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Thermodynamics of Na^+ binding to coagulation serine proteases

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Abstract

The sodium binding to serine proteases triggers a conformational change in the proteins that enhances the catalytic activity of the enzymes. The interaction of the cation with the protein is mediated by the hydrogen-bonding network of water molecules that embed the Na^+ site. We pointed out the crucial role of the insertion loop 186a–d and the I16-D194 ion pair in the stabilization of sodium binding pocket in thrombin. This paper contributes to better explain the molecular mechanism of sodium binding for different serine proteases leading to the identification of the structural changes necessary to engineer a functional Na^+ site and regulate catalytic activity in serine proteases.
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1. Introduction

The discovery that the allosteric properties of the serine proteases are linked to sodium binding [1,2] and strongly regulated by residue 225 of the proteins [3] has revealed a new molecular mechanism controlling the activity and the specificity of these enzymes [1]. Proteases that have a proline in position 225 have no requirement for Na^+ or other monovalent cations. In contrast, those proteases with a tyrosine in position 225 show catalytic activity enhanced by Na^+ binding [1]. The distribution of the different allosteric forms under physiological conditions is crucial to understanding the role of the transition in the biological system. In thrombin, sodium binds to a site that is approximately 15 A° away from the catalytic triad and 5–15 A° away the specificity sites [4]. The sodium binding site of factor Xa has also

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been identified [5]. The thermodynamic parameters describing the system provide detailed structural information on the molecular basis of protein–ligand interaction and molecular recognition [6].

The binding of sodium to serine proteases was studied by recording intrinsic protein fluorescence, reflecting the conformational change due to binding of cations to the protein in the absence of substrates, effectors — interaction or transition to the active site of the protein. In this study, we have characterized the temperature-dependence of sodium binding for several serine proteases showing a common mechanism of the interaction of cation with the protein. An unanticipated effect of the pH on sodium affinity has revealed the crucial importance of an ion pair in the stabilization of the architecture of the sodium binding site. The fluorescence study was extended to other cations, confirming the high selectivity of serine proteases over cations. This study explains the molecular mechanism of sodium binding to serine proteases, providing the first step in engineering allosteric enhancement of catalytic activity in proteases that do not bind Na^+ and provides a new therapeutic approach in the design of drugs acting on the allosteric transition of enzymes.

2. Materials and methods

Human thrombin and thrombin mutants were expressed, purified and tested for activity as previously described [7,8]. All other serine proteases of the highest commercially-available purity and activity were obtained from enzyme research (South Bend, IN). Fluorescence titrations were carried out as described [9], with the protein emission spectra recorded between 300 and 450 nm and an excitation wavelength of 280 nm. The temperature dependence of Na^+ binding to serine proteases was studied under experimental conditions of ionic strength 800 mM, 5 mM Tris, 0.1% PEG, and pH 8.0 (1 mM EDTA or 5 mM Ca^{2+}) over the temperature range from 5 to 45°C. In order to keep the ionic strength constant, the inert cation used for thrombin and aPC studies was choline and that for hFXa was ammonium, as it has previously been reported that choline may bind to hFXa and interfere with Na^+ binding [9].

The standard free energy for protein–cation interaction depends on other thermodynamic quantities derived from integration of the Gibbs–Helmholtz equation assuming a constant heat capacity change [6,10]:

$$\begin{aligned}\Delta G &= RT\ln K_d = \Delta H - T\Delta S \\ &= \Delta H_0 - T\Delta S_0 + \Delta C_p(T - T_0) \\ &\quad - T\Delta C_p \ln(T/T_0\ ) \end{aligned} \tag{1}$$

where $T_0 = 298.15$ K is the reference temperature, ΔH_0 and ΔS_0 are the values of binding enthalpy and entropy change at the reference temperature.

2.1. pH Measurements

Measurements of Na^+ binding over the pH range of 6.0–10.5 were carried out at 25°C under conditions of 10 mM CHES, 5 mM Tris, 5 mM bisTris, 0.1% PEG, (1 mM EDTA or 5 mM $CaCl_2$). The triple buffer ensured buffering over the entire pH range with minimal perturbations in the ionic strength of the solution [11]. The stability of the enzyme was checked over the entire temperature and pH range examined, as previously described [12].

