Antithrombin is a major proteinase inhibitor of the blood coagulation system. Its inherited deficiency or abnormality is often associated with thromboembolism. Antithrombin “Northwick Park,” a functionally inactive variant antithrombin, has recently been shown by us (Lane, D. A., Flynn, A., Ireland, H., Erdjument, H., Samson, D., Howarth, D., and Thompson, E. (1987) Br. J. Haematol. 65, 451--456) to be present in plasma, in part, as a high M, (~120,000) component which has a characteristic electrophoretic mobility in agarose gels in the absence of denaturing agents. In this communication, we present evidence that this M, ~120,000 variant component is comprised of an antithrombin-albumin covalent disulfide-linked complex. This proposal is supported by results of: (a) fast atom bombardment mass spectrometry of the isolated reduced, S-carboxymethylated, trypsin-digested M, ~120,000 complex; (b) sodium dodecyl sulfate-polyacrylamide gel electrophoresis of this complex and its reduced and S-carboxymethylated constituents; (c) immunoblotting of these polyacrylamide gels with antisera specific for antithrombin and albumin; (d) NH4-terminal sequence analysis of one of the isolated, S-carboxymethylated proteins that comprise the M, ~120,000 complex; and (e) fast atom bombardment mass spectrometry of its tryptic peptides.

Antithrombin is a major physiological inhibitor of serine proteinases generated by the coagulation system which plays an important role in the regulation of hemostatic balance (1, 2). This single-chain plasma glycoprotein is composed of 432 amino acid residues with an estimated M, of ~58,200 by calculation from the amino acid sequence and carbohydrate structure (3-6). Congenital deficiency of antithrombin is associated with familial thromboembolism and has been reported since 1965 (7). Sas et al. (8) described the first antithrombin variant with a normal amount of circulating antigen, but an impaired functional activity. Many congenital antithrombin variants, characterized either by defective interaction with serine proteinases or binding to heparin, have subsequently been reported, but in only three have the molecular abnormalities been identified (9-11). A family with an antithrombin variant, “Northwick Park,” associated with inherited thromboembolism was shown to have an impaired ability to inhibit thrombin (12-15). Our preliminary characterization (15) has demonstrated that this variant antithrombin circulates in part as an inactive high M, component with increased affinity for heparin. In this communication, we present evidence that this high M, component is a covalent disulfide-linked antithrombin-albumin complex.

**MATERIALS AND METHODS**

Previously collected and frozen citrated plasma from normal volunteers and the propositus (14, 15) was subjected to preliminary precipitation with dextran sulfate and applied to heparin-Sepharose (Pharmacia Biotechnology, Inc.) equilibrated with 0.1 M Tris/HCl, pH 7.4, containing 0.14 M NaCl (16). Antithrombin was eluted from the column with 2.5 M NaCl in 0.1 M Tris/HCl, pH 7.4, after overnight washing with 0.4 M NaCl in 0.1 M Tris/HCl, pH 7.4 (17). Antithrombin was further purified by anion-exchange chromatography using a monobead column, Mono Q HR 5/5 (Pharmacia Biotechnology, Inc.), and fast protein liquid chromatography (FPLC)1 delivery system (Pharmacia Biotechnology, Inc.) with NaCl elution (15).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions with dithiothreitol (DTT, Sigma) and nonreducing conditions using 10-15% gradient Phast Gels (Pharmacia Biotechnology, Inc.). Immunoblotting was essentially as described by Bowen et al. (18), with the extremely thin gels used in the Phast System (Pharmacia Biotechnology, Inc.) and the sensitive development technique allowing blotting onto nitrocellulose to take place by diffusion. Rabbit anti-human albumin (Behring Diagnostics) and rabbit anti-human antithrombin (Dako) were employed, the former being subjected to a preliminary adsorption step with normal antithrombin. Antiserum diluted in 0.1 M Tris/HCl, 0.15 M NaCl, pH 7.4, containing 5% skimmed milk powder were employed in the first immunological stage following transfer of the protein bands onto the nitrocellulose. Visualization of specific antiserum was achieved with peroxidase-conjugated swine anti-rabbit immunoglobulin (Dako) followed by final peroxidation of diaminobenzidine (Sigma) hydrochloride, essentially as described by Hadfield and Glynn (19).

S-Carboxymethylation of antithrombin was carried out under nitrogen in 0.6 M Tris/HCl buffer, pH 8.5, after initial reduction with 5-fold molar excess (over cysteine residues) of DTT followed by incubation at 37 °C for 30 min (20). Iodoacetic acid (Sigma, 20-fold molar excess over cysteine residues) was used for alkylation at 37 °C for 1 h followed by acidification to pH 4 using glacial acetic acid. Desalting of the S-carboxymethylated protein was achieved by reverse-phase HPLC (see below), followed by lyophilization.

