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γ–γ Cross-Linking Sites in Human and Bovine Fibrin*

Renné Chen† and Russell F. Doolittle‡

ABSTRACT: The amino acid sequence of the tryptic peptides involved in the γ–γ cross-linking of human fibrin has been studied. Comparison with the previously reported bovine sequences indicated two amino acid differences in the acceptor peptide. One of these changes, a histidine/glutamine change, occurred at position 10, a residue we previously thought was the acceptor half of the ϵ-(γ-glutamyl)lysine cross bridges. Accordingly, a reexamination of the bridge position in the bovine γ–γ cross-linking unit was also undertaken. The sequence of the bovine acceptor peptide was confirmed, but experiments with a radioactive substitute donor (glycine ethyl ester) indicate that the reciprocal cross bridges in both species involve the glutamine residues at position 7. As a consequence, the bridges which link the overlapping antiparallel chains are actually eight residues apart instead of the five previously reported. Characterization of the adjacent carboxy-terminal peptide has revealed it to be a pentapeptide.

The transformation of vertebrate fibrinogen molecules into a fibrin gel is a spontaneous self-assembly process following upon the removal of the fibrinopeptides by thrombin. The resulting gel, which is held together by a variety of weak forces and cooperative effects, is readily dissolved by dispersing agents such as concentrated urea or guanidine solutions. Under appropriate conditions, and certainly *in vivo*, the gelation can be reinforced by a transamidase-catalyzed introduction of ϵ-(γ-glutamyl)lysine covalent bonds. Fibrin gels containing significant numbers of these cross-links are no longer dispersable in urea or guanidine solutions. A series of reviews dealing with various aspects of fibrinogen and fibrin chemistry has recently appeared (Blombäck and Blombäck, 1970; Doolittle, 1970; Loewy, 1970; Lorand, 1970; Mihalyi, 1970; Pisano *et al.*, 1970).

Recently we reported the occurrence of two distinct bridging systems in cross-linked fibrin involving different constituent polypeptide chains, although in both cases presumably employing ϵ-(γ-glutamyl)lysine bridges (Chen and Doolittle, 1969). In one system, γ chains are dimerically[1] linked to other γ chains (Chen and Doolittle, 1969, 1970; Takagi and Iwanaga, 1970). In the second kind, we initially thought that γ chains were hooked to α chains. McKee *et al.* (1970) subsequently have shown that the latter system actually involves only α chains; in contrast to the γ–γ dimers, the α-chain cross-linking process continues at a slow but persistent rate to the formation of multimers.

The γ–γ cross-linking unit consists of overlapping antiparallel carboxy-terminal γ-chain segments which are reciprocally bridged. Originally, we reported that the ϵ-(γ-glutamyl)lysine cross-links were situated only five residues apart in cross-linked bovine fibrin. At the time, however, we noted that there were at least two amino acid replacements in the corresponding human peptides. Completion of the human amino acid sequence in this region has revealed that one of these changes is the replacement of the glutamine residue (glutamine-10) we had supposed was the acceptor portion of the cross-links. As a result we have reinvestigated the bovine structure also and now report that, although the proposed amino acid sequence of the bovine acceptor peptide was indeed correct, the principle site of incorporation of a radioactive substitute donor is at glutamine-7 in both species, indi-

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[1] It has been pointed out to us that the existence of γ–γ dimers was first suggested by Wilkinson (1967).

