Resistance to Inhibition by α-1-Anti-Trypsin and Species Specificity of a Chimeric Human/Bovine Protein C

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ABSTRACT: Human activated protein C (APC) has been shown to be physiologically susceptible to inhibition by the abundant serpin inhibitor α-1-anti-trypsin (AAT). Studies on the inactivation by AAT [Heeb, M. J., & Griffin, J. H. (1988) *J. Biol. Chem. 263*, 11613–11616] have shown that the calculated rate of this inactivation matches that of the observed half-life of APC *in vivo* [Wydro, R., Oppenheimer, C., Rodger, R., & Miemi, S. (1988) *Clin. Res. 36*, 329A] and complex formation therefore probably represents a physiologic regulation process for APC. In this study we observed that bovine APC, in contrast to human APC, is nearly completely resistant to inactivation by human AAT. An additional difference between human and bovine APC is that human APC is a potent anticoagulant in human plasma, whereas bovine APC is only minimally active in human plasma. These functional differences exist despite considerable structural similarity between the human and bovine molecules. In order to identify specific molecular regions responsible for function, a chimeric molecule consisting of the light chain of human protein C (PC) and the heavy chain of bovine PC was constructed, expressed, and characterized. The activated chimeric PC is similar to human APC in having potent anticoagulant activity in human plasma, but displays nearly identical resistance to AAT inhibition with the bovine molecule. The similarity between the chimeric and bovine molecules in resistance to AAT inhibition indicates that the structural determinants for inhibitor interactions reside within the heavy chain (serine protease) domain. The conservation of anticoagulant activity in human plasma between the chimeric and human molecules further indicates that structural determinants which confer species specificity to APC reside within the light chain of the molecule.

Protein C is the zymogen form of a serine protease present in plasma which plays an important physiological role in the regulation of blood coagulation pathways (Esmon, 1987). Human protein C undergoes extensive co- and post-translational modification during its biosynthesis in the liver. These modifications include proteolytic removal of a prepeptide and a propeptide (Foster et al., 1987) and endoproteolytic internal cleavage which converts the M_r 62 000 single-chain precursor to a light chain (M_r 21 000) and a heavy chain (M_r 41 000) which are held together by a disulfide bond (Kisiel et al., 1976; Stenflo, 1976).

The domain structure of protein C resembles that of several other serine protease blood coagulation factors in the vitamin-K-dependent class, including factor X, factor IX, and factor VII. The light chain consists of a Gla domain and two repeats of an EGF-like domain, while the heavy chain is a single domain consisting of an activation peptide followed by the catalytic, trypsin-like protease domain. The correlation between specific domain structures and biological functions is incomplete, but the serine protease domain is understood to be the catalytically active unit, while the Gla and EGF-like domains of the light chain are thought to mediate modulatory functions, such as interactions with phospholipid surfaces and other macromolecules such as cofactor proteins (Furie & Furie, 1988).

In this paper, we extend observations that bovine APC differs considerably in its biological properties from human APC. The bovine molecule is completely resistant to inactivation by human AAT and is nearly completely inactive as an anticoagulant in human plasma. The marked structural similarities between human and bovine protein C (Foster & Davie, 1984) suggests that these major physiologic differences may be traceable to relatively minor structural differences. It is reasonable to assume that the majority of contacts between human APC and AAT reside in the catalytic heavy chain, since serpins are known to form covalent complexes with the active site serine of serine proteases. It is much more difficult to predict which domain(s) might be involved in determining biologic potency toward human plasma. On the basis of the observation (Weinstein & Walker, 1991) that bovine protein S restores potency to bovine APC in human plasma, one might predict that a domain involved in interactions with protein S would mediate species-specific effects. Protein S binding sites have not been identified in APC, but by analogy with factor VIIa/tissue factor (Ruf et al., 1991), one might postulate involvement of one or both EGF-like domains of the light chain in interactions with cofactor proteins.

We have taken the first steps toward such a structure/function domain analysis by constructing and analyzing a chimeric protein C in which the heavy chain of human APC has been replaced by the corresponding region of bovine APC. Such a chimeric protein offers the opportunity to assign certain biological parameters to specific structural domains. Here we describe the construction, purification, and characterization of this chimeric protein in terms of its inhibition by AAT and also in terms of its biological anticoagulant activities in human and bovine plasma.

