Characterization of Human, Bovine, and Horse Antithrombin III†

Kotoku Kurachi, Gottfried Schmer, Mark A. Hermodson, David C. Teller, and Earl W. Davie*

ABSTRACT: A comparison of the physical-chemical properties of human, bovine, and horse antithrombin III has been made. These three plasma proteins are strong inhibitors of bovine factor X_a and form a 1:1 molar complex with this coagulation enzyme. Human, bovine, and horse antithrombin III are glycoproteins containing hexose, hexosamine, and neuraminic acid. The total carbohydrate was 9, 12, and 16% for human, bovine, and horse antithrombin III, respectively. These proteins have a similar amino acid composition, although some minor variations were noted. Each antithrombin III is composed of a single polypeptide chain with an amino-terminal histidine residue. Of the first 17 amino-terminal residues, only three differences were noted between the three proteins. These occur in position 2 which is occupied by Gly, Arg, and Trp in human, bovine, and horse, respectively; position 6 which has a deletion in human antithrombin III; and position 8 where Ile in human and horse antithrombin III has been replaced by Val in the bovine preparation. The remainder of the first 17 residues is the same in all three proteins. The molecular weights for the bovine and horse preparation were 56 600 and 52 500, respectively, as determined by sedimentation equilibrium in the presence of guanidine hydrochloride. Some immunological cross-reactivity was also observed between the three different proteins.

Antithrombin III is an α_2-glycoprotein present in mammalian plasma. It is a strong inhibitor of a number of plasma serine proteases such as thrombin or factor X_a, and this inhibitory activity is stimulated 50-100-fold in the presence of heparin (Monkhouse et al., 1955; Waugh and Fitzgerald, 1956; Abildgaard, 1968; Yin et al., 1971). Individuals that have an excess of antithrombin III may have a bleeding tendency (Robinson et al., 1967), while an absence of this protein may lead to recurrent thromboses (Egeberg, 1965; Abildgaard and Egeberg, 1968; Marciniak et al., 1974; Sas et al., 1974).

Human antithrombin III has been extensively purified by Fagerol and Abildgaard (1970), Heimburger et al. (1971), and Rosenberg and Damus (1973) by procedures which employ primarily ion-exchange chromatography and gel filtration. Recently, human, bovine, and canine antithrombin III have been isolated by affinity chromatography employing heparin agarose (Miller-Anderson et al., 1974; Damus and Wallace, 1974; Thaler and Schmer, 1975). With this procedure, it is possible to prepare large amounts of highly purified protein which is suitable for chemical and biological analyses.

In this manuscript, we wish to report a comparison of the physical-chemical properties of human, bovine, and horse antithrombin III prepared by affinity chromatography (Thaler and Schmer, 1975). In an accompanying manuscript, we describe experiments dealing with the mechanism of inhibition of factor IX_a and factor X_a by this serine protease inhibitor (Kurachi et al., 1976).

Experimental Section

Materials

Bovine factor X_1 was prepared according to Fujikawa et al. (1972) and antithrombin III from human, bovine, and horse plasma by method B of Thaler and Schmer (1975). Bovine serum albumin, ovalbumin, carbonic anhydrase, cephalin (rabbit brain extract), mannose, galactose, glucosamine, galactosamine, thiobarbituric acid, *p*-dimethylaminobenzaldehyde, dithiothreitol, diisopropyl phosphorofluoridate were purchased from Sigma Chemical Co., St. Louis, Mo. Phosphorylase *b* was kindly provided by Dr. E. H. Fischer.

Sephadex G-100 was a product of Pharmacia Fine Chemicals, Piscataway, N.J. 2-Mercaptoethanol and *N,N,N',N'*-tetramethylethylenediamine were obtained from Eastman Kodak Co., Rochester, N.Y. Acrylamide was obtained from Matheson Coleman and Bell, Norwood, Ohio. Sodium dodecyl sulfate was obtained from British Drug House, Poole, England. Guanidine hydrochloride (Gdn·HCl)[1] (Spectroscopic grade) was purchased from Schwarz/Mann, Van Nuys, Calif. Phosphatidylcholine and phosphatidylserine were purchased from Applied Science Laboratories, Inc., State College, Pa. Bovine factor X deficient plasma was made by the method of Bachmann et al. (1958). All other chemicals were commercial preparations of the highest qualities available.