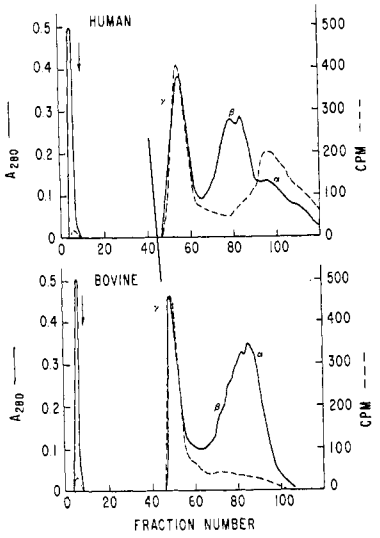

FIGURE 1: Carboxymethylcellulose chromatography of sulfitolyzed human (top) and bovine (bottom) fibrins which had been clotted in the presence of 0.03 M [^{14}C]glycine ethyl ester, 0.025 M calcium chloride, and 0.0125 M cysteine for 40 min. In both cases about 100 mg of protein was applied to the column (2.5 × 20 cm). Elution was obtained with a linear concentration gradient of sodium acetate at pH 5.2; the flow rate was 60 ml/hr. Solid lines represent the absorbance at 280 mμ; broken lines depict radioactivity incorporated from the [^{14}C]GlyOEt. Fraction size = 10 ml. Aliquots for counting radioactivity = 0.1 ml.

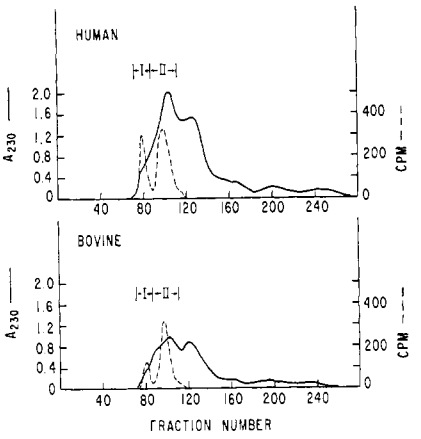

FIGURE 2: Gel filtration on Sephadex G-25 (5.5 × 70 cm) of tryptic digests of human (top) and bovine (bottom) γ chains prepared from [^{14}C]GlyOEt–fibrin. Approximately 80 mg of digest was applied; columns were equilibrated with 0.1 M ammonium bicarbonate and developed at a flow rate of 100 ml/hr. Solid lines represent absorbance at 230 mμ; broken lines depict radioactivity from incorporated [^{14}C]GlyOEt. Fraction size = 10 ml. Aliquots for counting radioactivity = 0.3 ml.

cating that the reciprocal ε-(γ-glutamyl)lysine bridges are actually eight residues apart.

Previously we had reported (Chen and Doolittle, 1970) that the tryptic peptide adjacent to the donor–acceptor segment consisted only of aspartic acid and valine. Further characterization has revealed that this peptide, which represents the carboxy terminus of the γ chain, is actually a pentapeptide in both the human and the bovine.

Materials and Methods

Human fibrinogen was prepared from blood bank blood by a modified Cohn ethanol fractionation procedure (Doolittle *et al.*, 1967). Purified bovine fibrinogen was prepared from Armour fraction I by ammonium sulfate precipitation (Laki, 1951). Thrombin was purchased from Parke-Davis, and [^{14}C]glycine ethyl ester was obtained from New England Nuclear. Thioacetylthioglycolic acid (TATG)[2] was prepared according to the method of Jensen and Pedersen (1961).

With the major exception of the novel stepwise degradation referred to below, most other methods used in this study were described in our previous articles (Chen and Doolittle, 1969, 1970). The degradation method (Mross, 1971; Mross and Doolittle, 1971) involves attachment of peptides to derivatized polystyrene resins and subsequent stepwise removal of residues using TATG. The major advantage of the method lies in the ready regeneration of the free amino acid by routine acid hydrolysis, making it possible to quantify each step on the amino acid analyzer. The resin attachment also makes it possible to continue a degradation through to the last residue of a peptide. In several cases peptides were also degraded by the three-cycle Edman method (Blombäck *et al.*, 1966). In the case of the bovine γ-chain carboxy-terminal peptide, the DNS-Edman method was used as described by Hartley (1970).

[2] Abbreviations used are: GlyOEt, glycine ethyl ester; TATG, thioacetylthioglycolic acid; PTH, phenylthiohydantoin; DNS, 5-dimethylamino-1-naphthylsulfonyl.