MATERIAL AND METHODS

Cloning Bovine PC cDNA. A bovine liver cDNA library was purchased from Clonetech Inc. A total of 60 000 plaques were screened by probing ultraviolet-irradiated nylon membrane lifts with a 1.7-kb human PC cDNA that was random prime labeled with [γ-^{32}P]ATP (Amersham random prime

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labeling kit). The hybridization was done with 1×10^6 cpm/mL of probe in a hybridization mixture consisting of 3X SSC, 10X Denhart's, and 0.02% SDS at 63 °C overnight. The lifts were washed once at room temperature and twice at 63 °C in 3X SSC plus 0.02% SDS before putting them on film. Two positive plaques were picked and the plaques purified. Phage DNA was prepared by ammonium sulfate precipitation of a liquid lysis culture (Mukesh, 1989). One of the clones was found to have a 1.7-kb insert, and the cDNA was subcloned into pUC19 for further work.

Chimeric PC Construction. A 1.0-kb *Taq*1 to *Eco*R1 fragment was isolated from the bovine PC cDNA and ligated to the *Eco*R1 to *Sst* II 700 bp human PC fragment using a synthetic *Sst* II to *Taq*I adapter pair, and a *Eco*R1-cut ZMB-3 vector previously described (Foster et al., 1991). This construction encodes the light chain, activation peptide, and first amino acid of the serine protease domain of human PC joined in frame to the second codon of the bovine heavy chain sequence. The chimeric PC cDNA is driven by the adenovirus middle late promoter and the selectable marker gene, *neo*, is driven by the SV40 late promoter. The human PC expression plasmid has been previously described (Foster et al., 1991).

Transfection/Selection. BHK570 cells were grown, transfected with PC expression plasmids, and cloned in selective medium essentially as previously described (Foster et al., 1991). Briefly, 50% confluent 10-cm plates of cells were transfected with a calcium phosphate-precipitate of 10 μg of plasmid DNA and 10 μg of salmon sperm DNA. Clonal cell lines resistant to 50 μg/mL G418 (chimeric PC) or 250 nM methotrexate were cylinder cloned following approximately 10 days growth in selective medium. Clones were expanded and the supernatant of confluent cultures was collected by replenishing the medium every 2–4 days with DME containing 2 mM L-glutamine, 5 μg/mL vitamin K, and 1% FBS. The PC was recovered by immunoaffinity purification using a Ca^{2+}-dependent anti-human PC monoclonal antibody (Foster et al., 1991).

Activation of PC. Bovine plasma PC (Enzyme Research Labs) and human recombinant and chimeric PC were activated by a purified activator (ACC-C, from the venom of *Agkistrodon contortrix* contortrix) which was a gift from Dr. Walt Kisiel. The activator was used at a ratio of 1:100 to 1:200 w/w to the PC at 37 °C for 2–3 h in TBS (25 mM Tris pH 7.4, 150 mM NaCl) with 1 mg/mL BSA (Sigma). The activation time course was followed at 405 nm using a synthetic APC substrate (Spectrozyme PCa, American Diagnostica).

Anticoagulant Activity Assay. A 100-μL sample of human plasma was mixed with 100 μL of kaolin/cephalin suspension (1% kaolin/1 mg/mL rabbit brain cephalin in TBS) and incubated at 37 °C for 2 min. Then 100-μL of activated protein C samples at various concentrations was added and the mixture incubated an additional 2 min at 37 °C. Finally, 100 μL of 25 mM $CaCl_2$ was added and the clotting time recorded. In samples containing human protein S, 8 μg of protein S was added to the plasma at the start of the assay. Purified human protein S was a gift from Dr. Walt Kisiel.

Inactivation of Human, Bovine, and Chimeric PC by Human AAT. Human, bovine, and chimeric PC were activated by ACC-C and then diluted to 10 μg/mL in 0–800 μg/mL human AAT in TBS plus 1 mg/mL BSA. Following overnight incubation at 37 °C, 20-μL samples were diluted into 100 μL of 1 mM Spectrozyme PCa in a 96-well plate and the absorbance at 405 nm measured in a single time point assay (10 min).

