Design and Synthesis of a Helix Heparin-Binding Peptide†

Dulce Soler Ferran,‡,§ Michael Sobel,‖ and Robert B. Harris*,‡

Department of Biochemistry and Molecular Biophysics and Department of Surgery, Division of Vascular Surgery, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Received August 23, 1991; Revised Manuscript Received February 11, 1992

ABSTRACT: Elaboration of heparin–protein-binding interactions is necessary to understand how heparin modulates protein function. The heparin-binding domain of some proteins is postulated to be a helix structure which presents a surface of high positive charge density. Thus, a synthetic 19-residue peptide designed to be α-helical in character was synthesized, and its interaction with heparin was studied. The peptide was shown to be 75% helix by circular dichroism (CD) spectrometry in neutral pH buffer (at 2 °C); helicity increased to nearly 85% under high ionic strength conditions or to nearly 100% in 75% ethanol. Increasing the temperature of the solution caused a change in the spectral envelope consistent with a coil transition of the peptide. The midpoint of the transition (i.e., the temperature at which the helix content was determined to be 50%) was 25 °C, and the determined van't Hoff enthalpy change (ΔH_{vH}) was 3.2 kcal/mol of peptide. By CD, heparin increases the helix content of the peptide to 100% and increases the apparent thermal stability of the peptide by about 1 kcal/mol. The melting point for the helix/coil transition of the heparin–peptide complex was 50 °C. The thermal coefficient of the transition ($\approx$300 deg·cm^2·dmol^{-1}·°C^{-1}) was essentially the same for the peptide alone or the peptide–heparin complex. Dissociation of the complex under high ionic strength conditions was also observed in the CD experiment. Biological assays showed less heparin-binding activity than expected (micromolar K_D values), but this was attributed to the absence of critical lysyl residues in the peptide. Nonetheless, this type of helix peptide can serve as a framework for the design of other peptides which might bind heparin(s) with unique, specific biological properties.

The interaction of heparin and other glycosaminoglycans with proteins regulates a broad spectrum of biological processes in both normal and disease states. Examples of physiological processes regulated by heparin–protein interactions are hemostasis (Rosenberg, 1977), cell attachment, growth, and differentiation (Hassel, 1986), and smooth muscle proliferation (Castellot, 1981). Heparin is by nature a heterogeneous acidic mucopolysaccharide which varies enormously in unit molecular weight, charge density, and sequence (Bienkowski & Conrad, 1985; Merchant et al., 1985). The structural heterogeneity of heparin is postulated to contribute to the specificity of its interactions with various proteins. Thus, elucidation of the chemical and structural features involved in these interactions is important for a full understanding of how various heparins regulate protein function.

On the basis of the primary sequences of the heparin-binding domains of characterized proteins, we previously designed and synthesized a "consensus sequence" peptide which bound heparin with high affinity and specificity (Sobel et al., 1992). We then used the consensus sequence to identify the likely heparin-binding domain within human von Willebrand factor (vWF)[1] protein, a plasma glycoprotein which plays a critical role in regulating hemostasis through its interactions with factor VIII, platelets, and vascular endothelium (Moroose et al., 1986; Ruggieri & Zimmerman, 1987). Far-ultraviolet CD spectrometry of the synthesized peptide encompassing this domain revealed that it was essentially devoid of α-helix character but that upon binding heparin a marked displacement in the spectral envelope and position of the absorption minima occurred, consistent with a conformational change that occurs upon binding heparin.

Although protein conformation is postulated to be important for heparin binding, little is known about the secondary or tertiary structures of putative heparin-binding domains. No three-dimensional structures of heparin–protein complexes have been solved, and the only heparin-binding protein for which the three-dimensional structure is known is bovine platelet factor 4, a tetramer composed of identical dimers (St. Charles et al., 1989). In this structure, four lysines which are known to be involved in heparin binding (Lys-76, -77, -80, and -81) lie on one side of the only helix in the monomer. This helix is opposite and antiparallel to the helix of the second monomer of each of the two dimers of the tetramer. Thus, the four lysines of one helix are opposite to the helix of the neighboring monomer. It is postulated that heparin binds to the two helices either by passing along a shallow hydrophobic groove between the two helices of each dimer or by crossing over the two helices at right angles. In either case, in this model, the two α-helices would be involved in binding heparin.

The interaction of heparin and antithrombin III is probably the most thoroughly studied heparin–protein interaction. Antithrombin III is a serine proteinase inhibitor of the serpin family (Carrel & Travis, 1987). Heparin enhances the inhibition of thrombin by antithrombin III several thousandfold,

† This work was supported by USPHS Grants NIH NS 24933 (R.B.H.) and HL39903 (M.S.), by Grant-in-Aid 89646 from the American Heart Association (R.B.H.), and by the Veterans Administration Research Program (M.S.) and was done under the tenure of an Established Investigatorship Award to R.B.H. from the American Heart Association. This work comprises part of the Ph.D. Thesis of D.S.F. to the Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Aug 1991.

