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Amino Acid Sequence Studies on the α Chain of Human Fibrinogen. Isolation and Characterization of Two Linked α-Chain Cyanogen Bromide Fragments from Fully Cross-Linked Fibrin†

R. F. Doolittle,* K. G. Cassman,‡ B. A. Cottrell, and S. J. Friezner§

ABSTRACT: Fully cross-linked human fibrin was digested with cyanogen bromide and the resulting fragments were characterized and compared with the fragments produced upon cyanogen bromide treatment of fibrinogen α chains. The largest molecular-weight fraction isolated by gel filtration on Sephadex G-150 was reduced and alkylated, and upon rechromatography on Sephadex G-150 it eluted at the same place as the original material. This large molecular weight fraction was subjected to amino acid analysis and the amino-terminal sequences of its constituent chains were determined by both dimethylaminonaphthyl sulfonation (Dns) and thioacetylation procedures. The identified sequences corresponded to two cyanogen bromide fragments previously found in α chains isolated from fibrinogen, one of which has a molecular weight of about 30 000 and the other 6000. The latter is thought to be the carboxy-terminal penultimate cyanogen bromide fragment of the α chain.

Under physiological conditions, the thrombin-catalyzed transformation of fibrinogen to fibrin is accompanied by a fibrin stabilization phenomenon whereby factor XIII—itself activated by thrombin—introduces a series of ε (γ-glutamyl) lysine covalent cross-links. One set of these leads to the existence of γ-γ dimers in fibrin after it is reduced to its constituent chains (Chen and Doolittle, 1969; Takagi and Iwanaga, 1970), the attachment being reciprocal in nature and the connections being interposed between the antiparallel carboxy-terminal segments of neighboring molecules (Chen and Doolittle, 1970, 1971). Another set of cross-links involves α chains, and—in contrast to γ-γ dimer formation—the ensuing product is multimeric in nature (McKee et al., 1970). β chains are not involved in fibrin cross-linking (Chen and Doolittle, 1969).

The existence of α-chain multimers in cross-linked fibrin implies that more than one connection site exists in α chains, and Pisano et al. (1971) have shown definitively that as many as 6 mol of cross-link can exist per mol of starting fibrinogen in fully cross-linked fibrin, 2 of which are presumably involved in γ-chain cross-linking and 4 in α-chain connections. Since the fibrinogen molecule is a dimer, this reduces to one acceptor site on γ chains and two on α chains. Indeed, evidence for two different α-chain *acceptor* sites has been brought forth by several laboratories (Chen, 1970; Doolittle et al., 1972; Takagi and Doolittle, 1975; Ferguson et al., 1975). One of these has been found to be located in a midsection region between residues 200 and 250 (Takagi and Doolittle, 1975); the other is located more distally toward the carboxy terminus (Takagi and Doolittle, 1975; Ferguson et al., 1975).

Knowing the amino acid sequence around the midsection acceptor site, and knowing that this acceptor site was situated in a cyanogen bromide fragment which consists of only three residues (Pro-Gln-Met), we undertook an investigation to find that tripeptide in a cyanogen bromide digest of fully cross-linked fibrin to see if we might find a donor fragment attached to it. We also knew from other studies ongoing in our laboratory that none of the cyanogen bromide peptides of the γ chain and probably none of the β chain have amino-terminal proline. Accordingly, we thought that we could locate the cross-linked material on the basis of amino-terminal analysis alone. Unfortunately, the only amino-terminal proline we found was in the smallest molecular weight fractions, where it would be expected if it were not cross-linked. Instead, we found large molecular weight material involving two other α-chain cyanogen bromide fragments, both of which are described individually in a preceding paper in this issue (Doolittle et al., 1977). One of these, if not disulfide bound or involved in cross-linking, ought to emerge in a small-medium molecular-weight fraction. The other is the largest molecular-weight fraction remaining after cyanogen bromide fragmentation of α chains.

Experimental Procedures

Materials and Methods. With the following exceptions, the materials used and the analytical procedures employed have

† From the Department of Chemistry, University of California—San Diego, La Jolla, California 92093. *Received September 29, 1976.* Supported by United States Public Health Service Grants HE-12,759 and GM-17,702.

‡ Present address: Department of Soil Science, University of Hawaii, Honolulu.
§ Present address: Department of Biochemistry, University of Washington, Seattle.

