based on estimates from molecular modeling (see Binding Analysis). High concentration master stocks of heparin (~10 mM saccharide) were dialyzed versus buffer CB + 1.0 mM KCH_3CO_2. The UV absorption of heparin at 280 nm was very low (A_{280} of a 1 mM heparin solution was 0.003) indicating negligible protein contamination, and there was also no detectable fluorescence (λ_{ex} = 280 nm, λ_{em} = 350 nm) for heparin concentrations up to ~1 mM. The heparin concentration of the stock solution was determined by titration with azure A (Jacques, 1978), which binds to the ionizable groups on the heparin polysaccharide. Although, on average, there are 3.5 charges per heparin disaccharide, there is considerable heterogeneity of heparin (Casu, 1985). For these reasons as well as theoretical considerations related to the analysis of equilibrium binding isotherms for the nonspecific binding of peptides to heparin presented below, heparin concentrations are given in units of monosaccharide, rather than the disaccharide.

Fluorescence Titrations. Fluorescence titrations were performed with an SLM 8000C fluorometer (SLM-Aminco, Urbana, IL) by addition of heparin to a constant concentration of oligopeptide, as described for our previous studies with nucleic acids (Overman et al., 1988; Mascotti & Lohman, 1990; Lohman & Mascotti, 1992b), using excitation and emission wavelengths of 280 and 350 nm, respectively, which are the excitation and emission maxima for these peptides. The excitation and emission bandpasses were 2 and 8 nm, respectively. All fluorescence measurements were corrected for dilution as described (Lohman & Mascotti, 1992). Photobleaching was negligible under these conditions, and inner filter corrections were not necessary due to the negligible UV absorbance of heparin at the excitation and emission wavelengths. The emission fluorescence spectra shown in Figure 1 show a shoulder at ~390 nm resulting from the Wood's anomaly in our grating monochromator. This shoulder is eliminated if spectra are taken with polarizers placed at the "magic angle". However, since we do not use polarizers in the fluorescence titrations performed to obtain the peptide−heparin binding isotherms, the spectra in Figure 1 are shown to indicate the signal to noise level observed routinely in our experiments.

Determination of Equilibrium Binding Isotherms. The enhancement of the oligopeptide tryptophan fluorescence which accompanies binding to heparin was used to measure the fraction of bound peptide as indicated in eq (1).

$$E_{obs}/E_{max} = L_B/L_T \quad (1)$$

L_B and L_T are the concentrations of bound and total peptide, respectively, and the observed and maximum fluorescence enhancements are given by $E_{obs} = [(F_{obs} - F_i)/F_i]$ and $E_{max} = [(F_{max} - F_i)/F_i]$, where F_{obs} is the observed fluorescence intensity at each heparin concentration, F_i is the fluorescence intensity of the free peptide before addition of heparin, and F_{max} is the fluorescence intensity of the peptide when saturated by heparin. Equation 1 assumes that the fractional peptide fluorescence enhancement is directly proportional to the fraction of bound peptide. Although, in principle, a binding density function (BDF) analysis (Bujalowski & Lohman, 1987; Lohman & Bujalowski, 1991; Lohman & Mascotti, 1992b) could be used to test this assumption, it was not possible to cover the large range of peptide binding densities required for this analysis due to the very low binding densities that were used in these studies (<6% saturation of the heparin). However, we have verified that the value of K_{obs} determined at constant solution conditions on the basis of this assumption is independent of peptide concentration from 1.5 to 5.8 μM (data not shown). This result indicates that eq 1 is valid at the low binding densities used in these studies.

Analysis of Equilibrium Binding Isotherms. Equilibrium binding isotherms were analyzed to determine equilibrium association binding constants, K_{obs} (M^{-1}), using a model which assumes peptides bind noncooperatively to heparin, which is modeled as an infinite one-dimensional homoge-

neous lattice (McGhee & von Hippel, 1974). This model incorporates overlap of potential peptide binding sites on the heparin. In this analysis, we have assumed each heparin monosaccharide is the start of a potential oligopeptide binding site. Although the disaccharide is the structural repeating unit of heparin, there is no evidence that these positively charged peptides bind in register with the disaccharide unit. In fact, it is likely that the disaccharide unit is not important for peptides that bind nonspecifically and mainly electrostatically to heparin. Therefore, we have used the monosaccharide to calculate the heparin concentration in order to more simply account for the overlap of nonspecific peptide binding sites on the linear heparin lattice. If a disaccharide unit is used to calculate the heparin concentration, then the resulting value of K_{obs} would be increased by a factor of 2 compared to the values reported here. With these assumptions, the expression for the isotherm is given in

$$\nu/L = K_{obs}(1 - n\nu)[(1 - n\nu)/(1 - (n - 1)\nu)]^{(n-1)} \quad (2)$$

where ν is the oligopeptide binding density (peptides bound per saccharide unit) and n is the number of saccharides occluded per bound peptide. K_{obs} [the association constant in units of M^{-1} (monosaccharides)] is defined in eq 3,

$$K_{obs} = [PH]/[P][H] \quad (3)$$

where [PH] is the peptide−heparin complex, [P] is the free peptide concentration, and [H] is the free heparin concentration (in units of saccharides).