Methods

Protein concentration was measured by absorption at 280 nm employing an $E_{280}(1\%)$ of 5.7, 6.0, and 7.7 for human, bovine, and horse antithrombin III, respectively. The $E_{280}(1\%)$ was determined by fringe displacement according to Richards et al. (1968) and Babul and Stellwagon (1969) using a Beckman Model E analytical ultracentrifuge with a double sector cell. Amino acid analyses were performed with a Durrum Model D500 amino acid analyzer according to Moore and Stein (1963) and Spackman et al. (1958). Protein samples were hydrolyzed in 6 *N* HCl at 110° for 24, 48, 72, and 96 hr in evacuated tubes. Threonine and serine values were determined by extrapolation to zero time

† From the Departments of Biochemistry and Laboratory of Medicine, University of Washington, Seattle, Washington 98195. *Received August 19, 1975.* This work was supported in part by Grants HL 16919-01A1 and GM 15253 from the National Institutes of Health.

[1] Abbreviation used is: Gdn·HCl, guanidine hydrochloride.

hydrolysis. Valine and isoleucine values were calculated from the 96-hr hydrolysates. Tyrosine values were those determined from the 48-hr hydrolysates. Hexosamine partially overlaps with tyrosine in the amino acid analyzer, but is almost completely decomposed after 40 hr of hydrolysis. Tryptophan was determined after alkaline hydrolysis according to Hugli and Moore (1972). Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation of the proteins according to Hirs (1967). Determination of free sulfhydryl groups was performed by the method of Ellman (1959) in the presence of 6 *M* guanidine hydrochloride.

Neutral sugar was determined by the phenol method of Doubois et al. (1956). Hexosamine was determined by the Elson and Morgan reaction according to Gardell (1957). Neuraminic acid was determined by the thiobarbituric acid method according to Warren (1959).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of protein samples was performed by a modification of the method of Weber and Osborn (1969). Electrophoresis was performed at room temperature in 7.5 or 10% polyacrylamide gels for about 2.5 hr at a current of 7 mA/gel. The buffer solution for the electrophoresis was 0.1 *M* Tris-phosphate (pH 7.0) containing 0.1% sodium dodecyl sulfate. Gels were stained for protein with Coomassie Brilliant Blue R according to Fairbanks et al. (1971). The molecular weight of the various samples was estimated from a semilogarithmic plot of the apparent molecular weight vs. distance of migration using phosphorylase *b* (95 000), bovine serum albumin (68 000), ovalbumin (45 000), and bovine carbonic anhydrase (29 000) as standards.

Immunoelectrophoresis in 2% agarose on microscope slides (25 × 75 mm) was performed according to the method of Scheidegger (1955). About 7 μg of sample was applied and run for 80 min with three slides at 150 V. Antibody was then added to the center trough and allowed to diffuse for 24 hr.

Ultracentrifugation was carried out with a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control. Sedimentation equilibrium was performed according to Yphantis (1964). A six-channel Kel-F centerpiece was employed, and Rayleigh patterns were recorded on Kodak II-G photographic plates. The plates were read on a modified Nikon microcomparator and computation was performed on a PDP-12 computer according to Harris et al. (1969) using programs developed by Teller et al. (1969).

Sedimentation equilibrium measurements with denatured human, bovine, and horse antithrombin III were performed at three different concentrations (0.75, 0.5, and 0.25 mg/ml) in 0.05 *M* sodium acetate–6 *M* Gdn·HCl (pH 5.5, ρ = 1.1425). The density of 6 *M* Gdn·HCl was determined with an Abe's refractometer. All samples were dialyzed for at least 4 days prior to ultracentrifugation, and the appropriate sample concentration was obtained by dilution with dialysate. Following each equilibrium run, a baseline run was performed to correct for window distortion. All experiments were carried out at 20° at a rotor speed of 26 000 rpm for at least 42 hr to attain complete equilibrium. Apparent partial specific volumes of ϕ' = 0.722, 0.719, and 0.710 ml/g for human, bovine, and horse antithrombin III, respectively, were determined by amino acid and carbohydrate analyses as described by Cohn and Edsall (1943) and Longsworth (1953), respectively, and corrected for 6 *M* Gdn·HCl by the method of Lee and Timasheff (1974).