Results

Incorporation of [^{14}C]GlyOEt into Human and Bovine Fibrins. The execution of the experiments described in this article depended largely on the use of the radioactive substitute donor glycine ethyl ester (Lorand and Jacobsen, 1964) which competes with particular lysyl side chains for incorporation into acceptor sites. Human fibrin, prepared under conditions suitable for activating the cross-linking enzyme, incorporated significantly more [^{14}C]GlyOEt substitute donor than did bovine preparations treated the same way. The carboxymethylcellulose chromatographic profiles for incorporated radioactivity reflect this difference (Figure 1). Under the conditions employed, the human preparation incorporated [^{14}C]GlyOEt into both γ chains and α chains, whereas the bovine fibrin, as previously reported (Chen and Doolittle, 1969), was labeled exclusively in the γ chains.

In all these experiments, however, the cross-linking of fibrin units or the incorporation of substitute donors into them is dependent on the presence of contaminating amounts of the precursor form of the plasma transamidase, factor XIII, in the starting fibrinogen. In the presence of calcium ions and a suitable sulfhydryl reagent, the transamidase is activated by the thrombin added to bring about clot formation. It is likely that the human fibrinogen, purified by the cold ethanol procedure, had more surviving transamidase activity than did the bovine fibrinogen purified by the ammonium sulfate method, rather than there being any fundamental difference in the mode of incorporation attributable to differences in fibrin structure in the two species. In this regard, Lorand and Chenoweth (1969) have reported that similarly prepared bovine material can also be labeled in both the γ-chain and α-chain regions if the incorporation is allowed to continue for extended periods.

The regions containing radioactivity (Figure 1) were

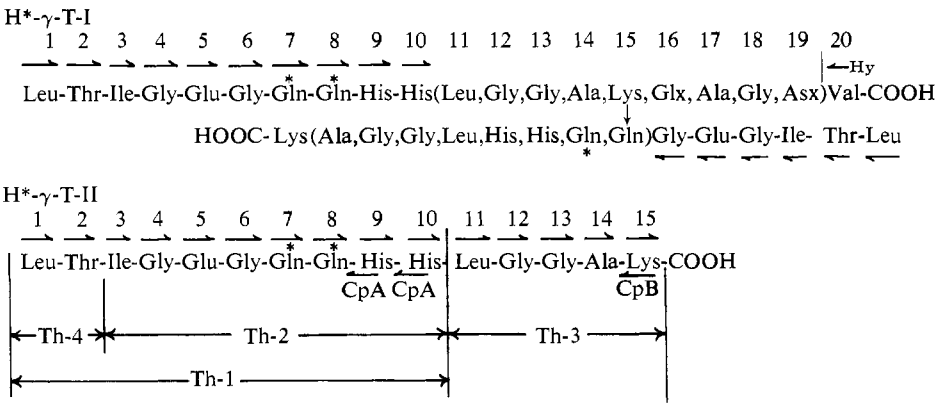

FIGURE 3: Summary of data used to deduce the structure of the tryptic fragments involved in the formation of the human cross-linked γ–γ dimer. Arrows directed from amino-terminal end indicate stepwise degradations using either phenyl isothiocyanate or thioacetylthioglycolic acid (TATG). Other symbols: Th = thermolysin; CpA = carboxypeptidase A; CpB = carboxypeptidase B; Hy = hydrazinolysis. The residues marked with * are labeled fully or in part with the [^{14}C]glycine ethyl ester substitute donor. The vertical arrow in H*-γ-T-I represents the ε-(γ-glutamyl)lysine cross bridge. H*-γ-T-I and H*-γ-T-II were isolated from gel filtration pools I and II, respectively (Figure 2).

TABLE I: Amino Acid Compositions of [^{14}C]GlyOEt Labeled Peptides Isolated from Tryptic Digests of Human and Bovine γ Chains.[a]