The number of saccharides occluded per bound peptide (n) was estimated to be 2, based on comparison of the structures of heparin and the oligopeptide, KWK-CO_2. The length of a fully extended tetrasaccharide was determined to be 20.4 Å (Diakun et al., 1978), and we estimate that the fully extended length of KWK-CO_2 is ~10−13 Å. However, at the low peptide binding densities used in our experiments (<6% saturation of the heparin by oligopeptides), accurate determination of the equilibrium binding constant, K_{obs}, does not require precise knowledge of the occluded site size, since the statistical effects of overlap are minor (<±2% difference in K_{obs} for $1.3 < n < 2.6$) (data not shown).

Our use of an infinite lattice model to interpret the peptide−heparin isotherms reported here is appropriate due to the small site size of the peptide ($n \sim 2$), its noncooperative binding properties, and the low binding densities used in our experiments. The fractional saturation of the heparin did not exceed ~0.06 saccharides bound (~6%) in any of our experiments. As discussed by McGhee and von Hippel (1974), in the limit of zero binding density, the correction due to finite lattice effects $[1 - (53 - n + 1)/53]$ is <2%. In fact, use of a model in which overlap is ignored would only introduce an error of ~3% for an $n = 2$ ligand at these low binding densities. Thus the error introduced by the use of an infinite lattice model (≤3%) is well within our experimental uncertainty.

The dependence of K_{obs} on KCH_3CO_2 concentration was determined by performing "salt-back" titrations of preformed peptide−heparin complexes in a manner analogous to that previously described for ligand−nucleic acid complexes (Overman et al., 1988; Lohman & Mascotti, 1992b). We have determined that E_{max} and the fluorescence of the free peptide do not vary with KCH_3CO_2 concentration (data not shown), which allows us to analyze the salt-back titrations quantitatively to obtain K_{obs} as a function of KCH_3CO_2 concentration.

Table 1: Temperature Dependence of Oligopeptide Fluorescence Enhancement upon Binding to Heparin[a]

peptide	temperature (°C)	E_{max} (%)[b]
KWK-CO_2	15	67 (±3)
RWR-CO_2	15	63 (±3)
KWK-CO_2	25	61 (±3)
RWR-CO_2	25	60 (±3)
KWK-CO_2	45	41 (±3)
RWR-CO_2	45	40 (±3)

[a] Buffer CB + KCH_3CO_2, pH 6.0. [b] Based on the average of duplicate titrations.

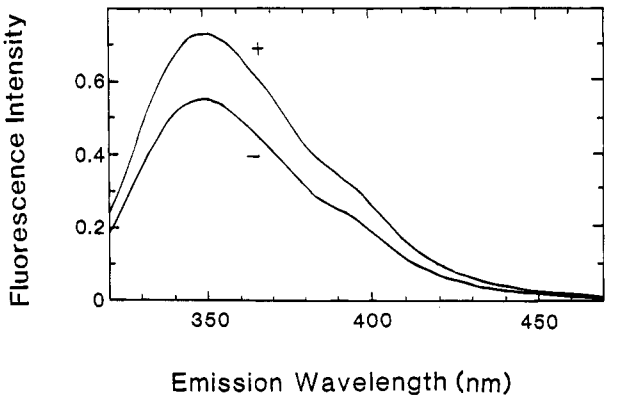

FIGURE 1: Fluorescence emission spectra (corrected for variation of lamp intensity as a function of wavelength) of 6.0 μM KWK-CO_2 in the absence (−) and presence of (+) of 951 μM (monosaccharide) heparin in buffer CB + 12 mM K^+ (as KCH_3CO_2), 25.0 °C, pH 6.0. We note that the shoulder present at ~390 nm in both spectra is the result of the so-called Wood's anomaly (Lakowicz, 1983) and is not due to a contribution from tryptophan or due to a fluorescent contaminant.

Titrations of the peptide KWK-CO_2 with K_2SO_4, KCH_3CO_2, or K_2HPO_4 were performed by pre-equilibrating 1.5 μM KWK-CO_2 in buffer CB + 1 mM KCH_3CO_2 at 25.0 °C, followed by titration with either 0.60 M K_2SO_4, 2.0 M KCH_3CO_2, or 1.0 M K_2HPO_4. The pH of the peptide solutions did not change upon titration with these salts. This was important to check since, above pH 6.0, the fluorescence intensity of KWK-CO_2 increases due to deprotonation of the α-amino group (Brun et al., 1975).

RESULTS

Fluorescence Properties of Oligolysines and Oligoarginines Containing a Single Tryptophan. The binding of the synthetic tripeptides L-lysyl-L-tryptophanyl-L-lysine carboxylate (KWK-CO_2) and L-arginyl-L-tryptophanyl-L-arginine carboxylate (RWR-CO_2) to heparin is accompanied by a significant increase in tryptophan fluorescence. The degree of fluorescence enhancement upon saturation with heparin is essentially identical for each peptide. At 25 °C, the maximum fluorescence enhancement, E_{max}, is 60 ± 3%, although this is dependent on temperature as indicated in Table 1. The fluorescence emission spectra of KWK-CO_2 in the absence and presence of nearly saturating amounts of heparin are shown in Figure 1. Upon binding heparin, the peptide fluorescence spectrum is enhanced with no observed shift. Although the fluorescence emission intensity of both KWK-CO_2 and RWR-CO_2 increases upon binding heparin (see Table 1), the UV absorbance spectra of the peptides remain unchanged between 240 and 320 nm (data not shown).

