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# Large Heat Capacity Change in a Protein−Monovalent Cation Interaction†

Enriqueta R. Guinto and Enrico Di Cera*

*Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, St. Louis, Missouri 63110*

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ABSTRACT: Current views about protein−ligand interactions state that electrostatic forces drive the binding of charged species and that burial of hydrophobic and polar surfaces controls the heat capacity change associated with the reaction. For the interaction of a protein with a monovalent cation the electrostatic components are expected to be significant due to the ionic nature of the ligand, whereas the heat capacity change is expected to be small due to the size of the surface area involved in the recognition event. The physiologically important interaction of $Na^+$ with thrombin was studied over the temperature range from 5 to 45 °C and the ionic strength range from 50 to 800 mM. These measurements reveal an unanticipated result that bears quite generally on studies of molecular recognition and protein folding. Binding of $Na^+$ to thrombin is characterized by a modest dependence on ionic strength but a large and negative heat capacity change of $-1.1 \pm 0.1$ kcal mol$^{-1}$ K$^{-1}$. The small electrostatic coupling can be explained in terms of a minimal perturbation of the ionic atmosphere of the protein upon $Na^+$ binding. The large heat capacity change, however, is difficult to reconcile with current views on the origin of this effect from surface area changes or large folding transitions coupled to binding. It is proposed that this change is linked to burial of a large cluster of water molecules in the $Na^+$ binding pocket upon $Na^+$ binding. Due to their reduced mobility and highly ordered structure, water molecules sequestered in the interior of a protein must have a lower heat capacity compared to those on the surface of a protein or in the bulk solvent. Hence, a binding or folding event where water molecules are buried may result in significant heat capacity changes independent of changes in exposed hydrophobic surface or coupled conformational transitions.

Monovalent cation binding to proteins is a widespread phenomenon and plays a particularly important role in the enhancement of catalytic activity for over a hundred enzymes (Suelter, 1970). This effect is typically accomplished by $K^+$ through essentially two mechanisms. In one mechanism, found predominantly in ATPases, $K^+$ forms a ternary complex with the enzyme and the substrate. In the other mechanism, documented by pyruvate kinase, $K^+$ binds to a distinct site and influences the activity of the enzyme in an allosteric fashion. $Na^+$-activated enzymes are fewer in number and are involved in the blood coagulation and complement cascades (Dang & Di Cera, 1996). In thrombin, the mechanism of activation is allosteric because $Na^+$ binds to a site that is about 15 Å away from the catalytic triad and 5−15 Å away from the specificity sites (Di Cera et al., 1995). Binding of $Na^+$ induces a transition from the slow to the fast form (Wells & Di Cera, 1992) and contributes to the physiologically crucial conversion of thrombin from an anticoagulant to a procoagulant factor (Dang et al., 1995).

Although the kinetic aspects linked to the allosteric activation of enzymes by monovalent cations have been studied for a long time and in a number of systems, remarkably little is known on the energetics of the binding interaction of $K^+$ or $Na^+$ with proteins. These studies are absolutely necessary to understand structure−function relations in detail but face intrinsically difficult problems. First, specific effects need to be distinguished from nonspecific ionic strength effects. Second, the equilibrium components of the interaction need to be derived from the kinetic quantities that are accessible to experimental measurements. Third, the paucity of structural data on monovalent cation binding sites makes it difficult to identify the molecular origin of the effects.

The first problem is overcome by studying the properties of the enzyme in the presence of suitable "inert" monovalent cations, typically tetraalkylammonium salts, that are too bulky to bind specifically to the protein (Collins & Washabaugh, 1985; Collins, 1995). The activity measured in the presence of the inert cation gives a reference for what is to be expected from purely nonspecific ionic strength effects (Suelter, 1974; Wells & Di Cera, 1992). The second problem has been much harder to overcome. Typically, the binding of $K^+$ or $Na^+$ to a protein does not produce suitable spectral changes and occurs with dissociation constants in the millimolar range, which makes the experimental approach based on calorimetry practically impossible. Hence, a thermodynamic analysis must pursue the effects of monovalent cations on the kinetic properties of the enzyme that can

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* Address correspondence to this author.

