Factor XIIIa-Catalyzed Cross-Linking of Recombinant αC Fragments of Human Fibrinogen[†]

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ABSTRACT: Direct measurements of the structure and function of the COOH-terminal portion of the Aα chain (residues 220−610) of human fibrinogen have been hampered by the difficulty of isolating intact fragments derived from this protease-sensitive region. Here, we overcame this problem by expressing two fragments, αC45K (Aα221−610) and a truncated version of it, αC30K (Aα368−610), in *Escherichia coli*. Both proteins were purified to homogeneous state, and their integrity was confirmed at protein level by sequencing. Upon treatment with factor XIIIa, the αC45K fragment but not the αC30K fragment formed polymers similar to those derived from fibrin clots. Sequence analysis of cross-linked αC45K polymers revealed involvement in the cross-linking reaction of at least three Gln residues (221, 237, 328) in the NH_2-terminal region of the fragment and four Lys residues (539, 556, 580, 601) located in the COOH-terminal part of the molecule. In addition, a fraction of αC45K fragment was found in an intramolecular cross-linked form, suggesting a high level of flexibility of its polypeptide chain and consistent with the location of its donor and acceptor residues in clusters near the ends of the molecule. The αC30K fragment, lacking the NH_2-terminal Gln residues, was not able to form polymers or internally cross-linked monomers. Thus, the C-terminal part of the Aα chain comprises an autonomous, functionally active, and flexible region that plays a key role in α polymer formation and stabilization of fibrin clots by factor XIIIa.

Fibrinogen is a multidomain protein involved in blood coagulation, fibrinolysis, and other important aspects of hemostasis. It consists of three pair of nonidentical polypeptide chains, Aα, Bβ, and γ, forming two identical subunits. Both the subunits and the chains are linked together by disulfide bonds to form a symmetrical trinodular structure (Doolittle, 1973, 1984, 1994). Each subunit comprises a number of structural and functional domains which determine the multifunctional character of this complicated molecule. Among them are the αC domains formed by the C-terminal two-thirds of each Aα chain which are situated on the surface of the molecule and are involved in fibrin assembly and other processes (Mosesson et al., 1967; Medved et al., 1985). Their importance is evident from the fact that congenital defects in this region including single amino acid substitutions in fibrinogens Caracas II and Dusart (Maekawa et al., 1991; Koopman et al., 1993) or deletion of the Aα461−610 region in fibrinogen Marburg (Koopman et al., 1992) are associated with a family history of recurrent thrombosis. These fibrinogens exhibited abnormalities in polymerization and defective *in vivo* thrombolysis (Maekawa et al., 1991; Soria et al., 1983). The latter was connected with the importance of αC domains for enhancing the activation of plasminogen by tPA on the fibrin surface (Lijnen et al., 1984). The αC domains are also known to interact with thrombospondin (Tuszynski et al., 1985) and integrin-type platelet and endothelial cell receptors (Cheresh et al., 1989). In addition, these regions control activation of factor XIII (Credo et al., 1981) and serve subsequently as its substrate, becoming cross-linked to each other and to fibronectin, α_2-antiplasmin, thrombospondin, and von Willebrand factor (Mosher, 1976; Sakata & Aoki, 1980, 1982; Hada et al., 1982; Bale et al., 1985).

Although the importance of the αC domains is well established, not much is known about the mechanism of α polymer formation and its structure. It is also not known whether fXIIIa-catalyzed α polymer formation requires the presence of the rest of the fibrin(ogen) molecule or if αC domains could act as independent units. Very limited information is available about the number and exact location of reactive Lys residues participating in fXIIIa-catalyzed cross-linking. The fact that the COOH-terminal portions of Aα chains are easily degraded by proteases (Kirschbaum & Budzynski, 1990) was a major obstacle for preparation and investigation of this very important fragment of fibrinogen. Thus, most of what we know about structure and functions of αC domains has been deduced by comparing the behavior of intact fibrin(ogen) with proteolytic derivatives lacking this portion of the Aα chains (X fragments). In the present study, we overcame this problem by expressing in *Escherichia coli* two recombinant fragments from the C-terminal portion of human fibrinogen Aα chain, the αC45K fragment (Gln^{221} through Val^{610}) and its truncated version, αC30K fragment (His^{368} through Val^{610}). We have shown that αC45K but not αC30K could be effectively cross-linked by factor XIIIa with formation of polymers. Upon treatment of αC45K with fXIIIa, a significant fraction was found to be internally cross-linked. Direct analysis of fXIIIa-stabilized αC45K polymers revealed the involvement at least three Gln residues (221, 237, and 328) and four Lys residues (539, 556, 580, and 601) in the cross-linking reaction.