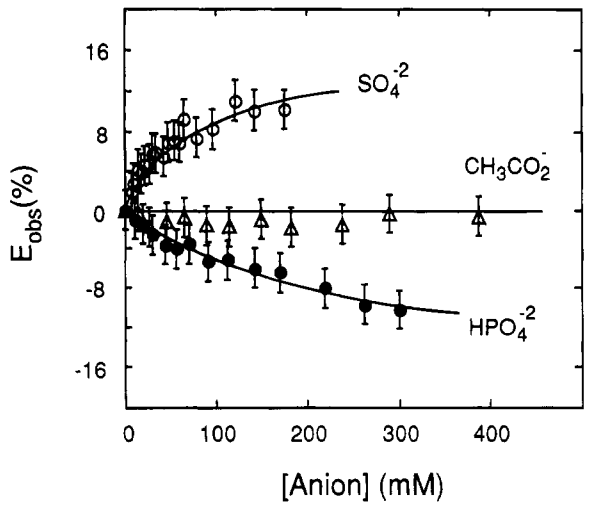

FIGURE 2: Tryptophan fluorescence emission intensity of KWK-CO_2 is enhanced by sulfate, but quenched by phosphate. The percent change in fluorescence intensity is plotted as a function of the added anion concentration. K_2HPO_4 (●), KCH_3CO_2 (△), K_2SO_4 (○).

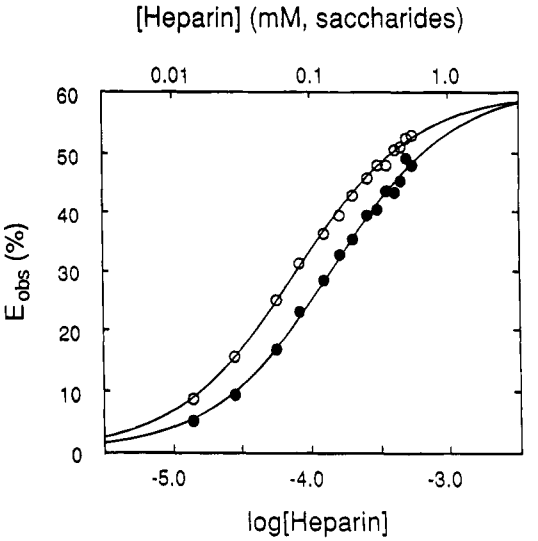

FIGURE 3: Dependence of tryptophan fluorescence enhancement of KWK-CO_2 (●) and RWR-CO_2 (○) on log [heparin saccharides] in buffer CB + 12 mM K^+ (as KCH_3CO_2) pH 6.0, 25.0 °C. Peptide concentrations were 1.6 μM. The titrations are fit to a theoretical isotherm (McGhee & von Hippel, 1974) for large ligands binding nonspecifically to an infinite linear lattice (eq 2). Parameters used were as follows: $n = 2.0$, $E_{max} = 60\%$, and $K_{obs} = 1.3 \times 10^4$ M^{-1} for RWR-CO_2; and $n = 2.0$, $E_{max} = 61\%$, and K_{obs} 7.0×10^3 M^{-1} for KWK-CO_2.

We were unable to obtain isotherms for the binding to heparin of peptides with higher net positive charges (e.g., +4 and +6), since binding of these peptides to heparin formed complexes which scatter light, even at low binding densities (unpublished data). The molecular basis for this light scattering is unknown. However, no light scattering was detected with the lower charged (+2) peptides, under the low binding density conditions of our experiments (data not shown).

The observation that the peptide tryptophan fluorescence is enhanced upon binding heparin was initially surprising since previous studies of the binding of these same peptides to single-stranded or duplex nucleic acids showed that the peptide tryptophan fluorescence is quenched partially upon binding (Helene & Dimicoli, 1972; Mascotti & Lohman, 1990). We therefore performed experiments to determine the basis for this qualitatively different behavior. Since heparin and single-stranded and duplex nucleic acids are all polyanions, it seemed most likely that the ability of heparin to enhance the peptide fluorescence was dependent on the presence of carboxylate or sulfate groups on the heparin which are not present on the polynucleotides. We therefore determined the effects of K_2HPO_4, KCH_3CO_2, and K_2SO_4 on the fluorescence of free KWK-CO_2. The results in Figure 2 indicate that phosphate slightly quenches the tryptophan fluorescence, consistent with previous observations (Steiner & Kirby, 1969; Alev-Behmoaras et al., 1979) and with the fact that polynucleotides quench the peptide fluorescence. However, sulfate enhances the fluorescence of KWK-CO_2. On the other hand, acetate shows relatively little effect. The qualitative correlation between the anion type and the ability to enhance (heparin or SO_4^{2-}) or quench (nucleic acids or PO_4^{2-}) the tryptophan fluorescence of KWK-CO_2 is striking and suggests that enhancement is due to an interaction of tryptophan with sulfate groups on heparin.

The magnitude of the effects of sulfate and phosphate in Figure 2 are much smaller than those observed with heparin or polynucleotides; however, this is likely due to the lower affinity of the peptide for the small ions vs the polyanions. Consistent with this, the fluorescence of *N*-acetyltryptophanamide (NATA) is also enhanced upon titration with sulfate, although the enhancement is only $\sim 1/3$ that observed with KWK-CO_2 (data not shown). This is likely due to a higher affinity of sulfate for the positively charged tripeptide.