Inactivation of Human and Chimeric APC in Human Plasma. Human and chimeric PC were activated with ACC-C and then diluted to 4 μg/mL in citrated human plasma. Samples were removed at time points from 0 to 60 minutes and diluted 5-fold in cold TBS. Samples of 60 μL each were added to 50 μL of 1 mM Spectrozyme PCa in a 96-well plate, the absorbance at 405 nm was measured immediately and after 15 min, and the difference was used to calculate relative activity to the zero time point.

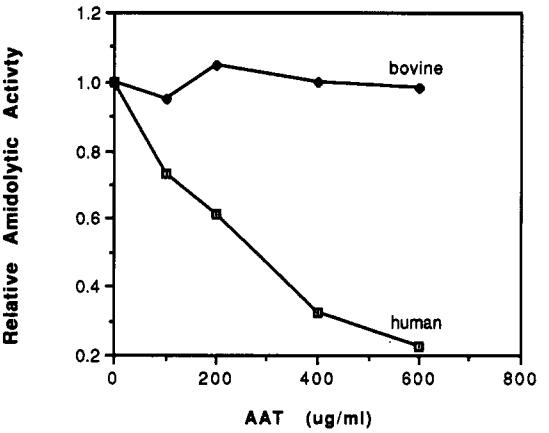

FIGURE 1: Inactivation of human and bovine APC by human AAT. Human and bovine PC were activated by ACC-C as described in Materials and Methods and then diluted to 10 μg/mL in human AAT at various concentrations and incubated at 37 °C overnight. Twenty-microliter samples were diluted into 100 μL of 1 mM Spectrozyme PCa in TBS in a 96-well plate. The absorbance at 405 nm was measured after a 3-min incubation at room temperature, and the data were normalized to the zero AAT point. In the absence of an inhibitor the amidolytic activity is not affected by overnight incubation at 37 °C (data not shown).

RESULTS

Since bovine PC is well-characterized and is 80% identical to human PC, we tested the ability of human AAT to inactivate bovine APC and found no measurable inactivation, even at AAT concentrations which inhibited >80% of human recombinant APC (Figure 1). It has been reported previously (Kisiel et al., 1977) that the specific anticoagulant activity of bovine APC is very low in human plasma. We reasoned that the structural differences between human and bovine APC molecules which account for their differential inhibition by human AAT would probably reside in the serine protease domain, and that the structural differences which give rise to the low activity of bovine APC in human plasma may reside in either chain, but might possibly be localized to specific domains.

This line of reasoning led us to construct a cDNA encoding a chimeric PC composed of the human PC light chain and activation peptide coding sequences joined to the bovine PC heavy chain coding sequence (Figure 2). This chimeric molecule was used as a tool to probe the differences in inactivation of human and bovine APC by AAT, and additionally to localize domains involved in species specificity.

Human PC and the chimeric PC were expressed by transfecting BHK cells with mammalian cell expression vectors containing the respective PC cDNAs and selecting for resistance to methotrexate (for human PC) or G418 (for chimeric PC), as described in the Material and Methods section. The proteins were purified from the conditioned media using a calcium-dependent, anti human PC light chain monoclonal antibody column. Figure 3 shows an SDS–polyacrylamide gel of plasma-derived bovine PC, purified recombinant chimeric PC, and purified recombinant human PC. The human recombinant heavy chain is distributed in three bands as previously reported for both plasma and

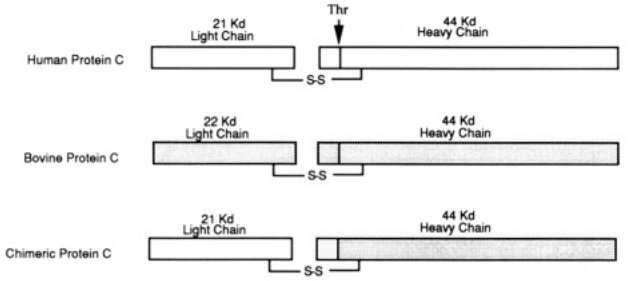

FIGURE 2: Schematic structures for human, bovine, and chimeric protein C. Structures corresponding to human sequences are shown in white boxes. Bovine structures are shown as shaded boxes. The arrow indicates the site of thrombin activation, releasing the 12 amino acid peptide from the N-terminus of each heavy chain.