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be extraordinarily complex. Important theoretical progress made recently in this area shows that the binding properties of a monovalent cation acting as an allosteric effector can be derived from measurements of the specificity constant $k_{cat}/K_m$ of the enzyme in a relatively straightforward manner (Di Cera et al., 1996). These developments simplify the study of the binding energetics and open the way to a detailed characterization of the physical basis of $K^+$ and $Na^+$ binding to enzymes in general. Finally, much progress has also been made in the structural characterization of binding sites for $K^+$ and $Na^+$. $K^+$ binding sites have been documented for pyruvate kinase (Larsen et al., 1994), dialkylglycine decarboxylase (Hohenester et al., 1994; Toney et al., 1993), the molecular chaperone Hsc70 (Wilbanks & McKay, 1995), and fructose 1,6-diphosphatase (Villeret et al., 1995). The $Na^+$ binding site of thrombin has been identified (Di Cera et al., 1995; Nayal & Di Cera, 1996) and so has the $Na^+$ binding site of factor Xa (Tulinsky, personal communication).

These recent developments enable a quantitative analysis of the energetic basis of monovalent cation specificity in proteins. In this study we present a detailed characterization of thrombin interaction with $Na^+$. Here we unravel unanticipated aspects of the energetics of thrombin−$Na^+$ interaction that may be paradigmatic of protein−monovalent cation interactions in general and also bear on studies of molecular recognition and protein folding.

## MATERIALS AND METHODS

Human α-thrombin was purified and tested for activity as described (Wells & Di Cera, 1992; Dang & Di Cera, 1994). The chromogenic substrates FPR[1] and VPR were synthesized by solid phase by Drs. John Tomich and Takeo Iwamoto (Kansas State University, Kansas City, MO), whereas S2238 and Spectrozym-TH were obtained from Chromogenix and American Diagnostica. The hirudin N-terminal fragment 1−49 was prepared, purified, and tested for activity as described (Ayala et al., 1995). Measurements of substrate hydrolysis were carried out under experimental conditions of 5 mM Tris and 0.1% PEG, pH 8.0, over the temperature range from 5 to 45 °C. The ionic strength was kept constant at 200 mM. The concentration of $Na^+$ was changed by diluting NaCl with ChCl. The pH was precisely adjusted at room temperature to obtain the value of 8.0 at the desired temperature. Tris buffer has a $pK_a = 8.06$ at 25 °C, and a temperature coefficient of $\Delta pK_a/\Delta T = -0.027$ (Stoll & Blanchard, 1990). These properties ensured buffering over the entire temperature range examined. Possible complicating effects due to ionization of Tris buffer were tested by carrying out similar measurements in the presence of HEPES, which has a lower heat of ionization (Stoll & Blanchard, 1990). No significant difference was found between results obtained in the two buffers. The stability of thrombin was checked over the entire temperature range examined, as described (Ayala et al., 1995). Measurements of substrate hydrolysis were also carried out as a function of ionic strength, from 50 to 800 mM, under experimental conditions of 5 mM Tris and 0.1% PEG, pH 8.0, at 25 °C. At each value of ionic strength, the concentration of $Na^+$ was changed by diluting NaCl with ChCl.

The release of *p*-nitroaniline following hydrolysis of FPR by thrombin was quantified from analysis of progress curves (Dang et al., 1995) using KINSIM and FITSIM (Barshop et al., 1983; Zimmerle & Frieden, 1989). The values of $k_{cat}/K_m$ derived from analysis of progress curves of FPR hydrolysis as a function of $Na^+$ concentration were analyzed according to the equation (Di Cera et al., 1996)

$$\frac{k_{cat}}{K_m} = s = \frac{s_0 + s_1\frac{x}{K_d}}{1 + \frac{x}{K_d}} \quad (1)$$

where $s_0$ and $s_1$ are the values of the specificity constant $s$ in the slow and fast forms, i.e., in the absence and under saturating concentrations of $Na^+$ (Wells & Di Cera, 1992), $K_d$ is the equilibrium dissociation constant for $Na^+$ binding, and $x$ is the $Na^+$ concentration. The derivation of eq 1, which simplifies considerably the analysis of allosteric effects in serine proteases like thrombin, is given elsewhere (Di Cera et al., 1996). Independent estimates of $K_d$ were obtained with the other chromogenic substrates VPR, S2238, and Spectrozym-TH and also from the equilibrium linkage between $Na^+$ binding and the inhibition of thrombin activity by the hirudin N-terminal fragment 1−49, as described in detail elsewhere (Ayala & Di Cera, 1994; Ayala et al., 1995). These estimates agreed quite well with those derived from eq 1 for FPR and with results of fluorescence and steady-state measurements reported previously under identical solution conditions (Wells & Di Cera, 1992).