HPLC of reduced, S-carboxymethylated protein was performed using C8 ProRPC 5 μm HR 5/5 column (Pharmacia Biotechnology, Inc.) with the FPLC delivery system. A gradient of 0-45% acetonitrile (HPLC grade, BDH Chemicals) with 0.1% trifluoroacetic acid (Spectrograde, BDH Chemicals) was used for elution, initial equilibration being achieved with water purified using a Millipore Q2 system and buffered with 0.1% trifluoroacetic acid. Detection was at 214 nm and

1 The abbreviations used are: FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; FAB, fast atom bombardment; DTT, dithiothreitol.
RESULTS AND DISCUSSION

Initial isolation of antithrombin from plasma was achieved by stepwise NaCl elution from heparin-Sepharose, a procedure adopted in preference to gradient elution in order to maximize the yield of antithrombin for further purification. Normal antithrombin isolated in this fashion produced a single elution peak on anion-exchange chromatography. Antithrombin from the propositus, however, could be resolved into two heterogeneous peaks, designated here as Northwick Park peak 1 (NWP peak 1) and Northwick Park peak 2 (NWP peak 2), in order of elution from the column with NaCl. Both of these peaks have been shown previously to react with anti-antithrombin antiserum by crossed immunoelectrophoresis (15) (see also below).

NWP peak 1 has been shown to have \( M_r \) indistinguishable from that of normal antithrombin (\( M_r \sim 60,000 \)) on SDS-PAGE, while NWP peak 2 has mainly \( M_r \sim 120,000 \) (see also below). It elutes later than normal antithrombin from heparin-Sepharose when gradient NaCl elution is employed, and is functionally inactive regarding thrombin inhibition (15). Following reduction, S-carboxymethylation, and trypsin digestion of NWP peak 2, numerous peptide signals not characteristic of antithrombin were observable on FAB mapping of the digest. Preliminary NH\(_2\)-terminal sequence analysis of a component isolated from S-carboxymethylated NWP peak 2 not subjected to trypsin digestion suggested a similarity of this component to albumin (see also below). We therefore compared the signals from FAB mapping of the tryptic digest of S-carboxymethylated NWP peak 2 that could not be assigned to antithrombin to the primary structure of human albumin (23). It was found that these peptide signals could account for more than 70% of the primary structure of albumin (Table I). Taken together with our previous finding that NWP peak 2 reacted with anti-antithrombin antiserum (15), these observations suggested that NWP peak 2 contains a complex of albumin and antithrombin.

This suggestion was corroborated and elaborated using SDS-PAGE in combination with immunoblotting with antisera raised against human albumin and human antithrombin. Under nonreducing conditions, NWP peak 1 had similar electrophoretic mobility to that of normal antithrombin, migrating as a single protein band with Coomassie staining (Fig. 1, lanes 1c and 1a) and reacting with anti-antithrombin antiserum and minimally with anti-albumin antiserum (Fig. 1, lanes 2c and 3c, respectively); see legend to Fig. 1 for comment on the reactivity of normal antithrombin with anti-antithrombin antiserum. NWP peak 2 was mainly comprised of a major band that migrated with \( M_r \), exceeding that of normal antithrombin (~120,000 versus ~60,000) on Coomassie staining (Fig. 1, lane 1b), but also contained a minor band with the same mobility as normal antithrombin. These major and minor components along with other high \( M_r \) bands reacted with antisera specific to both albumin and antithrombin (Fig. 1, lanes 3b and 2b, respectively).

The same antithrombin preparations were also studied by SDS-PAGE when electrophoresis was carried out in the presence of a reducing agent, DTT. As expected, reduced, normal antithrombin and NWP peak 1 both migrated as single major bands on Coomassie staining (Fig. 2, lanes 1a and 1c, respectively), with indistinguishable mobility, which reacted only

| Fragment size (M + H) | Amino acid sequence in human albumin | Fragment size (M + H) | Amino acid sequence in human albumin |
|-----------------------|-------------------------------------|-----------------------|-------------------------------------|
| 951                   | 13-20                               | 1386†                 | 263-274                             |
| 2490                  | 21-41                               | 2974                  | 297-313                             |
| 1149                  | 42-51                               | 1623                  | 324-336                             |
| 1017                  | 65-73                               | 1467                  | 337-348                             |
| 934                   | 74-81                               | 984                   | 352-359                             |
| 1076                  | 99-106                              | 1439†                 | 360-372                             |
| 940                   | 107-114                             | 2044                  | 373-388                             |
| 2649                  | 115-136                             | 1658                  | 390-402                             |
| 927                   | 138-144                             | 960                   | 403-410                             |
| 1742                  | 146-159                             | 1639                  | 414-428                             |
| 773                   | 175-181                             | 1511                  | 415-428                             |
| 645                   | 182-186                             | 1264                  | 446-466                             |
| 508                   | 206-209                             | 674                   | 467-472                             |
| 673                   | 213-218                             | 1140                  | 476-484                             |
| 503                   | 219-222                             | 1911                  | 485-500                             |
| 391                   | 223-225                             | 1000                  | 526-534                             |
| 880                   | 226-233                             | 1342                  | 546-557                             |
| 789                   | 234-240                             | 1013                  | 575-585                             |
| 2031†                 | 241-257                             |                       |                                     |