Amino-Terminal Sequence. Automated Edman degradations were performed with a Beckman sequencer, Model 890C. The mode of operation of the instrument and the method of sequenator analysis are adaptations (Hermodson et al., 1972) of the technique of Edman and Begg (1967).

Antibody Preparation. Rabbits were immunized against human, bovine, or horse antithrombin III by multisite subcutaneous injection of the purified proteins (1 mg) suspended in 2 ml of Freunds complete adjuvant. After 2 weeks, a second injection of the protein suspended in Freunds incomplete adjuvant was given, and a third injection was given in the following week. The rabbits were then bled 1 week later by heart puncture, and the blood was allowed to clot and retract overnight in the refrigerator. The sera were treated with $BaSO_4$ (100 mg/ml) for 30 min at room temperature and then centrifuged. Saturated ammonium sulfate was added to 33% saturation. The pellet obtained by centrifugation was dissolved in half of the original volume of 0.15 *M* NaCl. This procedure was repeated twice and the solutions were dialyzed extensively against 0.001 *M* phosphate buffer (pH 7.4). The precipitated euglobulin fraction was removed by centrifugation, and NaCl was added to the supernatant until the final concentration was 0.15 *M*. The supernatant thus obtained was frozen until further use.

Activation of Bovine Factor X and Purification of Factor $X_{a\beta}$. Bovine factor X (50 mg in 100 ml of 0.025 *M* Tris-HCl–0.15 *M* NaCl, pH 8.0) was activated by a protease from Russell's viper venom (0.5 mg) in the presence of 5 mg of phospholipid (an equal mixture of phosphatidylcholine and phosphatidylserine suspended in Tris buffer) and 0.005 *M* $CaCl_2$ for 30 min essentially according to Jesty et al. (1974). The reaction was stopped by adding 1 ml of 1 *M* EDTA. The reaction mixture was then concentrated to about 5 ml using an Amicon concentrator and applied to a Sephadex G-100 column (2.5 × 100 cm) equilibrated and eluted with 0.025 *M* Tris-HCl–0.15 *M* NaCl (pH 7.4). Factor $X_{a\beta}$ which was present in the second peak was pooled and concentrated until the absorbance at 280 nm was about 1.0. The enzyme was stored at −80° until further use.

Clotting Assay. For the assay of factor $X_{a\beta}$ activity, 10 μl of the incubation mixture was added to 1.0 ml of Michaelis buffer and the sample was further diluted from 1000- to 100 000-fold depending upon the amount of factor $X_{a\beta}$. The Michaelis buffer contained 3.6×10^{-2} *M* sodium acetate, 3.6×10^{-2} *M* sodium barbital, and 1.45×10^{-1} *M* sodium chloride (pH 7.4) containing 0.1 mg/ml of bovine serum albumin. An aliquot (0.05 ml) of the final dilution was incubated at 37° for 30 sec with 0.05 ml of phospholipid (cephalin) solution and 0.05 ml of factor X deficient plasma. A 0.05-ml aliquot of 0.025 *M* $CaCl_2$ was then added to the incubation mixture and the clotting time measured and compared to a standard curve.

Results

Polyacrylamide Gel Electrophoresis of Human, Bovine, and Horse Antithrombin III. Sodium dodecyl sulfate gel electrophoresis patterns of human, horse, and bovine antithrombin III showed single sharp protein bands in the presence and absence of reducing agents (Figure 1). Samples 1 and 2 are human antithrombin III in the absence and presence of reducing agent, and samples 3 and 4 are bovine antithrombin III in the absence and presence of reducing agent. Samples 5 and 6 are horse antithrombin III in the absence and presence of reducing agent. A small amount of

