Localization of Thrombin Cleavage Sites in the Amino-terminal Region of Bovine Protein S*

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Protein S is a vitamin K-dependent plasma protein. It functions as a cofactor to activated protein C in the inactivation of factors Va and VIIIa, by limited proteolysis. Protein S is very sensitive to proteolysis by thrombin which reduces its calcium ion binding and leads to a loss of its cofactor activity. We have now determined the sequence of the 100 amino-terminal amino acid residues and localized the thrombin cleavage sites. Protein S contains 11 γ-carboxyglutamic acid residues in the amino-terminal region (residues 1–36). This part of protein S is highly homologous to the corresponding parts in the other vitamin K-dependent clotting factors, whereas the region between residues 45 and 75 is not at all homologous to the other clotting factors. Thrombin cleaves two peptide bonds in this part of protein S, first at arginine 70 and then at arginine 52. The peptide containing residues 53–70 is released from protein S after thrombin cleavage. The amino-terminal fragment, residues 1–52, is linked to the large carboxyl-terminal fragment by a disulfide bond, which involves cysteine 47. After residue 78, protein S is again homologous to factors IX and X and to proteins C and Z, but not to prothrombin. Position 95 is occupied by a δ-hydroxyaspartic acid residue.

Protein S is a vitamin K-dependent plasma protein purified and characterized from bovine and human plasma (1–4). Unlike the vitamin K-dependent clotting factors (factors II, VII, IX, and X), it is not a proenzyme of a serine protease (2, 4). It is a single chain molecule (M, approximately 75,000) with 11 γ-carboxyglutamic acid (Gla) residues and 2–3 erythro-δ-hydroxyaspartic acid residues in the NH₂-terminal part of the polypeptide chain (1–3, 5, 6). The vitamin K-dependent plasma proteins, including protein S, bind Ca²⁺ ions and have a high affinity for negatively charged phospholipid (7, 8). The concentration of protein S in human plasma is approximately 25 μg/liter (4), half of which occurs as a monomolecular complex with C4b-binding protein (5, 9). C4b-binding protein is involved in the regulation of the complement system (10). The function of protein S in the C4b-binding protein-protein S complex has not yet been established (11, 12).

Protein S is a cofactor for activated protein C in the inactivation of factor Va (13–15), and recent evidence suggests that protein S is also required for the rapid degradation of factor VIIIa by activated protein C (16). It has been proposed that protein S augments the binding of activated protein C to phospholipid vesicles (17). The physiological importance of the protein S cofactor function (18–21) is indicated by a predisposition to venous thrombosis in patients with hereditary protein S deficiency.

Human and bovine protein S were recently found to be very sensitive to proteolysis by thrombin (4, 22). The single chain protein was converted to a two-chain structure, with the two chains (75 and 8 kDa) linked by disulfide bonds (4). Of particular interest was the finding that the thrombin-cleaved protein S had a lower affinity for calcium ions than the intact protein and that it appeared to have lost its affinity for negatively charged phospholipid surfaces (4). Furthermore, the thrombin-cleaved protein S had lost its cofactor activity (8, 23). The cleavage of protein S by thrombin may be an important regulating step in blood coagulation. We have, therefore, determined the amino acid sequence of the NH₂-terminal part of protein S and localized the thrombin-cleavage sites. The accompanying paper (24) deals with the Ca²⁺ ion-binding properties of intact and thrombin-modified protein S.

MATERIALS AND METHODS

RESULTS

Thrombin Cleavage of Protein S Correlated with a Change in Calcium Binding—Purified protein S was incubated with thrombin, and aliquots were drawn at intervals and analyzed by SDS-polyacrylamide gel electrophoresis. Agarose gel electrophoresis of the same aliquots was performed both in the anodic and in the cathodic direction on SDS-polyacrylamide gel electrophoresis. Agarose gel electrophoresis of the same aliquots was performed both in the presence and in the absence of calcium ions to correlate the peptide bond cleavages, observed on SDS gels, with changes in electrophoretic mobility due to reduced calcium binding (Fig. 1). An 18-kDa peptide that was present also prior to the addition of thrombin, due to slight degradation of the purified material, increased in concentration and was then cleaved to a 16-kDa species which, inexplicably, appeared as a doublet. Both the 18-kDa peptide and the 16-kDa doublet appeared to be linked to the large COOH-terminal fragment by disulfide bond(s). The first proteolytic cleavage correlated with a change in mobility of protein S in the anodal direction on

1 Portions of this paper (including "Materials and Methods," Fig. 2, and Tables 1–IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-3865, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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concentration) and heparin (30 units/ml) to stop the reaction and aliquots drawn and mixed with antithrombin were drawn and analyzed by SDS-polyacrylamide slab gel electrophoresis.

Incubated with thrombin, 10 pg/ml, at 37 C, calcium binding. Protein L, S, and unreduced samples; A, B, and C. Pool B contained a high concentration of Gla residues, and the amino acid sequence of the peptide was identical to that of intact protein S. Thus, this pool represents the NH2 terminus of protein S. Pools A and C had low Gla contents, and the amino acid sequences were different from that of intact protein S.