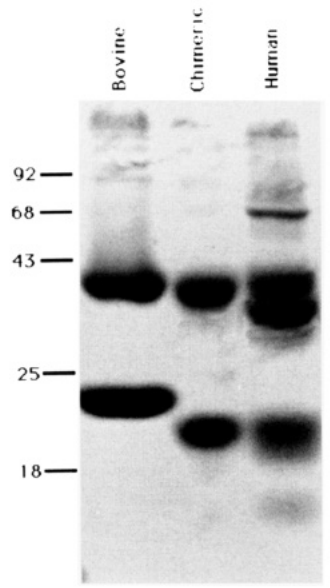

FIGURE 3: SDS–PAGE of bovine, chimeric, and human protein C. Twenty micrograms of each protein preparation was denatured with β-mercaptoethanol and run on 5–15% SDS–PAGE, fixed, and stained with Coomassie Blue.

recombinant human PC, which has been shown to be due to heterogeneity in the carbohydrate (Miletich & Broze, 1990; Grinnell et al., 1991). The human light chain runs at a lower molecular weight than the bovine light chain and stains less intensely. As expected, the chimeric PC heavy chain comigrates with the bovine heavy chain and the chimeric PC light chain comigrates with the human light chain. The chimeric PC was also subjected to 20 cycles of automated Edman degradation (data not shown), which confirmed the presence of the human light chain sequence and a heavy chain sequence beginning with the human activation peptide for the first 12 residues, followed by the bovine heavy chain sequence, which differs from the human heavy chain in positions 13, 14, 17, 18, 19, and 20 of the region sequenced.

In order to characterize the biological properties of the chimeric PC, the recombinant human PC, chimeric PC, and plasma-derived bovine PC preparation were activated with the venom activator (McMullen et al., 1989) ACC-C (Figure 4). All three PC's were activated to similar amidolytic activities, demonstrating that the chimeric protein was recognized by the activator and was catalytically active toward the chromogenic substrate. The bovine PC was activated somewhat faster by ACC-C than either the human or chimeric molecules, suggesting that sequences which affect the relative rates of activation by the venom activator reside in the light chain. The apparent activity at zero time in the bovine curve is due to extremely rapid activation of this sample and not

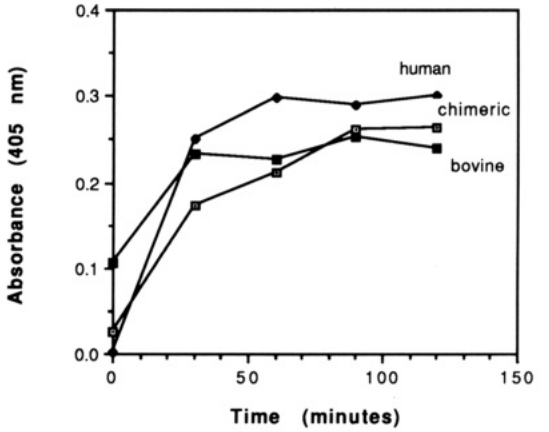

FIGURE 4: Activation of bovine, chimeric, and human protein Cs by ACC-C. The activation was carried out as described in the experimental procedures. Samples of the reactions were removed over a 2-h time course, diluted 10-fold in cold TBS/BSA, and then assayed for amidolytic activity with Spectrozyme PCa at 405 nm in a 96-well plate reader in a single point assay.

already-activated material, since control experiments without addition of the activator show no activity (data not shown).