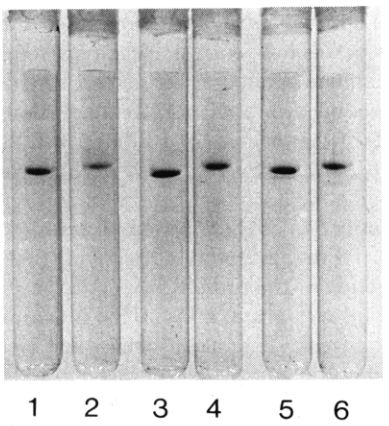

FIGURE 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of human, bovine, and horse antithrombin III. Ten microliters of human, bovine, and horse antithrombin III (10 μg of protein) were mixed with 10 μl of 0.1 *M* Tris-phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate and heated at 100° for 1 min in the presence and absence of 10 μl of 12.8 *M* 2-mercaptoethanol. The samples were applied on a 7.5% gel column, and electrophoresis was carried out as described in the Experimental Section. Gels 1 and 2 are nonreduced and reduced human antithrombin III. Gels 3 and 4 are nonreduced and reduced bovine antithrombin III. Gels 5 and 6 are nonreduced and reduced horse antithrombin III. Standard gels with rabbit phosphorylase *b*, bovine serum albumin, ovalbumin, and bovine carbonic anhydrase are not shown. The anode was at the bottom of the gel.

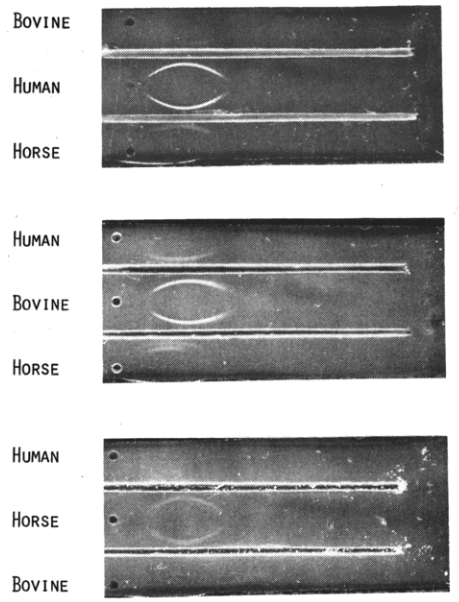

FIGURE 2: Immunoelectrophoresis of human, bovine, and horse antithrombin III. Five microliters of human antithrombin III (8 μg of protein), 5 μl of bovine antithrombin III (10 μg of protein), and 5 μl of horse antithrombin III (10 μg of protein) were placed in the appropriate well identified on the left of each of the three microscope slides. Electrophoresis was then carried out in 0.05 *M* sodium barbital buffer (pH 8.6) for 1 hr at room temperature with 150 V and 2 mA/slide. Following electrophoresis, 70 μl of rabbit antibody to human antithrombin III was placed in each of the two troughs of the slide shown in the top panel. Rabbit antibody to bovine antithrombin III (70 μl) was placed in each of the two troughs of the slide shown in the middle panel, and rabbit antibody to horse antithrombin III (100 μl) was placed in each of the two troughs of the slide shown in the lower panel. After 40 hr at 4°, the slides were photographed without staining. The anode is at the right of the photograph.

a faster moving component is evident in this preparation. In each case, the reduced proteins migrated slower than the nonreduced proteins. These results are consistent with the concept that antithrombin III isolated from the three different sources is composed of a single polypeptide chain.

Molecular weights estimated from 7.5% polyacrylamide gels were 54 000 and 65 000 for nonreduced and reduced human antithrombin III, 51 000 and 61 000 for nonreduced and reduced bovine antithrombin III, and 53 000 and 58 000 for nonreduced and reduced horse antithrombin III. Molecular weights estimated from 10% acrylamide gels were 54 000 and 56 000 for nonreduced and reduced human antithrombin III, 51 000 and 57 000 for nonreduced and reduced bovine antithrombin III, and 55 000 and 58 000 for nonreduced and reduced horse antithrombin III.

