Thrombin is an allosteric serine protease existing in two forms, slow and fast, targeted toward anticoagulant and procoagulant activities. The slow → fast transition is induced by Na\(^+\) binding to a site contained within a cylindrical cavity formed by three antiparallel β-strands of the B-chain (Met\(^{180}\)-Tyr\(^{184a}\), Lys\(^{224}\)-Tyr\(^{228}\), and Val\(^{231}\)-Gly\(^{239}\)) diagonally crossed by the Glu\(^{188}\)-Glu\(^{192}\) strand. The site is shaped further by the loop connecting the last two β-strands and is located more than 15 Å away from the catalytic triad. The cavity traverses through thrombin from the active site to the opposite surface and contains Asp\(^{189}\) of the primary specificity site near its midpoint. The bound Na\(^+\) is coordinated octahedrally by the carbonyl oxygen atoms of Tyr\(^{184a}\), Arg\(^{221a}\), and Lys\(^{224}\), and by three highly conserved water molecules in the D-Phe-Pro-Arg chloromethylketone thrombin. The sequence in the Na\(^+\) binding loop is highly conserved in thrombin from 11 different species and is homologous to that found in other serine proteases involved in blood coagulation. Mutation of two Asp residues flanking Arg\(^{221a}\) (D221A/D222K) almost abolishes the allosteric properties of thrombin and shows that the Na\(^+\) binding loop is also involved in direct recognition of protein C and antithrombin.

Thrombin is a serine protease playing a key role in hemostasis (1, 2). The enzyme is allosteric and exists in two forms, slow and fast (3). The slow form plays an anticoagulant role because it activates protein C (PC) \(^2\) more specifically, generating a potent inhibitor of blood coagulation; the fast form has a procoagulant role because it cleaves fibrinogen with higher specificity, promoting the formation of a blood clot (4). The slow → fast transition is triggered by the specific binding of a Na\(^+\) ion (3, 5). Other monovalent cations are less effective and bind with much lower affinity. Under physiological conditions of Na\(^+\) concentration, pH, and temperature, the slow and fast forms are almost equally populated (3) and help maintain the balance between procoagulant and anticoagulant activities that is crucial for effective hemostasis. Furthermore, the slow → fast equilibrium in vivo is under conditions where allosteric regulation is most efficient and can be exploited in much needed therapeutic treatments of thrombotic and bleeding disorders (2, 6).

Specific monovalent cation effects in proteins appear to be widespread and have been documented for a long time (7, 8). In the realm of blood coagulation, these effects were first reported for factor Xa (9) and then for thrombin (10) and activated PC (11). Monovalent cations can act as allosteric effectors, influencing the overall structure of an enzyme, or as cofactors that interact directly with substrates. Detailed structural information on the molecular basis underlying these effects has recently emerged for enzymes with specific requirement for K\(^+\) (12–14). In the case of Na\(^+\) specific effects, as seen for thrombin, identification of the bound Na\(^+\) ion in the crystal structure is complicated by the comparable electron density of a water molecule and this monovalent cation. For this reason, the exact position of the Na\(^+\) binding site of thrombin has remained elusive, notwithstanding a number of crystallographic structures of the enzyme complexed with various ligands and inhibitors that have been reported in the presence of NaCl (15).

Given the crucial physiological effect of Na\(^+\) on thrombin (4), identification of the Na\(^+\) binding site should reveal key aspects of structure-function relationships for this important enzyme.

**MATERIALS AND METHODS**

Crystals of thrombin inhibited at the active site with PPACK were grown from sodium phosphate and PEG 8000 in the presence of NaCl as described previously (16, 17). The mother liquor solution containing PPACK-thrombin crystals was exchanged with K\(^+\) ion solution of identical composition replacing Na\(^+\) with K\(^+\) ions (0.1 M potassium phosphate buffer, pH 7.3, 32% PEG 8000, 0.187 M KCl), by adding a 2-µl aliquot to a 10-µl hanging drop and withdrawing 2 µl from the other side of the drop. This was done four times over 2 days. To complete the Na\(^+\)-K\(^+\) exchange, the crystals were transferred directly into 1 ml of the potassium solution. The Na\(^+\)-Rb\(^+\) exchange was accomplished using a capillary soaking method (18) with the potassium phosphate solution containing RbCl instead of KCl. The crystal was placed in a capillary tube used to mount crystals for diffraction purposes. Then 20 µl of a similar solution that contained 20 µl of a similar solution, that contained 1.88 M RbCl, followed by another 20-µl layer of the potassium phosphate solution. Diffusion was allowed to take place for 90 h in the solvent and mounting the crystal in a usual manner. The final concentration of the Rb\(^+\) ion in the capillary is estimated to be 0.625 M. The x-ray diffraction pattern of the Na\(^+\) ion exchanged crystal was measured with an R-AXIS imaging plate detector and used to determine and refine the Rb\(^+\) ion PPACK-thrombin structure (R = 0.19 without solvent structure).

