Sulfation of a Tyrosine Residue in the Plasmin-binding Domain of \( \alpha_2 \)-Antiplasmin

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Glen Hortin‡§, Kam F. Fok∥, Paul C. Toren**, and Arnold W. Strauss†‡‡

From the Departments of ‡Biological Chemistry and ∥Pediatrics, §Division of Laboratory Medicine, Washington University School of Medicine, St. Louis, Missouri 63110 and the Monsanto Corporation, ‡Chesterfield, Missouri 63198 and **St. Louis, Missouri 63167

Sulfation of human \( \alpha_2 \)-antiplasmin, the major plasma inhibitor of fibrinolysis, was examined using both protein isolated from human plasma and protein synthesized and biosynthetically labeled with \([35S]\)sulfate by a human hepatoma-derived cell line. Linkage of sulfate to tyrosine was demonstrated by recovery of labeled tyrosine sulfate after base hydrolysis of sulfate-labeled \( \alpha_2 \)-antiplasmin. Analysis by reverse-phase high performance liquid chromatography of peptides released from \( \alpha_2 \)-antiplasmin by cleavage with trypsin or cyanogen bromide indicated that sulfate is linked to a single segment of the protein. A cyanogen bromide peptide corresponding to the sulfate-labeled peptide was prepared from \( \alpha_2 \)-antiplasmin isolated from human plasma. Consistent with the presence of tyrosine sulfate in this peptide, its chromatographic elution was altered by treatment with acid under conditions which release sulfate from a tyrosine residue. No peptide in the total digest of \( \alpha_2 \)-antiplasmin by cyanogen bromide eluted at the position of the peptide following desulfation, suggesting that all of the protein is in a sulfated form. The sequence of the sulfate-containing cyanogen bromide peptide as determined by sequential Edman degradation, amino acid composition, and fast atom-bombardment-mass spectrometry was: Glu-Glu-Asp-Tyr(\(SO_4\))-Pro-Gln-Phe-Gly-Ser-Pro-Lys-COOH. This peptide is a segment of the previously identified plasmin-binding domain of \( \alpha_2 \)-antiplasmin.

Occurrence of tyrosine sulfate as an amino acid residue in proteins was considered to be vary rare, limited to fibrinogen, until 1982, when Huttner reported evidence that every tissue in rats contains a number of proteins with sulfate groups. Subsequent studies by Huttner (2) demonstrated, further, that sulfation of tyrosine residues in proteins occurs in a diverse range of organisms. These findings stimulated efforts to identify proteins that contain tyrosine sulfate. Ensuing studies have identified a substantial number of proteins that contain tyrosine sulfate. Examples of human proteins noted to contain this amino acid residue are: the fourth component of complement (3, 4), fibrinogen (5), fibrinectin (6), \( \alpha \)-fetoprotein (5), and heparin cofactor II (7). Although a considerable number of proteins containing tyrosine sulfate have been identified, in most cases, the sites and stoichiometry of sulfation have not been determined. Structural characterization of sulfation sites has been reported for only three proteins, fibrinogen (in a number of animal species) (8), the fourth component of human complement (3), and bovine coagulation factor X (9). Studies by our laboratory (3, 7, 10) have been directed at expanding the limited structural data on sites of sulfation by identifying human plasma proteins that contain sulfate and by performing detailed structural analysis of their sulfation sites. Major objectives are to clarify the structural specificity of the sulfation of proteins and to determine the effect of sulfation on protein function. A continuing effort has been made to identify a 75,000-Da protein which was previously noted to be one of the major sulfate-containing proteins secreted by a human hepatoma-derived cell line (10). Possible identity of the product was suggested by a recent report by Lijnen and co-workers (11), who detected sulfate linked to \( \alpha_2 \)-antiplasmin. The present paper describes a detailed analysis of the sulfation of this protein, which functions as the major physiological inhibitor of plasmin circulating in blood (12-14). Structural analysis of \( \alpha_2 \)-antiplasmin indicated that sulfate is linked to a single site, to the tyrosine residue nearest the COOH terminus of the protein. The site of sulfation of \( \alpha_2 \)-antiplasmin is within a 26-residue segment of the protein that is of particular functional significance because it comprises a plasmin-binding domain (15).

MATERIALS AND METHODS AND RESULTS

DISCUSSION

The present study performs a detailed characterization of the sulfation of human \( \alpha_2 \)-antiplasmin, identifying stoichiometric sulfation of a single tyrosine residue. Obtaining structural data on proteins containing tyrosine sulfate, as presented here, is an important step in understanding the site specificity and the biological function of this widespread modification of proteins. Previously, the positions of sulfate-containing tyrosine residues have been determined in only a few proteins (3, 7-9). The present study clearly demonstrates the high degree of site specificity of the sulfation of tyrosine residues. Only one of several tyrosine residues in \( \alpha_2 \)-antiplasmin

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† Supported by an Individual National Research Service Award from the National Institutes of Health. To whom correspondence should be addressed.

