N-Terminal Sequences of Bovine Fibrinogen*

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ABSTRACT: The N-terminal sequence, H-Tyr-Val-Ala-Thr-Arg-Asp-Asn-, for the C chain of bovine fibrinogen was determined, using the phenyl isothiocyanate method of Edman. Stepwise degradation of whole fibrinogen corroborated the known fibrinopeptide A sequence as N terminal for the A chain and, since the B chain normally has a blocked N terminal, permitted deduction by difference of the C-chain sequence, except position 4. The fourth C-chain residue was identified and the deduced structure was confirmed directly by analysis of a fibrin derivative whose C chain alone was accessible to Edman degradation. This derivative was prepared by coupling fibrinogen with the degradation reagent, phenyl isothiocyanate, to give a phenylthiocarbamyl protein.

The degradation procedure was then interrupted in order to digest off with thrombin the phenylthiocarbamylated fibrinopeptide A and fibrinopeptide B, uncovering glycines as new N terminals of the A and B chains. Next, the terminal glycines were blocked by acetylation, while the C chain, its tyrosine protected from acetylation by prior coupling with phenyl isothiocyanate, remained degradable. The normal procedure for sequence analysis was then resumed.

The mammalian fibrinogen molecule is thought to be comprised of three different peptide chains (Lorand and Middlebrook, 1952; Blombäck and Yamashina, 1958), designated A, B, and C (Iwanaga *et al.*, 1966), probably cross-linked through disulfide and perhaps other bonds (Henschen, 1962, 1963; Clegg and Bailey, 1962). The A and B chains are cleaved by thrombin at single arginylglycyl bonds near their amino termini (Bailey *et al.*, 1951; Gladner *et al.*, 1958), releasing the corresponding fibrinopeptides A and B (Lorand, 1952; Bettelheim and Bailey, 1952). Complete amino acid sequences have now been determined for the isolated fibrinopeptides of several species (Folk *et al.*, 1959; Sjöquist *et al.*, 1960; Blombäck *et al.*, 1966b). If, as the evidence strongly indicates (Lorand, 1952; Bettelheim and Bailey, 1952; Blombäck and Yamashina, 1958; Blombäck *et al.*, 1965a,b), the fibrinopeptides are N-terminal fragments, then the N-terminal sequences of the A and B chains are known for several fibrinogens.

The N-terminal residue of the B chain of bovine fibrinogen is probably pyroglutamic acid (Blombäck and Doolittle, 1963) which, lacking a free amino group, does not react with N-terminal reagents. The A and C chains, on the other hand, have reactive N terminals (Blombäck and Yamashina, 1958) and therefore should be accessible to stepwise degradation by the Edman phenyl isothiocyanate method. If the A and C chains were degraded simultaneously, as in whole fibrinogen, then the C-chain sequence should be deducible by difference, given that the A-chain sequence is known.

In the present work, Edman degradation of whole bovine fibrinogen yielded amino acids corresponding to the A fibrinopeptide sequence and a series of second amino acids thought to represent the N-terminal sequence of the C chain. The deduced C-chain sequence was then confirmed by analysis of a fibrin derivative whose C chain alone was accessible to stepwise degradation.

Experimental Section

Fibrinogen. The glycine method of Blombäck and Blombäck (1956) was applied to fraction I, prepared by method 6 of Cohn *et al.* (1946) from fresh bovine plasma. Each batch of plasma was obtained from two or three animals. Fibrinogen prepared from three different batches of plasma was used in the present work. The purified fibrinogen was 97% coagulable with thrombin by the spectrophotometric method of Blombäck (1958). It was stored at −20° in 0.3 M sodium chloride at a protein concentration of 1% and, before use, was dialyzed for 22 hr at 4° against several changes of 0.3 M sodium chloride in order to remove free glycine left from the purification procedure.