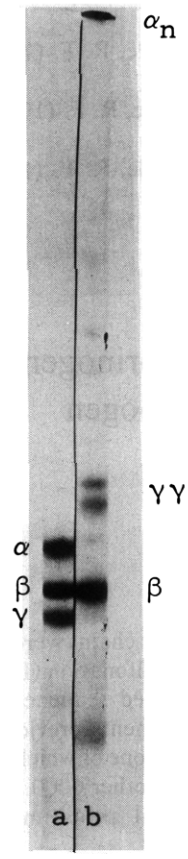

FIGURE 1: Sodium dodecyl sulfate gel electrophoresis (5% acrylamide) of (a) reduced human fibrinogen and (b) fully cross-linked fibrin.

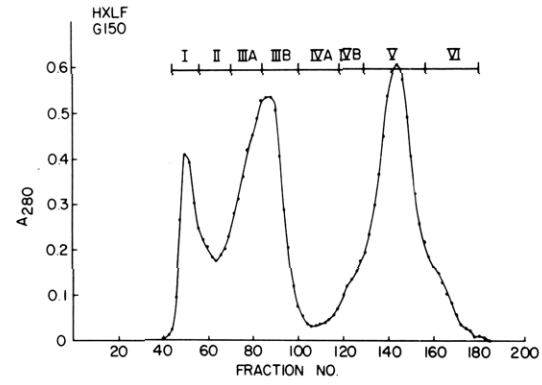

FIGURE 2: Gel filtration of cyanogen bromide digested fully cross-linked fibrin on Sephadex G-150 (2.5 × 90 cm) equilibrated and developed with 10% acetic acid. Approximately 60 mg of material was applied on eight separate occasions. Flow rate = 15 mL/h; fraction size = 2.5 mL.

been described in a preceding paper in this issue (Doolittle et al., 1977). Streptokinase (ca. 100 units/mg) was obtained from Nutritional Biochemicals. Lysine–Sepharose was prepared according to the procedure described by Deutsch and Mertz (1970). Thrombin (bovine, topical) was obtained from Parke-Davis. With regard to analytical procedures, the amino-terminal identification of some poorly soluble peptides and proteins was accomplished using the Dns-Cl[1] procedure as modified by Gros and Labouesse (1969), in which cases the preparations were dissolved in concentrated urea solutions. Cyanoethylation as a procedure for determining lysines involved in side-chain amide or ester bonding was conducted according to the procedure of Pisano et al. (1969).

Preparation of Plasminogen-Free Fibrinogen. The long incubation time necessary to ensure complete cross-linking of the fibrin necessitated that the starting fibrinogen be plasminogen-free in order to minimize proteolytic damage to the system. To this end, 433 mg of human fibrinogen prepared by a modified cold-ethanol procedure (Doolittle et al., 1967) was dissolved in 22 mL of 0.3 M NaCl–0.005 M sodium phosphate buffer, pH 7.2, and passed over a lysine–Sepharose column (bed volume ca. 5 mL) along the lines suggested by Matsuda et al. (1972). Aliquots of the fibrinogen solution were tested for plasminogen contamination before and after passage over the lysine–Sepharose by the addition of streptokinase. Thus, duplicate incubation mixtures of the following composition were prepared in both instances: 0.2 mL of fibrinogen solution (5 mg/mL), 0.1 mL of streptokinase (2 mg/mL), and 0.2 mL of thrombin (0.1 mg/mL). The preparations were incubated at 37 °C, and clotting occurred in about 1 min. The fibrin prepared from unabsorbed fibrinogen was completely lysed in 16 h, whereas the fibrin made from the fibrinogen which had been passed over the lysine–Sepharose was still solid after a week's incubation.

The bulk of the plasminogen-free fibrinogen (390 mg/27.5 mL) was clotted by the addition of an equal volume of a solution containing thrombin (0.1 mg/mL), 0.025 M cysteine, and 0.05 M $CaCl_2$, the latter components being present in order to activate contaminating factor XIII present in the fibrinogen preparation. The fibrin clot was let stand for 18 h, after which time the clot liquor was pressed out and the fibrin washed thoroughly with water. An aliquot was removed for dissolution in 8 M urea–2% dodecyl sulfate containing 0.1 M mercaptoethanol. After incubation at 37 °C for 24 h, the preparation was examined by sodium dodecyl sulfate gel electrophoresis and found to have its α chains fully cross-linked in the multimeric mode (Figure 1).