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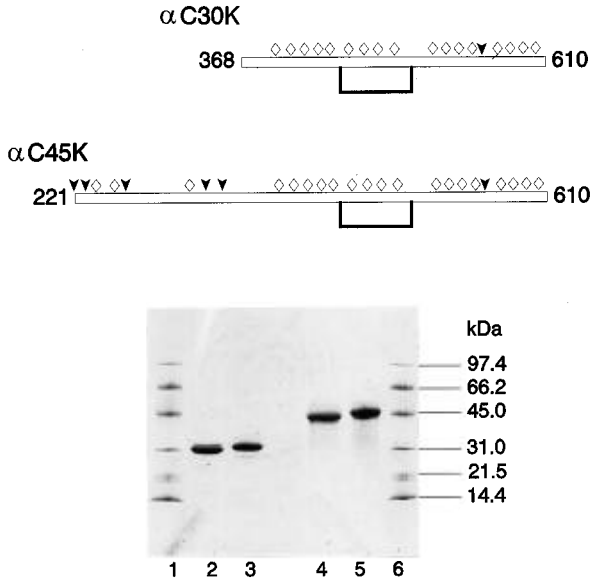

FIGURE 1: (Top) Schematic representation of the recombinant αC fragments and the location of Gln and Lys residues within them. The bold line depicts the single disulfide bond. Gln residues are indicated by dark arrows and Lys residues by open diamonds. (Bottom) SDS−PAGE (8−25% gel) of the expressed fragments αC30K (lanes 2 and 3) and αC45K (lanes 4 and 5) in nonreduced (lanes 2 and 4) and reduced (lanes 3 and 5) conditions. Lanes 1 and 6 contain molecular mass standards as indicated.

MATERIALS AND METHODS

Expression of Recombinant αC Fragments. Two recombinant fragments comprising residues 221−610 (αC45K fragment) and 368−610 (αC30K fragment) of the fibrinogen Aα chain (Figure 1) were produced in *E. coli* using the pET-20b expression vector (Novagen Inc.). To clone the designated regions, we designed polymerase chain reaction (PCR) primers shown in detail in Table 1. The forward and reverse primers contained 21 and 20 bases, respectively, corresponding to the 5′- and 3′-terminal sequences of the desired coding segment. The forward primer incorporated the *Nde*I restriction site immediately before the coding region; the final three bases of the *Nde*I site, ATG, code for the fMet initiation residue. Because of the difficulties with direct cloning of PCR products into the pET-20b vector using *Nde*I−*Hin*dIII sites, we added a *Bam*HI site immediately before the *Nde*I site, which allowed the corresponding cDNA segment to be cloned into the pUC19 plasmid for amplification. The reverse primers included a TGA stop codon immediately after the coding segment, followed by a *Hin*dIII site. Not shown in Table 1 is the presence of six additional bases at the free 5′ or 3′ ends of each restriction site to facilitate binding of the restriction enzymes.

The specific cDNA fragments were generated by PCR using *Pfu* DNA polymerase (Stratagene) and a template consisting of full-length cDNA encoding the human fibrinogen Aα chain kindly provided by Dr. S. Lord (Binnie et al., 1993). The reaction was cycled 15 times with denaturation at 94 °C, annealing at 50 °C, and extension at 72 °C. The amplified cDNA fragments were purified by electrophoresis in agarose gel, digested with *Bam*HI−*Hin*dIII restriction enzymes and ligated into the pUC19 vector. After *in vivo* amplification, the resultant plasmids were digested with *Nde*I and *Hin*dIII, and the 1.17- and 0.73-kilobase pair fragments were purified by agarose gel electrophoresis. These cDNA fragments were ligated into the pET-20b expression vector.