The double mutation D221A/D222K was obtained by amplification of the BglII/EcoRI sequence of pNUT-hII (19) using appropriate primers for the polymerase chain reaction. The double mutant was expressed in baby hamster kidney cells (BHK21) as prethrombin-1 using the pNUT vector constructed with the bovine factor V signal peptide and a 12- amino acid HPC4 epitope (20). The prethrombin-1 mutant preceded by a 12-amino acid HPC4 epitope was purified on an HPC4 Affi-Gel 10 column. The zymogen form was activated, and the thrombin form was purified and tested for activity as described (21).

Human α-thrombin was purified and tested for activity as described (3, 21). Recombinant desulfatohirudin variant 1 (Revasc™) was ob-
The concentration of hirudin was determined by amino acid analysis. Human fibrinogen, PC, antithrombin (AT), and rabbit lung thrombomodulin (TM) were from Hematologic Technologies (Essex Junction, VT). Heparin was from Sigma. The release of fibrinopeptides A and B (FpA and FpB), binding of hirudin, and cleavage of PC by the slow and fast forms of wild-type thrombin and the ARK mutant were studied and analyzed as described (4, 5). Inhibition of thrombin activity by AT was studied using the procedure of Olson (22) and was analyzed using KINSIM and FITSIM (23, 24) to obtain the apparent second-order rate constant for formation of the thrombin-antithrombin complex ($k_{on}$ in Table II). Experimental conditions were: 5 mM Tris, 0.1% PEG, pH 8.0, 25°C, and 0.2 M NaCl for the fast form, or 0.2 M choline (Ch$_1$) chloride for the slow form. The concentration of TM was 10 nM, and heparin was used at concentrations of 1 unit/ml.

RESULTS AND DISCUSSION

The Na$^+$ binding site of thrombin has been unequivocally identified here by exchanging Na$^+$ with Rb$^+$ ions in crystals of PPACK-thrombin. The site displays octahedral coordination involving Tyr$^{184a}$, Arg$^{221a}$, Lys$^{224}$, and three highly occupied water molecules (Fig. 1). It is located more than 15 Å away from the N terminus of the chain at the bottom, and the C terminus is at the end of the α-helix at the top. Important structural domains of the enzyme are noted as follows: 1, Na$^+$ binding loop; 2, autolysis loop; 3, fibrinogen binding loop; 4, heparin binding site. The Na$^+$ ion is shown as a red ball in the loop connecting the last two β-strands of the B-chain terminating into the heparin binding site. Binding of Na$^+$ to this loop induces the slow → fast transition and controls allosterically both the activity (3) and specificity (4) of the enzyme. The highly conserved fragment Cys$^{220}$-Gly$^{226}$ of this loop (see Table I) is in yellow.

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**FIG. 1.** The Na$^+$ binding site of PPACK-thrombin. Electron density of PPACK-thrombin at 2.5 Å resolution in blue (dotted) contoured at 1.20 σ (A. Tulinsky, unpublished results); Rb$^+$ ion difference electron density at same resolution in red at 4σ, calculated using Fourier coefficients of the form: $(F_{Rb} - F_{T}) \exp(-i\alpha T)$, where $F_{Rb}$ is the observed structure amplitude of Rb$^+$ ion soaked PPACK-thrombin, $F_{T}$ that of PPACK-thrombin, and $\alpha T$ the phase angle of PPACK-thrombin without water molecules included; distal water is designated WAT in correct sixth octahedral position, but about 3.4 Å from Na$^+$ position. The average Na$^+$-O distance is 2.9 Å. Numbering is based on topological similarities with chymotrypsin (25).