3. Results

3.1. Serine protease fluorescence

Serine proteases can be classified by their ability to bind Na^+ [1]. The increase in intrinsic fluorescence upon cation binding is compatible with the burial of tryptophan residue(s), representing the allosteric conformational change. The tryptophan residues responsible for the spectroscopic change remain unidentified.

In proteases with P225, e.g. human plasmin, tPA, trypsin, haptoglobin, human protein S, complement factor B, thrombin mutant Y225P and Y225W, in which the catalytic activity is not en-

hanced by Na^+ binding [4,13], there is no demonstrable change of protein spectroscopic properties between different cations. In contrast, proteases with a tyrosine in position 225, e.g. factor Xa and activated protein C along with thrombin and thrombin mutants Y225F and Y225H, do show a global change in fluorescence (10–30%) after cation binding. In other proteases expected to bind sodium, e.g. human factor IXaβ (Y225), human factor VIIa (F225), C1r (Y225) and C1s (Y225), we did not observe changes in fluorescence properties of the system, indicating either that a different structural allosteric transition of the enzyme is occurring or that the residue(s) responsible for the spectroscopic changes are hidden by the overall conformation of the protein.

The activity of serine proteases is physiologically regulated by calcium ion [14–17]. Experimental data show that the presence of this divalent cation influences the behavior of the proteins in solution and their spectroscopic properties. To check the linkage between the Na^+ binding and the divalent cation, all the measurements were carried in a buffer containing saturating amount of Ca^{2+} or EDTA. The sodium affinity for thrombin and thrombin mutants, as expected, was found to be independent of the calcium concentration, whilst Factor Xa shows a slight difference in sodium affinity depending on the Ca^{2+} concentration. For activated protein C the effect of Na^+ binding on the fluorescence signal is partially hidden by the presence of Ca^{2+}, allowing the experimental measurements to be recorded only in the presence of EDTA.

3.2. Temperature study

Figs. 1 and 2 show the van't Hoff plots for the different serine proteases and thrombin mutants studied. The temperature dependence of K_d for sodium for thrombin and aPC is significantly non-linear, indicating the presence of a large and negative heat capacity change. The data for thrombin wild-type are in perfect agreement with reported measurements derived from experiments on the specificity constant of the enzyme [6]. The thrombin mutant Y225F has a slightly higher K_d than wild type. The mutation Y225H significantly decreases the affinity of the protein for Na^+. The thermodynamic parameters reflecting Na^+ binding are listed in Table 1: the heat capacity change is in the order of 1.2 kcal mol^{-1} K^{-1} for thrombins, the ΔC_p value is lower but still negative for aPC. The associated enthalpy and entropy changes are in the order of 20 kcal/mol and 50 cal/mol, respectively. Factor Xa shows a different behavior and the temperature-dependence of the sodium affinity for this enzyme is almost linear.

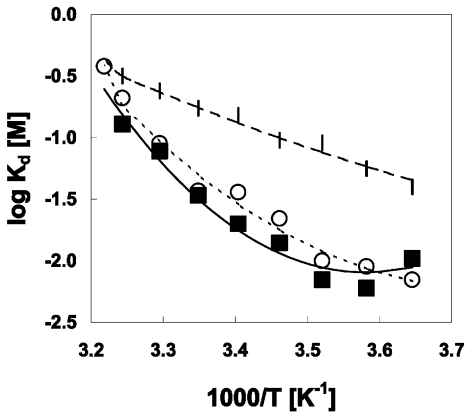

Fig. 1. van't Hoff plot for the binding of Na^+ to thrombin (■), activated protein C (○) and Factor Xa (|). The curves were drawn according to Eq. (1) in the text (see Section 2).