The standard free energy for thrombin−$Na^+$ interaction depends on other thermodynamic quantities derived from integration of the Gibbs−Helmholtz equation assuming a constant heat capacity change as

$$\Delta G = RT \ln K_d = \Delta H - T\Delta S = \Delta C_p\left(T - T_H - T\ln\frac{T}{T_S}\right) \quad (2)$$

The characteristic temperature values $T_H$ and $T_S$ are the values of $T$ where the binding enthalpy or entropy vanishes respectively. In particular, $T_H$ is the absolute temperature where the equilibrium dissociation constant assumes its minimum value.

The ionic strength dependence of the thrombin−$Na^+$ interaction was analyzed according to the expression

$$-\ln K_d = A_0 + \Gamma \ln I \quad (3)$$

This is essentially the Taylor expansion of the function $-\ln K_d$ around the logarithm of $I = 1$ M, where $-\ln K_d = A_0$. $\Gamma$ is the phenomenological coefficient quantifying the change in $-\ln K_d$ due to a change in $\ln I$ and represents a rigorous thermodynamic measure of the effect of salt concentrations on binding equilibria (Record & Anderson, 1995; Di Cera, 1995). The value of $\Gamma$ in the case of $Na^+$ binding depends solely on electrostatic components and screening effects.

## RESULTS

The dependence of the specificity constant for the hydrolysis of FPR, VPR, S2238, and Spectrozym-TH by

[1] Abbreviations: Ch, choline; FPR, H-D-Phe-Pro-Arg-*p*-nitroanilide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, poly(ethylene glycol); S2238, H-D-Phe-pipecolyl-Arg-*p*-nitroanilide; Spectrozym-TH, H-D-hexahydrotyrosyl-Ala-Arg-*p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane; VPR, H-D-Val-Pro-Arg-*p*-nitroanilide.