1 Portions of this paper (including "Materials and Methods," "Results," Figs. 2-6, and Table 1) 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. 86M-3358, 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.
min is sulfated. The issue of site specificity has been analyzed in detail for only two other proteins, the fourth component of complement (3) and heparin cofactor II (7), with similar results. In C4, sulfate is transferred only to 3 closely grouped tyrosine residues out of a total of more than 50 tyrosine residues in the protein (3), and, in heparin cofactor II, sulfate is specifically added to 2 out of a total of 12 tyrosine residues (7). High specificity of the process of sulfation is also inferred from the experimental observation that no sulfate is transferred to some proteins such as albumin and the third component of complement in detail for only two other proteins, the fourth component of complement.

The unique site of sulfation is located within the 8000-Da peptide which is excised from α2-antiplasmin when this protein forms a covalent complex with plasmin. Also, as α2-antiplasmin circulates in blood, it is converted to an inactive form lacking the plasmin-binding domain (22). The inactive form is about 8000 Da smaller than the active form; the precise COOH terminus of the inactive form has not been identified. Normally, about 70% of α2-antiplasmin circulates as the active form and 30% as the inactive form (22). Sulfate-labeled protein would serve as an ideal substrate for monitoring the conversion from the active to inactive form and for examining the peptide released by this process.

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Sulfation Site of ac-Antiplasmin

Glen Horst, Karen F. Ink, Paul C. Gyurts, and Arnold W. Strauss

MATERIALS AND METHODS

Antiserum to α2-antiplasmin was purchased from Calbiochem-Behring. α2-Antiplasmin was purified from human plasma by affinity chromatography as described by Wiseman (22). Also, purified α2-antiplasmin was generously provided by Dr. H. R. Libby (Cincinnati Thrombosis and Vascular Research, Cincinnati, Ohio). Tryptsin was purchased from Calbiochem-Behring. Antiserum against human α2-macroglobulin from Bethesda Research Laboratories, and antiserum for HPLC from Beckman. Carrier-free 35S-labeled and 3H-labeled α2-macroglobulin (Calf-Serum) were obtained from ICN and American Radiolabeled Chemicals, respectively. HepG2 cells were obtained from Dr. Barbara Knowles (Wisconsin Institute). Cysteine bromonicotinate was purchased from Sigma Chemical Company.

Methods used for cell culture, immunoprecipitation of radioiodinated proteins, and polyacrylamide gel electrophoresis have been described previously (3). Tryptic digestion of α2-antiplasmin was performed on protein in fragments of a polyacrylamide slab gels as previously described (19). A new procedure for cleavage of proteins with cyanogen bromide was developed to minimize release of sulfated bond to tyrosine residues. Protein was dissolved in 6 M guanidine hydrochloride containing 1 M acetic acid. Cyanogen bromide was dissolved in the solution and added to the sample to give a final concentration of 10 mM. Sample tubes were gassed with N2 and incubated at 37 °C (3 h). Samples were then diluted with 100 times their original volume. Following centrifugation at 10,000 x g for 1 h to remove particulate material, samples were analyzed by reverse-phase high-performance liquid chromatography (HPLC) using a 3.9 mm X 30 cm Bondapak C18 column (Waters Associates) eluted with a gradient of acetonitrile in water (3). The high viscosity of samples containing 4 M guanidine hydrochloride raised a temporary increase in column pressure. Accordingly, the flow rate was increased to 0.5 ml/min during sample injection. After the column pressure returned to normal, the flow was increased to 1 ml/min and the elution gradient was begun.

Basic hydrolysis of protein utilizing 1 M NaOH. Conditions for hydrolysis were similar to those described for analysis of carbodiimide-acid in protein (2). Briefly, samples were diluted in 0.5 ml of base of polypropylene tubes and heated at 100 °C for 30 min. Samples were cooled in an ice-bath and desalted by passing through a 1 ml column of Dowex 50 X 8 (105) form which was eluted with water. The column was washed with 10 ml of water. The concentration of 0.1 M NaH2PO4 adjusted to pH 3.0 with H3PO4. A gradient of buffer concentration was used for elution: 0.05 M, 0.15 M, 0.25 M, then to 0.3 M, 0.4 M, linear increase to 130 ml. Fractions of 0.5 ml were collected.

Sequential tryptic digestion of labeled protein was performed with a Beckman 99C sequencer using a 0.31 M Quilidex program (3). Sequence analysis of unlabeled protein was carried out with Applied Biosystems 473A Sequencer (24). Amino acid composition of peptides was determined with a Beckman System 6300 amino acid analyzer after vapor-phase hydrolysis with 6 N HCl at 120 °C for 24 h. The amino acid analyzer was a model 6300 equipped with a model 2205 injector. The samples were hydrolyzed and analyzed for 4 h. After the samples were hydrolyzed, the column was reequilibrated with distilled water for 24 h. The samples were then analyzed for tryptic peptide content in the sample. The sequence analysis of the labeled protein was performed as the same way.