* Address correspondence to this author of the Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Box 614, Richmond, VA 23298-0614.

‡ Department of Biochemistry and Molecular Biophysics.

§ Present address: Harvard University Medical School, Seeley G. Mudd Building, 250 Longwood Ave., Boston, MA 02115.

‖ Department of Surgery.

[1] Abbreviations: CD, circular dichroism; HPLC, high-performance liquid chromatography; Succ, succinyl; vWF, von Willebrand factor.

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and the specific activating heparins which bind to antithrombin III are known (Loganathan et al., 1990).

The three-dimensional structure of another serpin, α_1-antitrypsin inhibitor, has been solved and serves as a prototype for all serpins (Loberman, 1984). On the basis of extensive sequence homology between α_1-antitrypsin and antithrombin III, a theortical model for antithrombin III has been created, and this structure reveals that the heparin-binding domain of antithrombin III lies on a helix. Furthermore, the positive side chains of the cationic residues in this helix face one side of the amphipathic helix, forming a positive electrostatic surface for binding heparin (Huber & Carrell, 1990). On the basis of chemical modification studies and secondary structure predictive methods, Villanueva (1984) proposed a similar helix motif for heparin binding to antithrombin III involving lysines-282, -286, and -299.

In similar studies, where protein C inhibitor (another serpin) was modeled, a two-helix motif for heparin binding was proposed. In this model, the heparin-binding site is formed by two distinct helices, both of which are different from the helix identified by Huber and Carrell in antithrombin III (Kuhn et al., 1990).

On the basis of these studies, we designed and synthesized a peptide predicted to assume a helical structure in solution in which the cationic residues of the peptide were suitably spaced so that their positive side chains would be oriented on the same side of the helix. The conformation of the peptide was examined by CD spectroscopy, and the effect on conformation upon binding heparin was examined. The ability of this peptide to bind heparin was assessed in two different biological assays. This peptide model forms a framework for the study of protein–heparin interactions mediated by helical structures, as well as being a versatile tool for the isolation of heparins with unique affinities or biological activities.

Materials and Methods

Peptide Synthesis. The helix peptide was synthesized by solid-phase methods essentially as detailed previously [e.g., see Stewart and Young (1984) and You et al. (1991)]. The peptide was succinylated directly on the synthesis resin prior to cleavage in three successive 30-min reactions with 0.4 M succinic anhydride in dimethylformamide. Following cleavage and extraction, the peptide was purified to N-terminal homogeneity by preparative reverse-phase HPLC (Shimadzu LC 8A system). An aliquot of the peptide–resin was intentionally left unacetylated for subsequent sequence analysis of the cleaved, extracted, and purified peptide. The purity of the peptide was assessed by analytical reverse-phase HPLC, by amino acid compositional and sequence analyses. The expected residue was obtained at each cycle.

Circular Dichroism Spectrometry. CD spectra were obtained with a Jasco J-500C spectropolarimeter scanning from 240 or 260 nm to 190 nm at scan rates of 5 or 10 nm/min. The temperature of the cell holder was maintained at ±0.1 °C by means of a circulating water bath. A 1-mm path-length optical cell was used. The observed ellipticity was normalized to units of degrees centimeter squared per decimole, the precise peptide concentration was determined by amino acid compositional analysis of the stock solutions from which the experimental samples were diluted and of an aliquot of the actual solutions placed in the CD optical cell prior to the start of the experiment and after the spectra were obtained. This was necessitated by the fact that the helix peptide tended to adsorb to the walls of the test tube (glass or various plastics) containing the stock solutions but did not adsorb to the walls of the CD cell. Heparin essentially eliminated this adsorptive loss of peptide. Unless otherwise indicated, spectra were generally obtained in 10 mM phosphate buffer, pH 7.0, and all dichroic spectra were smoothed and corrected by background subtraction for the spectrum obtained with buffer alone or buffer containing heparin. At least three independent measurements were done for each relevant experiment. There was <5% variation in the observed ellipticities between solutions of the same peptide based on the precise concentration of peptide determined by compositional analysis.

The spectra were analyzed for percent secondary structural elements by two programs based on comparison to the spectra obtained for the structures of known proteins (Chang et al., 1978).

Determination of Heparin Binding by Dot Blot Assay. The ability of the helix peptide to bind heparin was assessed by radioligand dot blot assay using ^{125}I-fluorescent porcine mucosal heparin (Sigma, grade I) (Suda et al., 1990), performed as previously described (Sobel et al., 1992). Binding data were analyzed using weighted, nonlinear least-squares curve-fitting with the microcomputer-based version of LIGAND (McPherson, 1985). Negligible amounts of heparin (<0.1% of total counts) bind to nitrocellulose filters dotted either with buffer alone or with 1 mM ovalbumin.