	Human		Bovine	
Amino Acid	H*-γ-T-I	H*-γ-T-II	B*-γ-T-I	B*-γ-T-II
Lysine	1.9 (2)	1.0 (1)	1.8 (2)	1.2 (1)
Histidine	3.5 (4)	1.6 (2)	1.7 (2)	0.9 (1)
Aspartic acid	1.1 (1)	<0.1 (0)	1.2 (1)	<0.1 (0)
Threonine	1.7 (2)	1.0 (1)	<0.1 (0)	<0.1 (0)
Glutamic acid	7.1 (7)	3.1 (3)	9.4 (9)	4.0 (4)
Glycine	11.1 (9)[b]	6.0 (4)[b]	10.3 (9)[b]	4.9 (4)[b]
Alanine	3.1 (3)	1.1 (1)	5.0 (5)	2.1 (2)
Valine	0.9 (1)	<0.1 (0)	0.9 (1)	<0.1 (0)
Isoleucine	1.7 (2)	1.0 (1)	1.9 (2)	1.1 (1)
Leucine	4.0 (4)	1.6 (2)	3.8 (4)	1.9 (2)
Total Residues	36.1 (35)[b,c]	16.4 (15)[b]	36.0 (35)[b,c]	16.1 (15)[b]

[a] Values are expressed in residues per mole of peptide or peptide pair (calculated as molar ratios of amino acids recovered). Duplicate determinations were made on a Spinco Model 120B amino acid analyzer after total acid hydrolysis. Numbers in parentheses are integer values established by further study of peptide fragments and/or sequential degradations. [b] Whole number values do not include glycines contributed by substitute donor [^{14}C]GlyOEt. [c] The total of 35 residues actually represents a 15-residue peptide cross-linked to a 20-residue peptide.

pooled, dialyzed extensively against water, and freeze-dried. Accumulated material from a large number of columns was stored up for tryptic digestion and isolation of labeled peptides.

Tryptic Digestion of γ Chains and Separation of Radioactive Peptides. The radioactive γ-chain pools from human and bovine fibrin preparations were digested with trypsin and passed over a large Sephadex G-25 column (Figure 2). In both cases two radioactive peaks were obtained. These were pooled, freeze-dried several times, and subjected to further purification by paper electrophoresis at pH 2 and/or pH 4.1, 300 V for 3–4 hr. The amino acid compositions of the purified substances were determined after total acid hydrolysis (5.7 N HCl, 110°, 24 hr *in vacuo*) and are listed in Table I. The data are in accord with our previous finding (Chen and Doolittle, 1970) that the radioactive material in peak I is a linked dimer of the peptide isolated from pool II. Because one of the lysyl side chains in the dimer is involved in the cross-link formation and is therefore resistant to trypsin, the dimer also contains molar amounts of the adjacent carboxy-terminal pentapeptide, which fortuitously represents the carboxyl terminal of the γ chain and has a carboxy-terminal valyl residue.

Amino Acid Sequence of Human Acceptor Peptide (H-γ-T-II).* The amino acid sequence of the radioactive peptide isolated from pool II of the human γ-chain digests was determined using procedures similar to those reported for the corresponding bovine peptide (Chen and Doolittle, 1970). Its amino acid composition was similar to the bovine peptide except for the replacement of one glutamine and one alanine

TABLE II: Amino Acid Compositions of Thermolysin Fragments of Human and Bovine Cross-Link Acceptor Peptides.[a]

Amino Acid	From H*-γ-T-II			From B*-γ-T-II	
	Th-1 + Th-2[b]	Th-3	Th-4	Th-1	Th-2
Lysine		1.0 (1)			0.9 (1)
Histidine	1.7 (2)			0.8 (1)	
Threonine	0.6 (f)[b]		1.1 (1)		
Glutamic acid	2.9 (3)			4.0 (4)	
Glycine	3.4 (2)[c]	2.1 (2)		3.1 (2)[c]	2.1 (2)
Alanine		1.0 (1)		1.0 (1)	1.0 (1)
Isoleucine	0.9 (1)			1.0 (1)	
Leucine	0.6 (f)[b]	0.9 (1)	0.9 (1)	0.8 (1)	0.9 (1)
Total residues	10.1 (9)[b,c]	5.0 (5)	2.0 (2)	10.7 (10)[c]	4.9 (5)

[a] Expressed in molar ratios of amino acids recovered after total acid hydrolysis. [b] Th-1 and Th-2 (from H*-γ-T-II) were very difficult to separate and were usually isolated together. In this preparation about 40% of the terminal dipeptide had been removed by the thermolysin treatment; the symbol f = fractional. [c] Integer values do not include glycines contributed by substitute donor [^{14}C]GlyOEt.

residue by one histidine and one threonine residue. The human peptide also had a higher level of glycine reflecting increased incorporation of the [^{14}C]GlyOEt (Table I).