We then tested the anticoagulant activity of each PC in both human and bovine plasma (Figure 5). As previously observed (Kisiel, 1979), human APC showed potent anticoagulant activity in human plasma, but relatively poor activity in bovine plasma (Figure 5a). We could reverse this species specificity by the addition of human plasma protein S (PS) to the bovine plasma. Bovine APC showed the opposite pattern of activity with relatively high anticoagulant activity in bovine plasma and low activity in human plasma (Figure 5c), also as previously observed (Kisiel et al., 1977). The addition of human protein S did not affect the activity of bovine APC in bovine plasma. The chimeric APC showed an activity pattern similar to that of human APC, exhibiting much greater anticoagulant activity in human plasma than in bovine plasma, and high activity could be restored in bovine plasma when human protein S was added (Figure 5b). These results demonstrate the functional integrity of the chimeric molecule with respect to both amidolytic and anticoagulant activities. They further serve to localize the domains involved in species specificity of anticoagulant activity and the interactions with human protein S to the light chain, since the human and chimeric proteins become active in bovine plasma only after the addition of human protein S.

Since the chimeric PC appeared to be a fully functional molecule, the relative rate of its inactivation by human AAT was examined. Figure 6 shows the relative rates of inactivation of human, bovine, and chimeric APC by human AAT. At high AAT concentrations where the human APC was substantially inhibited, both the bovine and chimeric APC's were almost completely resistant to inhibition. These results indicate that the structural determinants which account for the species specificity observed in the differential inactivation of human and bovine PC by human AAT reside in the serine protease domain.

From these results one would predict that if inactivation by AAT is primarily responsible for the half-life of APC in plasma, as suggested by Heeb and Griffin (1988), then the plasma half-life of the chimeric APC would be longer than the plasma half-life of human APC in human plasma. Inactivation experiments using citrated human plasma demonstrated that the chimeric APC was indeed inactivated more slowly than human APC (Figure 7) but was nevertheless subject to substantial, albeit slower, inactivation. The residual inactivation of chimeric APC observed in human plasma is likely

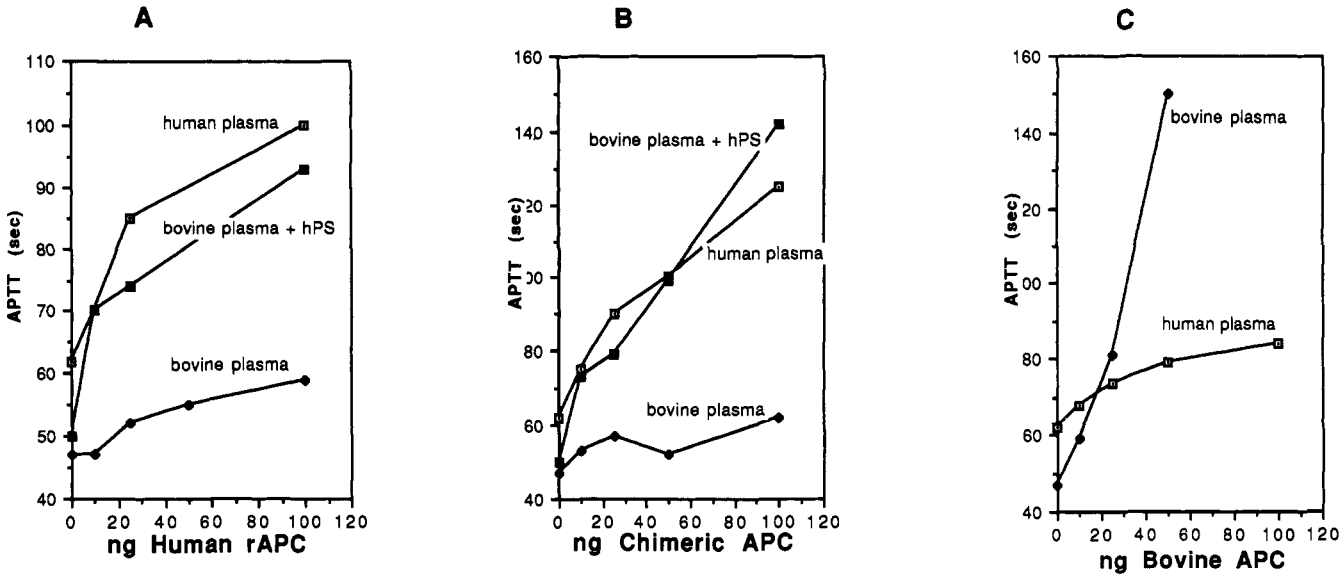

FIGURE 5: Anticoagulant activity of human, chimeric, and bovine APC in human and bovine plasmas. Samples of each protein C type were activated with the activator ACC-C as in Figure 4. Aliquots of these activated preparations were assayed in plasma in a two-stage protein C assay as described in Material and Methods.