Cyanogen Bromide Fragmentation. The fully cross-linked fibrin was snipped into small pieces with scissors and suspended in 42 mL of 70% formic acid, following which 700 mg of cyanogen bromide was added. After gentle stirring at room temperature (22 °C) for 17 h, the preparation was diluted with 10 volumes of cold distilled water and immediately freeze-dried.

Fractionation of CNBr Fragments. The freeze-dried powder was dissolved in 10% acetic acid (60 mg/6 mL in each batch) and chromatographed on Sephadex G-150 (Figure 2). Altogether, six different columns were run, and all yielded profiles essentially the same as that shown in Figure 2. In each case, the effluent was pooled into eight fractions and freeze-dried. Aliquots of each of these fractions were subjected to amino acid analysis and amino-terminal identification by the Dns-Cl procedure or thioacetylation or, in the cases of pools I and II, both.

Amino-Terminal Identification and Amino Acid Analyses. The amino terminals identified for each of the fractions are listed in Table I together with a tabulation of amino terminals identified for cyanogen bromide fragments of the individual chains. It is noteworthy that the only amino-terminal proline found was in pool VI, the smallest molecular-weight fraction. Beyond that, the presence of both cysteine and glucosamine in pool IIIA, as well as the identification of tyrosine and glycine amino terminals, indicates the position of the amino-terminal disulfide knot. Its molecular weight is 48 000, and in cyanogen bromide digests of noncross-linked fibrin it is the leading

[1] Abbreviations used are: TATG, thioacetylthioglycolic acid; Dns, 8-dimethylamino-1-naphthalenesulfonyl; CNBr, cyanogen bromide.

TABLE I: Distribution of Amino-Terminal Amino Acids in Cyanogen Bromide Fragments of Isolated α, β, and γ Chains of Human Fibrin Compared with Fully Cross-Linked Fibrin Fragments.

	Isolated Chains			CNBr Pools of Cross-Linked Fibrin[a]							
Amino Acid	α[b]	β[c]	γ[d]	I	II	IIIA	IIIB	IVA	IVB	V	VI
Asp	1	2	1							+	+
Thr											
Ser											
Glu	2	2	1	+					+		
Pro	1									+	+
Gly	1	3				+	+			+	+
Ala	1	1								+	+
Cys											
Val											
Ile			1							+	+
Leu	1		1	+						+	+
Tyr		2	1			+	+	+	+		
Phe		1	1						+	+	
His		1							+	+	
Lys	3	2	3				+	+	+		+
Arg	1									+	+
Total	11	14	9								

[a] See Figure 2 for pool designation. [b] From Doolittle et al. (1977). [c] From Takagi and Doolittle (unpublished observations). [d] From Doolittle, Takagi, Bouma, Cottrell, and Friezner (unpublished observations).

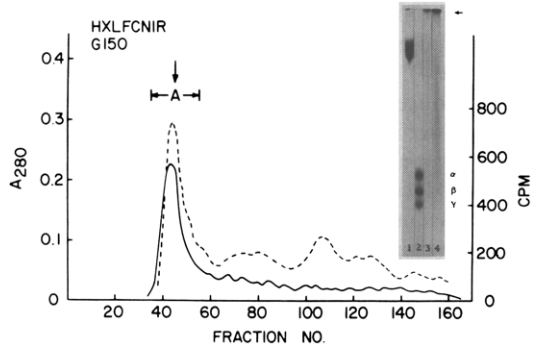

FIGURE 3: Rechromatography of peak I (from Figure 1) material which had been reduced and ^{14}C carboxymethylated. The column and conditions are the same as in Figure 1. The inset shows sodium dodecyl sulfate gels (4% acrylamide) of the rechromatographed peak I material. Gel 1, reference material (unreduced human fibrinogen); gel 2, reference material (reduced human fibrinogen); gel 3, high-molecular-weight peak I material; gel 4, same as gel 3, but double load. All four gels were stained with Coomassie blue.

component in gel filtration. Accordingly, the larger molecular-weight material found in pools I and II (Figure 2) was subjected to further study.