Measurement of Heparin Inhibition of the von Willebrand Factor–Platelet Interaction. vWF normally binds to platelets in the presence of ristocetin, but this binding is inhibited by heparin because heparin binds to vWF (Sobel et al., 1992). Thus, the ability of synthetic peptides to compete with vWF for binding heparin can be measured by their suppression of heparin inhibition of ^{125}I-labeled vWF platelet binding (Sobel et al., 1992). This assay method was used to assess the heparin-binding activity of the helical peptide.

Results

Design of a Heparin-Binding α-Helix Peptide. The heparin-binding domain of several well-studied proteins, including antithrombin III, is predicted to form an α-helix that presents a face of high positive charge density for heparin binding (Villanueva, 1984; Cardin & Weintraub, 1989). Thus, a cationic peptide was designed that was predicted to form an α-helix in which the cationic residues were suitably spaced so that their positive side chains would be oriented on the same side of the helix.

The sequence of the peptide, Succ-A-E-A-A-A-R-A-A-A-R-R-A-A-R-R-A-A-A-R-NH_2, incorporates many features known to promote helix formation and stability. Because interaction of charged residues with the helix dipole can either stabilize or destabilize an α-helix (Shoemaker et al., 1987), the termini of the peptide were blocked with appropriate groups designed to eliminate unfavorable electrostatic interactions between the positive amino terminus and the negative carboxyl terminus of the peptide with the helix macrodipole. Inclusion of a negatively charged functional group at the amino terminus of the peptide (Succ) is predicted to interact favorably with the positive pole of the helix macrodipole while positively charged groups at the C-terminal end of the peptide are predicted to interact favorably with the negative pole of the helix macrodipole.

Ion pairs stabilize α-helices in proteins and peptides, and the orientation, composition, and spacing of the ion pair all contribute to the stabilizing effect (Sundaralingam et al., 1985). On the basis of extensive work with synthetic peptides, the optimal spacing between members of the ion pair was found to be i+4, where the side chains of the two amino acids forming the ion pair are spaced by three amino acids. The optimal orientation is with the anionic residue toward the

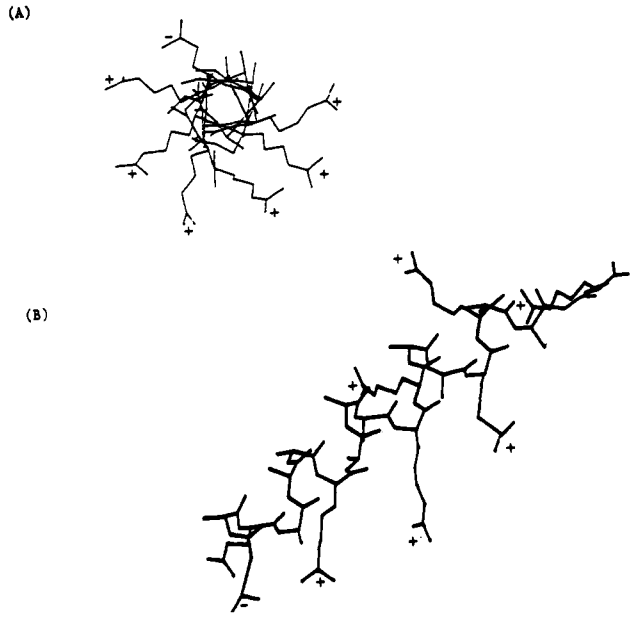

FIGURE 1: Three-dimensional representations of the helix peptide. To create these figures, the residues of the helix peptide were constrained to the atomic coordinates of the D-helix of α_1-antitrypsin. The homologous domain in antithrombin III, another serpin, has been implicated in binding heparin (Huber & Carrel, 1989). (A) Looking down the axis of the helix, highlighting the surface of high positive charge density. (B) Side view of the helix peptide.

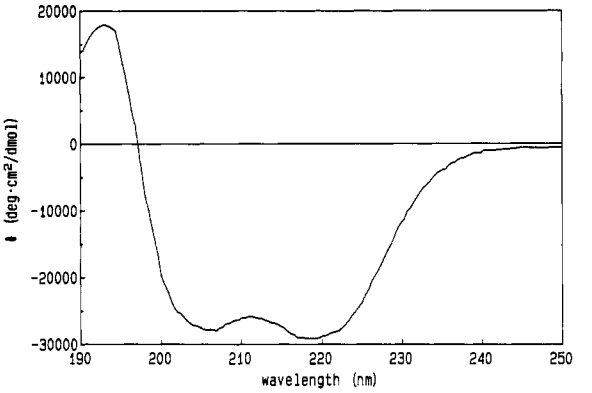

FIGURE 2: Far-UV CD spectrum of 58.8 μM helix peptide taken in 10 mM phosphate buffer, pH 7.0, at 2 °C.