Digestion of this 15-residue peptide with thermolysin yielded four peptides, one of which was an overlap peptide (Th-1) composed of two of the others (Th-2 and Th-4) (Figure 3). The amino acid composition data (Table II) indicated that the alanine/threonine (bovine/human) change occurred at the second residue from the amino terminal and that the only other change was a replacement of one of the three glutamines in the bovine acceptor peptide by a histidine in the human peptide. Digestion of peptides Th-1 and Th-2 (from H*-γ-T-II) with carboxypeptidase A released about 2 moles of histidine/mole of peptide, indicating that the glutamine which had been replaced was at position 10.

Th-3 was a pentapeptide with the same composition as a lysine-containing peptide previously isolated from a thermolysin digest of the bovine acceptor peptide and corresponds to the carboxyl terminus of H*-γ-T-II. This arrangement was confirmed by the observation that carboxypeptidase B treatment revealed that lysine was the carboxyl-terminal residue of H*-γ-T-II.

The three residues at the amino-terminal end of Th-3 were originally determined using a modified subtractive Edman method. The same peptide was subsequently studied using the TATG–resin method and all five residues were identified. A mixture of Th-1 and Th-2 was also examined by this method, and a sequence was determined which was compatible with all the other data.

Structure of the Linked Donor–Acceptor Units. Even in the presence of the substitute donor, a certain amount of natural cross-linking occurs between γ chains during clotting. As a consequence, tryptic digests contain a set of cross-linked peptide pairs in which one acceptor has incorporated the radioactive substitute donor whereas the reciprocal acceptor site is bonded to the native lysyl side chain donor, which happens to be the carboxy-terminal lysyl residue of the trypsin-derived acceptor peptide. Since the cross-linked lysyl side chain is invulnerable to trypsin cleavage, the adjacent carboxy-terminal peptide is also present in these fragments in a ratio of 1:2 compared with the acceptor peptides.

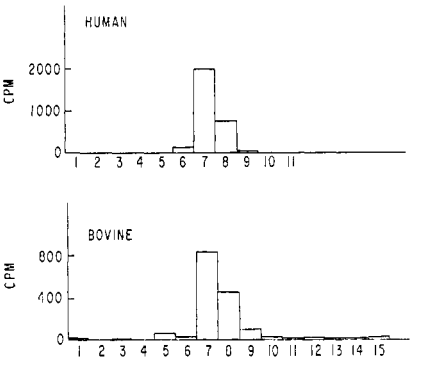

FIGURE 4: Histograms depicting release of incorporated radioactivity from human and bovine acceptor peptides. In the case of the human donor–acceptor unit (top), the purified material (H*-γ-T-I) was carried through 11 Edman cycles and all extractant solutions were counted. Only the radioactivity soluble in the organic phase after cleavage and conversion is plotted, however. The bovine pentadecapeptide (bottom) is the tryptic acceptor peptide (B*-γ-T-II) which was subjected to 15 consecutive degradations by the TATG method.

Previously (Chen and Doolittle, 1970) we had presumed that the adjacent peptide consisted of only aspartic acid and valine, since, apart from our demonstration of carboxy-terminal valine, total amino acid analysis indicated appropriate amounts of aspartic acid and valine in addition to the known compositions of the individual acceptor peptides. Subsequent isolation of the labeled acceptor fragment from cyanogen bromide digests of γ chains[8] has allowed us to obtain the adjacent tryptic peptide in full yield, however, and we have determined that it is actually a pentapeptide. The additional three amino acids were represented by those appearing most often in the acceptor peptide, and recalculation of our original amino acid compositions using the appropriate number of total residues has improved the integral values (molar ratios) for amino acids in the linked donor–acceptor units

[8] R. F. Doolittle, G. L. Wooding, and J. J. Sharp, unpublished experiments.