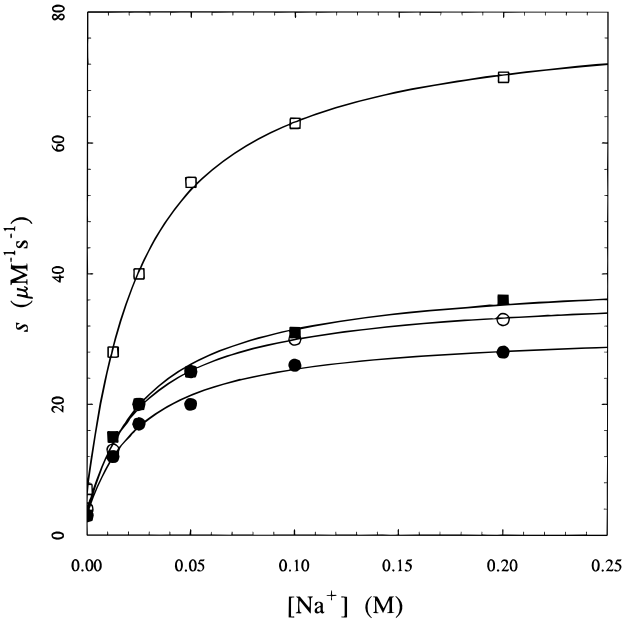


FIGURE 1: Effect of $Na^+$ on the specificity constant $s = k_{cat}/K_m$ for the hydrolysis of FPR (●), VPR (○), Spectrozym-TH (■), and S2238 (□) by thrombin. Experimental conditions are 5 mM Tris, 0.1% PEG, pH 8.0, 25 °C, and $I = 0.2$ M kept constant with ChCl. The continuous lines were drawn according to eq 1 in the text, with best-fit parameter values: $s_0 = 3.1 \pm 0.9$ $\mu M^{-1}$ $s^{-1}$, $s_1 = 32 \pm 1$ $\mu M^{-1}$ $s^{-1}$, $K_d = 28 \pm 5$ mM (FPR); $s_0 = 3.8 \pm 0.6$ $\mu M^{-1}$ $s^{-1}$, $s_1 = 37 \pm 1$ $\mu M^{-1}$ $s^{-1}$, $K_d = 29 \pm 3$ mM (VPR); $s_0 = 3 \pm 1$ $\mu M^{-1}$ $s^{-1}$, $s_1 = 40 \pm 1$ $\mu M^{-1}$ $s^{-1}$, $K_d = 31 \pm 5$ mM (Spectrozym-TH); $s_0 = 6.7 \pm 0.8$ $\mu M^{-1}$ $s^{-1}$, $s_1 = 80 \pm 1$ $\mu M^{-1}$ $s^{-1}$, $K_d = 29 \pm 2$ mM (S2238).

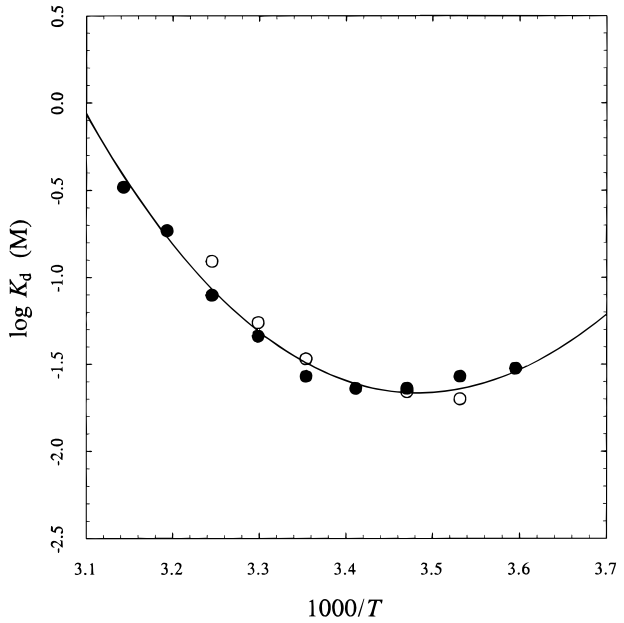


FIGURE 2: van't Hoff plot for the binding of $Na^+$ to thrombin spanning the temperature range from 5 to 45 °C. Experimental conditions are 5 mM Tris, 0.1% PEG, pH 8.0, and $I = 0.2$ M. Data points were obtained from kinetic linkage analysis of the effect of $Na^+$ on the hydrolysis of FPR (●), or equilibrium linkage analysis of the effect of $Na^+$ on the binding of the hirudin N-terminal fragment 1−49 (○). The curvature in the plot indicates the presence of a large and negative heat capacity change linked to $Na^+$ binding. The continuous line was drawn according to eq 2 in the text, as the best fit to both data sets with parameter values: $\Delta C_p = -1.1 \pm 0.1$ kcal $mol^{-1}$ $K^{-1}$, $T_H = 287.1 \pm 0.9$ K, and $T_S = 289.0 \pm 0.9$ K.

thrombin on the concentration of $Na^+$ is shown in Figure 1. In all cases the data obey eq 1 and yield an unequivocal determination of the $K_d$ for $Na^+$ binding, which is also in good agreement with previous determinations obtained from extensive linkage studies and fluorescence titrations (Wells & Di Cera, 1992; Ayala & Di Cera, 1994). The temperature dependence of the $K_d$ for $Na^+$ binding to thrombin is shown in Figure 2. These data extend previous work on the temperature dependence of $K_d$ that was limited to a narrower temperature range (15−35 °C) because of technical difficulties with fluorescence and steady-state determinations (Wells & Di Cera, 1992). The van't Hoff plot is significantly nonlinear, signaling the presence of a large and negative heat capacity change. Independent determinations of $K_d$ from equilibrium linkage analysis of the binding of the hirudin N-terminal fragment 1−49 are also given in Figure 2 and agree very well with those determined from kinetic measurements using eq 1. A fit to both data sets yields a value of $\Delta C_p = -1.1 \pm 0.1$ kcal $mol^{-1}$ $K^{-1}$ and characteristic temperatures $T_H = 287.1 \pm 0.9$ K and $T_S = 289.0 \pm 0.9$ K. Fitting of the separate data sets yields $\Delta C_p = -1.19 \pm 0.09$ kcal $mol^{-1}$ $K^{-1}$, $T_H = 288.3 \pm 0.8$ K, and $T_S = 290.2 \pm 0.7$ K for the kinetic data and $\Delta C_p = -1.2 \pm 0.2$ kcal $mol^{-1}$ $K^{-1}$, $T_H = 287 \pm 1$ K, and $T_S = 288 \pm 1$ K for the equilibrium data, confirming the consistency of the results.