Amino Acid Sequence of the 100 Amino-terminal Residues in Protein S—The sequence of the 100 NH2-terminal residues of protein S was deduced (Fig. 3) from amino acid sequence analysis of peptides isolated after digestion of protein S with thrombin, Staphylococcus aureus V8 protease, and chymotrypsin, and after cleavage with cyanogen bromide. The fragments used in the derivation of the sequence are shown in Fig. 4. From the chromatography illustrated in Fig. 2, pools A, B, and C corresponded to the fragments TH3, TH1, and TH2, respectively. The COOH-terminal Arg residue in TH1 was identified by carboxypeptidase Y digestion. Several other fragments were also analyzed in the sequenator, and the results were in agreement with those presented. The positions of the Gla residues are given in Fig. 3. Position 95 is occupied by a beta-hydroxyaspartic acid residue. Presumably due to its asymmetric beta-carbon atom, the phenylthiohydantoin derivative of Hya appeared as a typical doublet peak (38) early in the high pressure liquid chromatogram after sequenator degradation of fragment CB1 in the Beckman Sequencer. Its position was confirmed by Hya analysis of a fragment containing residues 95–105, isolated after tryptic digestion of fragment GL3.

Thrombin cleaves two peptide bonds in protein S. On polyacrylamide gel electrophoresis analysis of reduced samples (Fig. 1) from the thrombin digestion of protein S, the 18-kDa peptide that appeared first was further cleaved to the 16-kDa species, demonstrating that the first cleavage is between Arg 70 and Ser 71 and the second between Arg 52 and Ala 53. The Gla peptide (residues 1–52) contains three cysteine residues, whereas the peptide, residues 53–70, has no cysteine residue and is released from thrombin-cleaved protein S under nonreducing conditions. However, the small molecular weight difference between intact and thrombin-modified protein S was not detected on polyacrylamide gel electrophoresis before reduction of disulfide bonds (Fig. 1).

then analyzed by agarose gel electrophoresis in the presence of 2 mM calcium to study the effect on calcium binding.
Thrombin Cleavage Sites in Protein S

Ala Asn Thr Leu Leu Gla Thr Lys Lys Gly Asn Leu Gla Cys Ile Gla Gla Cys Lys Gla Gla Ala Arg Gla Ile 40
Phe Gla Asn Asn Pro Gla Thr Glu Tyr Phe Tyr Pro Lys Tyr Leu Gly Cys Leu Gly Ser Phe Arg Ala Gly Leu Phe Thr Ala Ala Arg
Leu Ser Thr Asn Ala Tyr Pro Asp Leu Arg Ser Cys Val Asn Ala Ile Ser Asp Gln Cys Asn Pro Leu Pro Cys Asn Glu Asp Gly Phe 70
Met Thr Cys Lys Hya Gly Gin Ala Thr Phe

Fig. 3. Amino acid sequence of the amino-terminal part of protein S.

DISCUSSION

We now demonstrate that two peptide bonds in protein S, both located close to the Gla-rich NH₂-terminal portion of the molecule, are cleaved by thrombin. Our conclusions are drawn from analysis of the isolated peptides formed by thrombin digestion of protein S and positioning of the cleavage sites at Arg 52-Ala 53 and at Arg 70-Ser 71. Recently, it was suggested that the thrombin cleavage site in protein S was close to the C-terminal end of the protein (8). This conclusion was based on end group analysis of intact protein S and of the isolated heavy chain of thrombin-modified protein S. Our results show this proposal to be untenable. On reduced polyacrylamide gels we could demonstrate that a peptide with an apparent molecular weight of 18 kDa is formed early during the reaction and that it is further processed to a 16-kDa species. Sequence analysis indicated that the first peptide bond to be cleaved is between Arg 70 and Ser 71. The anomalously high molecular weight of the peptide on SDS-polyacrylamide gel electrophoresis is presumably due to the high

Protein S
Protein C
Factor IX
Factor X
Protein Z
Prothrombin

FTAA R L S T N A Y P D LR S C V N A I S D Q C N P 60
S D G D Q C E D R P S G P C D L P C C G R G K C I S G L G G P 70
V D G D Q C E S N P C L N G G M C K S D I N S Y 80
K D G D Q C E G H P C L N G G H C K S I G D Y 90
M G G S P C A S Q P C L N G G S C O S S I R G Y 100

Fig. 5. Sequence comparison of the amino-terminal 105 amino acid residues of protein S with the corresponding regions in the vitamin K-dependent clotting factors. The numbers refer to protein S, and the standard IUPAC one-letter code for amino acid residues was used. γ, γ-carboxyglutamic acid residues; δ, δ-hydroxyaspartic acid residues. The blocks enclose residues identical with protein S. The sequences of protein C, factor IX, factor X, protein Z, and prothrombin were from Refs. 31, 41, 42, 43, and 44, respectively.
negative charge of the peptide interfering with its SDS binding.

