Characterization of an Interaction between Protein C and Ceruloplasmin*

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Coagulation factors V and VIII are substrates for activated protein C. Binding sites for the protease have been localized to homologous sequences within the terminal A domains of these proteins. Since ceruloplasmin contains significant sequence homology to these domains, a study was undertaken to determine whether ceruloplasmin was an activated protein C-binding protein. Ceruloplasmin was observed to inhibit the activated protein C-catalyzed inactivation of both factor Va and factor VIII. Searches of the ceruloplasmin sequence revealed a decapeptide sequence, HAGMETTYTV, that shares 60 and 40% sequence identity with the activated protein C binding sites in factors VIII and V, respectively. This peptide also inhibited factor Va inactivation and in addition was observed to enhance the amidolytic activity of activated protein C. The ferrous oxidase activity of ceruloplasmin was stimulated 5-fold by activated protein C, and this effect was negated by the peptide HAGMETTYTV. These results indicate that these conserved sequences of ceruloplasmin and factors V and VIII interact with activated protein C and suggest that this region may be important in the regulation of this anticoagulant protein.

Protein C is a vitamin K-dependent zymogen of a serine protease (1). When activated, it is a potent inhibitor of blood coagulation through the selective inactivation of coagulation factors Va (2–4) and VIII (4–6). The mechanism of inactivation is through the proteolysis of the heavy chains of both of these proteins. Previously, it was shown that activated protein C could bind to the light chains of factor Va (7) and factor VIII (8) and that exogenously added light chain could prevent the inactivation of factor VIII (8). These results suggested that binding of the protease to the light chain(s) of cofactor(s) represented an obligate step for their proteolytic inactivation. Recent data have localized the activated protein C binding sites on the cofactor light chains to regions that correspond to residues 2009–2018 in factor VIII and residues 1865–1874 in the factor V sequence (9). Peptides that were synthesized from these regions, RAGMQTFPLF from the factor V sequence and HAGMSTFLFIV from the factor VIII sequence, were found to inhibit the anticoagulant activity of activated protein C and to inhibit the inactivation of factor Va and factor VIII by activated protein C.

Factors V and VIII are members of a family of structurally related proteins that include the copper binding protein ceruloplasmin (10). One characteristic of these proteins is the tripeptide sequence of 350-residue sequence (designated as the A domain) that shares 30–40% sequence identity between proteins (11, 12). In this report we have examined the effects of ceruloplasmin and a peptide prepared from the ceruloplasmin sequence on the amidolytic and proteolytic activities of activated protein C. The peptide contains significant sequence homology to activated protein C binding sites in factors V and VIII. Results obtained indicate that ceruloplasmin is an activated protein C-binding protein.

EXPERIMENTAL PROCEDURES

Preparation of Proteins—Bovine activated protein C (13), bovine, factor Va (13), and human factor VIII (14) were prepared as previously described. Bovine ceruloplasmin was purchased from Sigma. The protein was predominantly single chain with some of the characteristic fragments derived by proteolysis. Ceruloplasmin was further purified using a Mono Q column using a linear gradient (0–0.4 M NaCl) containing 0.02 M Tris-HCl, pH 7.5, and 5 mM EDTA.

Preparation of Peptides—Synthetic peptides HAGMETTYTV and RQGAILFLPT were produced by Multiple Peptide Systems using tert-butoxycarbonyl chemistries. The peptides were at least 85% pure as judged by reversed-phase high pressure liquid chromatography.

Assays—Inactivation of factors Va and VIII by activated protein C was carried out as previously described (5). Lipid concentrations were chosen such that the rate of inactivation was independent of lipid concentration. The amidolytic activity of activated protein C was determined using the synthetic substrate S2366 (KabiVitrum) and monitoring the change in absorbance at 405 nm. All reactions were carried out at 15°C in 1.0 ml reaction volume. The rate of hydrolysis was measured in a Beckman DU 7 spectrophotometer equipped with the automatic kinetics module for data acquisition from multiple samples. Ceruloplasmin was assayed by the method of Osaki et al. (15). Ceruloplasmin was added to a cuvette containing 9.2 mM p-phenylenediamine and 0.05 M imidazole buffer, pH 6.0. The total reaction volume was 1.2 ml, and the change in absorbance at 540 nm was determined at 37°C.