Table I: Residue Molar Ellipticity at 222 nm of the Helix Peptide at 2 °C under Various Experimental Conditions[a]

experiment	$[\theta]_{222nm}$	% helix
10 mM sodium phosphate, pH 7.0	−27000	75
10 mM sodium phosphate−1 M NaCl, pH 7.0	−32300	85
75% ethanol	−35900	100
10 M sodium phosphate, pH 7.0, + 50 μM heparin	−34800	98

[a] 50 μM peptide was used for each experiment. The average ellipticity value for three different experiments is given; the helix content is calculated according to Chakrabartty et al. (1991).

N-terminus and the cationic residue toward the C-terminus (Marqusee & Baldwin, 1987). Furthermore, of all the possible ion pairs formed between the various charged amino acids, the most stabilizing pair was formed by Glu-Arg with Glu placed N-terminal to Arg (Merutka & Stellwagen, 1991). With respect to the model peptide, then, the potential ion pair Glu^2–Arg^6 was introduced close to the N-terminus with Glu^2 N-terminal to Arg^6.

The relative helix-forming propensities of amino acids in peptides have been extensively studied (Lyu, 1990; Marqusee et al., 1989; Padmanabhan et al., 1990; O'Neill & DeGrado, 1990). Although the rank order of preference obtained from those peptide studies differs from the order deduced from the frequency of occurrence of each amino acid in protein helices (Levitt, 1978; Fasman, 1989) or from studies in which the contribution of each amino acid to α-helix stability was studied empirically (Scheraga, 1978), Ala was found to be the most helix-favoring amino acid for proteins or peptides. In particular, Ala appears to have a stronger stabilizing effect in short-chain α-helices as they occur in peptides (Lyu et al., 1990) than as they occur in globular proteins. For this reason, Ala was chosen over other amino acids to be the backbone residue for the model peptide.

Arg was chosen to provide the positive surface because Arg is a better helix-forming residue than Lys (O'Neill & DeGrado, 1990). At 3.6 residues per turn of the helix, the Arg residues were spaced so that they would fall on the same side of the helix.

Finally, the distribution of polar residues (Glu and Arg) in the peptide was arranged so as to avoid formation of a true amphipathic helix. In amphipathic helices, helix formation is stabilized by intermolecular interactions through the hydrophobic core of the peptide, and thus the helix content may be concentration (aggregation) dependent (Marqusee & Baldwin, 1987).

Three-dimensional representations of the model peptide highlighting the features brought to its design are presented in Figure 1. Although Ala-containing peptides as short as 16 amino acids have been shown to possess α-helical character at low temperature (Marqusee & Baldwin, 1989), to our knowledge, a helical peptide with the characteristics described here has not been reported.

CD Examination of the Helix Peptide. The secondary structure of the peptide was examined by CD spectrometry. The far-UV CD spectrum of this peptide was taken in 10 mM sodium phosphate buffer, pH 7.0, at 2 °C (Figure 2). The spectra obtained exhibit two minima at ≈204 and 222 nm and an absorption maximum at 195 nm, indicative of helix structure. The spectra observed were independent of concentration in the range of 15–300 μM, indicating that helix formation in this peptide is likely to be intramolecular.

From several different dichroic spectra of the peptide, the mean ellipticity value, $[\theta]$, at 222 nm was found to be −27 000 ± 1000 deg·cm²·dmol⁻¹. Thus, as expected, this peptide possesses a significant percentage of helix structure.

The fractional percent helix content for the peptide can be determined from $[\theta]_{222nm}$ (Chakrabartty et al., 1991). Accordingly, the theoretical ellipticity at 222 nm for any peptide which possesses 100% α-helix character will be equal to $-40000(1 - 2.5/n)$, where n is the number of amino acid residues in the peptide, including additional amide bonds at each blocked terminus (in a protein, where $n \gg 2.5$, the maximum, attainable theoretical $[\theta]_{222nm} = -40\,000$ deg·cm²·dmol⁻¹). A peptide which is devoid of α-helix content has $[\theta]_{222nm} = 0$ deg·cm²·dmol⁻¹.

In the helix peptide under study, $n = 21$, and thus, if the peptide were 100% α-helix, $[\theta]_{theor}$ would be −35 400 deg·cm²·dmol⁻¹. The observed $[\theta]_{222nm} = -27\,000$ deg·cm²·dmol⁻¹, and so the peptide can be considered 75% helix in character. As expected, the helicity of the peptide increases in 1 M NaCl or 75% ethanol (Table I).