**FIG. 2.** MOLSCRIPT plot (27) of the B-chain of human α-thrombin, derived from the coordinates of the thrombin-hirudin complex (26), with the inhibitor removed. The residues of the catalytic triad are shown at the interface between the two six-stranded β-barrels. The N terminus of the chain is at the bottom, and the C terminus is at the end of the α-helix at the top. Important structural domains of the enzyme are noted as follows: 1, Na$^+$ binding loop; 2, autolysis loop; 3, fibrinogen binding loop; 4, heparin binding site. The Na$^+$ ion is shown as a red ball in the loop connecting the last two β-strands of the B-chain terminating into the heparin binding site. Binding of Na$^+$ to this loop induces the slow → fast transition and controls allosterically both the activity (3) and specificity (4) of the enzyme. The highly conserved fragment Cys$^{220}$-Gly$^{226}$ of this loop (see Table I) is in yellow.

**FIG. 3.** Value of the specificity constant for the hydrolysis of S2238 (H-o-Phe-pipocyl-Arg-p-nitroanilide) by wild-type thrombin and the ARK mutant, as indicated. Experimental conditions are: 5 mM Tris, 0.1% PEG, pH 8.0, 25°C. The salt concentration was kept constant at 200 mM with the chloride salts indicated on the abscissa. The large difference seen between Na$^+$ (fast form) and Ch$_1$ (slow form) for the wild type, is not observed in the ARK mutant.

**TABLE I**
Sequence homology in the Na$^+$ binding loop of thrombin (residues 220–226)

| Thrombin from different species | Human serine proteases |
|-------------------------------|------------------------|
| Human                         | CDRDGKYG               |
| Mouse                         | CDRUGXYG               |
| Rat                           | CDRUGKYG               |
| Rabbit                        | CDRUGXYG               |
| Bovine                        | CDRUGYG                |
| Chicken                       | CDRUGYG                |
| Gekko                         | CDRUGYG                |
| Newt                          | CDRUGYNG               |
| Rainbow trout                 | CDRUGYG                |
| Sturgeon                      | CDRUGYNG               |
| Hagfish                       | CDRUGYNG               |

**TABLE II**

| Thrombin from different species | Human serine proteases |
|-------------------------------|------------------------|
| Human                         | CDRDGKYG               |
| Mouse                         | CDRUGXYG               |
| Rat                           | CDRUGKYG               |
| Rabbit                        | CDRUGXYG               |
| Bovine                        | CDRUGYG                |
| Chicken                       | CDRUGYG                |
| Gekko                         | CDRUGYG                |
| Newt                          | CDRUGYNG               |
| Rainbow trout                 | CDRUGYG                |
| Sturgeon                      | CDRUGYNG               |
| Hagfish                       | CDRUGYNG               |
have evolved to exploit \( \text{Na}^+ \) pancreatic serine proteases. Proteases active in the blood might accomplish their multifunctional roles, taking advantage of molecular strategy shared by other members of the blood coagulation cascade, like factors VII, IX, X, and PC, but not by the ARK mutant. Examination of different crystal structures of thrombin reveals an octahedrally coordinated water molecule at the \( \text{Na}^+ \) site in over 20 of them, most with exceptionally small contacts to the central water molecule (2.2–2.4 Å).

The sequence Cys\(^{220}\)-Gly\(^{226}\) involving the \( \text{Na}^+ \) binding loop and part of the last \( \beta \)-strand of the B-chain is highly conserved in thrombin from 11 different species (Table I), thereby supporting the fundamental role of \( \text{Na}^+ \) binding in thrombin function. While Asp\(^{221}\) is the only variable residue, Arg\(^{221a}\) is replaced by a Lys in the hagfish (28). Comparison with analogous sequences in other serine proteases (Table I) indicates a high degree of homology, especially with factors IX and X and to a lesser extent with plasmin, PC, and factor VII. Homology is weaker between thrombin and pancreatic serine proteases like trypsin, chymotrypsin, and elastase. Hence, the architecture of the \( \text{Na}^+ \) binding site of thrombin and the allosteric regulation based on the slow ↔ fast equilibrium may be paradigmatic of a molecular strategy shared by other members of the blood coagulation cascade, like factors VII, IX, X, and PC, but not by pancreatic serine proteases. Proteases active in the blood might have evolved to exploit \( \text{Na}^+ \)-dependent allosteric transitions to accomplish their multifunctional roles, taking advantage of the availability of this monovalent cation in the extracellular environment.