* Mass (m + 1) corresponding to the tryptic fragment of albumin containing incompletely S-carboxymethylated cysteine residues.
with anti-antithrombin (Fig. 2, lanes 2a and 2c, respectively). In contrast, reduced NWP peak 2 migrated as two closely spaced, nonidentical bands on Coomassie staining (Fig. 2, lane 1b), the most mobile of which had $M_r$ indistinguishable from that of normal antithrombin (Fig. 2, lane 1a). This band of NWP peak 2 reacted only with anti-antithrombin antiserum, Fig. 2, lane 2b, while the higher $M_r$ component reacted only with anti-albumin antiserum, Fig. 2, lane 3b.

The electrophoretic and immunoblotting results in Figs. 1 and 2 all corroborated the suggestion that NWP peak 2 found in the propositus' plasma, is predominantly a covalent disulfide-linked complex of antithrombin and albumin of $M_r \sim 120,000$. The covalent complex may also have a small amount of albumin and antithrombin noncovalently associated with it. Such noncovalently associated proteins could explain the minor immunoreactive bands of NWP peak 2 observable on SDS-PAGE without reduction (see Fig. 1, lane 3b) that have $M_r \sim 60,000$. Noncovalent association of albumin and antithrombin could be caused either as a consequence of the molecular abnormality of the variant antithrombin or by copurification of trace amounts of albumin with the variant. Support for this latter view is provided by occasional trace contamination of normal antithrombin preparations with albumin, visible only on immunoblotting with anti-albumin antisera (not illustrated). However, it should be noted that high $M_r$ complexes ($M_r \sim 120,000$) of the type illustrated in Fig. 1, reacting with both anti-antithrombin and anti-albumin antisera, have been observed in no normal antithrombin preparation, nor indeed in antithrombin purified from another distinct variant familial antithrombin, antithrombin “Glasgow” (17).

Further evidence to support the proposal of a covalent antithrombin-albumin complex in the patient’s plasma was obtained in experiments designed to separate the component parts of NWP peak 2. Chromatography of reduced, S-carboxymethylated normal antithrombin on a ProRPC 5/5 column produced a single peak (Fig. 3a). Reduced S-carboxymethylated NWP peak 2, however, could be readily resolved into two major components, designated here as NWP peak 2.1 and NWP peak 2.2 (Fig. 3b). Minor components of variable concentration also eluted around and between NWP peaks 2.1 and 2.2, Fig. 3b.

SDS-PAGE and immunoblotting were used to identify these two major S-carboxymethylated protein peaks from NWP peak 2. S-Carboxymethylated normal antithrombin migrated as a doublet (possibly caused by incomplete S-carboxymethylation) on Coomassie staining (Fig. 4, lane 1a), which reacted only with anti-antithrombin antiserum (Fig. 4, lane 2a). NWP peak 2.2 also migrated as two bands that were immunoreactive with anti-antithrombin antisera (Fig. 4, lane 2b). A trace component, migrating faster than the antithrombin bands, barely visible on Coomassie staining, was highlighted with anti-albumin antisera (Fig. 4, lane 3b). NWP peak 2.1 migrated as a heterogeneous band on Coomassie staining (Fig. 4, lane 1c), faster than the antithrombin doublet and reacted only with anti-albumin antisera (Fig. 4, lane 3c).
Formation of an Antithrombin-Albumin Complex

4, lane 3c; see also legend to Fig. 4 for comment on the mobility of NWP peak 2.1.

FAB mapping of a trypsin digest of NWP peak 2.1 produced almost identical peptide signal assignments to those of human albumin listed in Table I, while most of the tryptic peptides signals obtained from NWP peak 2.2 FAB mapping were characteristic of normal antithrombin (this mapping of NWP peak 2.2 is not yet complete). Finally, NH₂-terminal sequence analysis of NWP peak 2.1 performed by means of a gas-phase Edman degradation revealed that the sequence Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Ala-Phe-Lys-Asp, which is identical to the NH₂-terminal sequence of human serum albumin (23).

