Tracking Structural Features Leading to Resistance of Activated Protein C to α1-antitrypsin[†]

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Received October 11, 1999; Revised Manuscript Received November 23, 1999

ABSTRACT: Activated protein C (APC) is a multi-modular anticoagulant serine protease, which degrades factor V/Va and factor VIIIa. Human APC (hAPC) is inhibited by human α1-antitrypsin (AAT), while the bovine enzyme (bAPC) is fully resistant to this serpin. Structural features in the catalytic domains between the two species cause this difference, but detailed knowledge about the causal molecular difference is missing. To gain insight into the APC−AAT interaction and to create a human protein C resistant to AAT inhibition, we have used molecular modeling and site-directed mutagenesis. First, a structural model for bAPC based on the Gla-domainless X-ray structure of hAPC was built. Screening the molecular surface of the human and bovine APC enzymes suggested that a hAPC molecule resistant to AAT inhibition could be constructed by substituting only a few amino acids. We thus produced recombinant hAPC molecules with a single mutation (S173E, the numbering follows the chymotrypsinogen nomenclature), two mutations (E60aS/S61R) or a combination of all these substitutions (E60aS/S61R/S173E). Amidolytic and anticoagulant activities of the three mutant APC molecules were similar to those of wild-type hAPC. Inhibition of wild-type hAPC by AAT was characterized by a second-order rate constant (k_2) of 2.71 M^{-1} s^{-1}. The amino acid substitution at position 173 (S173E mutant) led to partial resistance to AAT (k_2 = 0.84 M^{-1} s^{-1}). The E60aS/S61R mutant displayed mild resistance to AAT inhibition (k_2 = 1.70 M^{-1} s^{-1}), whereas the E60aS/S61R/S173E mutant was inefficiently inactivated by AAT (k_2 = 0.40 M^{-1} s^{-1}). Inhibition of recombinant APC molecules by the serpin protein C inhibitor (PCI) in the presence and absence of heparin was also investigated.

Protein C (PC)[1] is an anticoagulant vitamin K-dependent plasma serine protease zymogen, which is activated after limited proteolysis by the thrombin−thrombomodulin (T−TM) complex on the lumenal surface of endothelial cells. Activated PC (APC) functions as an anticoagulant by selectively inactivating factor V/Va (FV/FVa) and factor VIIIa (FVIIIa) [reviewed in *1*−*7*]. During these reactions, the activity of APC is potentiated by the presence of a nonenzymatic multi-modular vitamin K-dependent cofactor named protein S. Because of its unique anticoagulant properties, protein C is a potential therapeutic compound that has been shown to be efficient in model as well as human thrombotic diseases (*8*−*13*).

The domain composition and organization of PC/APC resembles that of several other blood coagulation serine proteases, including factors X, IX, or VII. The light chain consists of a γ-carboxy glutamic acid (Gla) containing module (essential for membrane binding) and two epidermal growth factor (EGF)-like modules, while the heavy chain consists of a trypsin-like serine protease (SP) domain. Recently, the three-dimensional structure of Gla-domainless human APC (hAPC) was determined by X-ray crystallography (*14*), thus facilitating structure−function studies of this important enzyme. The serine protease domain of APC was found to be structurally very similar to those of other serine proteases, with the exception of some surface-exposed loops (e.g., a long 148-loop as compared to trypsin).

Two serpin molecules, α1-antitrypsin (AAT) and protein C inhibitor (PCI), are the main inhibitors of APC. AAT is present at very high concentration in plasma (2.5 mg/mL), whereas PCI circulates at much lower level (5 μg/mL). Since PC circulates at a concentration of about 4 μg/mL, it is clear that AAT is a major physiologic inhibitor of APC. Inhibition of APC by PCI is stimulated by heparin, whereas inhibition by AAT is heparin-independent (*15*−*17*). It has been shown that bovine APC (bAPC) is nearly completely resistant to inactivation by human AAT and that the structural determinants for this species difference reside within the serine protease domain (*18*). Comparison of hAPC and bAPC amino acid sequences suggests that the two proteins should have very similar three-dimensional structures as they share about 80% sequence identity. On the sole basis of amino

[†] This work was supported by grants from the Swedish Medical Council, National Network for Cardiovascular Research and research funds from Malmö University Hospital.

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[1] Abbreviations: APC, activated protein C; PC, protein C; PCI, protein C inhibitor; AAT, α1-antitrypsin, also called α1-proteinase inhibitor; T, thrombin; TM, thrombomodulin; serine protease domain, SP domain. The chymotrypsinogen nomenclature for APC is used in the text. P1, P2... and P1′, P2′.. designate inhibitor residues amino- and carboxy-terminal to the scissile peptide bond, respectively, and S1, S2.. and S1′, S2′.. the corresponding subsites of the protease.

10.1021/bi992357p CCC: $19.00
Published on Web 02/24/2000

acid comparison, an interesting difference between hAPC and bAPC involves the 148-loop (chymotrypsinogen nomenclature described in ref *14*) as the corresponding segment in the bovine species is four amino acids shorter than that of the human protein. Recently, we probed this segment via interspecies loop grafting and found that it is not responsible for the resistance of bAPC to inhibition by AAT (*19*). Besides the difference in AAT inhibition between hAPC and bAPC, it is also known that the interaction between APC and protein S demonstrates certain species specificity. Thus, the anticoagulant activity of bAPC is potentiated by bovine, but not by human protein S. In contrast, hAPC functions with protein S from both species (*20*−*22*). Most likely, the light chain of APC plays a critical role in the interaction with protein S and we expect that the molecular surface of the hAPC light chain differs from that of the bovine enzyme.