Amino Acid Sequence Analysis. The three-stage phenyl isothiocyanate method (Edman, 1957, 1960) was used as described by Blombäck *et al.* (1966a). Solutions containing 100 mg of dialyzed fibrinogen were brought to about 4 ml by pervaporation before coupling with phenyl isothiocyanate. To the fibrinogen solution were added 1.5 volumes of freshly distilled pyridine and 0.12 volume of dimethylallylamine (synthesized according to Cope and Towle, 1949). The pH was then adjusted to 9.5 with 10% trifluoroacetic acid. After the first degradation step the protein was

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insoluble and therefore, after vacuum drying, was powdered or broken up before the next coupling. In this case, the dry protein was just moistened with 10% trifluoroacetic acid and then 5–15 ml of pyridine–water–dimethylallylamine (15:10:1.2, v/v) was added to the insoluble protein until the pH of the solution–suspension was 9.5. Next, the insoluble protein was allowed to settle and phenyl isothiocyanate[1] (vacuum distilled) was added just to saturation (persistent turbidity of the supernatant solution). This required about 50 μl of phenyl isothiocyanate/ml of reaction mixture.

Spectrophotometry of PTH-amino Acids.[2] Absorption spectra between 235 and 340 mμ were recorded with a Beckman DB spectrophotometer on the products from each step dissolved in varying amounts (50–200 μl) of ethyl acetate. For spectrophotometry 5 μl of ethyl acetate solution was added to 1 ml of 100% ethanol. This was done in order to establish normal spectral curves for the PTH derivatives obtained and to estimate, from the absorbance at 269 mμ, the appropriate amount of sample for thin-layer and paper chromatography. Direct quantitation of PTH-amino acids from their ultraviolet absorbance was not possible since the degradation products often included unknown proportions of two PTH-amino acids with different extinction coefficients and, following the first degradation step, usually included a side product, diphenylthiourea, which absorbs strongly in the region of 274 mμ.

Chromatographic Identification of PTH-amino Acids. Thin-layer chromatography was performed on silica gel layers, using Eastman type K301R sheets with solvent D of Edman and Sjöquist (1956), E. Merck AG F254 precoated plates with solvent E of Edman and Sjöquist (1956), and solvent 7 and modified solvent 8 of Brenner *et al.* (1961). Modified solvent 8, prepared by mixing chloroform and 88% formic acid (100:15, v/v) and discarding the aqueous phase, gave increased migration of aspartic acid and glutamic acid. Reference mixtures of PTH-amino acids,[3] dissolved in ethyl acetate, were applied in 0.007-μmole amounts in duplicate to each chromatogram on which four unknowns were analyzed. Spots on the chromatograms were located with an ultraviolet lamp[4] and their intensities were estimated visually on an arbitrary scale from 1 to 5. Depending on the preparation and the step, the first seven steps each yielded one or two unequivocal, major N-terminal residues. Occasional traces of other identifiable PTH-amino acids or unidentifiable derivatives were encountered, but there were no appreciable overlaps between successive steps.

Paper chromatography (Whatman No. 1 paper and solvent I of Sjöquist, 1960) was used to distinguish PTH-valine from PTH-phenylalanine and PTH-methionine from diphenylthiourea. Spots were visualized directly by ultraviolet transillumination of the papers (Pirkle, 1967).

Identification of PTH-arginine. The water-soluble Edman degradation products, spotted on filter paper, was tested with the Sakaguchi reaction as described by Block *et al.* (1958).

Synthesis of PTH-dehydrothreonine.[5] PTH-dehydrothreonine (mp 228–233°) was prepared as described by Blombäck and Yamashina (1958). It was chromatographically homogeneous in the four thin-layer systems mentioned above.

Preparation and Degradation of Fibrin Phenylthiocarbamylated in the Tyrosine and Acetylated in the Glycine α-Amino Groups (C Fibrin). Dialyzed fibrinogen (100 mg) was coupled with phenyl isothiocyanate and extracted with benzene in the usual way. Residual benzene was removed with a nitrogen stream. The solution–suspension of PTC-fibrinogen was mixed with phosphate buffer to give pH 6.35, ionic strength 0.15, and final volume 12 ml (0.005 M Na_2HPO_4, 0.010 M KH_2PO_4, and 0.12 M NaCl). Bovine thrombin[6] was added to a final concentration of 5 NIH units/ml, and the mixture was left for 16 hr at room temperature. No clotting or visible change in the amount of undissolved protein was observed. The digest was dialyzed for 48 hr at 4° against several changes of distilled water. Acetylation of the thrombin-digested, PTC-protein was carried out in an ice–water bath. Solid sodium acetate was added to a final concentration of 2.5 M. The final volume was 25 ml. Acetic anhydride was then added in two 0.3-ml portions at an interval of 6 min, the pH being continuously adjusted to 7.5 to 8.5 with 3 M sodium hydroxide. By 20 min after the last addition of acetic anhydride the pH had stabilized at 8.1. The reaction mixture was dialyzed for 96 hr at 4° against several changes of distilled water, concentrated by prevaporation, and freeze dried. The step 1 Edman degradation procedure was then resumed.