Reduction and Carboxymethylation of Pool I. The freeze-dried material from the largest molecular-weight fraction (pool I in Figure 2) was redissolved in 6 M guanidinium chloride and reduced and alkylated with radioactive iodoacetic acid according to the scheme detailed in a preceding article in this issue (Doolittle et al., 1977). After thorough dialysis and lyophilization, the preparation was dissolved in 10% acetic acid and rechromatographed on the same Sephadex G-150 column as had been used in the original isolation. The material emerged from the column at the void volume, just as it had before reduction and carboxymethylation, only now it was radioactive since its constituent cysteine had been converted to carboxymethylcysteine (Figure 3). The reduced and carboxymethylated material, now designated pool IR, was pooled appropriately and freeze-dried; aliquots were subjected

TABLE II: Amino Acid Compositions of Pool IR and Corresponding CNBr Fragments from Isolated α Chains.[a]

Amino Acid	HXLF[b] Pool I	HXLF[c] Pool IR	αCNI[b]	αCNIVB[d]	αCN[e] I + IVB
Asp	9.5	8.2	9.3	5.0	8.5
Thr	10.2	10.5	11.4	6.7	10.5
Ser	13.6	15.3	16.1	25.0	17.8
Glu	11.4	10.3	10.1	13.4	10.7
Pro	6.8	7.6	7.4	3.3	6.6
Gly	13.6	16.5	16.9	10.0	15.6
Ala	4.1	3.7	3.3	1.7	3.0
Cys	1.3	0.6	0.8		0.6
Val	4.5	4.6	4.1	1.7	3.6
Ile	2.4	1.9	1.4	3.3	1.8
Leu	3.9	2.7	2.9	1.7	2.7
Tyr	2.3	1.5	1.1	3.4	1.5
Phe	2.6	2.3	1.6	8.4	2.9
His	2.2	2.2	1.6	3.4	1.9
Lys	4.7	4.6	4.2	6.7	4.7
Arg	4.6	5.1	5.2	5.0	5.2
Trp	1.9	1.8	2.4		1.9
Hse	0.4	0.4	0.3	(1.3)	0.5

[a] Given as mole percent. [b] Duplicate analyses of 24-h hydrolysates. [c] Duplicate analyses of 24- and 48-h hydrolysates. [d] Based on sequence data. [e] Calculated on a ratio of 260:60 residues.

to amino acid analysis, amino-terminal identification, sodium dodecyl sulfate gel electrophoresis, and cyanoethylation.

When the pool IR material was subjected to sodium dodecyl sulfate gel electrophoresis on 4% acrylamide gels, none of the protein could penetrate the gel (Figure 3), indicating a very large molecular weight (>500 000). These observations were consistent with the emergence of the material with the void volume on Sephadex G-150. On the other hand, the results of amino-terminal analysis by the modified Dns-Cl procedure indicated that the approximate yield of end groups was consistent with a much smaller unit molecular weight; the amino terminals determined by this method were glutamic acid and

TABLE III: Lysine Determination after Cyanoethylation of Various CNBr Fractions from Cross-Linked Fibrin and Isolated α Chains.[a]

		Lysine Content[b]		
		Inaccessible	Accessible	Total
Cross-linked materials[c]	HXLF-IR	6	9	15
	HXLF-I	4	11	15
Noncross-linked materials[c]	HXLF-II	1	15	16
	HXLF-IIIA	3	19	22
	HαCNI	2	11	13

[a] In each case approximately 100 μg of material was subjected to cyanoethylation and then to total acid hydrolysis. Amino acid analysis was performed and the amount of lysine found compared with the amount found in the same material which was not subjected to cyanoethylation, the difference being equal to lysines accessible to cyanoethylation. [b] Residues of lysine per 320 residues. [c] Based on gel filtration and sodium dodecyl sulfate gel electrophoresis.