In this study, we have investigated which structural features could lead to the different inactivation of hAPC as compared to bAPC by human AAT. We first built a 3D model for bAPC using the X-ray structure of the human enzyme as an initial template. The molecular surfaces of the two proteins were scanned, searching for key amino acid difference within the active site area. This search also took into account the results of recently reported mutagenesis studies, which indicated that APC residues K37−K38−K39 and K62−K63 play important role in both heparin binding and interaction with PCI (*23*, *24*). Computer comparisons of the hAPC and bAPC serine protease domains enabled identification of a few residue differences between the two enzymes. The molecular modeling data were then used to direct mutagenesis experiments. Our goal was to design a limited number of mutant APC molecules carrying amino acid substitution(s) in the area of the active site cleft, but in regions that would likely not damaged the sensitive catalytic machinery (i.e., amino acids not in direct contact with the catalytic triad). Structural analysis suggested that hAPC S173 could be replaced by a glutamic acid as observed in bAPC (mutant S173E). Human APC residues E60a and S61 could be changed by two residues present in the bovine enzyme, S and R, respectively (mutant E60aS/S61R). A mutant APC combining these mutations was also constructed. The hAPC mutant containing three mutations (E60aS/S61R/S173E) was not inhibited efficiently by AAT. Inhibition of recombinant APC molecules by PCI in the presence and absence of heparin was also investigated.

MATERIALS AND METHODS

Reagents. Highly purified human AAT and PCI were kindly provided by Dr. Carl-Bertill Laurell (Department of Clinical Chemistry, University Hospital, Malmö, Sweden) and Dr. Bonno N. Bouma (Department of Haematology, University of Utrecht, Netherlands), respectively. Sulfopropyl (SP)-sepharose was purchased from Pharmacia (Sweden). Unfractionated Heparin was from Lövens (Denmark). The synthetic substrate Spectrozyme PCa was from American Diagnostica Inc. (Greenwich, CT). The APTT reagent Platelin LS was from Organon Teknika (Belgium).

Modeling Gla-domainless Bovine APC. The X-ray structure of the Gla-domainless human APC (*14*) [2.8 Å resolution, Protein Data Bank (*25*), entry 1aut]) was used as framework to derive a model for bAPC. Conservative side-chain replacements were modeled in conformations similar to the ones present in the human APC structure. Other residue replacements were modeled using hAPC as initial template, but optimized, if needed, using low-energy rotamer conformations (*26*). The four residues deletion within the loop 148 of bAPC with respect to hAPC was built by first removing the appropriate residues from the human loop and second, by energy minimization using Discover (Biosym-MSI). A calcium ion was added interactively at the level of the loop 70 and a sodium ion at the level of the loop 220, according to previously reported experimental data (*5*, *27*, *28*). The human and bovine APC structures were then briefly energy minimized.

Screening the Molecular Surface of Human and Bovine APC Molecules. We first color-coded the side chains and backbone atoms of both molecules using the following criteria: positively charged residues (R, K, H), blue; negatively charged (D, E), red; polar and backbone atoms (white); hydrophobic/aromatic (remaining residues), yellow. Computation of the solvent accessible surface was then carried out within Insight II (Biosym-MSI). Second, the electrostatic potential of bovine and human APC molecules as computed with DelPhi (*29*, *30*) was mapped onto the solvent exposed surface. A standard set of formal charges was assigned to the titratable residues (e.g., Arg, +1e; Lys, +1e; His, +0.5e; Asp, −1e; Glu, −1e), with calcium, +2e; and sodium, +1e.

Primers for Protein C Mutagenesis. The following primers were used: A, 5′-AAA TTA ATA CGA CTC ACT ATA GGG AGA CCC AAG CTT-3′ (corresponding to sense of nucleotides 860−895 in the vector pRc/CMV, including the *Hind*III cloning site); B, 5′-GCA TTT AGG TGA CAC TAT AGA ATA GGG CCC TCT AGA-3′ (antisense to nucleotides 984−1019 in the vector pRc/CMV including the *Xba*I cloning site); C, 5′-GCC CAC TGC ATG GAC *AGC CGC* AAG AAG CTC CTT GTC-3′ (corresponding to sense strand encoding amino acids 56−66 in the human protein C serine protease domain, with the E60aS/S61R mutation in italics) D, 5′-GAC AAG GAG CTT CTT *GCG GCT* GTC CAT GCA GTG GGC-3′ (complementary to the primer C); E, 5′-TGC AGC GAG GTC ATG *GAA* AAC ATG GTG TCT GAG-3′ (corresponding to sense of amino acids 168−178 in human protein C serine protease domain with the S173E mutation in italics); F, 5′-CTC AGA CAC CAT GTT *TTC* CAT GAC CTC GCT GCA-3′ (complementary to the primer E).

In Vitro Mutagenesis, Expression, and Purification of Recombinant Protein C Molecules. Human protein C mutants were created using the polymerase chain reaction (PCR) mutagenesis method as described previously (*31*). To obtain mutant E60aS/S61R, full-length human protein C cDNA was cloned in the pRc/CMV vector using *Hind*III and *Xba*I sites. PCR amplification and mutagenesis of the target DNA was performed by the following steps: primers A and D were used to amplify the 5′ part of protein C (up to codon for amino acid at position 66 of the serine protease domain), whereas primers B and C were used to amplify the 3′ part (from the amino acid 56 codon in the serine protease domain to the end of protein C cDNA). These two fragments were then used as templates to amplify the full-length E60aS/S61R mutant protein C cDNA with primers A and B. The cDNA containing the two mutations was cleaved by *Sac*II and *Fse*I,

and the *Sac*II and *Fse*I fragments replaced corresponding fragment in wild-type protein C by ligation of the fragment to *Sac*II and *Fse*I cleaved protein C cDNA in pUC18. The protein C cDNA mutants were recovered from pUC18 after *Hind*III and *Xba*I cleavage and cloned into pRc/CMV vector for expression as described previously (*31*). Human protein C containing S173E was mutated in a similar way, but with primers E and F instead of primers C and D, and the restriction enzyme used were *Fse*I and *Apa*I instead of *Sac*II and *Fse*I. To obtain human protein C E60aS/S61R/S173E, the PCR fragment E60aS/S61R (*Sac*II−*Fse*I) was used to replace the corresponding section in the human protein C S173E mutant. All mutations were confirmed by DNA sequencing before transfection. Protein C expression in human 293 cells, purification, quantification, and activation by thrombin were performed as described (*31*).

Hydrolysis of Small Synthetic Substrates. Hydrolysis of substrates Spectrozyme PCa, by the wild-type and mutant APC, was performed at room temperature in TBS buffer containing 2 mM Ca^{2+} and 1 mg/mL BSA. The concentration of chromogenic substrates ranged from 0 to 2 mM. The APC concentration was 10 nM. The K_m and k_{cat} values for substrate hydrolysis were calculated using the Michaelis−Menten equation.