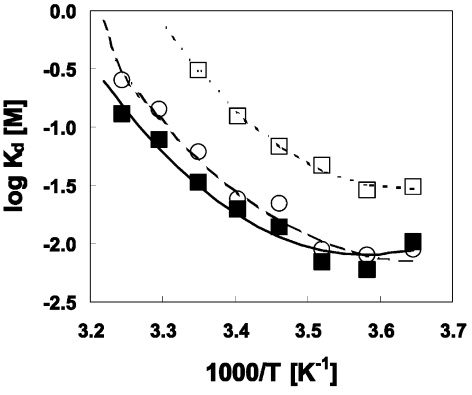

Fig. 2. van't Hoff plot for the binding of Na^+ to thrombin (■), Y225F (○) and Y225H (□). The curves were drawn according to Eq. (2) in the text (see Section 2).

Table 1
Thermodynamic parameters for sodium binding to different serine proteases under experimental conditions of ionic strength 800 mM, 5 mM Tris, 0.1% PEG 8000 and pH 8.0

Protein	ΔC_p [kcal mol^{-1} K^{-1}]	ΔH_0 [kcal mol^{-1}]	ΔS_0[kcal mol^{-1}]
Thrombin WT	-1.191 ± 0.283	-17.869 ± 1.677	-0.052 ± 0.006
Activated protein C	-0.631 ± 0.159	-18.248 ± 0.912	-0.054 ± 0.003
Y225F	-1.018 ± 0.284	-21.709 ± 1.684	-0.066 ± 0.006
Y225H	-1.393 ± 0.208	-26.478 ± 1.803	-0.085 ± 0.006
Factor Xa	-0.053 ± 0.050	-4.432 ± 0.443	-0.015 ± 0.001

3.3. pH Study

The pH dependence of K_d of sodium for serine proteases is shown in Fig. 3. The experimental points were fitted according to:

$$K_d = K_d^{opt}\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right) \quad (2)$$

where K_d^{opt} is the optimized (pH independent) value of K_d, while K_1 and K_2 are the acid dissociation constant of the two groups [18,19]. The equation assumes that the complex ligand-protein is formed only when one of the two groups is protonated and the other is deprotonated [19]. The results are summarized in Table 2.

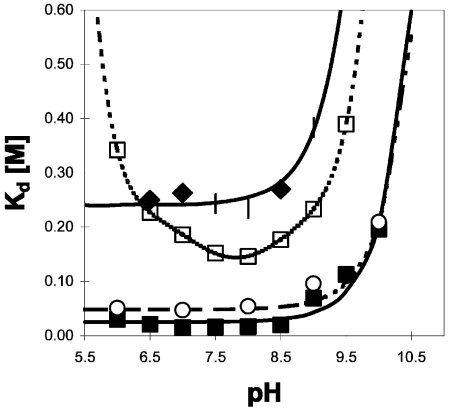

Fig. 3. pH Dependence for the binding of Na^+ to thrombin (■), Y225H (□), Factor Xa (|) and activated protein C (○). The curves were drawn according to Eq. (2) in the text (see Section 3).

Kinetic experiments were carried out to check the overall integrity of the protein with regard to pH (data not shown). We did not measure a significant decrease of the enzyme activity over the pH range of this study. Consequently, the enzymes remain active in all those pH conditions, which suggests that the folding of the protein is maintained and proves that the measured effect on the sodium affinity is due to specific ionizable groups.

For thrombin, aPC and FXa the plot shows the presence of only one ionizable group controlling the cation binding in this pH range. A value of p$K \sim 9.2$ for this residue was estimated for all the proteases. The deprotonation of this residue disrupts Na^+ binding. These experimental data support the hypothesis of the presence of an ion pair that, if disrupted, decreases the affinity for cations.