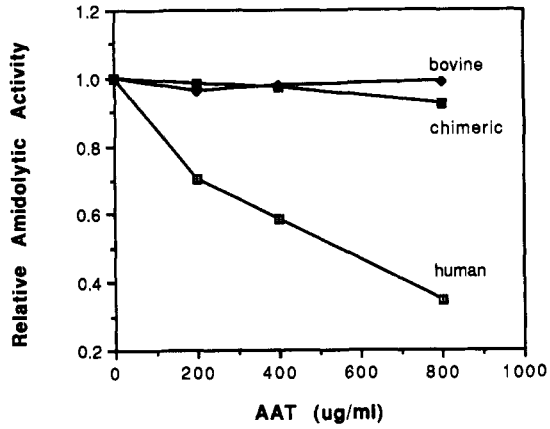

FIGURE 6: The activation of human, bovine and chimeric APC's by human AAT. Human, bovine, and chimeric PC's were activated then added to various concentrations of AAT as described in Material and Methods. After overnight incubation the remaining amidolytic activity was measured at 405 nm using the spectrozyme PCa substrate in a single time point assay. The activity is expressed relative to the activity with no inhibitor.

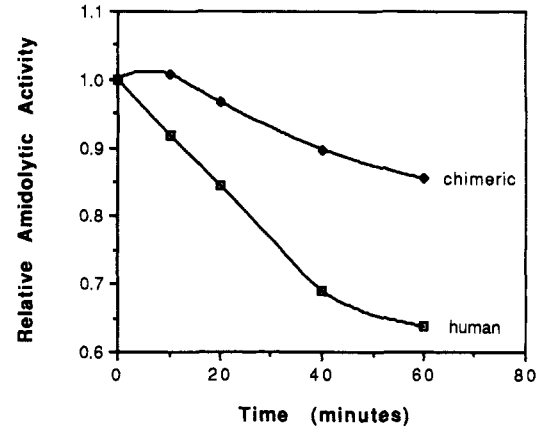

FIGURE 7: Plasma inactivation of human and chimeric APC's. The PC's were activated as described and then diluted into citrated human plasma and time points taken as described in the experimental procedures. The remaining amidolytic activity was normalized to the initial activity at time zero.

due to its interaction with additional inhibitors present in plasma, such as PCI-1 (Meijers et al., 1988).

DISCUSSION

Protein C interacts with a large number of other proteins in its biological functions, including interactions with cell surface activation complexes, substrates, cofactors, and inhibitors. In seeking to identify protein C domains involved in macromolecular interactions, it would be feasible to consider a systematic examination or replacement of structural domain elements throughout the protein. We were aided considerably in this respect by the observation that a closely related structural homolog (bovine APC) did not share two key biologic properties with its human counterpart. Therefore we reasoned that we could gain insight into the function of specific domains by the simple substitution of a bovine domain for the corresponding human domain (Figure 1), resulting in a limited structural deviation from the human molecule. Since bovine and human PCs carry out equivalent functions in their respective plasmas, it is reasonable to assume that substitutions of homologous parts of human PC with bovine PC would not affect the global structure or function of the molecule but would serve as a map to localize any species-specific differences observed in any of APC's interactions with other proteins.

As a beginning toward such domain-function analysis, a cDNA was constructed which encoded a chimeric protein which included the human light chain and activation peptide and the bovine serine protease domain. The cDNA was expressed in a mammalian cell system identical to that employed for native human r-protein C (Foster et al., 1989) and was purified with a monoclonal antibody which recognized a human light chain epitope. The recombinant chimeric PC yielded the expected bands on SDS gels, was amidolytically active, and was fully functional as an anticoagulant in human plasma. After demonstrating that the chimeric PC was functionally active, its biologic properties were examined relative to its human bovine counterparts.