Activated Partial Thromboplastin Time (APTT) Assay. The anticoagulant activities of wild-type and mutant APC molecules were tested in an APTT assay using Platelin LS (Organon Teknika) as APTT reagent. In this assay, 50 μL of human citrated normal plasma was incubated with 50 μL APTT reagent at 37 °C. After 200 s, 100 μL of $CaCl_2$ (12.5 mM) containing APC (final concentrations in assay of 0−40 nM) were added. The clotting time was measured using an Amelung-Coagulometer KC 10 (Swedish Labex AB). All dilutions were made in TBS buffer in the presence of 1 mg/mL BSA. In the comparison of the anticoagulant activity of the various APC forms, the APC concentrations were adjusted using their amidolytic activity against Spectrozyme PCa.

Inactivation of APC Molecules in Human Plasma. Wild-type and mutant APC (final concentration of 70 nM) were added to human citrated plasma and the mixtures were incubated at 37 °C from 0 to 60 min and then diluted 5-fold in TBS buffer. One hundred microliters of the diluted samples were added to 50 μL of Spectrozyme PCa (1 mM) in a microplate. The rate of hydrolysis of PCa by APC was recorded for 10 min at 405 nm in a Vmax kinetic microplate reader (Victor VPC IIe, U.S.A.).

Inactivation of APC Molecules by AAT. To investigate the inhibition of APC by AAT, the procedure described by Holly and Foster was followed after slight modifications (*18*). Wild-type or mutant APC molecules (final concentration of 170 nM) were incubated overnight with 0−16 μM human AAT in TBS buffer containing 0.1% BSA at 37 °C. A 20 μL sample of the incubation mixtures was added to 100 μL of the synthetic substrate Spectrozyme PCa (1 mM) in a microplate, and the rate of hydrolysis was monitored at 405 nm. In another set of experiments, the kinetic analysis of APC inhibition by AAT was performed under pseudo-first-order rate conditions. APC variants (170 nM) were incubated with human AAT (16 μM) at room temperature in 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM $CaCl_2$, containing 0.1% PEG 8000 for a period of time (0−16 h). Spectrozyme PCa was added to a final concentration of 0.2 mM and the rate of chromogenic substrate hydrolysis was measured with a Vmax kinetic microplate reader. Second-order rate constants (k_2) were calculated as $-\ln a/t$ [I] where a is the fractional proteinase activity remaining relative to the uninhibited control, t is the time of incubation, and [I] is the AAT concentration. All inhibition assays were performed by the time course analysis to ensure that steady-state kinetics were at hand and that at least 50% APC inhibition was achieved in values use to calculate the kinetic parameters.

To exclude the possibility that AAT functioned as a substrate for bAPC, AAT (50 μg/mL final concentration) was incubated with human or bovine APC (10 μg/mL final concentration) in TBS up to 24 h at room temperature. Aliquots of the incubation mixture were drawn at intervals and then applied to 10% SDS-PAGE run under nonreducing conditions. Parallel gels were stained with silver or analyzed by Western blotting using antibodies against human or bovine APC and AAT.

Inactivation of APC Molecules by PCI. The rate of inhibition of wild-type and APC mutants in the absence of heparin was measured under pseudo-first-order rate conditions, as reported in a previous study (*24*). In brief, APC variants were incubated between 0 and 60 min with a 20-fold molar excess of PCI at room temperature in 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM $CaCl_2$ containing 0.1% PEG 8000. Spectrozyme PCa was added to a final concentration of 0.2 mM and the rate of chromogenic substrate hydrolysis was measured with a Vmax kinetic microplate reader. Second-order rate constants (k_2) were calculated as $-\ln a/t$ [I]. The rate of inactivation of APC in the presence of heparin was too fast to be measured under the conditions described above. Instead, PCI (25−50 nM final concentration) was mixed with APC (16 nM) and heparin (5 IU/mL) in a final volume of 50 μL. After 2 min incubation, 50 μL Spectrozyme PCa (final concentration was 0.2 mM) was added and the color development immediately measured with Vmax kinetic at 405 nm. The following equation was used to calculate the k_2 value:

$$k_2 t = \frac{1}{([A]_0 - [B]_0)} \ln \frac{[B]_0 ([A]_0 - x)}{[A]_0 ([B]_0 - x)}$$

where $[A]_0$ and $[B]_0$ were the initial concentrations of APC and PCI, and x was the molar concentration of APC−PCI complexes formed after time t; k_2 was calculated from data points demonstrating 30−70% proteinase inhibition. As the color development was linear in time, it was concluded that ongoing inhibition of APC during the measuring phase was not a practical problem. All experiments were performed in triplicate.

Elution of APC Molecules from Heparin-Sepharose. The interaction between APC (wild-type or mutants) and heparin was evaluated using a 2 mL heparin-sepharose column as reported previously (*24*). A 200 μL sample of a 200 nM APC (wild-type or mutant) was applied to the heparin column, and the bound APC was eluted by a NaCl gradient (20 mM Tris-HCl, 50−500 mM NaCl, 0.1% gelatine, pH 7.4). The column was run at 36 mL/hour with a collection time for each fraction of 20 s (200 μL) in a Waters 625 LC system. Fifty microliters of each fraction were transferred to a microplate, and 50 μL of Spectrozyme PCa (final

concentration was 0.2 mM) was added to each APC fraction. After 10 min incubation at room temperature, 50 μL of 20% acetic acid was added to stop the reaction. The amidolytic activity of APC was measured at 405 nm at room temperature. The NaCl concentrations in the fractions were measured in the local hospital routine clinical chemistry laboratory.