Table 1: Definition of Primers used in This Study

αC45K (Gln 221-Val 610)[b]	Fwd:	GG ATC CAT ATG CAG CTT CAG AAG GTA CCC CCA *Nde* I *Bam* HI
	Rev compl:	AAG CTT TCA GAC AGG GCG AGA TTT AGC AT *Hind* III
αC30K (His 368-Val 610)[b]	Fwd:	GG ATC CAT ATG CAC TCT GAA TCT GGA AGT TTT *Nde* I *Bam* HI
	Rev compl:	AAG CTT TCA GAC AGG GCG AGA TTT AGC AT *Hind* III

[a] Forward (Fwd) and reverse complement (Rev compl) DNA sequences are indicated. [b] Both expressed fragments contain an extra NH_2-terminal fMet residue (see text).

The resulting plasmids were used for transformation of DH5α and then BL21 *E. coli* host cells. Both cDNA fragments were sequenced in both directions to confirm the integrity of the entire coding sequence. For expression of the αC fragments, BL21 cells were grown at 37 °C in Luria broth medium containing ampicillin (50 μg/mL). Overnight cultures were diluted 1:100 with fresh Luria broth medium, grown for 2 h at 37 °C (mid-log phase), induced with isopropyl-1-thio-β-D-galactopyranoside (0.4 mM) for 2−3 h, harvested by centrifugation, and lysed by the freeze/thaw method.

Purification of the Recombinant Fragments. The αC45K fragment was purified from the insoluble pellet of the bacterial lysate. The pellet was washed three times with TBS, pH 7.4, containing 5 mM EDTA and 0.5% Triton X-100, resulting in significant purification from *E. coli* membrane proteins. The pellet was then solubilized at room temperature during 20 min in TBS, pH 7.4, containing 8 M urea. Residual insoluble material was removed by centrifugation, and the soluble protein was further purified and the urea removed by size exclusion chromatography on a column of Superdex 75 (Pharmacia) equilibrated in TBS, pH 7.4.

The αC30K fragment was prepared from the soluble fraction of the bacterial lysate. After precipitation with 30% ammonium sulfate, the pellet was dissolved in 20 mM Tris, pH 8.0, containing 25 mM NaCl, and applied on a Q-Sepharose ion exchange column (Pharmacia) equilibrated with the same buffer. The material that eluted from the column at 170 mM NaCl represented mainly the αC30K fragment with variable amounts of its degradation products as revealed SDS−PAGE and NH_2-terminal sequence analysis. These were mostly removed by size exclusion chromatography on the Superdex 75 column equilibrated with TBS, pH 7.4. Both αC30K and αC45K fragments were concentrated and stored frozen at −70 °C.

Protein concentrations were determined spectrophotometrically using extinction coefficients calculated from the amino acid composition by the following equation: $E_{280,1\%} = (5690W + 1280Y + 120S{-}S)/0.1M$, where W, Y, and S−S represent the number of Trp and Tyr residues and disulfide bonds, respectively, and M represents molecular mass (Edelhoch, 1967; Gill & vonHipple, 1989; Pace et al., 1995). The following values of $E_{280,1\%}$ were obtained for recombinant fragments: αC45K, 11.6; and αC30K, 3.9.

Factor XIIIa Mediated Cross-Linking of αC Fragments. Cross-linking of the recombinant αC fragments was initiated by addition of 1 unit/mL of thrombin (Sigma) to a solution containing 0.6 mg/mL fragment and 30 μg/mL recombinant factor XIII obtained from Dr. Paul Bishop of ZymoGenetics

(Bishop et al., 1990). The reaction was carried out in TBS, pH 7.4 containing 10 mM $CaCl_2$ for varying times at 25 °C. The incubation mixture was then transferred into a solution containing 2% SDS and 10% β-mercaptoethanol at 95 °C and analyzed by SDS−PAGE. Alternatively, the reaction mixture was fractionated on Superose 12 (Pharmacia), and the polymeric or internally cross-linked fractions were subjected to NH_2-terminal sequencing.