Heparin–Tripeptide Equilibrium Binding Isotherms. Figure 3 shows typical binding isotherms obtained from titrations of KWK-CO_2 and RWR-CO_2 with heparin at constant solution conditions (buffer CB + 12 mM K^+, pH 6.0, 25.0 °C). From these data it is clear that the arginine peptide, RWR-CO_2, binds with slightly higher affinity than the lysine peptide. The smooth curves through the data are simulated isotherms based on eq 2 using the following binding parameters: $K_{obs} = 7.0 \times 10^3$ M^{-1}, $E_{max} = 60.0\%$, and $n = 2$ for KWK-CO_2 and $K_{obs} = 1.3 \times 10^4$ M^{-1}, $E_{max} = 61.0\%$, and $n = 2$ for RWR-CO_2. We used KCH_3CO_2 as the added salt since we have shown previously that acetate does not interact strongly with these peptides (Mascotti & Lohman, 1990). The experiments were performed at pH 6.0 so that the peptides would be fully protonated with a net charge of $z = +2$ and also to ensure that the heparin remained fully deprotonated [based on pK_a values of 0.5–1.5 for the sulfates (Casu & Gennaro, 1975) and 2.8–3.1 for the carboxyls (Wang et al., 1991)].

For the most accurate determination of the dependence of the equilibrium binding constants on [KCH_3CO_2] "salt-back titrations" were performed (see Materials and Methods) (Overman et al., 1988; Lohman & Mascotti, 1992) in which complexes of KWK-CO_2 or RWR-CO_2 with heparin were preformed at low [K^+] and then dissociated in steps by addition of aliquots of buffer CB + 2.0 M KCH_3CO_2. The decrease in fluorescence which accompanies peptide dissociation was used to calculate the fraction of peptide bound to heparin from eq 1 and K_{obs} from eq 2 at each [KCH_3CO_2]. The results of these "salt-back" titrations (at 25.0 °C, pH 6.0) are shown in Figure 4 as plots of log K_{obs} vs log [K^+]. Two sets of data for each peptide are shown. The salt dependence of K_{obs} for the binding of KWK-CO_2 and RWR-CO_2 to heparin are listed in Table 2. For each peptide, K_{obs} decreases with increasing salt concentration, and the dependence of K_{obs} on [K^+] is the same for each peptide binding to heparin although the arginine peptide binds to

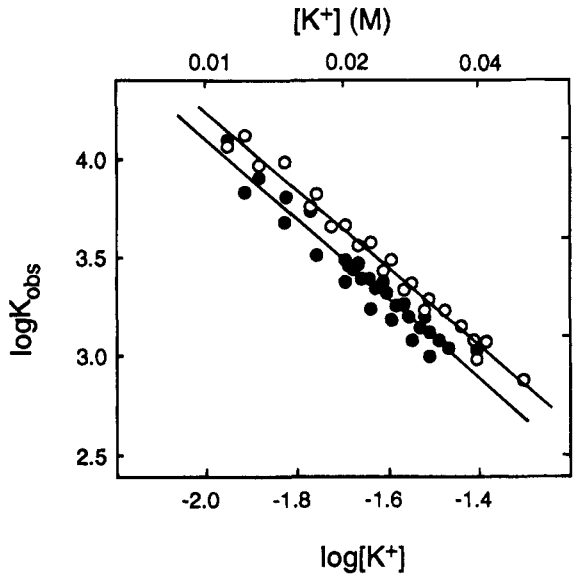

FIGURE 4: Association equilibrium constants, K_{obs}, for the interaction of KWK-CO_2 (●) and RWR-CO_2 (○) with heparin decrease with increasing monovalent cation concentration. Data are plotted as log K_{obs} vs log [K^+] and were determined from "salt-back" titrations (see Materials and Methods) at pH 6.0, 25.0 °C. Two sets of data are shown for each peptide. The slopes and intercepts of the linear least-squares lines are given in Table 2.

heparin with slightly higher affinity than the lysine peptide over the entire salt concentration range; the ratio of K_{obs} for KWK-CO_2 to RWR-CO_2 binding to heparin is 1.4 at 20 mM K^+, pH 6.0, 25.0 °C. This difference in stability is due to a more favorable $\Delta H°_{obs}$ for oligoarginine binding (see below and Table 2). The values of the slopes of these lines, (∂ log K_{obs}/∂ log [K^+]) = −2.0 (±0.3), indicate that 2.0 ± 0.3 ions (presumably K^+) are *released* from heparin upon binding each tripeptide.