Performic Acid Oxidized Fibrinogen. Oxidation was carried out essentially as described by Schram *et al.* (1954). Fibrinogen was precipitated with 7% ethanol at 0° in order to remove chloride ions, which have been shown to result in modification of tyrosine residues during performic acid oxidation (Hirs, 1956). Precipitate corresponding to 100 mg of fibrinogen was dissolved in 5 ml of 88% formic acid and cooled to 0°. Performic acid was prepared by adding one volume of 30% hydrogen peroxide to nine volumes of 88% formic acid. After 1 hr at room temperature, the mixture was cooled to 0°. The fibrinogen sample was mixed with 20 ml of performic acid, left for 4 hr at 0°, and then diluted with 75 ml of ice-cold water. The oxidized protein was dialyzed for 72 hr at 4° against several changes of distilled water. The final solution, concentrated to 8 ml by pervaporation, was yellow.

The following results are based on stepwise degradation of four samples of unmodified fibrinogen, one of

[1] Eastman Organic Chemicals, Rochester, N. Y.

[2] Abbreviations used: PTH-amino acids, phenylthiohydantoin derivatives of amino acids; PTC-fibrinogen, phenylthiocarbamyl derivative of fibrinogen; C fibrin, fibrin phenylthiocarbamylated in the tyrosine and acetylated in the glycine α-amino groups.

[3] Sigma Chemical Co., St. Louis, Mo.

[4] Ultra-Violet Products, Inc., San Gabriel, Calif.

[5] Common usage for $H_2NC(=CHCH_3)CO_2H$.

[6] Parke Davis and Co., Detroit, Mich.

which was originally dissolved in 8 M urea and another in 1% sodium dodecyl sulfate, two samples of C fibrin, and two samples of performic acid oxidized fibrinogen.

Results

Unmodified fibrinogen, degraded by the Edman method through seven cycles (Table I), yielded the PTH-amino acids that would be expected to come from the A chain (Folk *et al.*, 1959; Sjöquist *et al.*, 1960). In addition, second residues, thought to have come from the C chain (*vide supra*), were obtained at each step, except step 4. The thin-layer chromatograms for step 4 were complex, owing, at least in part, to the presence of multiple derivatives of serine from the A chain, formed during the conversion of thiazolinone into phenylthiohydantoin (Edman and Begg, 1967). The failure to identify a second residue at step 4 suggested the possibility that this position might be occupied by the same amino acid in both A and C chains. To investigate this question and to obtain, in any case, chromatograms uncomplicated by multiple serine derivatives from the A chain, we prepared C fibrin, whose C chain only was accessible to stepwise degradation.

TABLE I: Stepwise Degradation of A Fibrinopeptide, Fibrinogen, and Fibrin Phenylthiocarbamylated in the Tyrosine and Acetylated in the Glycine α-Amino Groups (C Fibrin).

	Amino Acids Found		
Step	A Peptide[a]	Fibrinogen[b]	C Fibrin
1	Glu	Glu, Tyr	Tyr
2	Asp	Asp, Val	Val
3	Gly	Gly, Ala	Ala
4	Ser	Ser, ?	Thr
5	Asp	Asp, Arg	Arg
6	Pro	Pro, Asp	Asp
7	Pro	Pro, Asn	Asn

[a] Included for comparison, from Folk *et al.* (1959) and Sjöquist *et al.* (1960). [b] Unmodified and performic acid oxidized.