The remarkable consequence of the temperature dependence of $Na^+$ binding to thrombin is that $K_d$ increases from 27 mM at 25 °C to 113 mM at 37 °C. This result has an important bearing on studies of structure−function relations for this enzyme. Under physiological concentrations of NaCl (145 mM) the fast form is only populated by 56% of the molecules at 37 °C, as opposed to nearly 85% at 25 °C. Hence, the properties of thrombin *in vivo* are the average of those of the slow and fast forms, while those measured at room temperature reflect predominantly the contribution of the fast form. Studies that fail to recognize the contribution of the slow and fast forms and the fact that this contribution changes with temperature and salt conditions can provide at best only a superficial understanding of structure−function relations for this important enzyme.

The $K_d$ for $Na^+$ was also measured as a function of ionic strength in the range from 50 to 800 mM. Below 50 mM the value of $K_d$ could not be measured accurately in view of the small difference between the specificity values $s_1$ and $s_0$ in eq 1 and the comparable values of $K_d$ and $I$. The results are shown in Figure 3. The value of $\Gamma$ in eq 4 is $-0.25 \pm 0.05$, implying a modest contribution from electrostatic forces. In fact, a change in $K_d$ by a factor of 10 requires a change in ionic strength by a factor of 10 000. Similar coefficients have been measured for the interaction of thrombin with hirudin (Stone et al., 1989) and fibrinogen and fibrin I (Vindigni & Di Cera, 1996) and are in the range from −2.5 to −1.5. These values are much smaller than the number of charges involved in the recognition event. A possible explanation is that the ionic atmosphere of thrombin is affected only slightly by these binding interactions. This conclusion is supported by extensive electrostatic calculations of thrombin interaction with hirudin, hirudin fragments, and $Na^+$ to be presented elsewhere (Nayal and Di Cera, in preparation).

## DISCUSSION

Current theories on the origin of monovalent cation specificity in proteins emphasize the role of electrostatic

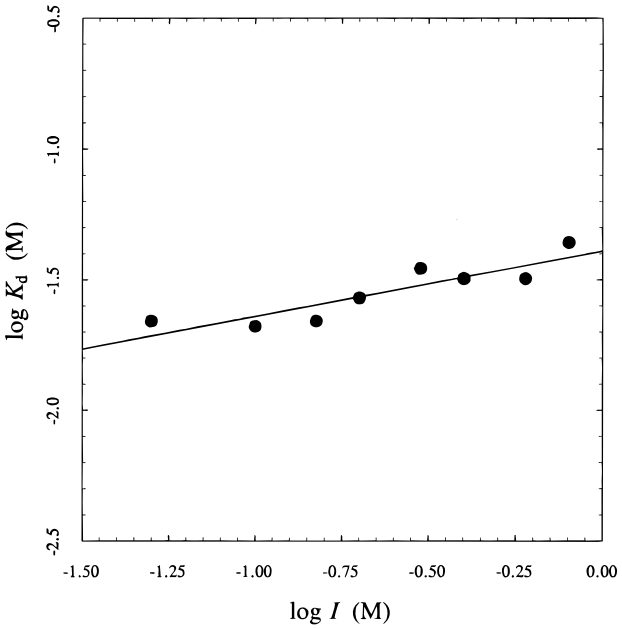


FIGURE 3: Effect of ionic strength on the binding of $Na^+$ to thrombin. Experimental conditions are 5 mM Tris, 0.1% PEG, pH 8.0, and 25 °C. The continuous line was drawn according to eq 3 in the text, with best-fit parameter values: $A_0 = 1.39 \pm 0.04$, $\Gamma = -0.25 \pm 0.05$.