From analysis of similar "salt-back" titrations performed at different temperatures, one can determine the temperature dependence of K_{obs} as a function of [K^+] (Lohman & Mascotti, 1992b). From van't Hoff analysis of these same data, one can determine the van't Hoff $\Delta H°_{obs}$ as a function of [KCH_3CO_2]. No curvature was apparent in the van't Hoff plots (over the range from 15 to 45 °C), suggesting that $\Delta C_p°$ is nearly zero for the interaction (data not shown). We find that $\Delta H°_{obs}$ is independent of [KCH_3CO_2] for each peptide, with values of −1.0 ± 1.5 for KWK-CO_2 and −3.5 ± 1.5 for RWR-CO_2 (see Table 2). Table 3 lists the equilibrium binding constants and their dependence on [KCH_3CO_2] for KWK-CO_2 and RWR-CO_2 binding to heparin at 15 and 45 °C. The thermodynamic parameters of KWK-CO_2 binding to heparin are shown as a function of [KCH_3CO_2] in Figure 5 (25.0 °C, pH 6.0). Although $\Delta H°_{obs}$ is independent of KCH_3CO_2 concentration, $\Delta S°_{obs}$ decreases with increasing [KCH_3CO_2]. This holds for both tripeptides and indicates that the salt dependence of $\Delta G°_{obs}$ is entropic in origin. This observation has also been made for the binding of oligolysines to duplex DNA (Lohman et al., 1980) and single-stranded polynucleotides (Mascotti & Lohman, 1992, 1993). We also note (see Table 2) that for oligolysines binding to heparin at 20 mM K^+, $\Delta G°_{obs}$ is dominated by $\Delta S°_{obs}$, whereas $\Delta G°_{obs}$ for the binding of oligoarginine is dominated by $\Delta H°_{obs}$.

The van't Hoff $\Delta H°_{obs}$ values for KWK-CO_2 and RWR-CO_2 binding to heparin are compared with the van't Hoff $\Delta H°_{obs}$ values determined previously for the binding of these same peptides to the single-stranded homopolyribonucleotide, poly(U) in Table 2 (Mascotti & Lohman, 1992). Although the salt dependencies for each peptide are nearly the same within error, the values of $\Delta H°_{obs}$ are slightly more favorable for the poly(U) interaction.

DISCUSSION

The equilibrium binding constant for the interaction of a protein with heparin, as for all macromolecular interactions, is a sensitive function of solution conditions (pH, temperature, salt concentration, and type) and thus an understanding of the origins of specificity and stability of these complexes requires quantitative studies of the thermodynamic linkages among these solution variables. Since heparin is a highly negatively charged linear polyelectrolyte, counterions (e.g., Na^+, K^+, Mg^{2+}, Ca^{2+}) will be sequestered nonspecifically in the local vicinity of heparin, as observed by ^{23}Na NMR (Delville & Laszlo, 1983). Furthermore, proteins that interact with heparin generally contain positively charged amino acids within the polyanion binding site (Cardin & Weintraub, 1989), and thus electrostatic interactions and the accompanying release of counterions from the polyanion are expected to play a major role in stabilizing the complex in much the same way as has been observed for protein−nucleic acid interactions (Record et al., 1976b, 1978, 1991; Lohman, 1986; Lohman & Mascotti, 1992a). As discussed below, the association equilibrium constants for several protein−heparin interactions have been shown to decrease with increasing salt concentration indicating that a net release of ions accompanies formation of these complexes, at least some of which is due to counterion release from the heparin. In some cases, the equilibrium constant decreases by a factor of nearly 10^6 upon increasing the monovalent salt concentration by a factor of 10 (Faller et al., 1992). Therefore, the influence of salt concentration and type on the stability of these complexes can be dramatic and thus potentially important in the regulation of these processes. From a practical viewpoint, it is clearly important to know whether and to what extent these equilibrium constants change with salt concentration in order to study them *in vitro*. Since the effects of salt concentration and type on heparin−protein equilibria are due primarily to differential binding of cations to the heparin and cations and anions to the protein, it is inappropriate to interpret these effects in terms of simple ionic strength or screening effects [see Record et al. (1978, 1991), Lohman (1986), and Lohman and Mascotti (1992a)].

By analogy with studies of charged ligand−polynucleotide interactions, recent studies of protein−heparin equilibria have attempted to resolve the electrostatic and nonelectrostatic components of the free energy of interaction through studies of the effects of salt concentration on these equilibria. In addition to the usual caveats associated with such an analysis [see Record et al. (1976b, 1991) and Lohman and Mascotti (1992a)], there have been no data available on the equilibrium binding to heparin of simple ligands such as charged oligopeptides that can be used to "calibrate" the analysis and compare with the results of protein−heparin studies. Such studies of oligolysines binding to linear nucleic acids have proven quite useful for interpreting the thermodynamic behavior of ligands that bind primarily electrostatically (Latt & Sober, 1967; Record et al., 1976b; Lohman et al., 1980; Mascotti & Lohman, 1990, 1992, 1993). One motivation for the current studies was to provide similar thermodynamic information for relatively simple positively charged peptides that should bind to heparin primarily electrostatically to be

Table 2: Comparison of the Dependence of K_{obs} on [KCH_3CO_2] for the Interaction of Oligolysines with Heparin and Poly(U)[a]

polyanion	peptide	$\partial \log K_{obs}/\partial \log [K^+]$	$\log K$ (1 M)[b]	K_{obs} (20 mM K^+) × 10^{-3} M^{-1} [c]	$\Delta H°_{obs}$ [d]
heparin	KWK-CO_2	−2.02 (±0.26)	0.07 (±0.35)	3.2 (±0.3)	−1.0(±1.5)
heparin	RWR-CO_2	−1.97 (±0.26)	0.31 (±0.35)	4.5 (±0.5)	−3.5 (±1.5)
poly(U)	KWK-CO_2	−1.68 (±0.20)	0.26 (±0.24)	1.3 (±0.1)	−2.2 (±1.5)
poly(U)	RWR-CO_2	−1.75 (±0.20)	0.16 (±0.24)	1.4 (±0.1)	−4.2 (±1.5)

[a] Buffer CB + KCH_3CO_2, pH 6.0, 25.0 °C, 1.6 μM peptide. [b] Obtained from a linear extrapolation of a plot of log K_{obs} vs log [K^+]. [c] Interpolated from best-fit dependence of log K_{obs} vs log [K^+]. [d] Average $\Delta H°_{obs}$ (kcal/mol) within the range of [K^+] examined.