C fibrin yielded only the six single amino acids deduced for the C chain[7] through seven degradation steps and, in addition, threonine in position 4 (Table I). Threonine was recovered entirely as PTH-dehydrothreonine, identified by thin-layer chromatography using synthetic PTH-dehydrothreonine as a reference substance. The chromatographic behavior of PTH-dehydrothreonine resembled that of valine except in solvent D where its R_F value was 0.25 as compared to 0.39 for PTH-valine, 0.33 for PTH-methionine, and 0.17 for PTH-alanine. The ultraviolet absorption spectrum of the step 4 PTH product from C fibrin exhibited, in addition to a principal maximum at 269 mμ, the second broad maximum around 320 mμ that would be expected from PTH-dehydrothreonine (Levy and Chung, 1955).

Sakaguchi spot tests on the water-soluble products from unmodified fibrinogen and C fibrin revealed arginine at step 5 with steps 4 and 6 serving as negative controls.

Performic acid oxidized fibrinogen was prepared in order to convert cystine into cysteic acid residues. This was prompted by decreased yields of 269-mμ-absorbing material at step 4 of unmodified fibrinogen and the initial failure to identify a C-chain amino acid at that point, suggesting that this residue might be cross-linked to another part of the molecule, preventing its release by degradative scission of its peptide bond. Degradation of oxidized fibrinogen gave essentially the same results as unmodified fibrinogen.

The expected A-chain residues were found as major N terminals through 12 steps in one sample of unmodified fibrinogen. However, beginning with step 8, preparations of all types gave uncertain results, either through the appearance of more than the expected number of N-terminal residues or through disagreement between replicate samples.

Discussion

From the present results the N-terminal sequence, H-Tyr-Val-Ala-Thr-Arg-Asp-Asn-, can be proposed for the C chain of bovine fibrinogen.

The interpretation that the dehydrothreonine identified in step 4 of C fibrin was derived from threonine is based on the following considerations. Dehydrothreonine has not been found to occur in nature (Greenstein and Winitz, 1961). PTH-threonine readily decomposes to PTH-dehydrothreonine under conditions which obtained in this work (Blombäck and Yamashina, 1958). Both PTH-threonine and PTH-dehydrothreonine spots were found by thin-layer chromatography for step 4 of unmodified fibrinogen if the analysis was carried out without delay following completion of the degradation procedure. However, even then the chromatograms were too complex to allow the confident, independent identification of a second residue in addition to serine.

Arginine as the fifth residue of the C chain was not recognized originally (Pirkle and Henschen, 1967), since its absorption maximum at 269 mμ was obscured by the spectral curves of water-soluble side products. As a result, tests for the water-soluble PTH-amino acids were not done at first.

Pozdnyakova (1965, 1966) proposed the N-terminal sequence, H-Tyr-Pro-Ala-, for the C chain on the basis of Edman degradation of the isolated chain and of whole bovine fibrin, for which it was claimed that steps 2 and 3 gave the same result for all three chains.

[7] This result indicates that, during the preparation of C fibrin, the A fibrinopeptide, at least, was removed by thrombin. Since the B fibrinopeptide has a normally blocked N terminal, its removal was not essential to our purposes and, for this reason, no proof of its removal was attempted.

Pozdnyakova noted much more nonspecific hydrolysis of the protein than we did, requiring substantial corrections for the results of steps 2 and 3 and making it impossible to continue the degradation further, but this does not explain the failure to find valine in step 2.

Gerbeck *et al.* (1967) have been able, by chromatography of sulfitolyzed fibrinogen on carboxymethylcellulose, to separate two components having N-terminal tyrosine.[8] The amino acid compositions of the two components were similar, however, indicating that they might have basically similar structures.[9] This possibility is supported by our finding of a single N-terminal sequence commencing with tyrosine.

A comparison of fibrinopeptides of various species has suggested that the fibrinogen molecule may have a quite variable structure (Blombäck *et al.*, 1966b). This may be true only for limited portions of the molecule. There is reason to suspect, for example, that the C chain may have a more constant structure, since its N-terminal residue in all of the seven mammals thus far reported was tyrosine (Blombäck and Yamashina, 1958), while the A and B chains have, respectively, three and five different end groups (Blombäck *et al.*, 1966b). It will be of interest, therefore, to examine the C-chain N-terminal sequences in other species.