Cyanogen Bromide and Enzymatic Cleavage. Approximately 0.1 mg of protein was dissolved in 0.5 mL of 70% formic acid, and 1 mg of CNBr was added to this solution. The reaction was allowed to proceed for 4 h at 37 °C in the dark. Samples were then dried down using a Savant SpeedVac concentrator, diluted to 1 mL with H_2O, and redried. The acceptor Gln and donor Lys residues involved in cross-linking were identified by isolation and sequencing of peptides from endoproteinase Lys-C or endoproteinase Glu-C (Boehringer-Mannheim) digests of the cross-linked αC45K fragment. Endoproteinase Lys-C digestion was performed in 50 mM TBS, pH 8.2, containing 5 mM EDTA, at 37 °C overnight; enzyme/substrate ratio was 1/60 (mass/mass). Glu-C digestion was performed in 50 mM NH_4HCO_3 buffer, pH 7.8, at room temperature overnight; enzyme/substrate ratio was 1/60 (mass/mass).

Peptide Separation. The digested fragments were fractionated on a RP300 Brownlee C-18 reverse-phase cartridge (30 × 2.1 mm) using a Waters HPLC system equipped with two model 510 pumps, a 490E variable wavelength detector, and a 712 WISP autoinjector. Data were collected at 220 and 280 nm and analyzed using Maxima 820 software. The column was equilibrated in solvent A (0.1% by volume of trifluoroacetic acid in H_2O) and eluted with various gradients of solvent B (0.1% by volume of trifluoroacetic acid in acetonitrile). A linear gradient from 0−10% B over 5 min followed by 10−50% B over 50 min was used for the endoproteinase Lys-C digest. For the CNBr digests, the gradient was from 0 to 50% B over 100 min. For the separation of the endoproteinase Glu-C digests, a concave gradient from 0−10% B over 20 min followed by a linear gradient from 10−15% B over 40 min followed by 15−40% B over 40 min was used.

Sequence Analysis. NH_2-terminal sequence analysis was performed with a Hewlett-Packard model G1000S sequenator. The NH_2 termini were determined by direct sequencing for several cycles. To determine the COOH termini, the corresponding material was digested with CNBr and separated by reverse-phase HPLC, and individual fragments were sequenced through the end. When analyzing various fractions from the proteolytic digests of cross-linked material, multiple residues were often found in each cycle indicating heterogeneity of the fractions from HPLC. Computer-assisted comparison of the residues in each cycle against the known sequence of αC45K allowed specific sequences to be identified.

RESULTS

Preparation and Characterization of Recombinant αC Fragments. The recombinant fragments, αC45K and αC30K comprising residues Gln^{221} through Val^{610} and His^{368} through Val^{610}, respectively, were directly produced in *E. coli* using the pET-20b expression vector. Both fragments contained one extra NH_2-terminal fMet residue that initiated translation

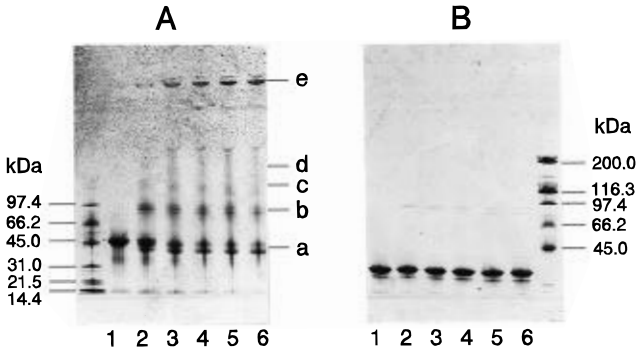

FIGURE 2: Treatment of the αC45K (panel *A*) and αC30K (panel *B*) with factor XIIIa analyzed by reduced SDS−PAGE (4−15% gel). Lanes 1 contain the starting material. Lanes 2−6 represent increasing times of incubation (10, 30, 60, 120, and 360 min) with factor XIIIa as described in Materials and Methods.

(Studier et al., 1995). The αC45K fragment was found predominantly in the insoluble fraction. It was recovered after solubilization in 8 M urea. Solubility was retained after removal of urea, and size-exclusion chromatography revealed no sign of aggregation. Attempts to recover αC45K from the soluble fraction resulted in extraction of highly degraded material (not shown). αC30K was purified intact from the soluble fraction. The final yield of purified fragments varied between 4 and 7 mg/L of bacterial culture.