Table 3: Dependence of K_{obs} on [KCH_3CO_2] for the Binding of Oligolysines and Oligoarginines to Heparin at 15 and 45 °C[a]

peptide	temperature (°C)	$\partial \log K_{obs}/\partial \log [K^+])$	$\log K$ (1 M)[b]	K_{obs} (20 mM K^+) × 10^{-3} M^{-1} [c]
KWK-CO_2	15.0	−1.88 (±0.26)	0.25 (±0.35)	2.8 (±0.3)
RWR-CO_2	15.0	−1.89 (±0.26)	0.57 (±0.35)	6.0 (±0.6)
KWK-CO_2	45.0	−2.02 (±0.26)	−0.05 (±0.35)	2.4 (±0.2)
RWR-CO_2	45.0	−2.14 (±0.26)	−0.13 (±0.35)	3.2 (±0.3)

[a] Buffer CB + KCH_3CO_2, pH 6.0; titrations performed with 1.6 μM peptide. [b] Obtained from a linear extrapolation of a plot of log K_{obs} vs log [K^+]. [c] Interpolated from best-fit dependence of log K_{obs} vs log [K^+].

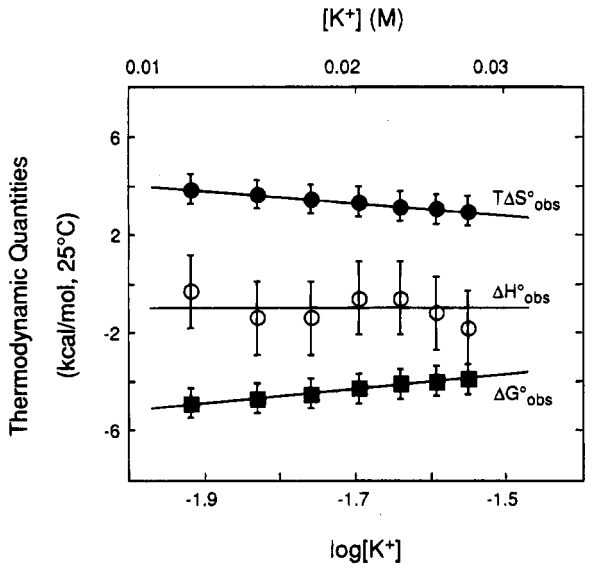

FIGURE 5: Dependence of $\Delta G°_{obs}$, $\Delta H°_{obs}$, and $T\Delta S°_{obs}$ on monovalent salt concentration for the interaction of KWK-CO_2 with heparin. The thermodynamic quantities are plotted as a function of log [K^+] (pH 6.0, 25.0 °C). KCH_3CO_2 was used to vary [K^+].

used to compare to studies of more complex protein–heparin interactions.

Thermodynamics of Positively Charged Oligopeptides Binding to Heparin. We find that the tripeptides KWK-CO_2 and RWR-CO_2 ($z = +2$) bind to heparin with thermodynamic properties that are nearly equivalent to those measured previously for binding of the same peptides to the single-stranded polynucleotide poly(U) (Mascotti & Lohman, 1990, 1992). Even the absolute values of the equilibrium constants, K_{obs}, for the binding of these tripeptides to heparin are very close to the values obtained for their binding to poly(U), *at the same KCH_3CO_2 concentration* (Table 2) (Mascotti & Lohman, 1990, 1992). This suggests that these peptides bind in primarily a nonspecific electrostatic mode and a prime factor determining stability is the charge density of the linear polyelectrolyte, which is nearly equivalent for both polyanions. Mattai and Kwak (1981) have calculated from structural models that the average charge density of heparin is one negative charge per 2.94 Å, which is similar to the value of one charge per 3.7 ± 0.6 Å estimated for poly(U) (Record et al., 1976a,b; Mascotti & Lohman, 1990, 1992).

Our studies of the dependence of K_{obs} on [KCH_3CO_2] indicate that ~2 monovalent ions are *released* upon binding either peptide to heparin (Record et al., 1978), which is consistent with the net charge of +2 for these peptides. Furthermore, we find that the $\Delta H°_{obs}$ for binding of both peptides to heparin is small [~zero for KWK-CO_2 and only slightly negative (−3.5 ± 1.5 kcal/mol) for RWR-CO_2], as would be expected for primarily nonspecific electrostatic interactions. We also find that the salt dependence of the free energy of binding ($\Delta G°_{obs}$) is entirely entropic for both peptides, consistent with the interpretation that counterion (K^+) release from heparin dominates the salt dependence (Record et al., 1976b; Lohman et al., 1980; Mascotti & Lohman, 1992). Although we have not examined anion effects for the binding of charged oligopeptides to heparin, these effects are likely to be minimal based on our studies of charged peptides binding to ss-polynucleotides (Mascotti & Lohman, 1990). Hence, the dependence of $\Delta G°_{obs}$ on [K^+] can be attributed primarily to cation (K^+) release from heparin. We also find that the values of K_{obs} are unity within error at 1 M KCH_3CO_2 [based upon a linear extrapolation of a plot of log K_{obs} vs log [KCH_3CO_2] to a 1 M KCH_3CO_2 standard state (Record et al., 1976b, 1978; Mascotti & Lohman, 1992)] indicating little affinity of these peptides for heparin in 1 M K^+ and suggesting that these interactions are driven at low salt concentration by counterion release (a free energy of dilution) (Record et al., 1976b, 1978, 1991; Lohman & Mascotti, 1992a).