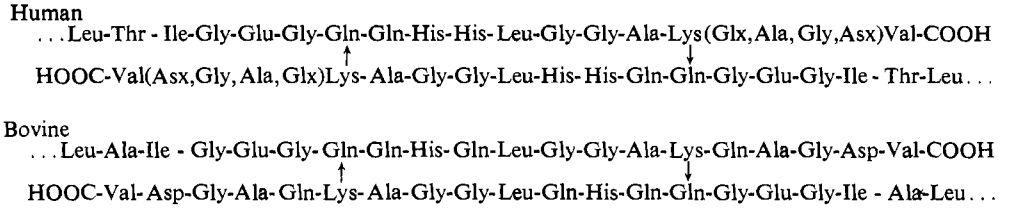

FIGURE 5: Antiparallel arrangement of carboxy-terminal segments of γ chains showing proposed location of reciprocal ϵ-(γ-glutamyl)-lysine cross bridges. The vertical arrows denote the bridge positions.

(Table I). In the case of the bovine carboxy-terminal pentapeptide, four successful cycles of the DNS-Edman method (Hartley, 1970) were performed. Also, extensive enzymatic hydrolysis with leucine aminopeptidase released equimolar amounts of glutamine, alanine, glycine, aspartic acid, and valine. It should be noted that Mills and Liener (1969) reported the carboxy terminal of human γ chains to be (Ser,Gly,Ala)Asp-Val. Serine is not present in our human linked peptides, however, the amino acid composition indicating that the human terminal pentapeptide has the same structure as the bovine.

Stepwise degradation of the human donor–acceptor unit (H*-γ-T-I) successfully identified ten consecutive residues from the amino terminus. A PTH derivative corresponding to a γ-glutamylglycine was found at position 7. At position 8, however, both PTH-glutamine and the γ-substituted derivative were found. PTH-histidine was identified at both positions 9 and 10. A summary of sequence information for the linked and unliked human peptides is presented in Figure 3.

Verification of Bovine Acceptor Peptide Sequence (B-γ-T-II).* The finding that the glutamine at bovine position 10 had been replaced by a histidine residue in the human acceptor peptide was surprising to us, since we had supposed that particular residue to be the acceptor site for cross-link formation. Accordingly, we undertook a reexamination of the bovine sequence using the TATG–resin method. About 0.2 μmole of the pentadecapeptide was attached to a derivatized polystyrene resin and subjected to 15 TATG degradative cycles. The regenerated amino acids were identified on a Spinco automatic amino acid analyzer. At positions 7 and 8 glycine from the substitute donor, [^{14}C]GlyOEt, was found in addition to the anticipated glutamic acid residues (these were glutamines before derivatization and hydrolysis). In all other cases only a single amino acid contributed significantly, including the fifteenth and final step, which yielded lysine in good yield.

Exact Position of [^{14}C]GlyOEt Substitute Donor and Natural Cross-Links in Human and Bovine γ-Chain Acceptor Peptides. Previously, we had reported (Chen and Doolittle, 1970) that the cross-links in the bovine γ–γ donor–acceptor unit occurred between glutamine-10 in one peptide and lysine-15 in its antiparallel partner. The discovery that the human acceptor peptide has a histidine at position 10 forced us to reconsider the problem of bridge positions. In the human case there were two acceptor possibilities, glutamine-7 and glutamine-8. The bovine has three glutamines, at positions 7, 8, and 10. Although it was possible that the bovine system might employ a different glutamine residue for its cross-link than does the human system, it seemed altogether unlikely. Accordingly we compared the two species with regard to exact position of the incorporated substitute donor.

The position of the radioactive substitute donor, [^{14}C]-GlyOEt, was established in the case of the human donor–acceptor unit (H*-γ-T-I) by counting the radioactivity of the extracted derivatives at each cycle of 11 Edman degradations. The largest amount of radioactivity was released at step 7 (Figure 4, top) in the form of a PTH-γ-glutaminylglycine derivative. A significant amount of this same radioactive material was also released at step 8, however, as well as a substantial amount of PTH-glutamine. The possibility that the stepwise degradative cycle was merely out of phase was rendered unlikely by the fact that only trivial amounts of PTH-glutamine appeared in step 9, which was clearly identified as PTH-histidine. In this linked peptide pair, then, it was clear that the major incorporation site was glutamine-7, but that the transamidase had also been able to incorporate substitute donor into glutamine-8. Stepwise degradations of thermolysin fragments of the unlinked human acceptor peptide (H*-γ-T-II) provided a similar picture.