Immunoelectrophoresis of Human, Bovine, and Horse Antithrombin III. Antithrombin III was subjected to electrophoresis on agarose slides in barbital buffer (pH 8.6) followed by immunodiffusion against rabbit antibody prepared from the purified human, bovine, and horse antithrombin III. The top panel in Figure 2 shows experiments employing an antibody to human antithrombin III. This antibody forms a strong precipitin line with human antithrombin III but a very weak precipitin line with bovine or horse antithrombin III. The middle panel shows similar experiments employing an antibody to bovine antithrombin III. This antibody forms a strong precipitin line with bovine antithrombin III but a weak precipitin line with human antithrombin III. It forms a strong and a weak precipitin line with horse antithrombin III. Antibody to horse antithrombin III (bottom panel) formed a weak and somewhat diffuse precipitin line with horse antithrombin III and no precipitin lines with human or bovine antithrombin III. The weak precipitin line with the horse preparation was also observed when various ratios of antibody and antigen were employed.

These experiments provide additional evidence of purity for human and bovine antithrombin III. They indicate, however, the presence of more than one form of antithrombin III in the preparation from horse plasma. These experiments also suggest some similarity in the antigenic sites of the three proteins in that some antigen–antibody cross-reaction is observed among the proteins isolated from the three different species.

Sedimentation Equilibrium Studies on Antithrombin III. Sedimentation equilibrium studies of bovine and horse antithrombin III in 6 *M* Gdn·HCl showed that these two proteins are homogeneous at all concentrations tested (Table I). The minimal molecular weights for the denatured proteins were 56 600 for bovine antithrombin III and 52 500 for horse antithrombin III. Preliminary studies on the human preparation gave anomalous results.[2] Whether this was due to aggregation or some other property of this protein has not been established.

Amino Acid and Carbohydrate Compositions of Human, Bovine, and Horse Antithrombin III. The amino acid and carbohydrate compositions of human, bovine, and horse antithrombin III are shown in Table II. A molecular weight of 56 600 was employed for human antithrombin III in these calculations. The composition of all three proteins is very similar except for a significantly higher carbohydrate content of horse antithrombin III. No free sulfhydryl group was detected in any of the three proteins employing Ellman's procedure.

[2] In the presence of 6 *M* Gdn·HCl, the following values were obtained for human antithrombin III: M_n = 70 300 ± 600, M_w = 66 900 ± 700, and M_z = 71 400 ± 400. In the presence of 6 *M* Gdn·HCl and 0.1 *M* dithiothreitol, M_n = 50 100 ± 1000, M_w = 58 700 ± 2200, and M_z = 74 900 ● 5200.

Table I: Molecular Weight of Bovine and Horse Antithrombin III by Sedimentation Equilibrium.[a]

Sample	M_1	M_n	M_w	M_z
Bovine antithrombin III	56 600 ± 300	56 100 ± 230	55 400 ± 200	56 700 ± 300
Horse antithrombin III	52 500 ± 170	53 400 ± 120	54 400 ± 110	54 400 ± 160

[a] M_1 refers to the smallest molecular weight calculated by the methods described by Teller et al. (1969). The M_n (number average), M_w (weight average), and M_z (Z average) molecular weights are values obtained by extrapolation to zero protein concentration employing three different initial protein concentrations, as described in the Experimental Section.

Table II: Amino Acid and Carbohydrate Compositions of Human, Bovine, and Horse Antithrombin III.

Components	Residues/56 600 g Glycoprotein		Residues/ 52 500 g Glycoprotein
	Human	Bovine	Horse
Amino acid			
Lysine	36.7	32.0	29.9
Histidine	5.3	6.4	7.7
Arginine	23.5	23.2	19.6
Aspartic acid	47.7	45.5	40.5
Threonine	25.5	28.1	23.4
Serine	32.9	33.9	27.1
Glutamic acid	54.1	43.9	48.1
Proline	21.9	18.2	20.5
Glycine	19.1	18.1	16.3
Alanine	32.5	29.0	25.8
Half-cystine[a]	6.1	6.3	6.1
Valine	27.5	29.2	19.9
Methionine[b]	10.9	8.9	9.3
Isoleucine	21.1	24.5	18.1
Leucine	39.5	42.4	29.6
Tyrosine	7.3	9.6	8.9
Phenylalanine	33.7	34.0	25.8
Tryptophan[c]	6.3	5.8	7.9
Carbohydrate (%)			
Hexose	3.0	3.9	6.5
Hexosamine	3.6	4.5	3.8
Neuraminic acid	2.4	3.1	5.6
Protein (%)	91.0	88.5	84.1
Carbohydrate (%)	9.0	11.5	15.9