Increasing the temperature of the solution causes a change in the spectral envelope consistent with unfolding of the peptide resulting in a larger fractional percent random coil and lower fractional percent α-helix. The nested set of spectra obtained at increasing temperatures (Figure 3) display an isodichroic

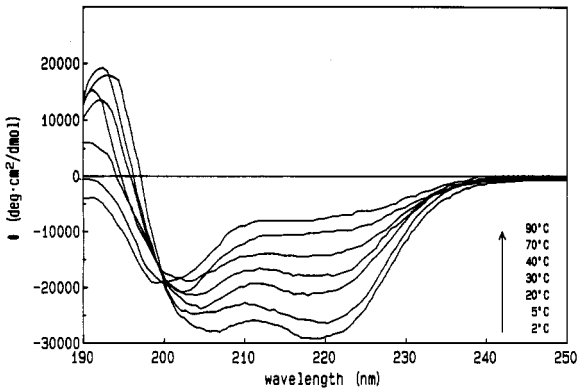

FIGURE 3: Effect of temperature on the far-UV CD spectra of the helix peptide. All spectra were obtained with 58.8 μM peptide in 10 mM phosphate buffer, pH 7.0. Prior to acquisition of the spectra, the sample cell was equilibrated for a minumum of 10 min at each temperature. Spectra were obtained every 5 °C in the range 2–95 °C, but for simplicity's sake, only representative spectra obtained at 2, 5, 20, 40, 50, 70, and 90 °C are presented.

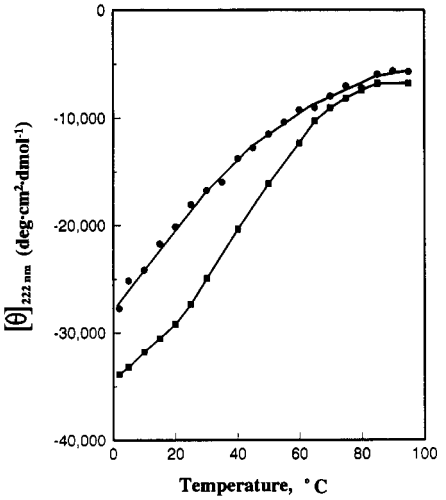

FIGURE 4: Effect of temperature on the $[\theta]_{222nm}$ of the (●) helix peptide and of the (■) helix peptide–heparin complex.

point at 202 nm with a shift in the minima from 205 to 201 nm at higher temperatures. At temperatures >70 °C, the peptide is predominantly random coil in character.

These features are characteristic of peptides which undergo a helix to coil transition in which a two-state equilibrium is assumed to exist between the two conformers (Shoemaker et al., 1987). The midpoint of the helix/coil transition can be taken as the temperature at which the peptide is 50% α-helix; for this peptide, this will be the temperature at which $[\theta]_{222nm}$ = −17 500 deg·cm²·dmol⁻¹. From the thermal transition curve (Figure 4), the transition temperature is about 25 °C. The thermal coefficient for the transition is 300 deg·cm²·dmol⁻¹·°C⁻¹. The observed thermal transition is quite broad and is characteristic of other thermal-induced transitions observed for short-chain helical peptides. However, denaturation of the peptide is completely reversible. Upon cooling the peptide solution heated to 95 °C, identical spectra were obtained for the peptide at all temperatures examined down to 2 °C.

According to the two-state model, an equilibrium exists between the helix and coil conformers of the peptide. The equilibrium constant governing this helix to coil transition at a given temperature can be calculated according to

$$K = [\text{helix}]/[\text{coil}] = ([\theta]_{obs} - [\theta]_{coil})/([\theta]_{helix} - [\theta]_{obs})$$

At high temperatures, where the peptide is completely unfolded

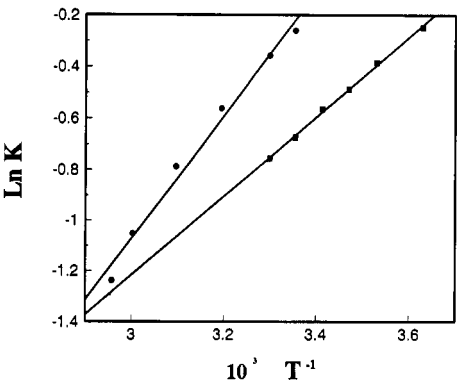

FIGURE 5: van't Hoff plot of the (●) helix peptide–heparin complex and of the (■) helix peptide. Points in the linear range of the temperature transition curve (Figure 4) were used to construct this plot.

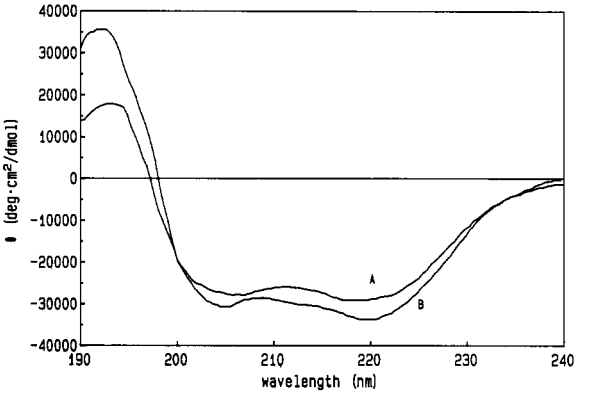

FIGURE 6: Normalized CD spectra of the helix peptide in the presence and absence of heparin. Spectrum A, 58.8 μM helix peptide; spectrum B, 45 μM helix peptide plus 50 μM unfractionated heparin. Spectra were acquired in 10 mM phosphate buffer, pH 7.0, at all temperatures in the range 2–90 °C, but only those spectra obtained at 5 °C are shown.