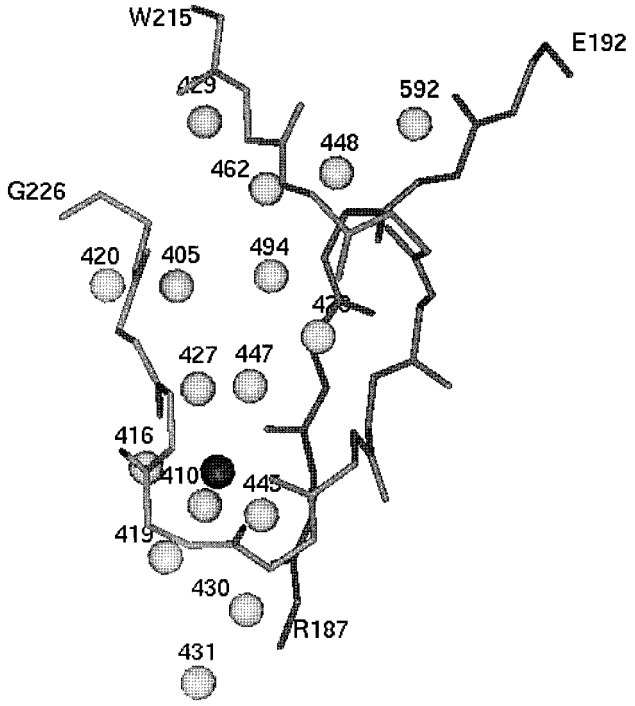


FIGURE 4: Molecular environment of the $Na^+$ binding site of thrombin (Protein Data Bank entry: 1hah.pdb). The sequence 215−226 comprises the $Na^+$ binding loop (Di Cera et al., 1995). The bound $Na^+$ (dark gray circle) is coordinated by the carbonyl O atoms of K224 and R221a (not shown) and four water molecules (light gray circles) numbered 416, 419, 445, and 447 (Nayal & Di Cera, 1996). Also shown is $\beta$-strand 187−192 that diagonally crosses the $Na^+$ binding site from behind. Sixteen water molecules (light gray circles) occupy the buried cavity delimited by the $Na^+$ binding loop and the 187−192 strand. These water molecules are engaged in extensive hydrogen-bonding interactions among themselves and with protein atoms and provide a network of communication among the $Na^+$ site, the specificity site S1 (D189 on the 187−192 strand), and other residues in and around the catalytic pocket defining the *allosteric core* of the enzyme (Guinto et al., 1995). Burial of these water molecules may be responsible for the large heat capacity change observed experimentally upon $Na^+$ binding.

forces (Suelter, 1974; Eisenman & Dani, 1987). Weak electrostatic fields from the protein preferentially dehydrate $K^+$ over $Na^+$ and account for the prevalence of $K^+$-activated enzymes. Specific binding of $Na^+$ over $K^+$ would require strong electrostatic fields to guarantee optimal dehydration and preferential interaction with the protein. In the case of a protein like thrombin, which binds $Na^+$ with an affinity 10 times higher than $K^+$ (Wells & Di Cera, 1992), these theories predict the existence of a strong electrostatic field at the level of the $Na^+$ binding site. The coupling between a charged ligand like $Na^+$ and a strong field from the protein should then produce a large effect of ionic strength on the binding interaction.

The results obtained in this study represent the first quantitative and complete characterization of the thermodynamic driving forces responsible for a protein−monovalent cation interaction and provide an experimental test for the predictions offered by current theories. Our results show that electrostatic forces do not play a major role in the recognition of $Na^+$ by thrombin. This finding, although surprising, must be considered as paradigmatic of protein−monovalent cation interactions in general. The thrombin−$Na^+$ interaction involves carbonyl O atoms and water molecules as typically seen in other proteins (Gursky et al., 1992; Hohenester et al., 1994; Larsen et al., 1994; Villeret et al., 1995; Wilbanks & McKay, 1995). Furthermore, other $Na^+$-activated enzymes like factor Xa, factor IXa, activated protein C, and complement factor C1r (Dang & Di Cera, 1996) bear high structural homology to thrombin. The rules underlying monovalent cation specificity will need to be reformulated as more experimental data are gathered on the role of electrostatic effects on monovalent cation binding to proteins.