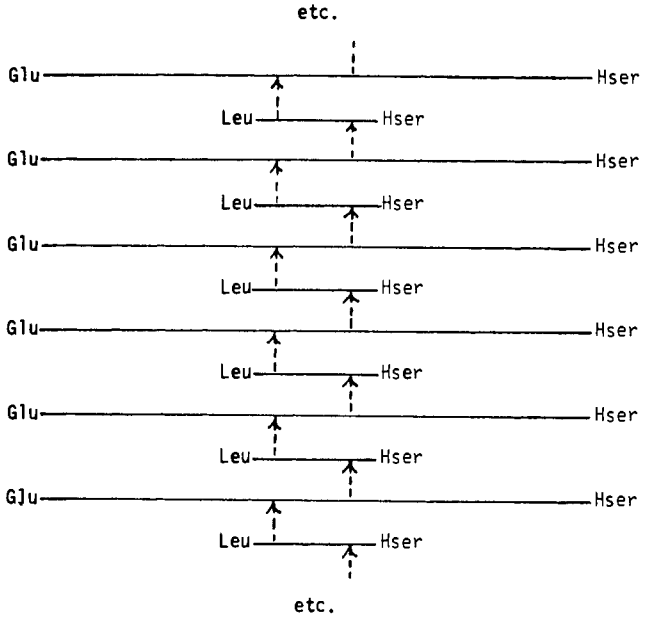

FIGURE 4: Schematic depiction of how cyanogen bromide fragments CNI and CNIVB may be linked to form polymer. The longer chain has amino-terminal glutamic acid and the shorter chain has amino-terminal leucine. The molecular weights of CNI and CNIVB are 29 000 and 6000, respectively, leading to a fundamental CNBr cross-linking fragment molecular weight of $(35\,000)_n$.

leucine. The amino acid composition of the material was similar to but distinguishable from HαCNI, the largest molecular-weight fragment produced upon fragmentation of isolated α chains (Table II). Finally, cyanoethylation indicated the presence of a significant number of inaccessible lysines which are presumably bound in peptide linkage through their ϵ-amino groups (Table III). Unfortunately, we are never able to achieve complete cyanoethylation, even in noncross-linked proteins, and the excess number of free lysines found in the putative cross-linked materials must be regarded as a qualitative index.

Three different preparations of pool I material were degraded by a manual thioacetylation procedure (Doolittle et al., 1977), including two preparations in free solution and one attached to glass beads by the lysine-attachment procedure. Although the background was high in the free-solution preparations and although the first residue is lost when using the lysine procedure, the results of all three degradations were consistent with the existence of equimolar amounts of two α-chain cyanogen bromide fragments being present in pool I. Clearly, the pool I material is an extensively cross-linked polymer composed of two different cyanogen bromide fragments (Figure 4). One of these is the largest molecular-weight fragment produced during cyanogen bromide degradation of isolated α chains; its molecular weight is approximately 30 000 and ordinarily it emerges behind the amino-terminal disulfide knot when produced from noncross-linked fibrin. Its beginning amino acid sequence is Glu-Leu-Glu-Arg-Pro. The other fragment is α-chain CNIVB (Doolittle et al., 1977). Its molecular weight is ordinarily about 6000, and if noncross-linked would be expected to elute in pools V or VI. Its amino acid sequence begins with Leu-Gly-Glu-Phe-Val (Table IV).

TABLE IV: Stepwise Degradation of Pool I Material from Cyanogen Bromide Fragmentation of Fully Cross-Linked Fibrin.

	Amino Acids Found[a]				
	Step 1	Step 2	Step 3	Step 4	Step 5
HXLF pool I	Glu	Leu	Glu	Arg	Pro
	Leu	Gly		Phe	Val
HαCNI	Glu	Leu	Glu	Arg	Pro
HαCNIVB	Leu	Gly	Glu	Phe	Val

[a] The amino acids were regenerated by mild acid hydrolysis from their thioacetyl derivatives after stepwise degradation with either thioacetylthioethane (TATE) or thioacetylthioglycolic acid (TATG). Three different preparations of HXLF-I were run on various occasions. Although the yields varied from preparation to preparation, the values at any given step were consistent with the two fragments being present in equimolar amounts. In these experiments, HαCN-I was run as a control; the sequence of HαCN-IVB was determined independently and is reported in a preceding article in this issue (Doolittle et al., 1977).

Discussion

The unravelment of the α-chain CNBr fragments provided a natural entreé for extending our investigations of α-chain cross-linking. In earlier experiments, we had precisely located an acceptor site in a midsection sequence and less definitively identified another in a large CNBr fragment located more distally toward the carboxy terminus (Takagi and Doolittle, 1975). Unfortunately, acceptor sites for artificial donors—as we have been cautious to point out in the past (Doolittle et al., 1972; Doolittle, 1973)—are not necessarily authentic cross-linking sites, a lesson we have learned well after searching for the acceptor peptide Pro-Gln-Hse which we expected to find in a cross-linking situation in fully cross-linked fibrin. When that search proved fruitless, we turned our attention to the high-molecular-weight fractions produced by the same CNBr fragmentation and promptly identified two α-chain CNBr peptides which had eluted from a Sephadex G-150 column in a position not at all consistent with their being independent. One of these fragments is the largest fragment produced upon CNBr digestion of free α chains and one in which we had previously located an acceptor site (Takagi and Doolittle, 1975). In advance of that, we had found the same site in a tryptic digest of the entire labeled α chain (Chen, 1970; Doolittle et al., 1972). The peptide was distinguished by its unusual amino acid composition, which included ten glycines