The effect of a positive charge in position 225 was studied in the case of the histidine mutant. In contrast to wild type thrombin, a bell-shaped curve is observed for thrombin mutant Y225H between pH 6.0 and 10.0. This indicates that Na^+ binding is controlled by an additional ionizable group. The calculated pK value of this group is 6.1,

Table 2
Best fit parameters of the pH dependence of the sodium dissociation constant (see Table 3 for details)

Protein	K_d^{opt} [M]	pK_1	pK_2
Thrombin WT	0.025 ± 0.003	9.14 ± 0.17	–
Activated protein C	0.048 ± 0.003	9.48 ± 0.04	–
Y225F	0.028 ± 0.003	9.20 ± 0.18	–
Y225H	0.153 ± 0.006	9.31 ± 0.04	6.11 ± 0.04
Factor Xa	0.188 ± 0.023	9.15 ± 0.04	–

corresponding to the expected proton affinity for histidine residues in a protein [20]. These interesting data indicate that the protonation of the histidine 225 decreases the affinity of the protein for the sodium at low pH.

In the case of the thrombin mutant Y225H and wild type, the experimental data also show a similar increase of the K_d value for Na^+ when pH increases. This result suggests no linkage between the hypothesized ion pair I16-D194 and residue 225.

3.4. Cation study

The dissociation constants for different cations were measured for all the proteins studied (see Table 3). Na^+, K^+, Li^+ have no quenching effect on proteins or L-tryptophan and L-tyrosine in the concentration range used in this work [21]. The increase in fluorescence recorded after changing the cation concentration, whilst the ionic strength is kept constant, can be attributed to the allosteric transition after the ligand binds to a specific site on the protein. All of the serine proteases are able to discriminate amongst the different cations. Na^+ is the most specific for all the proteins with the thrombin and Y225F maintaining a difference with K^+ of approximately 10-fold, aPC of approximately eightfold. FXa and thrombin mutant Y225H show a dissociation constant for potassium in the molar range. For the lithium salt no fluorescence properties change was recorded in the studied range.

Table 3
Sodium and potassium binding dissociation constant to different serine proteases under experimental conditions of ionic strength 800 mM, 5 mM Tris, 0.1% PEG 8000, pH 8.0 at 25°C[a]

Protein	Sodium K_d [mM]	Potassium K_d [mM]
Thrombin WT	20 ± 1	213 ± 18
Activated protein C	36 ± 5	374 ± 50
Thrombin Y225F	24 ± 1	220 ± 21
Thrombin Y225H	124 ± 16	> 500
Factor Xa	151 ± 18	> 500

[a] Previous studies [29,30] indicated similar K_d value for the interaction of sodium and potassium with aPC. The discrepancies could be addressed to a different protein used in that measures (bovine des-1-41 light chain activated protein C).

4. Discussion

Current theories on the origin of monovalent cation specificity in proteins propose that electrostatic forces drive the binding of charged species, and that the burial of hydrophobic and polar surfaces controls the heat capacity change associated with the reaction. In a previous study [6], the fundamental role of the burial of a large cluster of water molecules in the Na^+ binding pocket upon Na^+ binding was proposed to be the molecular mechanism responsible for the large, negative heat capacity change in thrombin.

The experimental results confirm that the thermodynamic properties of thrombin are typical of the other serine proteases except FXa. The value of ΔCp in the order of -1 kcal mol^{-1} K^{-1} observed for Na^+ binding to proteins is the result of van't Hoff analysis and not of direct calorimetric determination (which is practically impossible with ligand dissociation constant in the millimolar range [6]). The value is comparable with processes linked to the burial of extended hydrophobic surface or large folding transitions [22,23]. As described by Guinto and Di Cera [6], these mechanisms seem unrealistic if applied to serine proteases-cation interaction. We speculate that the predominant origin of the large heat capacity change upon Na^+ binding to serine proteases must be found in the conspicuous water channel in which the Na^+ environment is embedded [24]. The conservation of clusters of buried water molecules is a structural motif present throughout the serine proteases family. The complex network of hydrogen-bonding of the water molecules may be affected significantly upon release of Na^+. The heat capacity change should be derived by the difference in mobility and highly ordered structure of water molecules sequestered in the interior of a protein compared to those on the surface of the protein or in the bulk solvent. The different behavior of FXa, showing an almost linear temperature-dependence of the sodium

affinity, could be attributed to the absence of the insertion loop 186a–d, influencing the large heat capacity change linked to Na^+ binding in thrombin [24].