RESULTS AND DISCUSSION

Protein C as a Potential Therapeutic Compound and the Overall Rational of the Study. The specific anticoagulant properties of human APC makes it attractive as therapeutic compound in the treatment of disseminated intravascular coagulation, septic shock, or other thromboembolic conditions. Indeed, protein C infusions have already been used successfully in case of protein C deficiency and in the treatment of Meningococcemia (*8*, *9*, *11*, *12*). However, the presence of AAT in plasma impairs the use of APC as it inhibits this enzyme efficiently. Thus, molecular engineering of hAPC to eradicate its inactivation by AAT without inducing major decreases in enzyme activity should lead to a molecule with better anticoagulant properties as such modifications should reduce the required therapeutic dosages (*18*). The other APC inhibitor, PCI, may not be a significant obstacle for the use of APC in clinical settings (*18*). Yet, designing a recombinant APC molecule resistant to both serpins would certainly be valuable.

It is known that hAPC and bAPC exhibit several functional differences (*18*, *19*, *22*, *24*). Indeed, bovine and human APC molecules do not respond similarly to AAT as the bovine enzyme is fully resistant to inhibition by this serpin. To understand this difference, we first built a structural model for bAPC by comparative model building and then compared the molecular surfaces of the human and bovine enzymes. We expected that such structural analysis would guide our site-directed mutagenesis experiments as one of the goal of the present study was to create a human APC molecule partially or fully resistant to AAT.

Screening the Molecular Surfaces of the Human and Bovine APC Molecules. The lack of large insertions/deletions and the high degree of sequence identity (≈73% entire chain, 76% catalytic domain) between human and bovine APC molecules allow for accurate structural predictions of bAPC by homology modeling strategy. No obvious structural differences were observed in the bovine model as compared to the human structure, except for the loop 148 area, which is four residues shorter in the bovine enzyme (Figure 1). To identify key differences between human and bovine APC molecules and to rationally design mutations, we compared the molecular surfaces of the two proteins (Figure 2A and 2B). To simplify the screening, the serine protease domain was arbitrarily divided into two shells (Figure 1). The inner shell surrounds directly the catalytic triad, while the outer shell involves residues that where further away (Figure 1).

Within the inner shell (when looking down the active site as shown in Figure 1), only minor amino acid substitutions can be noticed when comparing hAPC and bAPC. These involve for instance A41 in hAPC, which is V in the bovine enzyme and T99 in hAPC, which is S in the bovine protein. Such changes were not considered significant enough to result in resistance of bAPC to inhibition by AAT. Within the second shell, only a few key changes were observed. For instance, M175 in hAPC is K in the bovine protein, hAPC has S at position 173, while the bovine enzyme has E. Human APC displays A at position 55, while the bovine enzyme has V, but this substitution can be considered as minor. In addition, hAPC has E at position 60a and S at position 61, while the bovine enzyme has S and R, respectively, at equivalent positions. L221a in hAPC is replaced by R in the bovine protein. Furthermore, the human loop 148 (including at least residues WGYHSSREKEA) is four residues longer than its bovine counterpart (WGY- - - - RDET).

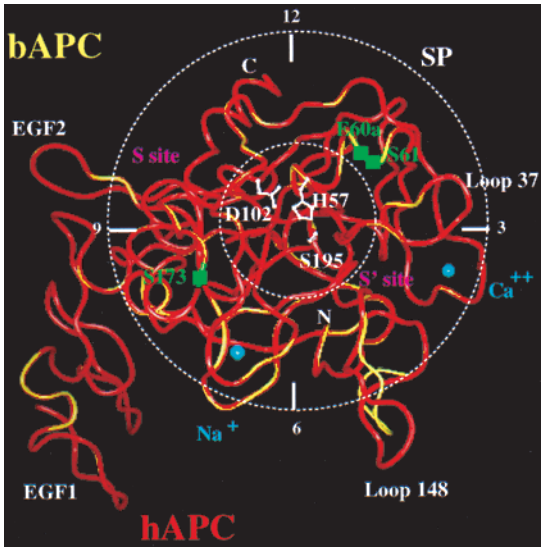

FIGURE 1: Human and bovine Gla-domainless APC. The X-ray structure of hAPC is shown in ribbon drawing with a view down the active site (red). The model structure for bAPC (yellow) has been superimposed onto the human protein. Residues belonging to the active site triad are colored white (S195, H57, D102). The protein C residues that are replaced by site-directed mutagenesis, in the present study, are highlighted in green. Some loops of functional importance are labeled for orientation. The blue spheres represent a sodium ion and a calcium ion.

When comparing the electrostatic potential of hAPC and bAPC in the overall region of the serine protease domain, the most striking difference was the environment of the catalytic triad, which is more electronegative in hAPC than in bAPC (Figure 2B). Also, the region running from 3 o'clock to about 6 o'clock is more electropositive in hAPC than in the bovine protein. These differences are essentially due to amino acid substitutions within the outer shell described above. Interestingly, one region that is involved in heparin binding (comprising at least K37−K38−K39 and K62−K63 but not K86) (*23*, *24*) in hAPC is essentially conserved in the bovine enzyme (Figure 2A and 2B).

Our principal aim was to create a human APC molecule resistant to AAT inhibition. The above structural analysis of the active site cleft area and more specifically of the outer shell helped us in the selection of residues to substitute by mutagenesis. This selection was also guided by previously reported experimental data. First, it is known that the serine protease domain is responsible for resistance of bAPC to AAT (*18*). Second, we have shown that replacing the loop 148 of hAPC by its bovine counterpart results in a human mutant enzyme that was inhibited by AAT in very similar