The modest effect of ionic strength on $Na^+$ binding to thrombin is contrasted by the conspicuous effect of temperature. The large and negative heat capacity change poses intriguing questions as to its possible molecular origin, because it cannot be reconciled with current theories on the role of the hydrophobic effect in molecular recognition and protein folding (Spolar & Record, 1994; Makhadatze & Privalov, 1995). The value of $\Delta C_p = -1.1$ kcal mol$^{-1}$ K$^{-1}$ observed for $Na^+$ binding to thrombin is the result of van't Hoff analysis and not of direct calorimetric determinations, which cannot be carried out in a system where the ligand binds in the millimolar range. Even allowing for a possible bias of 0.2−0.4 kcal mol$^{-1}$ K$^{-1}$ in the van't Hoff analysis, the value is comparable to that measured for the dimerization of the arc repressor (Bowie & Sauer, 1989), the tetramerization of melittin (Wilcox & Eisenberg, 1992), the folding of proteins like parvalbumin (Filimonov et al., 1978) and ribonuclease A (Privalov & Gill, 1989), or the binding of the hirudin N-terminal fragment 1−49 to thrombin (Ayala et al., 1995). In all these cases, the process is linked to burial of extended hydrophobic surface or large folding transitions.

A number of empirical expressions relate changes in polar and nonpolar surface area with observed heat capacity changes (Spolar et al., 1989, 1992; Makhadatze & Privalov, 1990, 1995; Livingstone et al., 1991; Murphy et al., 1992, 1993). For a change of −1.1 kcal mol$^{-1}$ K$^{-1}$, the predicted nonpolar surface buried upon binding must be on the order of 2600 Å$^2$ (Makhadatze & Privalov, 1995). This value is unrealistically high for the thrombin−$Na^+$ interaction, be-

cause it is nearly 35% of the entire accessible hydrophobic surface area of the enzyme (Ayala et al., 1995). Alternatively, a large heat capacity change can arise from coupled folding transitions (Spolar & Record, 1994). The predicted number of residues that must order upon $Na^+$ binding can be calculated to be about 70. One is then bound to conclude that the slow form of thrombin is more disordered than the fast form and that $Na^+$ binding causes nearly 25% of the enzyme to fold. This scenario is unrealistic in view of the highly constrained environment of the $Na^+$ binding site (Di Cera et al., 1995) and is also inconsistent with circular dichroism studies demonstrating that the fast form is actually more disordered than the slow form (Ayala & Di Cera, 1994; Di Cera et al., 1995).

We speculate that the predominant origin of the large heat capacity change upon $Na^+$ binding to thrombin must be found in the conspicuous water channel that embeds the $Na^+$ environment (see Figure 4). The hydrogen-bonding network of the water molecules in this region is remarkably complex and bridges residues of the enzyme that could not interact otherwise. This network of buried water molecules may be affected significantly upon release of $Na^+$. Indeed, the crystal structure of a thrombin mutant defective for $Na^+$ binding indicates that most of these buried water molecules are missing when $Na^+$ is not bound (Tulinsky and Di Cera, in preparation). The structure of the slow form ($Na^+$ free) of the wild type will test our hypothesis directly.

Solvation of charged and polar groups is a process accompanied by a negative heat capacity change, the magnitude of which is small and precisely known only for simple molecules (Edsall, 1935; Edsall & Wyman, 1958). The thermodynamic signatures of solvation of polar groups must depend on where these groups are located. In the interior of a protein, as is the case for the $Na^+$ binding site of thrombin, solvating water molecules experience a drastic entropy loss compared to bulk water. This is in contrast with water molecules solvating groups on the surface of a protein. Likewise, the heat capacity of water molecules sequestered in the interior of a protein must be significantly lower than that of water molecules in the bulk solvent, because of their reduced mobility and more ordered structure. Burial of water molecules linked to ligand binding or protein folding may then result in large and negative heat capacity changes and contribute significantly to the effects of thermal transitions observed experimentally. This possibility needs to be kept in mind when interpreting heat capacity changes due to ligand binding or protein folding, especially if there is structural evidence of buried water molecules.

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