The arginine peptide, RWR-CO_2, binds with slightly higher affinity to heparin than does the lysine peptide, KWK-CO_2, even though both of these tripeptides possess the same net charge ($z = +2$). The difference in $\Delta G°_{obs}$ is due primarily to differences in $\Delta H°_{obs}$, with RWR-CO_2 binding showing a more negative $\Delta H°_{obs}$. Interestingly, a more negative $\Delta H°_{obs}$ is also observed for ss-polynucleotide binding to oligoarginines vs oligolysines (Mascotti, 1992). In the case of polynucleotides this may reflect the ability of arginine to participate in more extensive hydrogen bonding interactions, and the same may be true for its interaction with heparin. In this context, it is interesting that arginines are essential for the binding of thrombin and antithrombin III to heparin (Pomerantz & Owen, 1978; Machovich et al., 1978).

The counterion condensation (CC) hypothesis (Manning, 1969, 1978) has proven to be a useful model to describe the effects of cation concentration on linear polynucleotide conformational transitions (Record, 1975; Record et al., 1976a; Manning, 1972) and the interactions of charged ligands with linear nucleic acids (Record et al., 1976b, 1991; Lohman et al., 1980; Mascotti & Lohman, 1990, 1992, 1993; Lohman & Mascotti, 1992a). The CC hypothesis predicts that the extent of counterion binding per polyion structural charge for linear polyelectrolytes, such as nucleic acids and

heparin, is dependent only upon the average polyion charge density. The ^{23}Na NMR line broadening measurements of Delville and Laszlo (1983) indicate that the interaction of Na^+ with heparin is consistent with the CC hypothesis. On the basis of this model and the average structural parameters of heparin (Diakun et al., 1978; Mattai & Kwak, 1981), the average extent of counterion binding per heparin charge is predicted to be 0.80 ± 0.02. Assuming further that a peptide with a +2 charge neutralizes two heparin charges upon binding, then 1.5−1.8 counterions are predicted to be released per peptide binding, which is close to the value determined experimentally ($\partial \log K_{obs}/\partial \log [K^+] = -2.0 \pm 0.1$; see Tables 2 and 3). We have also reanalyzed the data of Mattai and Kwak (1981, Figures 6 and 7 of that work) for Mg^{2+} binding to heparin, using the McGhee−von Hippel site overlap model (1974) and a site size of 2.5 charge equivalents (since their data are quoted in these units). We estimate ($\partial \log K_{obs}/\partial \log [M^+]) = -1.75 \pm 0.22$, which is within experimental error of the value that we obtain for the tripeptides, which also have $z = +2$.

Although the tryptophan contained within the peptides studied here was introduced as a spectrofluorometric probe to monitor the binding to heparin, it is interesting that a role for tryptophan has been proposed in the interaction of antithrombin III with heparin (Bjork & Nordling, 1979; Blackburn & Sibley, 1980; Lellouch & Lansbury, 1992). The interaction of two tryptophans with polysaccharides has also been observed in a crystal structure of an antibody−carbohydrate complex (Cygler et al., 1991). Furthermore, peptides constructed on the basis of the type I repeats of human endothelial cell thrombospondin, containing the consensus sequence, Trp-Ser-X-Trp, bind to heparin and also inhibit the binding of thrombospondin to heparin (Guo et al., 1992). However, because we have not examined peptides with variable numbers of tryptophans in this work, we cannot assess the contribution of tryptophan to the free energy of binding of these peptides to heparin. However, since log K_{obs} (extrapolated to 1 M KCH_3CO_2, 25 °C) is nearly zero for the binding of both KWK-CO_2 and RWR-CO_2 to heparin, it is likely that the *net* $\Delta G°_{obs}$ for the interaction of tryptophan with heparin is near zero.

Protein−Heparin Interactions. There have been a number of recent studies of the effects of salt concentration on the equilibrium constants for heparin binding to several proteins, including thrombin and antithrombin (Olson et al., 1991; Olson & Bjork, 1991), mucus proteinase inhibitor (MPI) (Faller et al., 1992), and basic fibroblast growth factor (bFGF) (Thompson et al., 1994). Although the *net* ion release is determined from the values of $\partial \log K_{obs}/\partial \log [M^+]$, a more detailed interpretation of the salt dependencies for protein−heparin interactions requires further studies. In particular, effects due to differential ion binding to the protein must be examined as well as the possibility that ion binding is thermodynamically linked to both pH and temperature effects [e.g., see Overman and Lohman (1994) and Lohman and Mascotti (1992a)].