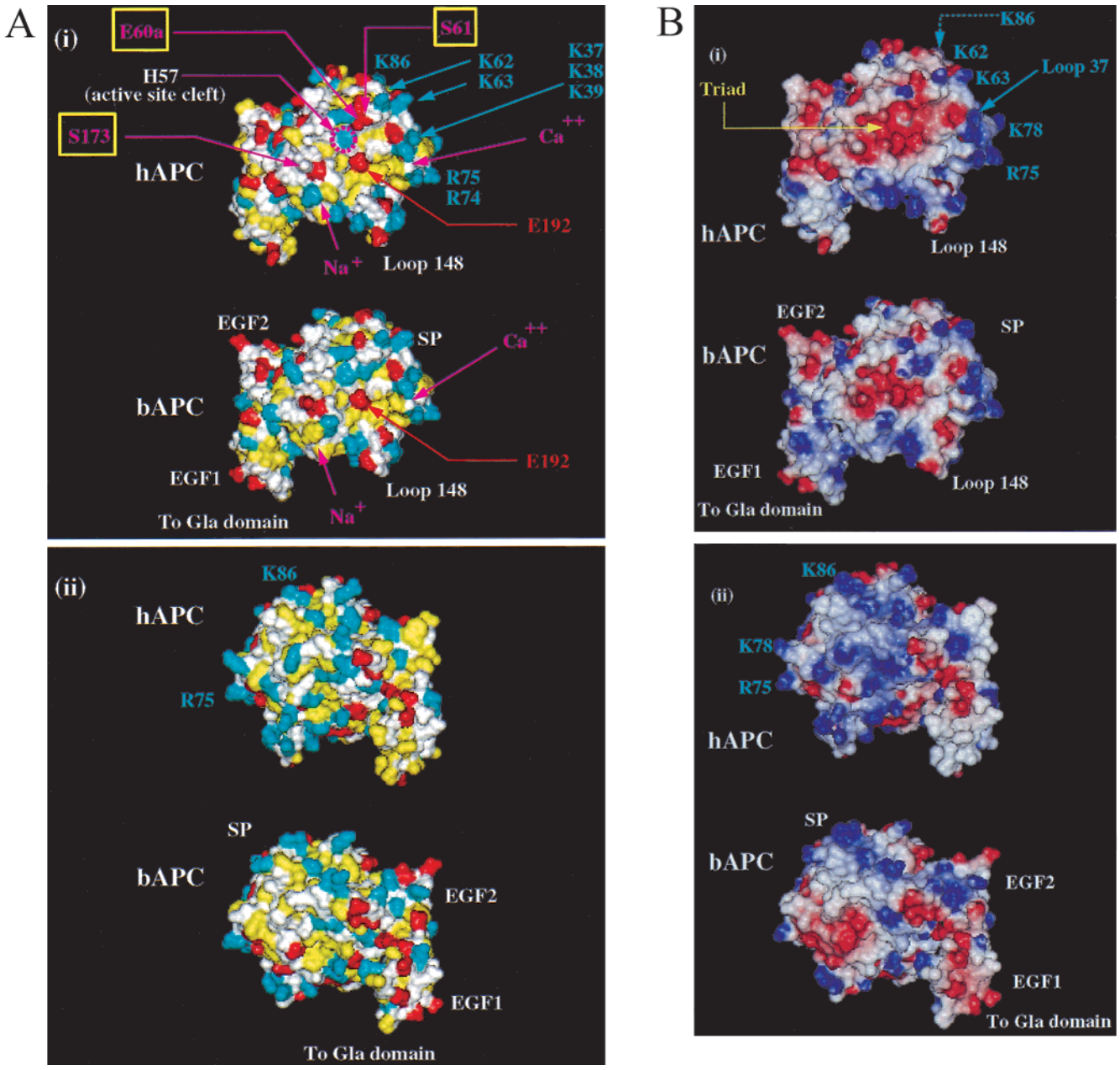

FIGURE 2: (A) Solvent accessible solid surface for hAPC and bAPC. The backbone atoms and glycine residues and the side chains of polar residues (N, Q, T, S) are colored in white, side chains of positively charged amino acids (K, R, H) are in blue, negatively charged (E, D) are in red and hydrophobic/aromatic residues (A, V, I, L, M, F, Y, W, P, C) in yellow. Top figure (i), the orientation is similar to the one presented in Figure 1. Bottom panel (ii), the molecule has been rotated 180 degrees in order to see the opposite face. Some residues are labeled for orientation. A few differences are noted in the area of the catalytic triad and residues of interest are discussed in the text. Some key differences between hAPC and bAPC which could be important for the interaction with cofactors or macromolecular substrates are also noted at the level of the light chain. For example, hAPC displays patches of hydrophobic residues at the level of its light chain, and it is known that such clusters often play a role in protein−protein interaction (*37*). (B) Electrostatic potential. The solvent-accessible molecular surfaces of hAPC and bAPC are shown in an orientation similar to one used in Figure 2A (i, view down the active site; ii, opposite face). The surfaces are now color-coded according to electrostatic surface potential; red, −3 kcal/mol/e; white, 0 kcal/mol/e; blue +3 kcal/mol/e. The positions of some charged side chains are labeled for orientation. When looking down the active site cleft, hAPC is more electronegative around the catalytic triad than bAPC. On the side opposite to the active site cleft and on both faces of the light chain, some interesting differences are noted when comparing hAPC and bAPC. For instance, bovine EGF1 is more electropositive than human EGF1 on one side of the molecule (side i) while on the other face (side ii), bovine EGF1 tends to be more electronegative than the hAPC EGF1 module, this latter being more neutral and displaying several hydrophobic side chains (Figure 2A). Also, the side opposite to the catalytic cleft (at the surface of the serine protease domain) is more electropositive in hAPC as compared to the bovine enzyme (side ii).

fashion than wt-hAPC. Third, it has been reported that PCI and heparin contact at least hAPC residues K37−K38−K39 and K62−K63 during the inhibition of APC by PCI (*23*, *24*). Collectively, these observations led us to create three hAPC mutants. The first mutant contained the S173E substitution as this residue could be well located to interact with the AAT reactive loop, while being at the same time at some distance away from the catalytic triad. Human APC residues E60a and S61 were changed to S and R (present in the bovine enzyme), resulting in the creation of a double mutant E60aS/S61R. This choice was supported by the fact that these residues are not in direct contact with the triad,

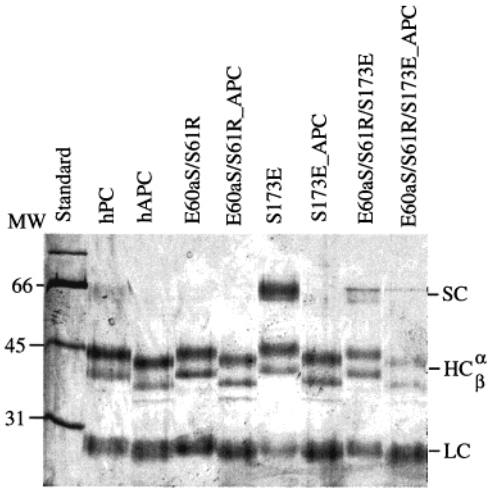

FIGURE 3: SDS-PAGE analysis of recombinant protein C molecules. The three recombinant protein C variants and wt- molecules were applied to 10% SDS-PAGE gel (1 μg in each lane) before and after activation by thrombin and run under reducing conditions. The proteins were visualized with silver staining. Each lane indicates the protein content. Thrombin activated APC molecules are denoted _APC at the end of the label, while the corresponding zymogen forms have no suffix added. SC stands for single chain, HC α and β denote the two heavy chain forms, and LC is the light chain.

but within an exosite that we have shown of importance for the interaction between APC and PCI. These substitutions would thus probe the 2 o'clock and 8 o'clock areas of hAPC. A mutant APC combining the three mutations was also constructed (mutant E60aS/S61R/S173E) with the hope that the multiple substitutions would have cumulative effects.