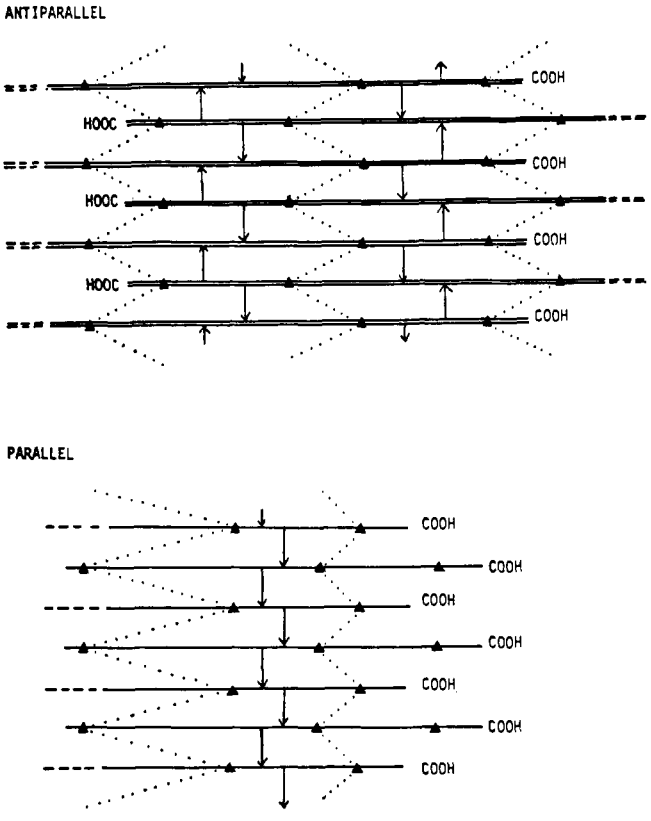

FIGURE 5: Schematic depiction of two ways by which α chains in fibrin may interact to form long polymers consistent with CNBr fragmentation pattern. In either case, the linkage could extend laterally from one intermediate polymer to another. Triangular symbols represent methionines in carboxy half of the α chain, and dotted lines outline expected CNBr fragmentation pattern.

and eight serines among its 32 residues. The other cyanogen bromide fragment involved is one which we have partially sequenced (Doolittle et al., 1977), somewhat more than half of the 55–60 residues having been positioned so far. The peptide is apparently located on the carboxy side of the other fragment involved (Figure 5), and we have tentatively assigned it to the penultimate position on the basis of a significant fraction of this material lacking homoserine, an observation in accordance with there being a very early plasmin cleavage site just before the final methionine of the α chain near the carboxy-terminal end (Cottrell and Doolittle, 1976).

The fact that a polymeric derivative involving two different peptides survives CNBr fragmentation indicates that at the very least two unique ε (γ-glutamyl) lysine bonds are involved in holding α chains together, and that each of the CNBr peptides must have either two acceptor sites *or* two donor sites *or* one of each. Beyond that, there are at least two fundamental ways in which the α chains of neighboring molecules can interact to form multimers (Doolittle, 1974). First, the chains could be oriented in an antiparallel manner in which their oppositely directed ends are intercalated (Figure 5). Alternatively, the interacting fibrin molecules may be nested in such a fashion that extended α chains are parallel but staggered (Figure 5). Either case would lend itself well to lateral cross-linking between fundamental fibrin polymers ("intermediate polymers"), a mode of attachment well suited in a structure-function sense (Doolittle et al., 1972) and for which some suggestive evidence has been reported (Doolittle, 1973).

It will be of considerable interest to find if the two different α-chain cross-linking sites have any sequence homology, in line with the suggestion that the α chain has been extended during its evolution by a series of elongating duplications (Doolittle, et al., 1977). It will also be of interest to find out if there is any structural resemblance with the γ-chain cross-linking segment. To this end, experiments to isolate the tryptic fragments containing these cross-linking units from the large molecular-weight material are currently under way.

Acknowledgments

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Supplementary Material Available

Supplementary data are available for all three papers of this series in this issue (63 pages). Ordering information is given on any current masthead page.

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