The value of ($\partial \log K_{obs}/\partial \log [NaCl]$) for the interaction of thrombin with heparin was determined to be -4.8 ± 0.2 (Olson et al., 1991), indicating a net release of ~5 ions upon complex formation. No differences in the salt dependence were observed upon substitution of acetate for chloride, although the binding constants in acetate were slightly higher than in chloride indicating a possible effect of anion binding to thrombin. Although specific cation effects were not examined it is possible that such effects exist in light of the recent evidence that thrombin is specifically activated by Na^+ binding (Wells & Di Cera, 1992). Olson et al. (1991) also determined that ($\partial \log K_{obs}/\partial \log [NaCl]) = -3.8 \pm 0.1$ for the interaction of antithrombin III with heparin indicating that ~4 ions are released upon formation of this complex. A value of ($\partial \log K_{obs}/\partial \log [NaCl]) = -5.8 \pm 0.2$ was determined for MPI binding to heparin (Faller et al., 1992) indicating that ~6 ions are released. However, the possibility that differential anion or cation binding to these proteins may contribute to these salt dependencies still remains. The interaction of bFGF with heparin has a considerably lower salt dependence, ($\partial \log K_{obs}/\partial \log [NaCl]) = -1.95 \pm 0.1$ (Thompson et al., 1994), in fact this is nearly the same as that observed for the tripeptides studied here. However, this low value is likely a reflection of contributions from cation or anion uptake, which would decrease the absolute value of ($\partial \log K_{obs}/\partial \log [NaCl]$).

Tryptophan Fluorescence Enhancement by Heparin Correlates with Enhancement by Sulfate Ion. Several studies of the binding of proteins to heparin have used changes in protein tryptophan fluorescence to monitor binding. Interestingly, in all cases, the tryptophan fluorescence is *enhanced* upon heparin binding; fluorescence enhancements ranging from 32 to 40% have been reported for the binding of antithrombin III to high molecular weight heparin (Jordan et al., 1980; Olson, 1988; Villanueva & Allen, 1983; Villanueva, 1984; Olson & Shore, 1981; Olson et al., 1991; Blackburn & Sibley, 1980; Bjork & Nordling, 1979; Villanueva et al., 1980). Much larger tryptophan fluorescence enhancements (~4−5-fold) have been reported for heparin binding to mucus proteinase inhibitor (MPI) (Faller et al., 1992). This is noteworthy in its contrast to the observation that tryptophan fluorescence of proteins and peptides is generally *quenched* upon binding duplex and single-stranded nucleic acids (Dimicoli & Helene, 1974; Kelly & von Hippel, 1976; Lohman & Overman, 1985; Mascotti & Lohman, 1990). Tryptophanyl fluorescence can be enhanced upon transfer of the indole ring from a polar to nonpolar environment, which is also accompanied by a blue-shift of the emission spectrum (Lakowicz, 1983). In fact, the tryptophan emission spectrum of MPI undergoes such a blue-shift upon MPI binding to heparin (Faller et al., 1992); however, for the peptides studied here (see Figure 1), as well as for antithrombin III (Villanueva & Allen, 1983), the emission spectrum does not shift upon binding heparin. A conformational change in antithrombin III occurs upon binding heparin (Olson & Shore, 1981; Olson et al., 1981), and this conformational change was postulated to account for the observed tryptophan fluorescence enhancement (Villanueva & Allen, 1983). Since the tryptophan fluorescence of the simple tripeptides that we examine here also is enhanced upon binding heparin, such interactions may contribute to the fluorescence enhancement observed in the binding of antithrombin III to heparin.

We show here that sulfate and phosphate exhibit qualitatively different effects on the tryptophan fluorescence intensities of the tripeptides studied here. While sulfate enhances the tryptophan fluorescence, phosphate quenches the tryptophan fluorescence. The monovalent anion acetate has very little effect. These effects are striking in light of the enhancement of tryptophan fluorescence observed for peptide binding to heparin, where sulfates account for approximately 70% of its charged groups (Diakun et al.,

1978), and the fluorescence quenching observed upon peptide binding to polynucleotides, which contain phosphate groups. The mechanism for quenching of tryptophan fluorescence by phosphates has been postulated to be collisional where the transient interaction of the fluorophore with an electron scavenger such as phosphate leads to the nonradiative decay of the excited state (Steiner & Kirby, 1969). In contrast, Alev-Behmoaras et al. (1979) have proposed that proton transfer from the phosphate to the indole ring is responsible. Steiner and Kirby (1969) also determined that acetate and sulfate had little effect on the quantum yield of acetyltryptophan, which we also observe. The larger effects of these salts on the tripeptides observed in our studies is likely due to the fact that these tripeptides have a net positive charge ($z = +2$). The increased magnitude of the fluorescence changes observed for the peptides binding to heparin (vs sulfate) and poly(U) (vs phosphate) are due in part to the higher affinity of these peptides for the polyanions, which is due partially to the increase in entropy that accompanies counterion release form the polyelectrolytes. It is also possible that the extent of Trp fluorescence enhancement and quenching by the sulfate and phosphate anions, respectively, are quantitatively different than those caused by the sulfonate and phosphodiester anions in heparin and polynucleotides, respectively. However, we conclude from these results that at least part of the tryptophan fluorescence enhancement of these peptides results from interaction with the sulfate groups on heparin, whereas tryptophan fluorescence quenching is associated with an interaction with the phosphate groups on the polynucleotides.

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BI941879W