Expression, Purification, and Characterization of Anticoagulant and Catalytic Activities of Recombinant APC Molecules. The mutant recombinant protein C molecules migrated as single bands on SDS-PAGE under nonreducing conditions, to positions similar to those of the wt-protein C and the plasma-derived protein C (not shown). Under reducing conditions, the heavy chain (HC) of human recombinant protein C variants migrated as double bands corresponding to the α- and β-forms of protein C, which differ in the degree of glycosylation (Figure 3). In all the recombinant protein C variants, the single chain (SC) form of protein C was to some extent present. For unknown reasons, the S173E mutant contained a high level of the single chain form. After activation by thrombin, the migration of the heavy chains shifted to slightly more anodal positions and the single chain forms disappeared. The pattern on SDS-PAGE was consistent with full activation of the recombinant protein C variants.

The chromogenic substrate Spectrozyme PCa was used to measure the amidolytic activity of wt-hAPC and the various mutants. Mutant E60aS/S61R and wt-hAPC manifested similar K_m values, whereas S173E had a lower K_m as compared to wt-hAPC (Table 1). This suggests that a glutamate residue at position 173 improves directly or indirectly the fitting of this small substrate into the catalytic cleft, while residues at positions 60a and 61 had no effect on binding. The k_{cat} values observed for the mutants were slightly lower than the one of wt-hAPC, while the specificity constants k_{cat}/K_m for wt- and mutant hAPC molecules were similar. In an APTT-based clotting assay, all APC mutants were found to express anticoagulant activities, which within

Table 1: Steady-State Kinetics of Spectrozyme PCa Hydrolysis by hAPC and Mutants

enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
wt-hAPC	0.24 ± 0.82	42.65 ± 0.54	178
S173E	0.15 ± 0.01	30.48 ± 0.46	203
E60aS/S61R	0.25 ± 0.01	37.46 ± 0.74	150
E60aS/S61R/S173E	0.17 ± 0.01	31.65 ± 0.62	186

Table 2: Second-Order Association Rate Constants for AAT Inhibition of Wt- and Mutant APC

enzyme	k_2(M^{-1} s^{-1})	fold acceleration (APC derivatives/hAPC)
wt-hAPC	2.71 ± 0.03	
S173E	0.84 ± 0.05	0.31
E60aS/S61R	1.70 ± 0.03	0.68
E60aS/S61R/S173E	0.40 ± 0.03	0.15
wt-bAPC	-0.10 ± 0.03	0.00

experimental errors, were indistinguishable from that of wt-hAPC (data not shown). Taken together, these data suggest that the introduced mutations did not damage the catalytic machinery nor the anticoagulant activity of the enzymes.

Inactivation of APC Mutants in Human Plasma and by Purified AAT. The rate of inhibition of wt- and mutant APC molecules in human plasma was investigated after addition of the different APC variants. The inhibition curves were found to be similar for all constructs tested and $t_{1/2}$ was found to be around 60 min (data not shown). In human plasma, not only the serpins PCI and AAT have the potential to inactivate APC but also the nonserpin molecular trap α_2-macroglobulin can play a role in this process. We did not investigate the relative distribution of the wt- and mutant APC variants between the different inhibitors in plasma, but rather investigated directly the inhibition by purified AAT.

Human APC and the three APC mutants were all inhibited by AAT, but at different rates, whereas bAPC was completely resistant to inhibition by AAT. The kinetics analysis is reported in Table 2. The second rate order constant for inhibition of wt-hAPC by AAT (k_2) was 2.71 M^{-1} s^{-1}. The S173E substitution was characterized by a k_2 of 0.84 M^{-1} s^{-1}, suggesting a 69% reduction in the rate of AAT inhibition. The E60aS/S61R mutant was also inhibited by AAT at lower rate than wt-hAPC ($k_2 = 1.70$ M^{-1} s^{-1}), but still at higher rate than the S173E mutant. The E60aS/S61R/S173E mutant was quite inefficiently inactivated by AAT ($k_2 = 0.40$ M^{-1} s^{-1}), although it was not as resistant as bAPC.

During the serpin−serine protease interaction, the reactive loop of the serpin inserts into the active site cleft of the protease, thereby forming the initial noncovalent Michaelis-like complex. In most situations, the reactive loop is cleaved after the P1 residue, and a covalent acyl enzyme intermediate is formed. Subsequent insertion of the reactive loop into the β-sheet A is associated with concomitant translocation of the enzyme, possibly up to the opposite end of the serpin as compared to its location within the initial Michaelis-like complex (*32*). To analyze further the above data, we found it necessary to investigate whether AAT could function as a substrate for bAPC, which could be a potential explanation for the apparent resistance of the enzyme to this serpin. Formation of APC−AAT complexes or AAT degradation products was investigated by Western blotting under nonreducing condition and as a function of time (from 0 to 24

Table 3: Second-Order Association Rate Constants for PCI Inhibition of Wt- and Mutant APC in the Absence and Presence of Heparin

enzyme	k_2($\times 10^3$M^{-1} s^{-1}) (−heparin)	k_2($\times 10^5$M^{-1} s^{-1}) (+heparin)	fold acceleration
wt-hAPC	0.74 ± 0.05	2.31 ± 0.19	312
S173E	0.95 ± 0.08	3.20 ± 0.18	337
E60aS/S61R	1.32 ± 0.36	6.93 ± 0.33	525
E60aS/S61R/S173E	1.65 ± 0.45	9.28 ± 0.48	562
wt-bAPC	1.03 ± 0.07	2.26 ± 0.13	219

h). Human and bAPC and AAT were detected using appropriate polyclonal antibodies (data not shown). Complex formation was observed between hAPC and AAT, but no cleaved AAT molecules were found during this period of time. No complexes between bAPC and AAT were detected and no AAT degradation products were observed, indicating that proper formation of the Michaelis-like complex does not take place between AAT and the bovine enzyme. Therefore, as expected, a few residues within the serine protease domain of bAPC are responsible for repulsion of AAT.