Finally, the complete TATG-resin degradation of the bovine acceptor peptide (B*-γ-T-II) allowed us to locate the substitute donor in this system also, and it yielded a similar profile (Figure 4, bottom). Virtually no radioactivity was released at position 10, establishing unequivocally that glutamine-10 is not an acceptor residue in the cross-linking system. It was also clear that the human and bovine systems were behaving similarly with regard to incorporation sites.

Discussion

Our original aim in studying fibrin cross-linking was to find out about the contact sites involved in fibrin polymerization. We reasoned that there is probably little or no rearrangement of individual molecules (fibrin monomers) during the covalent reinforcement of fibrin, and that if we could find out which polypeptide chains (α, β, or γ) were involved in cross-linking, we might be able to identify which chains are brought into contact during the initial polymerization process. It is now clear that in the case of the γ–γ cross-linking system, neighboring molecules[4] are reciprocally linked by two ϵ-(γ-glutamyl)lysine cross bridges near their carboxy-terminals (Figure 5). The question arises as to whether these antiparallel oriented carboxy-terminal γ-chain segments are in direct contact, or merely close juxtaposition, before the introduction of the covalent cross bridges. In order to gain some possible insight into this association, we constructed space-filling models of the γ-chain carboxy-terminal eicosapeptides, assuming that the cross-linking region is α helical. Significantly, if two of these peptide models are aligned in an antiparallel fashion, the two chains fit together quite naturally with the lysine-15 of one chain situated opposite the glu-

[4] Proof that the γ–γ links are intermolecular, as opposed to being formed between γ chains of the same fibrin monomer, was recently obtained by isolating donor–acceptor pairs from a mixed human–bovine fibrin system (Doolittle *et al.*, 1971).

tamine-7 of the other and *vice versa*. Moreover, leucine-11 sits comfortably next to leucine-11 of its neighbor, neatly filling the void between the bridge residues, whether or not the isopeptide linkages are actually formed. Furthermore, glutamine-8, which became significantly labeled in our incorporation experiments with [^{14}C]glycine ethyl ester (Figure 4), remains in a very exposed position, quite suitable for incidental labeling with a small molecular weight substitute donor, but quite remote from lysine-15. It seems reasonable to presume that the [^{14}C]GlyOEt introduced at glutamine-8 is spurious and does not reflect any ambiguity on the part of the cross-linking enzyme (activated factor XIII) when it is actually forming native ϵ-(γ-glutamyl)lysine cross bridges.

The antiparallel orientation of the γ–γ cross-linking segments is consistent with a fibrin polymerization mode involving either directly abutting end-to-end contacts or a staggered overlap of the type first suggested by Ferry (1952) which leads to an initial polymer two molecules thick (Figure 6). Either of these association modes is likely the type involved in the initial stages of fibrin assembly. It will be of interest to find if the slower forming α-multimer cross-linking system (McKee *et al.*, 1970) is associated with the latter stages of the fibrin polymerization process in which the fibers apparently pack laterally and assume their characteristic banded pattern.

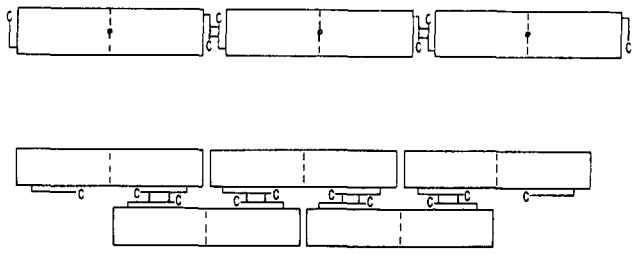

FIGURE 6: Schematic representation of two possible modes of early fibrin assembly process which are consistent with the formation of reciprocally bound γ–γ dimers.

Acknowledgments

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