[a] Determined as cysteic acid after performic acid oxidation (Hirs, 1967). [b] Determined as methionine sulfone after performic acid oxidation (Hirs, 1967). [c] Determined after alkaline hydrolysis according to Hugli and Moore (1972).

	5					10						15		
Human	His	Gly	Ser	Pro	Val	–	Asp	Ile	Cys	Thr	Ala	Lys	Pro	Arg	Asp	Ile	Pro
Bovine	His	Arg	Ser	Pro	Val	Glu	Asp	Val	Cys	Thr	Ala	Lys	Pro	Arg	Asp	Ile	Pro
Horse	His	Trp	Ser	Pro	Val	Glu	Asp	Ile	Cys	Thr	Ala	Lys	Pro	Arg	Asp	Ile	Pro

FIGURE 3: Amino-terminal sequence of human, bovine, and horse antithrombin III. A space, shown by a dash, has been inserted in human antithrombin III in position 6 to bring this protein into alignment for better homology with the bovine and horse preparations.

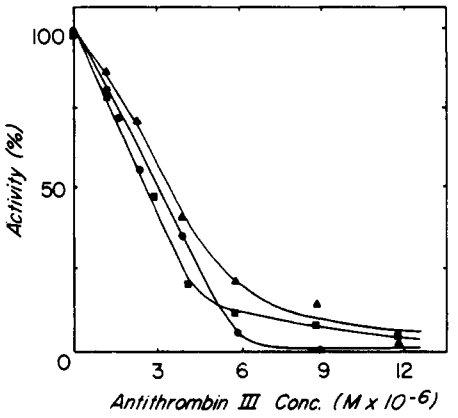

FIGURE 4: Inhibition curve of bovine factor $X_{a\beta}$ with human, bovine, and horse antithrombin III. The inhibition mixture contained 7.7×10^{-6} *M* bovine factor $X_{a\beta}$ in 0.025 *M* Tris-HCl buffer (pH 7.5) containing 0.15 *M* NaCl and various concentrations of either human (■), bovine (●), or horse (▲) antithrombin III. The total volume was 0.15 ml. The inhibition mixture was incubated at 38° for 5 min and aliquots (10 μl) were removed, diluted in Michaelis buffer, and assayed for residual factor $X_{a\beta}$ coagulant activity.

Amino-Terminal Sequences of Human, Bovine, and Horse Antithrombin III. Amino-terminal sequences of human, bovine, and horse antithrombin III are shown in Figure 3. Each protein was found to contain an amino-terminal histidine, a residue which is somewhat unique for plasma proteins. No other sequences were observed as contaminants in any of the preparations. The repetitive yields for the degradations were about 95%, and over 60% of the expected quantities of the phenylthiohydantoins based on the weight of lyophilized protein degraded was observed for each of the proteins. The amino acid residue in the second position was different for all three proteins. Human antithrombin III also shows a deletion in position 6, and Val replaced Ile in position 8 in bovine antithrombin III. The remainder of the first 17 residues is the same for all three proteins.

Inhibition of Bovine Factor $X_{a\beta}$ by Human, Bovine, and Horse Antithrombin III. Inhibition curves for bovine factor $X_{a\beta}$ by human, bovine, and horse antithrombin III are shown in Figure 4. Clotting activity of factor $X_{a\beta}$ was inhibited almost equally well by human, bovine, and horse antithrombin III. In these experiments, an enzyme concentration of 7.7×10^{-6} *M* was employed. Extrapolation of the inhibition curve for bovine antithrombin III to 100% inhibition gives a value of 6.1×10^{-6} *M*. This gives an enzyme:inhibitor ratio of 1.3. This value has ranged from 1.3 to 1.0 in different experiments. A similar value was obtained for human and horse antithrombin III. In these calculations, a molecular weight of 42 600 was employed for factor $X_{a\beta}$ (Fujikawa et al., 1975) and a molecular weight of 56 600 was employed for the antithrombin III. These data indicate that antithrombin III inhibits bovine factor $X_{a\beta}$ by forming a 1:1 molar complex with the enzyme. The apparent association constant of bovine antithrombin III and bovine factor $X_{a\beta}$ was calculated to be about 5×10^8 *M*. As shown in Figure 4, the affinity of human or horse antithrombin III for bovine factor $X_{a\beta}$ is much less than that for bovine antithrombin III.