The homogeneity of the fragments was checked by SDS−PAGE. Both exhibited a major band whose mobility was slightly decreased upon reduction indicating that the single disulfide bond was intact in both recombinant fragments (Figure 1). Several minor bands with higher mobility were sometimes observed suggesting impurities or proteolytic degradation of the fragments upon preparation. However, both fragments consistently displayed a single N-terminal sequence starting at Gln^{221} for αC45K and His^{368} for αC30K, both preceded by variable yields of Met. This suggests that the minor bands represent the same fragments with partially degraded COOH termini. To check this suggestion, a 27-kDa contaminant of αC30K (see Figure 2B) was isolated during further purification of the αC30K fragment by size exclusion chromatography, and its cyanogen bromide digest was compared with that of αC30K by reverse-phase HPLC (not shown). The digest of the αC30K fragment exhibited an extra peak that was missing in the digest of the 27-kDa fragment. The material from this peak was sequenced through the end and identified as the complete COOH-terminal CNBr fragment of the Aα chain. Sequence analysis of a total CNBr digest of αC45K showed the presence of all five expected CNBr fragments. Thus sequence analysis clearly indicated that, after purification, the NH_2 and COOH termini in both fragments were mainly preserved and that the minor impurities revealed in some preparations by SDS−PAGE occurred due to partial degradation of their COOH termini.

Factor XIIIa Catalyzed Cross-Linking of the αC Fragments. Since the NH_2-terminal portion of αC45K contains reactive Gln residues previously identified by Doolittle and co-workers (Cottrell et al., 1979) as well as the Aα518−584 region, where potential donor Lys residue(s) were predicted to occur (Doolittle et al., 1977), we checked if it could be cross-linked by factor XIIIa into polymeric structures similarly to those observed in cross-linked fibrin. Upon incubation of αC45K with factor XIIIa, the major band on SDS−PAGE corresponding to the monomeric fragment was

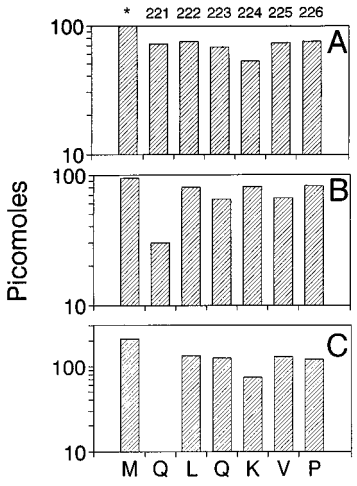

FIGURE 3: Repetitive yields of successive residues during NH_2-terminal sequencing of untreated αC45K (panel *A*), the internally cross-linked monomeric form of αC45K (panel *B*), and the polymeric fraction of αC45K (panel *C*). Numbers above panel A indicate the position of the residues in the Aα chain sequence. An extra fMet is denoted by the asterisk.

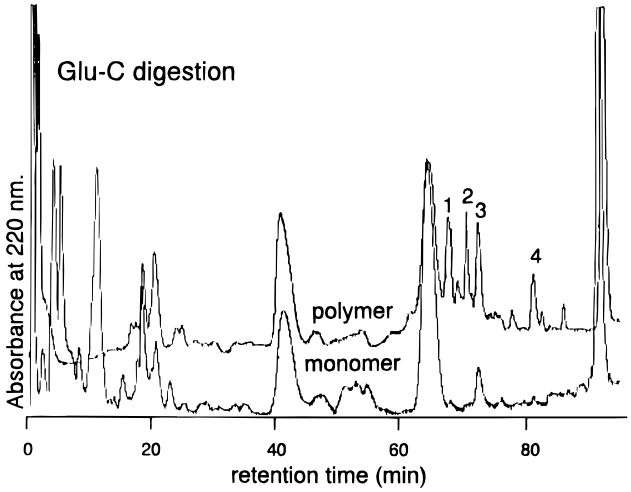

FIGURE 4: HPLC separation of cross-linked peptides obtained from αC45K polymers after Glu-C digestion. The numbers designate peaks that are observed in digest of the polymers but not the monomers.