The allosteric nature of thrombin makes it possible to control its function by interfering with \( \text{Na}^+ \) binding. A mutation involving the two Asp residues flanking Arg\(^{221a}\) (Table I) in the \( \text{Na}^+ \) binding loop was made to mimic the ARK sequence found in factor Xa, which has a reduced \( \text{Na}^+ \) affinity (\( K_D = 110 \text{ mM} \)) at 25 °C compared to thrombin (\( K_D = 22 \text{ mM} \) at 25 °C, see Ref. 3). The difference may be due to a reduction of the negative electrostatic potential at the \( \text{Na}^+ \) site due to the presence of Ala\(^{221}\) and Lys\(^{222}\), as opposed to Asp\(^{221}\) and Asp\(^{222}\) in thrombin. In the crystal structure (25, 29), Asp\(^{221}\) and Asp\(^{222}\) are ion-paired to Arg\(^{187}\) and make no contacts with synthetic substrates (25), hirudin (26), and most likely fibrinogen (30). This double mutant D221A/D222K (ARK) has lost the ability to bind \( \text{Na}^+ \) selectively (Fig. 3) and cleaves a synthetic substrate with nearly the same specificity in the presence of Li\(^+\), Na\(^+\), K\(^+\), or the bulky cation Ch\(^+\). The ARK mutant interacts with fibrinogen and hirudin with increased specificity in the slow form, as shown in Table II. The pathlength was 1 cm. The slow and fast forms of the wild-type enzyme show significant differences in the regions around 204 and 218 nm, that are not seen for the ARK mutant.

| Slow | Fast | r  |
|------|------|----|
| \( K_a \) values | \( k_{\text{up}}/K_{\text{mn}} \) values | \( k_{\text{up}}/K_{\text{mn}} \) values |
| Hirudin | FpA | PC | PC (+ TM) | FpA | PC | PC (+ TM) |
| 1.0 ± 0.1 pm | 5.3 ± 0.1 \( \mu \text{M}^{-1} \text{s}^{-1} \) | 53 ± 5 \( \text{mM}^{-1} \text{s}^{-1} \) | 242 ± 32 \( \text{mM}^{-1} \text{s}^{-1} \) | 4.4 ± 0.4 | 1.88 ± 0.04 | 2.00 ± 0.06 |

*Values are taken from Ref. 4.*
for the ARK mutant that behaves as an intermediate between the slow and fast forms of the wild type.

When PC interacts with the ARK mutant, the functional differences between the slow and fast forms are restored (Table II). Furthermore, the mutation has the noteworthy effect of slightly enhancing the catalytic properties of the slow form compared to the wild type. This result is also observed in the presence of TM. Protein C presumably makes favorable contacts with Lys222 of ARK, and this effect overwhelms the indirect effect of the mutation on the slow → fast equilibrium. In the absence of a direct effect, activation of PC by the ARK mutant would be decreased in the slow form and increased in the fast form compared to the wild type, consistent with the findings on S2238, fibrinogen, and hirudin. Binding of AT in the absence of heparin is perturbed exclusively as a result of the effect of the mutation on the slow → fast equilibrium (Table II). In the presence of heparin, however, binding of AT is decreased by more than 10 times. The contacts made by AT with thrombin are likely different when heparin is bound, as a result of heparin-induced conformational changes. Favorable contacts made by AT with Asp221 and Asp222 of the wild type in the presence of heparin may explain the loss of binding affinity in the ARK mutant. Hence, the Na⁺ binding loop of thrombin plays an important role in the direct recognition of PC and AT in the presence of heparin.

The architecture of the Na⁺ binding site of thrombin reveals important details on the molecular trigger for the slow → fast transition that contributes to the conversion of the enzyme from an anticoagulant to a procoagulant factor (4). The slow form has increased specificity for substrates like PC carrying an acidic residue at P3. Even in the absence of a structure of the slow form, the side chain of Arg221a, which is ion-paired to Glu146 in the fast form (25, 29), is oriented differently and makes a water mediated contact with the acidic side chain of the residue at P3 of substrates like the thrombin platelet receptor peptide (31, 32) and probably PC. The enhanced anticoagulant activity of the ARK mutant may well be a result of the D222K substitution, with the side chain of Lys222 mimicking or enhancing synergistically the role of Arg221a. Further mutagenesis analysis of the Na⁺ binding loop will reveal the exact origin of Na⁺ specificity and the trigger for the allosteric slow → fast transition.