In APTT coagulation assays the human rAPC had potent activity in human plasma and much less activity in bovine plasma (Figure 5a). This species specificity of human APC is consistent with that previously observed for human plasma APC (Kisiel, 1979). Bovine APC showed the opposite pattern of species specificity, with relatively high anticoagulant activity

in bovine plasma and low activity in human plasma (Figure 5c), also as previously observed (Kisiel et al., 1977). The chimeric APC showed an activity profile nearly identical to that of human APC, being a potent anticoagulant in human plasma and nearly inactive in bovine plasma. We could reverse this species specificity by the addition of human plasma protein S to the bovine plasma. This result indicates that the light chain contains structures which account for the differences in anticoagulant activity between species. Since both the human and chimeric molecules have nearly identical activity profiles and respond to the addition of human protein S, it follows that light chain domains are sufficient for interaction with human cofactors, and specifically with human protein S. This both confirms the observation (Weinstein & Walker, 1989) that protein S interactions determine species range and further localizes its interactions with protein C to domains in the light chain.

Having established that the chimeric PC was functionally active, its susceptibility to inactivation by human AAT was tested (Figure 6). In this respect, the chimeric PC was similar to bovine PC with no detectable inactivation by human AAT. Since both the chimeric and bovine proteins were similarly resistant to inhibition, this result indicates that the molecular determinants affecting inactivation by human AAT lie within the heavy chain of PC. A comparison of bovine and human heavy chain sequences (Foster & Davie, 1984) reveals a scattering of mostly conservative substitutions. In addition, there are four short segments in which the human heavy chain sequence is substantially different from the bovine heavy chain and therefore one or more of these may contribute the functional differences observed in the inactivation by human AAT. These include amino acid segments (from the N-terminus of the human heavy chain) 17–21, 146–153, 171–181, and 252–262. Amino acids 17–21 differ greatly in charge between the two PC molecules with the human sequence containing three basic residues and no acidic residues, while the bovine sequence contains no positive charges and one acidic residue. The human 146–153 sequence is extremely hydrophilic, and the corresponding bovine segment is four amino acids shorter. The last two segments noted each have substitutions at 7 of 11 amino acid positions. Future investigations into more precise localization of specific determinants involved in the AAT interaction will focus on more limited substitutions in these areas with the corresponding bovine sequences.

The localization of determinant(s) affecting the interaction of AAT with APC to the serine protease domain is consistent with reports of mutants in other serine proteases which affect their inactivation by specific serpins. Madison et al. (1989) demonstrated the critical role of three consecutive basic amino acids of tissue plasminogen activator (t-PA) in its inactivation by PAI-1 by creating mutants in t-PA based upon a model derived from the BPTI and trypsin complex. Both human and bovine PC have basic amino acids in equivalent positions (amino acids 22–24 from the activation site) and therefore these specific residues cannot explain the different responses of bovine and human APC by human AAT. However, APC is efficiently inactivated by PAI-1 (Fay and Owen, 1989) and these basic residues may be important in the inhibition of APC by PAI-1. This suggests that the two serpins may interact with the same target protease via independent sites. Future investigations into serpin inhibition of APC will include alteration of these residues and examination of the effect on PAI-1 interactions.

Since AAT has been shown to be one of two major inhibitors of APC in plasma (Heeb & Griffin, 1988), the relative rates of inactivation of the human and chimeric APCs were compared in citrated human plasma. While the chimeric APC is inactivated more slowly than human APC it nevertheless shows substantial inactivation. This suggests that the other major inhibitor of APC in plasma, PCI, is able to inactivate the chimeric APC.

The regulation of APC in plasma by AAT has been demonstrated in both experimental and clinical settings. In disseminated intravascular coagulation (DIC), a significant proportion of the total PC can be found in complexes with AAT and PCI. The potential therapeutic use of APC to treat DIC, septic shock, or other thromboembolic conditions is complicated by the presence in plasma of 2.5 mg/mL AAT as a virtually inexhaustible inhibitor of activated PC. The utilization of a mutant APC that is not inactivated by AAT would reduce the therapeutic dosages required, especially when the therapeutic interval is long relative to the plama half-life of APC. The other major plasma inhibitor of APC, PCI, is present at only 5 μg/mL in plasma and therefore is probably not a significant obstacle to APC clinical therapy.

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