Fibrinogen exhibits two striking and as yet unexplained biochemical properties which might be clarified by primary structural studies. (1) It is a substrate for thrombin, a proteolytic enzyme of remarkably narrow specificity. (2) Following limited proteolysis by thrombin, the parent molecules quickly aggregate into highly ordered, polymeric strands of enormous length. The structural features of fibrinogen which direct the attack of thrombin on the vulnerable arginylglycyl bonds might be sought first in the region of the cleavage site. In part, this has already been accomplished through the elucidation of fibrinopeptide structures. It remains, however, to characterize the region on the parent molecule side of the cleavage site.[10] Toward this goal, the C-chain sequence can be used as a "stepping stone" to the N-terminal sequences of fibrin (Henschen and Pirkle, 1967), just as the A-fibrinopeptide sequence was used to deduce the C-chain structure. Moreover, the possibility should not be rejected that the C chain might have some role in directing the action of thrombin or in fibrin polymerization.

Although the existing indirect evidence that the A fibrinopeptide is an N-terminal fragment of fibrinogen is substantial (Blombäck and Yamashina, 1958; Blombäck *et al.*, 1965a,b), it was, nonetheless, of some interest that stepwise degradation of the whole fibrinogen molecule from its amino termini did, in fact, yield residues corresponding to the A fibrinopeptide for 12 steps.

[8] A. Henschen (unpublished results) has also observed chromatographic evidence for heterogeneity of the C chain of pooled bovine fibrinogen, possibly amounting to as many as four molecular species.

[9] More recently Montgomery has found small compositional differences between the two C-chain components with respect to five amino acid residues, so that the components can now be described as similar but not identical (R. Montgomery, personal communication).

[10] Since the writing of this report, the results of Blombäck *et al.* (1967) on the N-terminal fragments of the A and B chains of human fibrinogen af er cleavage with plasmin and with cyanogen bromide have become available. See also Henschen and Pirkle (1967).

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Physical Evidence for Transferrins as Single Polypeptide Chains*

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ABSTRACT: Physical methods were used to evaluate the possibility that the two iron binding sites of transferrins are located in two subunits of the proteins. The disulfide bonds in chicken ovotransferrin, human serum transferrin, and rabbit serum transferrin were reduced, and the resulting sulfhydryls were carboxymethylated. These carboxymethylated transferrins were compared ultracentrifugally with similarly produced carboxymethylated derivatives of bovine serum albumin and porcine pepsin.

The s_{20} values of these derivatives observed in 8 M urea and in 6 M guanidine hydrochloride were proportional to the molecular weights of the native proteins as determined by sedimentation equilibrium. These results indicated that the transferrins had not been dissociated by reduction. The modified proteins in 8 M urea were analyzed using the relation $s/(1 - \bar{v}\rho) \propto M^{1/2}$ as predicted from theory for random coils. The minimum weight of the polypeptide chain of ovotransferrin as estimated from this relation is equivalent to its molecular weight. All three transferrins, therefore, appear to be monomers.

The transferrins are a group of homologous, iron binding glycoproteins present in vertebrate blood, mammalian milk, and avian egg whites (Feeney and Komatsu, 1966). The serum transferrins have been implicated in the transfer of iron from storage areas to immature red blood cells (Jandl *et al.*, 1959). Transferrins from several different sources have similar molecular sizes (mol wt 70,000–90,000), similar iron binding properties ($K_{diss} \simeq 10^{-29}$), and similar amino acid composition (Feeney and Komatsu, 1966; Osuga and Feeney, 1968). The two iron binding sites are apparently equivalent and noninteracting (Aisen *et al.*, 1966). The iron binding ligands of the proteins are contributed by amino acid side chains (Fraenkel-Conrat and Feeney, 1950; Windle *et al.*, 1963), and the characteristic color is dependent on bicarbonate binding (Aisen *et al.*, 1967).

Tryptic peptide maps of chicken ovotransferrin (OT)[1] (Williams, 1962) and human serum transferrin (HST) (Jeppsson, 1967) show much smaller numbers of spots than would be predicted from their lysine and arginine content. HST apparently contains two similar oligosaccharides (Jamieson, 1965). These data,

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966) are: OT, chicken ovotransferrin; HST, human serum transferrin; RST, rabbit serum transferrin; RCM, reduced carboxymethylated; BSA, bovine serum albumin.