REFERENCES
1. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, R. (1990) Blood 76, 1–16
2. Beck, W. S. (1991) Hematology, MIT Press, Cambridge, MA
3. Wells, C. M., and Di Cera, E. (1992) Biochemistry 31, 11721–11730
4. Dang, Q. D., Vindigni, A., and Di Cera, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5977–5981
5. Ayala, Y., and Di Cera, E. (1994) J. Mol. Biol. 235, 733–746
6. Ratnoff, O. D., and Forbes, C. D. (1991) Disorders of Hemostasis, W. B. Saunders, Philadelphia
7. Evans, H. J., and Sorg, G. J. (1966) Annu. Rev. Plant Physiol. 17, 47–76
8. Suelter, C. H. (1970) Science 168, 789–795
9. Orthner, C. L., and Kosow, D. P. (1978) Arch. Biochem. Biophys. 185, 400–406
10. Orthner, C. L., and Kosow, D. P. (1980) Arch. Biochem. Biophys. 202, 63–75
11. Steiner, S. A., Amphlett, G. N., and Castellino, F. J. (1980) Biochim. Biophys. Res. Commun. 94, 340–347
12. Toney, M. D., Hohenester, E., Cowan, S. W., and Janssens, J. N. (1993) Science 261, 756–759
13. Larsen, T. M., Laughlin, L. T., Holden, H. M., Rayment, I., and Reed, G. H. (1994) Biochemistry 33, 6301–6309
14. Wilbanks, S. M., and McKay, D. B. (1995) J. Biol. Chem. 270, 2251–2257
15. Stubbs, M. T., and Bode, W. (1993) Thrombosis Res. 69, 1–8
16. SKRZYPKCI-JANKUN, E., RYDEL, T. J., TULINSKY, A., FENTON, J. W., and MAN, K. G. (1989) J. Mol. Biol. 206, 755–757
17. Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) EMBO J. 8, 3467–3475
18. Lebioda, L., and Zhang, E. (1992) J. Appl. Crystallogr. 25, 323–324
19. Le Bonnefie, B. F., MacGillivray, R. T. A., and Esmann, C. T. (1991) J. Biol. Chem. 266, 13796–13803
20. Le Bonnefie, B. F., Guinta, E. R., and Esmann, C. T. (1992) J. Biol. Chem. 267, 19341–19348
21. Dang, Q. D., and Di Cera, E. (1994) J. Protein Chem. 13, 367–373
22. Olson, S. T., Bjork, I., and Shore, J. D. (1993) Methods Enzymol. 222, 525–559
23. Barshop, B. A., Wrenn, R. F., and Frieden, C. (1989) Anal. Biochem. 159, 1–14
24. Zimmerle, C. T., and Frieden, C. (1989) Biochem. J. 258, 381–387
25. Bode, W., Turk, D., and Karshikov, A. (1992) Protein Sci. 1, 426–471
26. Rydel, T. J., Tulinsky, A., Bode, W., and Huber, R. (1991) J. Mol. Biol. 221, 583–601
27. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
28. Barfield, D. K., and MacGillivray, R. T. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2779–2783
29. SKRZYPKCI-JANKUN, E., Carperos, V. E., Ravi Chandran, K. G., Tulinsky, A., Westbrook, M., and Maraganore, J. M. (1991) J. Mol. Biol. 221, 1379–1393
30. Stubbs, M. T., Oschinat, H., Mayr, I., Huber, R., Angliker, H., Stone, S. R., and Bode, W. (1992) Eur. J. Biochem. 206, 187–196
31. Matthews, I. F., Padmanabhan, K. P., Ganesh, V., Tulinsky, A., Ishii, M., Chen, J., Turk, C. W., and Coughlin, S. R. (1994) Biochemistry 33, 3266–3279
32. Ni, F., Ripoll, D. R., Martin, P. D., and Edwards, B. F. P. (1992) Biochemistry 31, 11551–11557