into the coil conformer, $[\theta]_{obs} = [\theta]_{coil}$, and $[\theta]_{obs}$ is the limiting ellipticity for the coil form. For the helix peptide under study, the limiting ellipticity of the coil form was approached at 95 °C (−5700 deg·cm²·dmol⁻¹) but never reached the theoretical value of $[\theta] = 0$ determined by others (Chakrabartty et al., 1991). For this peptide, $[\theta]_{helix}$ = −35 400 deg·cm²·dmol⁻¹.

To determine the enthalpy (ΔH_{vH}) of the helix to coil transition for the peptide alone (or for the peptide in the heparin–peptide complex, see below), K was calculated at increasing temperature. The van't Hoff plot of ln K versus $1/T$ yields ΔH_{vH} from the slope according to

$$\ln K = -(\Delta H/R)(1/T) + \Delta S/R$$

where T is the temperature in degrees kelvin and R is the gas constant (1.987 cal·mol⁻¹·K⁻¹). Least-squares analysis of the experimental data (Figure 5) gave an enthalpy of 3.2 kcal/mol of peptide for the helix/coil transition or 0.17 kcal/mol per residue.

Heparin Binding by the Helix Peptide. Addition of 50 μM heparin to a solution containing 45 μM peptide in 10 mM sodium phosphate buffer, pH 7.0, causes a significant change in the spectral envelope. At 5 °C, for example, in the presence of heparin, the spectrum is essentially the same, but the ellipticity values are more pronounced (more negative) (Figure 6). Five spectra were taken of the peptide plus heparin at 2 °C, and for these experiments, the average $[\theta]_{222nm}$ = −34 000 ± 1000 deg·cm²·dmol⁻¹, corresponding to ≈96% helix content. Thus, heparin increases the helicity of the peptide, and this change is likely to be a reflection of the peptide–heparin interaction.

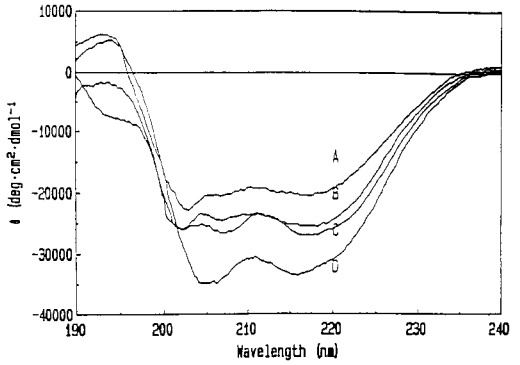

FIGURE 7: Dissociation of the peptide–heparin complex under high ionic strength conditions. All spectra were acquired using 50 μM helix peptide in 10 mM phosphate buffer, pH 7.0, 22 °C. (A) Helix peptide alone. (B) Helix peptide in phosphate buffer containing 1 M NaCl; note the increase in the absorption minima due to NaCl. (C) Solution of the helix peptide containing 50 μM unfractionated heparin plus 1 M NaCl. (D) Solution of the helix peptide containing 50 μM unfractionated heparin. In the presence of NaCl, the helix peptide–heparin complex dissociates, and the spectrum obtained is virtually identical to that of the peptide alone in the presence of 1 M NaCl.

As expected (and as we observed previously with the heparin-binding domain peptide of vWF; Sobel et al., 1992), addition of 1 M NaCl to the peptide–heparin solution causes a spectral change consistent with dissociation of the heparin–peptide complex (Figure 7). The spectrum obtained under high ionic strength conditions is essentially identical to the spectrum obtained for the peptide alone in the presence of 1 M NaCl.

The thermal coefficient for the helix/coil transition for the peptide in the presence of heparin is 320 deg·cm^2·dmol^{-1}·°C^{-1} (Figure 4), essentially identical with that determined for the peptide alone. However, the melting point of the transition for the heparin–peptide complex was estimated to be 50 °C, strongly suggesting that once bound, heparin "protects" the peptide from thermal-induced unfolding. The enthalpy (ΔH_{vH}) of the helix/coil transition for the heparin–peptide complex was calculated to be 4.5 kcal/mol of peptide and 0.24 kcal/mol per residue (Figure 5).

The ability of the helix peptide to bind heparin was also assessed using two different biological assays. The helix peptide competes with vWF for heparin binding, although concentrations as high as 40 μM were needed to achieve 80% supression of heparin-induced inhibition of vWF binding to platelets. From these experiments, the K_D for heparin binding was estimated to be $\approx$3 μM.