At this stage of our investigation, we could then attempt to correlate the structural and functional data. Human APC amino acid residues at positions 60a, 61, and 173 interact favorably with AAT during the formation of the initial Michaelis-like complex. When these residues are replaced by their bovine counterparts, they impair appropriate interaction between the two molecules. Detailed atomic explanation for this observation would require high-resolution X-ray structure of the Michaelis complex, while, at least in the case of the S173E substitution, after observing the X-ray structure of uncleaved AAT (33) (PDB entry 1psi), we suggest possible repulsion between E173 and the reactive loop P5 (E354) residue of AAT. Alternative candidates for impaired interaction with APC E173 could be AAT D202 or E199.

Inactivation of APC Variants by Human PCI and Increased Affinity of APC E60aN/S61R for Heparin. In the absence of heparin, hAPC as compared to bAPC is inhibited slightly less efficiently by PCI (Table 3). Overall, PCI inhibits bAPC and hAPC relatively inefficiently in the absence of heparin. Mutant S173E was inhibited slightly more efficiently by PCI as compared to wt-hAPC. Likewise, the mutant E60aS/S61R was inhibited about 2-fold more efficiently than wt-hAPC as the second-order rate constants were 1.32×10^3 M^{-1} s^{-1} and 0.74×10^3 M^{-1} s^{-1}, respectively. The triple mutant E60aS/S61R/S173E was also inhibited more efficiently than wt-hAPC by PCI (Table 3). The increased inhibition rates by PCI of the mutant APC variants may explain the observation that wt-hAPC and this mutant were inhibited at similar rates in human plasma.

Heparin accelerated the inhibition rates of wt-hAPC and the S173E mutant approximately 312−337-fold, whereas the addition of heparin resulted in a 525-fold increased rate of inhibition of the E60aS/S61R mutant. The second-order rate constant (k_2) for the inhibition by PCI of mutant E60aS/S61R/S173E increased about 4-fold as compared to wt-hAPC, when heparin was present. These results indicate that residues at positions 60a and 61 influenced the interaction between APC, PCI, and heparin, whereas the substitution at position 173 had only a small impact on the PCI interaction. Enhanced inhibition of some APC mutants by PCI, especially in the presence of heparin, is not desirable in terms of the design of an improved anticoagulant as heparin-like molecules are naturally present in blood vessels. Although the concentration of PCI is relatively low as compared to AAT, it will be important to design new APC mutants to eliminate this problem. The present investigation provides significant insights into which regions of hAPC need to be engineered and our S173E mutant represents an excellent basis for future mutagenesis studies.

The increased effect of heparin on a E60aS/S61R mutant as compared to wt-hAPC suggested the possibility that the mutation resulted in an increased affinity of APC for heparin. To probe this point further, the binding of wt- and mutant APC molecules to heparin was examined using a heparin-sepharose chromatography. In repeated experiments, the E60aS/S61R mutant was found to elute from the heparin-sepharose column at approximately 10% higher NaCl concentration (250 ± 10 mM) than wt-APC (221.5 ± 3.5 mM). The S173E substitution did not affect the binding to the heparin-sepharose column. The molecular difference between wt-hAPC and the E60aS/S61R APC mutant involves neutralization of glutamic acid 60a and addition of a positively charged arginine, adjacent to residues K61−K62 and K37−K38−K39, which are already suggested to form part of the heparin binding in APC (*23*, *24*). The modified elution profile resulting from the E60aS/S61R mutant indicates that indeed, the electropositive region in loops 37 and 60 in APC form a binding surface for heparin (Figure 2B). It was known that activated protein C interacts with heparin (*34*, *35*) and the localization of this binding site to loops 37 and 60 has been elucidated recently (*23*, *24*). However, some other regions could not be ruled out, e.g., when computing the electrostatic potential of hAPC (Figure 2B), several areas possess an electropositive character and could be expected to interact with the negatively charged heparin molecule. These areas are centered around loops 60, 37, 70, and 148 when looking down the active site (Figure 2B, side i). These positive regions are conserved in the bovine protein. However, when looking at the side opposite to the catalytic cleft, it can be noticed that hAPC is more electropositive than bAPC (Figure 2B, side ii). These observations together with previously reported mutagenesis studies (*23*, *24*) and the data that we report here clearly indicate that a key heparin-binding site in APC, important during inhibition by PCI, involves the region of APC residues K61−K62 and K37−K38−K39, but not the region of the 148 loop. Additional experiments are needed to clarify whether loops 37 and 60 define the entire heparin-binding site. It is, however, possible that this area contributes significantly to the binding of such glycosaminoglycan as a cluster of three to five positively charged residues are commonly observed in the region of a protein involved in heparin binding (*36*). Linear array of positively charged residues is not clearly observed on the side opposite to the catalytic cleft and the electropositive character observed in this region of APC is due essentially to the lack of negatively charged residues rather than the presence of closely packed positively charged residues. Thus, the present structural analysis suggests that heparin should not interact specifically with the side opposite to the active site cleft of APC.

CONCLUSION

We have reported a model for bovine APC and compared its molecular surface with the one of human APC. Clear differences were noticed at the level of the light chain and on the side opposite to the active site cleft. Closer structural analysis within the catalytic triad region suggested that at least three residues could be replaced in hAPC by their bovine counterparts in order to create a human APC molecule resistant to AAT inhibition. The triple hAPC mutant molecule (E60aS/S61R/S173E) was indeed inefficiently inhibited by AAT. However, this enzyme was more efficiently inhibited by PCI as compared to wt-hAPC, especially in the presence of heparin. Yet, our single S173E mutant is particularly interesting as it presents partial resistance to the inhibition of AAT, while it leaves the enzyme inhibited in similar fashion as wt-hAPC by PCI. Our data also confirms that a key heparin-binding site in hAPC involves loops 60 and 37. Overall, the present analysis helps to correlate the relationships between the structure and the function of activated protein C.