RESULTS

Effects of Ceruloplasmin on the Inactivation of Factors Va and VIII—We have recently localized the activated protein C binding sites to sequences on factor Va and VIII light chains using as an assay the inhibition of protease-catalyzed cofactor inactivation (9). If ceruloplasmin interacts with activated protein C, then it should affect the rates of cofactor inactivation by the protease. Examination of the activated protein C-catalyzed inactivation of factor Va (Fig. 1) and factor VIII (Fig. 2) performed in the presence and absence of ceruloplasmin indicated that ceruloplasmin protected both cofactors. Rates of inactivation were reduced as much as 3-fold with increasing levels of ceruloplasmin in the reaction mixture. These results suggested a direct interaction between ceruloplasmin and activated protein C.

Identification of an Activated Protein C Binding Sequence in Ceruloplasmin—The sequence of ceruloplasmin contains...
Interaction of Protein C with Ceruloplasmin

**Figure 1. Effect of ceruloplasmin on the inactivation of factor VA.** Factor Va inactivation by activated protein C was carried out in the presence of calcium and phospholipid as previously described (13). Assays were performed in the absence of ceruloplasmin (circles) and in the presence of 0.6 μM (triangles) or 1.2 μM (squares) ceruloplasmin.

**Figure 2. Effect of ceruloplasmin on the inactivation of factor VIII.** Factor VIII inactivation by activated protein C was carried out in the presence of calcium and phospholipid as previously described (5). Assays were performed in the absence of ceruloplasmin (squares) and in the presence of 0.6 μM (triangles) or 1.2 μM (circles) ceruloplasmin.

**Table I** Comparison of similar sequences in factor V, factor VIII, and ceruloplasmin

| Protein (domain) | Residue no. | Sequence | Ref. |
|-----------------|-------------|----------|------|
| Factor V (A3)   | 1865-1874   | RAGMQTPLFI | 17   |
| Factor VIII (A3)| 2009-2018   | HAGMSTLFIV | 12   |
| Ceruloplasmin (A1) | 336-345 | KAGLQAFFQV | 16   |
| Ceruloplasmin (A2) | 687-696 | TGGMKQKYTV | 16   |
| Ceruloplasmin (A3) | 1028-1037 | HAGMETTYTV | 16   |

a Based upon the nomenclature of Vehar et al. (12).

three internal repeat units (16) that share 30% similarity with the A domains of factors V and VIII (10). Each of these repeats was examined for sequence similarity with either RAGMQTPLFI or HAGMSTLFIV peptides from the factor Va and VIII light chain sequences, respectively, that have been shown to interact with activated protein C (9). From the alignment of the three repeats of the ceruloplasmin sequence, it was possible to identify three sequences that shared similarity with the protein C binding site in factors B and VIII (Table I). Comparison of these sequences indicated that the third repeat of ceruloplasmin, residues 1028-1037, shared the greatest degree of identity with the protein C binding sites on factor V (40%) and factor VIII (60%). This sequence, HAGMETTYTV, was synthesized and its effect upon factor Va inactivation by activated protein C examined. Like the peptides synthesized from factors V and VIII light chains, the peptide prepared from the ceruloplasmin sequence, HAGMETTYTV, also was found to inhibit the inactivation of factor Va (Fig. 3). A scrambled version of the activated protein C binding sequence in factor V, MRQGALPT, had no effect on the proteolytic inactivation of factor Va.

HAGMETTYTV was tested for its effects on the amidolytic activity of activated protein C. The peptide was found to enhance the rate of cleavage of the synthetic substrate S2366 by activated protein C approximately 5-fold (Fig. 4), suggesting that the peptide can interact with activated protein C. Double-reciprocal analysis of the data suggested an apparent $K_c$ of 15 μM. A scrambled peptide with the same composition as the activated protein C binding site in factor Va had no effect.

Alteration of Ceruloplasmin Activity by Interaction with Protein C—If protein C can bind to ceruloplasmin, it could potentially alter the ferrous oxidase activity of ceruloplasmin.