RESULTS

Using antiserum to α2-antiplasmin, a single labeled protein was isolated from culture medium of HepG2 cells treated with 35S-labeled α2-antiplasmin. The protein had an apparent molecular weight of 75,000 Daltons as measured by SDS-polyacrylamide gel electrophoresis in reducing conditions (Fig. 2). This agrees with previous electrophoretic estimations of the size of α2-antiplasmin (25). Antisera directed against other peptides did not precipitate this protein (see Refs 3, 10).

Fig. 2. Autoradiographs of sulfation-labeled α2-antiplasmin by polyacrylamide gel electrophoresis. Gels were run in the presence of 10 μg/ml α2-antiplasmin. Lanes labeled by sulfation-labeled α2-antiplasmin. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresis. A, lane L, lanes 1 and 3, lanes 2 and 4. Lanes labeled by polyacrylamide gel electrophoresi...
Sulfation Site of α2-Antiplasmin

Fig. 5. HPLC analysis of cyanogen bromide peptides from α2-antiplasmin.
Top panel. α2-Antiplasmin isolated from human plasma was reduced and carboxymethylated, cleaved with cyanogen bromide, and analyzed by reverse-phase HPLC. Bottom panel. 15Sulfate-labeled α2-antiplasmin secreted by HepG2 cells was analyzed similarly.

Table I

| Amino Acid Composition | Sequential Edase Degradation |
|------------------------|-----------------------------|
| Acid (m/z)             | Cycle | Residue Yield |
| Asp 1.1                | 1     | Gly 1.580 pmol |
| Ser 0.9                | 2     | Glu 1.735     |
| Glu 2.7                | 3     | Asp 1.773     |
| Pro 2.1                | 4     | Tyr 0.413     |
| Glu 0.9                | 5     | Pro 1.136     |
| Tyr 0.9                | 6     | Leu 0.852     |
| Pro 1.1                | 7     | Glu 1.027     |
| Asp 1.1                | 8     | Ser 0.745     |
| Ser 0.9                | 9     | Tyr 0.413     |
| Glu 2.7                | 10    | Pro 3.552     |
| Pro 1.1                | 11    | Gly 2.422     |

Table values for the amino acid compositions of the peptide were based on the sequence analysis and published values (11).

The sequence of the cyanogen bromide peptide with the attachment of sulfate to it were confirmed by fast atom bombardment mass spectrometry (Fig. 6, Miniprint). Three peaks at 1297, 1378, and 1398 daltons, were observed in the high molecular weight segment of the spectrum. The mass of the first peak matches the calculated molecular weight of a peptide with the sequence of the cyanogen bromide peptide from α2-antiplasmin with sulfate. The peak at 1380 daltons corresponds to the calculated mass of the same peptide with sulfate, and the peak at 1398 corresponds to the mass of the sodium salt of the sulfated form of the peptide. The greater abundance of the sulfated form of ion is not an indication that a major fraction of the peptide lacked sulfate, because there was considerable loss of sulfate from peptides during elution by fast atom bombardment. Under our analytical conditions, chymotryptic α2-antiplasmin, which contains a single tryptic sulfated residue, yielded a ratio of sulfated/unsulfated mass ions similar to that of the cyanogen bromide peptide from α2-antiplasmin (not shown). This comparison with chymotryptic suggests that the sulfated cyanogen bromide peptide is predominantly in the sulfated form. Two previous reports of the use of fast atom bombardment to analyze peptides containing tryptic sulfates also reported substantial loss of the sulfates from peptides during elution (16, 17). These two studies differ from ours in that negative ion detection was used. In our experiments, positive ion detection yielded higher sensitivity. Using the same quantity of peptide as analyzed in Fig. 6, negative ion detection resulted in less above background noise.

Fig. 6. Mass spectrum of the cyanogen bromide peptide from α2-antiplasmin. Human α2-antiplasmin was cleaved with cyanogen bromide and analyzed by HPLC as in Fig. 5. The peptide corresponding with the sulfated peptide was subjected to fast atom bombardment mass spectrometry with positive ion detection. Approximately 1 nmol of peptide was analyzed.

The stoichiometry of the sulfation of the cyanogen bromide peptide was investigated using en-NAME HPLC. Sulfated and unsulfated forms of the peptide were clearly separated. Removal of the sulfate by heating the peptide briefly in 0.1% trifluoroacetic acid increased its retention time by 3 min (not shown). Similarly, we did not observe any peak in the analysis of the total cyanogen bromide yield of α2-antiplasmin which corresponded to the position of the unsulfated peptide. Only a single sharp peak, corresponding to the position of the sulfated peptide, was found in this segment of the chromatogram (see Fig. 7. Miniprint). Similar results were obtained using α2-antiplasmin purified from a single individual and from plasma pooled from 20 donors. Thus, virtually all molecules of α2-antiplasmin are sulfated, and there is probably little variation among normal individuals in the sulfation of α2-antiplasmin.