In order to explain these findings, we propose that antithrombin Northwick Park (contained in NWP peak 2.2) contains an additional (or available) cysteine residue that reacts with the free cysteine in albumin, for example at residue 34 (23), during or at some time after synthesis of both molecules, to form a covalent disulfide-bridged complex of $M_r$ ~ 120,000. Degradation of this complex and/or its aggregation could further explain its heterogeneity evident upon anion-exchange chromatography and SDS-PAGE (with immunoblotting). It has been suggested that another antithrombin variant, "Milano" (24), is present in plasma as a disulfide-linked antithrombin dimer. This variant, which has many electrophoretic and chromatographic properties in common with the present case, was apparently not studied using immunoblotting with anti-albumin antisemur. Trace amounts of a dimer molecule can not be excluded in the case we have studied; however, the predominant component of the high $M_r$ complex is shown here to be comprised of albumin and antithrombin. To our knowledge, this is the first time a variant proteinase inhibitor-albumin complex of this type has been demonstrated. Given the similar structural features of those proteinase inhibitors originating from the same ancestor as antithrombin (for example $\alpha_1$-antitrypsin and $\alpha_2$-antiplasmin), the present findings may be of general importance to the inhibitor family as a whole.

A complete explanation of the nature of the disulfide bond between the antithrombin variant Northwick Park and albumin will require elucidation of the structural abnormality of the former, attempts at which are underway. This information will also illuminate the reason for its functional abnormality, consequential inherited thromboembolism, and increased heparin affinity of the antithrombin-albumin complex (14, 15).

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REFERENCES

1. Rosenblatt, R. D. (1972) Fed. Proc. 31, 10-18

2. Bjork, I. and Lindahl, U. (1982) Mol. Cell. Biochem. 48, 161-182

3. Petersen, T. E., Dudek-Wojcikowska, G., Sottrop-Jensen, L., and Magnusson, S. (1979) in The Physiological Inhibitors of Blood Coagulation and Fibrinolysis (Collet, D., Wiman, B., and Verstraete, M., eds) pp. 43-54, Elsevier/North-Holland, Amsterdam

4. Bock, S. C., Wison, K. L., Vedur, G. A., and Lawn, R. M. (1982) Nucleic Acids Res. 10, 8113-8125

5. Chandra, T., Stackhouse, R., Kidd, V. J., and Woo, S. L. C. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1845-1848

6. Prochownik, E. V., Markham, A. F., and Orkin, S. H. (1983) J. Biol. Chem. 258, 8396-8399

7. Egeberg, O. (1965) Thromb. Diath. Haemorrh. 13, 516-530

8. Sas, G., Hlasko, G., Banhegyi, D., Jako, J., and Palos, L. A. (1974) Thromb. Diath. Haemorrh. 32, 105-115

9. Koide, T., Odani, S., Takahashi, K., Oro, T., and Sakuragawa, N. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 289-293

10. Chang, J., and Tsw, T. H. (1986) J. Biol. Chem. 261, 1174-1176

11. Stenbury, A. W., Thalley, B. S., and Hirs, C. H. W. (1987) J. Biol. Chem. 262, 1044-1048

12. Burovči, M., Stirling, Y., and Hamlyn, A. N. (1985) Thromb. Haemostasis 54, 77-79

13. Saxon, D., Stirling, Y., Wolfe, L., Howarth, D., Seghatchian, M., and Dr Chazal, R. (1984) Br. J. Haematol. 56, 243-249

14. Howarth, D. J., Saxon, D., Stirling, Y., and Seghatchian, M. J. (1985) Thromb. Haemostasis 53, 314-310

15. Lane, D. A., Flynn, A., Ireland, H., Erdjument, H., Saxon, D., Howarth, D., and Thompson, R. (1987) Br. J. Haematol. 65, 421-456

16. McKay, E. J. (1981) Thromb. Res. 21, 375-372

17. Lane, D. A., Lowe, G. D. O., Flynn, A., Thompson, E., Ireland, H., and Erdjument, H. (1985) Br. J. Haematol., in press

18. Bowen, R., Steinberg, J., Laemmli, U. K., and Weinstenb, H. A. (1980) Nucleic Acids Res. 8, 1-20

19. Hadfield, S. G., and Gryn, A. A. (1984) Immunology 51, 615-621

20. Morris, H. R., Pasino, M., and Taylor, G. W. (1983) Biochem. Biophys. Res. Commun. 117, 296-305

21. Morris, H. R., Pasino, M., Barier, M., Bordoli, R. S., Sedgwick, R. D., and Tyler, A. N. (1981) Biochem. Biophys. Res. Commun. 104, 621-631

22. Mueckler, M., Caruso, C., Baldwin, S. A., Pasino, M., Hlencz, I., Morris, H. R., Allard, W. J., Lienhardt, G. E., and Lodish, H. F. (1985) Science 229, 941-945

23. Meloun, B., Merasek, L., and Kostka, V. (1975) FEBS Lett. 58, 134-137

24. Wolf, M., Boyer, C., Tripioli, A., Meyer, D., Larrieu, M. J., and Mannucci, P. M. (1985) Blood 65, 495-500