However, negligible amounts of heparin bound to the helical peptide in the dot blot assay compared with other heparin-binding peptides derived from known heparin-binding proteins. By this method, K_D was estimated to be $>1 \times 10^{-7}$ M. It is quite possible that the physical contraints inherent in immobilizing the peptide to nitrocellulose adversely affect the ability of this peptide to bind heparin.

Discussion

The study of the mode of interaction between proteins and heparin is important for an understanding of how heparin modulates protein function in a variety of physiological processes. The specificity of binding is likely to be governed by protein domains with a conformation which appropriately presents suitably oriented cationic residues for interaction with specific heparins.

CD analysis of the heparin-binding domain of vWF (Tyr^{565}–Ala^{587}; Sobel et al., 1992) shows that this peptide domain is devoid of α-helix structure and is predominantly β-strand and random coil in character. Nonetheless, this peptide shows high affinity and specificity for heparin. This binding is marked by significant changes in the shape and position of the CD spectral envelope. Furthermore, dissociation of the peptide–heparin complex was acomplished under high ionic strength conditions, and the dissociation event was also observed in the CD spectrum.

Thus, although a helix structure is not implicated in heparin binding to vWF, with other proteins, the putative heparin-binding domains are postulated to encompass a least one helix structure. To study the possible contribution of helix structure to heparin–peptide interactions and as an initial step in the development of specific heparin-binding peptides, a 19-residue peptide predicted to form an α-helix in solution was designed and synthesized. Arg residues were spaced throughout the sequence so that one side of the predicted helix structure would present a surface for binding heparin.

As determined by CD analysis, the peptide shows a very high fractional percent of α-helix content in aqueous solution at neutral pH. Furthermore, because there is no discernible dependence of ellipiticity (at 222 nm) on the concentration of the peptide (in the range 20–300 μM), we can conclude that helix formation is unimolecular and is not dependent on aggregation of the peptide.

It should be noted here that a two-state model in which the peptide exists as either fully helical or fully coil probably does not adequately describe the thermal-induced transition observed for the peptide. Other intermediate structures of the peptide likely exist which are not revealed by a global, averaging technique such as CD spectroscopy. In fact, ^{1}H nuclear magnetic resonance experiments with short-chain helical peptides reveal that these peptides may possess partial helix and partial coil character with an unequal distribution of helical structure between the termini and the center of the peptide (Lyu et al., 1990). A two-state equilibrium probably does not exist, but, nonetheless, the propensity for a given peptide to possess helix character was the same as shown by NMR or CD (Lyu et al., 1990). So, although a two-state model assumption may be a poor approximation for helix formation in short-chain peptides, analysis of CD data under the assumptions of this model is useful for comparison of the effects of heparin on helix stabilization. Even if a "multi-state" model is actually operative, qualitative analysis of the CD data in a two-state assumption compares very favorably with more complex multistate statistical methods (Lyu et al., 1990; O'Neill & De Grado, 1990; Merutka & Stellwagen, 1990). We thus used the simpler two-state model for comparison analysis of the effect of heparin on peptide structure.

Binding to heparin significantly increases the helix content of the peptide, and the binding event can be monitored in the CD. Heparin may increase the helical content of the peptide either by increasing the number of residues in the helical conformation or by increasing the number of peptide molecules in the helix versus the coil conformation. In any case, heparin clearly increases the stability of the peptide which is reflected in the higher melting temperature observed for the peptide–heparin complex compared to that for the peptide alone. There is a van't Hoff enthalpy difference of $\approx$1 kcal/mol for the helix/coil transition in comparing the peptide with the heparin–peptide complex, further indicating that binding heparin serves to stabilize the helix structure of the peptide.

The van't Hoff enthalpies determined for the peptide alone or the peptide–heparin complex (0.17 and 0.23 kcal/mol per residue, repectively) are similar to those determined by calorimetry and CD methods for a 50-residue helical peptide (0.22

kcal/mol; Scholtz et al., 1991). These authors showed that the enthalpy of the transition as determined by differential scanning calorimetry (ΔH_{cal}) was much greater than ΔH_{vH}, thus concluding that the helix/coil transition is far from being a two-state process. Similarly, the enthalpy values determined in our study are probably far from the real enthalpies of the transition, but the $\Delta\Delta H_{vH}$ values for the peptide–heparin complex compared to the peptide alone are indicative of the thermodynamic stability afforded to the peptide by heparin. This finding is being substantiated in titration and differential scanning calorimetric studies now in progress.

Heparin binding was also recently shown to dramatically stabilize acidic fibroblast growth factor against thermal or acid-induced denaturation (Copeland et al., 1991). It appears that heparin acts to stabilize the native conformation of this protein, facilitating its interaction with cellular receptors. There are numerous heparin-binding growth factors, and it may be that the role of heparin is to stabilize the conformation of each of these peptides.