**Figure 3. Effect of HAGMETTYTV on factor Va inactivation.** Factor Va was inactivated by activated protein C in the presence of calcium and phospholipid as described previously (13). The peptides HAGMETTYTV (100 μg/ml) (triangles), MRQGALPT (100 μg/ml) (squares), or no peptide (circles) were added to the reaction mixture.

**Figure 4. Effect of HAGMETTYTV and MRQGALPT on the amidolytic activity of activated protein C.** The amidolytic activity of activated protein C on the synthetic substrate S2366 was determined as described under “Experimental Procedures” in the presence of the indicated concentration of either HAGMETTYTV (squares) or MRQGALPT (circles).
The oxidation of p-phenylenediamine was measured at various concentrations of protein C. Protein C enhanced the rate of oxidation of substrate (Fig. 5). Protein C had no oxidase activity. Rate enhancement was proportional to the concentration of protein C. A 2-fold molar excess of protein C relative to ceruloplasmin resulted in a doubling of the oxidation rate.

The protein C effect was reversed by the addition of HAGMETTVTV. The peptide had no effect on the oxidase activity of ceruloplasmin in the absence of protein C. These results indicate that the interaction between the two proteins could be inhibited by blocking the ceruloplasmin binding site on protein C.

DISCUSSION

Factor V, factor VIII, and ceruloplasmin belong to a family of structurally related proteins (10). Searches for internal sequence homologies have revealed that each protein contains a triplicated unit (A domain) of about 350 amino acids. The A domains of the two coagulation cofactors share about 30% sequence identity with each other and about 30% sequence identity with the A domains of ceruloplasmin. In addition, these proteins are similar in that each requires metal ions to maintain their structure and/or function. Factors Va and VIII are noncovalent heterodimers with subunits linked by divalent metal ion(s) (reviewed in Ref. 18). Treatment of these proteins with chelating reagents dissociates the subunits and abolishes cofactor activity (19, 20). Reassociation of the isolated subunits and the restoration of cofactor activity is achieved in the presence of added Mn"+ of Ca2+ (19, 21, 22).

Ceruloplasmin is the principal copper transport protein in plasma, and it is this copper ion binding property that gives ceruloplasmin its oxidase activity (23). Factor V has also been shown to contain a tightly bound copper ion (24).

Based upon results presented in this report, we extend the similarities of these three proteins to include the capacity to bind (activated) protein C. Evidence in support of this conclusion includes: (i) the ability of ceruloplasmin to protect factors Va and VIII from activated protein C-catalyzed inactivation; (ii) a similar protective effect observed using the synthetic peptide HAGMETTV prepared from the ceruloplasmin sequence which shares similarity with the protease binding sites identified in the cofactors; (iii) the ability of this peptide to stimulate the amidolytic activity of activated protein C; and (iv) the ability of protein C to accelerate the ferrous oxidase activity of ceruloplasmin.

Although we have not rigorously examined the heavy chains of factors Va and VIII nor evaluated the sequences in ceruloplasmin that may potentially serve as activated protein C binding sequences, it is of interest to note that the sequences thus far identified as the protease binding sites map to the A3 domain of each protein. For factors Va and VIII, it is within these domains located on the light chains of the factors that activated protein C is postulated to bind, resulting in subsequent proteolytic cleavage of the heavy chains and inactivation of cofactor function (9). Neither the mechanism(s) by which protein C affects the ferrous oxidase activity of ceruloplasmin nor those by which the peptide HAGMETTVTV enhances the amidolytic activity of the protease are known at the present time.

The interactions between activated protein C and factors Va and VIII are important in the regulation of blood coagulation (25). The acute phase protein C4b-binding protein is an important regulator of the protein C pathway. This protein functions as an inhibitor of protein S (26), a protein cofactor required for the maximum expression of the anticoagulant activity of activated protein C (13). Ceruloplasmin also is an acute phase protein (27) and its plasma level increases several-fold in response to inflammation (28). In this paper we present evidence that ceruloplasmin can bind activated protein C at sites that are similar to the binding site C found in factors V and VIII. It is possible that the interaction between protein C and ceruloplasmin may play some role in the regulation of the protein C pathway.

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