ACKNOWLEDGMENT

We are very grateful to Astra Andersson for her help in culturing cells and for protein purification.

REFERENCES

1. Esmon, C. T., Ding, W., Yasuhiro, K., Gu, J. M., Ferrell, G., Regan, L. M., Stearns Kurosawa, D. J., Kurosawa, S., Mather, T., Laszik, Z., and Esmon, N. L. (1997) *Thromb. Haemostasis 78*, 70−74.
2. Kalafatis, M., Egan, J. O., van't Veer, C., Cawthern, K. M., and Mann, K. G. (1997) *Crit. Rev. Eukaryotic Gene Expression 7*, 241−280.
3. Dahlbäck, B. (1997) *Semin. Hematol. 34*, 217−234.
4. Rosing, J., and Tans, G. (1997) *Thromb. Haemostasis 78*, 427−433.
5. Di Cera, E., Dang, Q. D., and Ayala, Y. M. (1997) *CMLS, Cell. Mol. Life Sci. 53*, 701−730.
6. Aiach, M., Borgel, D., Gaussem, P., Emmerich, J., Alhenc Gelas, M., and Gandrille, S. (1997) *Semin. Hematol. 34*, 205−216.
7. Koeleman, B. P., Reitsma, P. H., and Bertina, R. M. (1997) *Semin. Hematol. 34*, 256−264.
8. Smith, O. P., White, B., Vaughan, D., Rafferty, M., Claffey, L., Lyons, B., and Casey, W. (1997) *Lancet 350*, 1590−1593.
9. Dreyfus, M., Maghey, J. F., Bridey, F., Schultz, H. P., Planche, C., Dehan, M., and Tchernia, G. (1991) *N. Engl. J. Med. 325*, 1565−1568.
10. Gruber, A., Griffin, J. H., Harker, L., and Hanson, S. R. (1989) *Blood 73*, 639−642.
11. Gresele, P., Momi, S., Berrettini, M., Nenci, G. G., Schwarz, H. P., Semeraro, N., and Colucci, M. (1998) *J. Clin. Invest. 101*, 667−676.
12. Okajima, K., Koga, S., Kaji, M., Inoue, M., Nakagaki, T., Funatsu, A., Okabe, H., Takatsuki, K., and Aoki, N. (1990) *Thromb. Haemostasis 63*, 48−53.
13. Taylor, F. B., Chang, A., Esmon, C. T., D'Angelo, A., Vigano-D'Angelo, S., and Blick, K. E. (1987) *J. Clin. Invest. 79*, 918−25.
14. Mather, T., Oganessyan, V., Hof, P., Huber, R., Foundling, S., Esmon, C., and Bode, W. (1996) *EMBO J. 15*, 6822−6831.
15. España, F., Gruber, A. G., Heeb, M. J., Hanson, S. R., Harker, L. A., and Griffin, J. H. (1991) *Blood 77*, 1754−1760.
16. Heeb, M. J., and Griffin, J. H. (1988) *J. Biol. Chem. 263*, 11613−11616.
17. Hermans, J. M., and Stone, S. R. (1993) *Biochem. J. 295*, 239−245.
18. Holly, R. D, and Foster, D. C. (1994) *Biochemistry 33*, 1876−1880.
19. Shen, L., Villoutreix, B. O., and Dahlbäck, B. (1999) *Thromb. Haemostasis 82*, 1078−1087.
20. Walker, F. J. (1981) *Thromb. Res. 22*, 321−327.
21. He, X., Shen, L., and Dahlbäck, B. (1995) *Eur. J. Biochem. 227*, 433−440.
22. He, X., Shen, L., Villoutreix, B. O., and Dahlbäck, B. (1998) *J. Biol. Chem. 273*, 27357−27366.
23. Neese, L. L., Gerlitz, B., Cooper, S. T., Grinnell, B. W., and Church, F. C. (1997) *Blood 90*, 146a.
24. Shen, L., Villoutreix, B. O., and Dahlbäck B. (1999) *Thromb. Haemostasis 82*, 72−79.
25. Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F. Jr., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T., and Tasmui, M. (1977) *J. Mol. Biol. 112*, 535−542.
26. Ponder, J. W., and Richards, F. M. (1987) *J. Mol. Biol. 193*, 775−791.
27. Rezaie, A. R., Mather, T., Sussman, F., and Esmon, C. T. (1994) *J. Biol. Chem. 269*, 3151−3154.
28. He, X., and Rezaie, A. R. (1999) *J. Biol. Chem. 274*, 4970−4976.
29. Gilson, M., Sharp, K., and Honig, B. (1987) *J. Comput. Chem. 9*, 327−335.
30. Klapper, I., Hagstrom, R., Fine, R., Sharp, K., and Honig, B. (1986) *Proteins 1*, 47−59.
31. Shen, L., Shah, A., Dahlbäck, B., and Nelsestuen, G. L. (1997) *Biochemistry 36*, 16025−16031.
32. Stratikos, E., and Gettins, P. G. W. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 453−458.
33. Elliott, P. R., Lomas, D. A., Carrell, R. W., and Abrahams, J. P. (1996) *Nat. Struct. Biol. 3*, 676−681.
34. Kazama, Y., and Koide, T. (1992) *Thromb. Haemostasis 67*, 50−55.
35. Petäjä, J., Fernández, J. A., Gruber, A., and Griffin, J. H. (1997) *J. Clin. Invest. 99*, 2655−2663.
36. Fromm, J. R., Hileman, R. E., Caldwell, E. E., Weiler, J. M., and Linhardt, R. J. (1995) *Arch Biochem Biophys 323*, 279−287.
37. Young, L., Jernigan, R. L. and Covell, D. G. (1994) *Protein Sci. 3*, 717−729.

BI992357P