The Gla region in protein S has a high degree of homology to the Gla regions of the other vitamin K-dependent plasma proteins (Fig. 5). The region in protein S between residues 45 and 74, which contains the two thrombin cleavage sites, has no counterpart in the other proteins, whereas after residue 74 the homology with factors IX, X, protein C, and protein Z is again pronounced. These parts of the molecules are also homologous to a domain in epidermal growth factor (39) and to a domain in the low density lipoprotein receptor (40). The cysteine residue in position 47 links the Gla domain to the C-terminal fragment. This proposal is based on the homology with the other vitamin K-dependent plasma proteins in which Cys residues corresponding to Cys 17 and Cys 22 have been shown to be disulfide linked. Furthermore, chymotryptic digestion of protein S, which results in several cleavages around position 40, also removes the Gla region, indicating that Cys 17 and Cys 22 are also disulfide linked in protein S (45).

One of the functions of protein S in plasma appears to be that of cofactor to activated protein C in the regulation of coagulation (15-16). The protein S cofactor function is completely lost after thrombin cleavage (8, 23). This is presumably due to the drastic loss in affinity of protein S for calcium ions. In agreement with this, Walker (8) recently demonstrated that thrombin-modified protein S, unlike intact protein S, had no affinity for negatively charged phospholipids. Although inhibited by calcium ions, the thrombin cleavage readily takes place during coagulation of platelet-rich plasma (4), and it may be an important regulatory mechanism of the anticoagulant effect of activated protein C.

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REFERENCES


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Molecular weight markers for polyacrylamide gel electrophoresis and Sepharose 4 B-2B were obtained from Pharmacia Fine Chemicals. Proteinase K (Boehringer) and trypsin (Calbiochem) were used under conditions specified by the suppliers. The specificity of the proteases was demonstrated using CNBr-digested prothrombin as described previously (31). Protein was assayed by the method of Lowry and co-workers (32) using bovine serum albumin as a standard.

Preparation of protein S fragments. Prothrombin (5 mg/ml), 12.5 mM Tris pH 8.3, 100 mM NaCl, 2 mM CaCl₂, was incubated at 37°C for 2 h in the absence of thrombin. The digest was separated on a column (1.6 x 55 cm) of Sephadex G-100 and the active fractions were used without further purification (33). The proteinase K was added to the reaction mixture to a final concentration of 250 μg/ml. After lyophilization, the protein was reduced and carboxamidomethylated as described previously (31). The proteinase K was then removed by passage through Sephadex G-100 (32%). The reduced protein was dissolved in a solution containing 50 mM Tris pH 8.3, 100 mM NaCl, 2 mM CaCl₂, and dialyzed against buffer A (50 mM Tris pH 8.3, 100 mM NaCl, 2 mM CaCl₂) for 2 h. The lyophilized protein (10 μg) was solubilized in 0.5 ml of buffer A containing 50 mM Tris pH 8.3, 100 mM NaCl, 2 mM CaCl₂, and 10% glycerol. After dialysis against the same buffer in the presence of 20% ethylene glycol, the protein was lyophilized and stored at -20°C.

MATERIALS AND METHODS

Localization of Thrombin Cleavage Sites in the Amino-Terminus Region of Prothrombin S

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Table I: Automated sequence analysis of peptides TH1 and TH2

<table>
<thead>
<tr>
<th>Degradation cycle</th>
<th>Identity</th>
<th>Yield (mol)</th>
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<tbody>
<tr>
<td>2.5 mmol TH1</td>
<td>Ala</td>
<td>0.22</td>
</tr>
<tr>
<td>2.5 mmol TH2</td>
<td>Ile</td>
<td>0.29</td>
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Fig. 8. Separation of protein S peptides formed by thrombin digestion. Reduced and carboxymethylated thrombin-digested fragments were analyzed by paper chromatography and by electrophoresis. The active fragments were separated by electrophoresis in a 25% polyacrylamide gel containing 0.1% SDS, which were then transferred to a nitrocellulose membrane. The protein was digested with trypsin and the fragments were eluted from the membrane with 0.1 N KCl. After dialysis the peptides were lyophilized and subjected to electrophoresis in a 25% polyacrylamide gel containing 0.1% SDS. The positions of the peptides were identified by the bars.

Table II: Automated sequence analysis of peptide TH1

<table>
<thead>
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<th>Degradation cycle</th>
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<th>Yield (mol)</th>
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<tbody>
<tr>
<td>10 mmol TH1</td>
<td>Ser</td>
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<tr>
<td>10 mmol TH2</td>
<td>Thr</td>
<td>0.84</td>
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</table>

Table III: Automated sequence analysis of peptide TH2

<table>
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<tr>
<th>Degradation cycle</th>
<th>Identity</th>
<th>Yield (mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 mmol TH1</td>
<td>Thr</td>
<td>0.84</td>
</tr>
<tr>
<td>40 mmol TH2</td>
<td>Thr</td>
<td>0.84</td>
</tr>
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