Discussion

Human, bovine, and horse antithrombin III have been shown to be glycoproteins composed of a single polypeptide

chain. They have similar amino acid and carbohydrate compositions, although the carbohydrate content for the horse preparation is slightly higher. The amino acid composition of the human preparation is similar to that reported by Heimburger et al. (1971) and Miller-Anderson et al. (1974). The latter investigators, however, reported no half-cystine in their preparations, in contrast to that of Heimburger and co-workers and the present studies. Also, Miller-Anderson and co-workers found no neuraminic acid in their human antithrombin III, in contrast to the present investigations and those of Heimburger et al. (1971).

The molecular weight for antithrombin III ranged from 53 000 to 56 000 as estimated by sedimentation equilibrium. This molecular weight may require some revision since there is always some uncertainty in the calculation of the partial specific volume for glycoproteins. This may account for the higher value reported by Miller-Anderson et al. (1974) for antithrombin III. These investigators reported a value of 67 000 by sedimentation equilibrium studies. Preliminary data on the number of cyanogen bromide fragments from human antithrombin III suggest the presence of about 11 peptides (M. A. Hermodson, unpublished results). This value is more consistent with a molecular weight of about 56 000 and 11 methionine residues rather than a molecular weight of 67 000 and 13 methionine residues. It is also consistent with the molecular weight estimated for the nonreduced proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The presence of an amino-terminal histidine in human antithrombin III confirms the results of Miller-Anderson et al. (1974). This amino acid was also found in the bovine and horse preparations. The human, bovine, and horse preparations show a marked similarity in their amino-terminal sequences. The differences in the amino acids in positions 2 and 8 can be attributed to single base changes in the triplet code. The marked similarity of the amino-terminal sequence between the three proteins is also consistent with the immunological cross-reactivity observed for the three different preparations.

The inhibition of factor $X_{a\beta}$ by antithrombin III by the formation of a 1:1 complex is analogous to that found by Rosenberg and co-workers for antithrombin III and thrombin and antithrombin III and plasmin (Rosenberg and Damus, 1973; Highsmith and Rosenberg, 1974). These investigators employed sodium dodecyl sulfate polyacrylamide gel electrophoresis and clearly demonstrated the formation of a 1:1 complex between the inhibitor and enzyme. A similar approach has been employed by Kurachi et al. (1975) with factor $X_{a\beta}$ and factor IX_a. These investigators also demonstrated a 1:1 complex between inhibitor and enzyme by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Acknowledgments

The authors express their sincere thanks to Dr. Takehiko Koide for many helpful discussions, and to Richard Granberg, Jenny Krys, and Judy Lane for excellent technical assistance. Thanks are also due to P. D. and J. Meats Co., Kent, Wash., for kindly providing the bovine blood, and the Florence Meat Packer Co., Florence, Wash., for kindly providing the horse blood.

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Inhibition of Bovine Factor IX_a and Factor $X_{a\beta}$ by Antithrombin III†

Kotoku Kurachi, Kazuo Fujikawa, Gottfried Schmer, and Earl W. Davie*

ABSTRACT: Factor IX_a and factor $X_{a\beta}$ are serine proteases which participate in the middle phase of blood coagulation. These two enzymes are inhibited by antithrombin III by the formation of an enzyme–inhibitor complex containing 1 mol of enzyme and 1 mol of antithrombin III. The complex was readily demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and loss of coagulant or esterase activity at increasing concentrations of inhibitor. The inactivation of factor IX_a by antithrombin III was relatively slow, but the reaction was greatly accelerated by the addition of heparin.