The high temperature-dependence of sodium affinity in the physiopathological range can contribute to explain the coagulation problems associated with temperature variations associated with different pathological conditions [14]. Indeed, the dissociation constant for the studied enzymes can increase by $\sim 10–20\%$ between 37 and 40°C, leading to an alteration of coagulation process.

The dependence of Na^+ binding to the pH suggests the presence of an ion pair to stabilize the cation binding site on the protein. The pH effect is due to a basic group that starts to deprotonate at approximately pH 9.0, whereas the comparable activity of proteases on synthetic substrates over the pH range studied indicates that the overall folding of the proteins is correct. We hypothesize that the ion pair involved in the stabilization of sodium binding pocket could be I16-D194 for thrombin, thrombin mutants and factor Xa, and L16-D194 for aPC. This salt bridge is formed after the activation of zymogens and has a key role in the conformational stability of the enzyme. The assigned pK_a value of this group determined by pH studies of the thrombin–fibrinogen complex is 8.80 [19,25]. Deprotonation of Ile16 causes the salt bridge to break and yields an initial disruption of sodium binding site, thereby decreasing the affinity for the cation. The pK of the basic group of the salt bridge I16-D194, responsible for the alkaline portion of pH response, is not altered in the case of Y225H mutant. This result points out that the presence of a positive charge in position 225 is not changing the protonation of the basic group, thereby indicating no linkage between position 225 and the salt bridge.

The sodium affinity of the thrombin Y225H mutant reveals the importance of the presence of a positive charge in position 225. At lower pH the protonation of the histidine significantly decreases the affinity for sodium.

Use of the thrombin mutants facilitated clarification of the mechanism of Na^+ binding. Of course, one component of the energetics of sodium binding could be the cation-π interaction, a general non-covalent binding force [26] derived from the interaction between cations and aromatic system. The theoretical models and experimental results on simple aromatic modeling aminoacid side chains predict that tryptophan may be especially important in cation-π interaction, followed by tyrosine, phenylalanine and histidine. This behavior is observed in all except the tryptophan residue, as Y225W does not show significant Na^+ binding affinity ([3] and this study). The disagreement could be explained by a totally different orientation of residue W225 in the quaternary structure of the protein, due to the highly hydrophobic ring and to the different dimensions of this aminoacid. The lower specificity of K^+ and Li^+ ion compared to the Na^+ reinforces the hypothesis of multiple mechanisms of cation binding, accounting for ligand dimension, charge density, and electrostatic and hydrophobic forces driving the interaction of the cation with thrombin. The observed effect [2] did not follow the lyotropic series and, therefore, cannot be accounted for by preferential hydration phenomena.

The binding constant for Na^+ and other monovalent cations can be measured accurately by fluorescence titration. Na^+ is bound with an affinity of approximately one order of magnitude higher than K^+, and Li^+ does not show any effect in the range studied (up to 400 mM). The origin of the higher specificity of Na^+ is due to steric factors in the cavity hosting the metal ion. Valence calculations show that the density occupied by Na^+ in the crystal structure has high Na^+-specific valence and a low valence specific for Li^+ or K^+ [27]. K^+ is too big to fit the cavity without disturbing the network of water molecules, while Li^+, on the other hand, appears to be too small to guarantee optimal bond strength with the surrounding water molecules. The results show that Na^+ has the optimal requirements of ionic radius and charge density to be accommodated in the cavity. The interaction of this cation with thrombin is linked to a modest free energy change resulting by two large enthalpic and entropic contributions that compensate each other. A consequence of the large enthalpy change is a strong

temperature dependence of the sodium affinity in physio-pathological condition. Moreover, the properties of the enzyme in vivo are the average of the properties of the two forms (bounded and free). For thrombin and activated protein C the proteins are optimally poised for allosteric regulation, suggesting a sophisticated mechanism of control based on linkage thermodynamics laws. On the other hand, the physiologic role of Na^+ in FXa remains still unclear and the cation may have only a small effect on the activity of the prothrombinase complex [28]. These properties suggest the importance of finding drugs able to act on the allosteric transition of the proteins in order to switch between the appropriate forms.

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