Heparin may stabilize the helix peptide by eliminating detrimental electrostatic interactions that may occur between arginyl side chains brought into close proximity by folding of the peptide and formation of the α-helix. The negatively charged sulfate (and possibly carboxylate) groups of heparin may neutralize Arg positive charges that might otherwise contribute to helix destabilization. Although the predictive algorithims used for designing the peptide suggest that it should be pure helix in character, the actual fractional percent helix of the peptide alone is somewhat lower than 100%. Thus, in the absence of heparin, the close proximity of the Arg side chains may actually "ruin" the helix structure. It may be that the phosphate ions in the buffer used to take the spectra also help to eliminate unwanted electrostatic interactions, but the helix content in water was still substantial (although somewhat lower than that in phosphate buffer). In this case, it may be that the acetate spectator ions released from the peptide (which was prepared as the polyacetate salt) may also help neutralize detrimental positive charges. Regardless of the structure assumed by the peptide in solution, rotational movement of the Arg side chains would be expected to seek the most thermodynamically stable conformation where unfavorable electrostatic interactions are minimized.

Dissociation of the peptide–heparin complex under high ionic strength conditions shows that heparin binding is mediated by electrostatic interactions. When examined by the dot blot and vWF/platelet competition assays, however, the helix peptide does not seem to be as effective in binding heparin as the vWF domain peptide (Sobel et al., 1992). This may merely indicate that the helix peptide has low affinity for the specific heparin which binds to vWF; the affinity of the helix peptide for other heparin unit structures cannot be extrapolated from this experiment. In fact, because the heparin-binding domain of antithrombin III is ostensibly helix in character, we expect that the helix peptide may have greater affinity for antithrombotic heparins than for anti-vWF heparins. These experiments are in progress.

There may be other simple explanations for the lackluster performance of the helix peptide in the biologic assays. It may be that adsorption of the helix peptide to the nitrocellulose filters used in the dot blot assay adversely affects the helix conformation of the peptide or even that the helix peptide does not adsorb to the nitrocellulose as efficiently as some of the other peptides tested. A radiolabeled helix peptide will be synthesized to quantitate the amount of helix peptide adsorbed to the nitrocellulose filters.

Both Arg and Lys residues are found in established heparin-binding domains of various proteins. In some instances, Arg is the exlusive cationic residue found in the binding domain (e.g., the heparin-binding domain of HIV GP 120 protein, Asn^{301}–Phe^{324}; Rusche et al., 1988; Palker et al., 1988; Cardin, 1990), but more often, Lys residues have been implicated as being essential for heparin recognition and binding. The nature of the side chain carrying the positive charge seems to be important for binding; guanidination of Lys residues, which changes the structure but preserves the charge, greatly reduces binding of heparin to platelet factor 4 (Handin & Cohen, 1986). This idea has been confirmed in our studies; acetylation of lysyl residues of heparin-binding domain peptides eliminates most of the heparin-binding activity, even though Arg residues are present (Sobel et al., 1992). Delocalization of the positive charge as it occurs in Arg may contribute to the inherent differences in affinity between Lys and Arg residues for heparin.

The helix peptide described may be considered the prototype of a new class of heparin-binding peptides. The affinity of such peptides for heparin may be improved by increasing the number of turns of the helix segment or by inclusion of Lys residues. The model peptide probably encompasses no more than four or five helical turns. At 5.4 Å per turn, the overall chain length of this helix would be about 25 Å, which could accommodate at most 6 monosaccharide units (4 Å/unit; Atkins, 1976). We predict that one of the Arg residues is involved in an intrahelix ion pair and it is well established that the probability of helix formation is greatly reduced at the termini of peptides. Thus, it is very likely that the ratio of six monosaccharide units per helix segment is an overestimate. Increasing the chain length of the helix by as few as one or two more turns may greatly increase the affinity for heparin.

The structural differences observed with heparin-binding peptides are probably a reflection of the heterogeneity in the heparin-binding domains in proteins. The multiplicity of heparin-binding strategies in proteins implies that different heparin structures interact with different protein-binding domains of distinct primary, secondary, and tertiary structures. Although heparin-binding domains in proteins are regions of high positive charge density, and the interaction is predominantly electrostatic, the diversity of interaction between heparin and proteins can be contributed by attractive interactions due to van der Waals forces and hydrogen bonding. The nature of the heparin-binding domain in proteins may determine the nature of the interaction. This interaction can be specific as it occurs in antithrombin III (Olson & Bjork, 1991) or nonspecific as it occurs in thrombin (Olson et al., 1991). Thus, by altering the composition and chain length of the model peptide, we may create peptides capable of binding specific heparin unit structures which may possess unique biological properties.

Acknowledgments

We gratefully acknowledge the expertise and help of Dr. John Kermode and Ms. Dalila Marques in performing the various biological assays used to assess heparin binding to the synthetic peptides.

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