Antithrombin III is a plasma protein which blocks the enzymatic activity of a number of plasma and pancreatic serine proteases. It inhibits several coagulation factors including thrombin, factor X_a, factor XI_a (Monkhouse et al., 1955; Waugh and Fitzgerald, 1956; Abildgaard, 1968; Biggs et al., 1970; Yin et al., 1971; Dombrose et al., 1971; Damus et al., 1973; Walsh et al., 1974; Highsmith and Rosenberg, 1974), as well as trypsin and chymotrypsin (Abildgaard and Egeberg, 1968).Antithrombin III accounts for the major portion of the antithrombin activity in human plasma as shown by immunoprecipitation experiments (Abildgaard et al., 1970; Rosenberg, 1974). Thus, it is probable that it plays an important physiological role in arresting the coagulation process which is triggered at a site of injury.

Abildgaard (1969) showed by gel filtration that thrombin and antithrombin III form an inactive complex. More recently, Rosenberg and Damus (1973) and Highsmith and Rosenberg (1974) reported that the inhibition of thrombin as well as plasmin by antithrombin III was due to the formation of a stable complex between the inhibitor and enzyme. These investigators employed sodium dodecyl sulfate polyacrylamide gel electrophoresis and identified a new slow moving component corresponding to a 1:1 molar complex of the enzyme and inhibitor. In this manuscript, we wish to report the formation of a similar 1:1 enzyme–inhibitor complex between antithrombin III and factor IX_a and factor $X_{a\beta}$ using similar techniques.

Experimental Section

Materials

Bovine factors IX and X were prepared according to Fujikawa et al. (1972, 1973). Factor $X_{a\beta}$ was prepared as described by Kurachi et al. (1976). Bovine antithrombin III was prepared by method B of Thaler and Schmer (1975). Bovine factor IX_a was kindly provided by P. A. Lindquist in our laboratory. This preparation migrated as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis and has been characterized in previous studies from our laboratory (Fujikawa et al., 1974a). Bovine serum albumin, ovalbumin, carbonic anhydrase, cephalin (rabbit brain extract), and diisopropyl phosphorofluoridate (Dip-F)[1] were purchased from Sigma Chemical Co., St. Louis, Mo. Phosphorylase *b* was a kind donation of Dr. E. H. Fischer. Sephadex G-100 was a product of Pharmacia Fine Chemicals, Piscataway, N.J. 2-Mercaptoethanol and *N,N,N′,N′*-tetramethylethylenediamine were obtained from Eastman Kodak Co., Rochester, N.Y. Acrylamide was obtained from Matheson Coleman and Bell, Norwood, Ohio. Sodium dodecyl sulfate was obtained from British Drug House, Poole, England. Phosphatidylcholine and phosphatidylserine were purchased from Applied Science Laboratories, Inc., Inglewood, Calif. Bovine factor X deficient plasma was made by the method of Bachmann et al. (1958). Benzoylarginyl ethyl ester labeled with [^{3}H]ethanol was kindly provided by E. Fodor of this department. The toluene scintillant was prepared by dissolving 15 g of Omnifluor in 3.8 l. of toluene. Sodium heparin (20 000 units/ml) was purchased from Invenex, San Francisco, Calif. All other chemicals were commercial preparations of the highest quality available.

Methods

Factor X concentration was determined by absorption at 280 nm employing an $E_{280}(1\%)$ of 11.5 (Fujikawa et al., 1974b), and factor $X_{a\beta}$ concentration was determined assuming an $E_{280}(1\%)$ of 10. Factor IX and factor IX_a concentrations were determined by absorption at 280 nm employing an $E_{280}(1\%)$ of 14.9 for factor IX and 14.3 for factor IX_a (Fujikawa et al., 1974b). Bovine antithrombin

† From the Departments of Biochemistry and Laboratory Medicine, University of Washington, Seattle, Washington 98195. *Received August 19, 1975.* This work was supported in part by Research Grant HL 16919-01A1 from the National Institutes of Health.

[1] Abbreviation used is: Dip-F, diisopropyl phosphorofluoridate.