rapidly depleted (Figure 2A). This was accompanied by the appearance of new slower mobility bands whose apparent molecular mass was consistent with the formation of dimers, trimers, tetramers, and, most prominently, large polymers which failed to enter the gel (bands b, c, d, and e, respectively). In addition, some of the material in the incubation mixture precipitated, suggesting the formation of even higher molecular mass polymers. The results clearly indicate that cross-linking of the αC45K fragment by factor XIIIa results in the rapid formation of multimers and polymers. By contrast, the αC30K fragment, when incubated with factor XIIIa under identical conditions, was unaffected (Figure 2B), consistent with what was suggested about the distribution of donors and acceptors in different parts of the fibrinogen Aα chain (Doolittle et al., 1977, 1979).

Figure 2A also reveals that incubation with factor XIIIa results in the gradual accumulation of an additional monomeric band (band a) with higher mobility than the original αC45K fragment. One possible explanation would be proteolytic degradation of the fragment. However, the appearance of higher mobility products was observed neither with the αC30K fragment, which was incubated with factor XIIIa under identical conditions (Figure 2B), nor with a separate sample of αC45K to which only thrombin was added, omitting factor XIII (not shown). If degradation with some other protease is responsible for the increased mobility of a portion of the αC45K fragment, this degradation should involve its NH_2-terminal part, the part missing in αC30K which does not show the effect. To check this suggestion, the fraction which exhibited higher mobility on SDS−PAGE after 360 min of cross-linking (band a, Figure 2A) was separated from soluble multimers by size exclusion chromatography and sequenced through seven cycles. The untreated fragment and the soluble multimer fraction were also sequenced for comparison. The analysis revealed the same single NH_2-terminal sequence in all three cases indicating that the NH_2-terminal part of αC45K is preserved during incubation with factor XIIIa. However, unlike the untreated material (Figure 3A), both band a (Figure 3B) and the multimer fraction (Figure 3C) exhibited a depressed yield of Gln^{221} (cycle 2) compared to Gln^{223} (cycle 4). The depressed yield of Gln^{221} would be expected if it were involved in cross-linking since it would not be liberated following Edman degradation. The results seem to indicate that this residue can participate in cross-linking not only intermolecularly to form αC multimers but intramolecularly to form a more compact structure which migrates faster through the gel. The intramolecular cross-linking may also occur in some of the dimers and multimers and could account for the diffuse nature of bands b, c, and d and their tendency to shift downward with increasing time of cross-linking (Figure 2A). These results suggest a high level of flexibility in the αC45K polypeptide chain which allows fXIIIa to introduce intramolecular amide bonds and increase the mobility on SDS−PAGE.

Identification of Cross-Linked Residues in αC45K Polymers. Both untreated αC45K monomers and the cross-linked polymer fractions were treated with protease Glu-C or protease Lys-C, and HPLC profiles of their digests were compared to identify the regions involved in cross-linking. An example of a Glu-C digest is provided in Figure 4 where several new peaks designated as 1, 2, 3, and 4 are visible in the HPLC profile of the cross-linked polymeric fraction but absent in that of the untreated monomeric fragment. Some of the new peaks were shown to arise merely from changes in the digestion pattern as a result of the cross-linking reaction, presumably because of changes in the steric accessibility of cleavable bonds or, in the case of Lys-C digests, failure of the enzyme to recognize a Lys residue that was cross-linked to a Gln. Other peaks represented cross-linked peptides whose retention times were increased because of the larger size. Upon sequencing, most peaks produced multiple residues in each cycle, indicating heterogeneity of the HPLC fractions or the presence of several peptides in a given cross-linked species. Comparison of the residues in each cycle against the known sequence of αC45K allowed several individual sequences to be identified. Figure 5 presents repetitive yield data for several peptides that appeared to be involved in cross-linking as evidenced by much lower than expected yields of either Gln or Lys in a given cycle. Some of the same peptides were also identified in peaks from a digest of untreated αC45K, in which case the yields of the corresponding Gln and Lys residues were

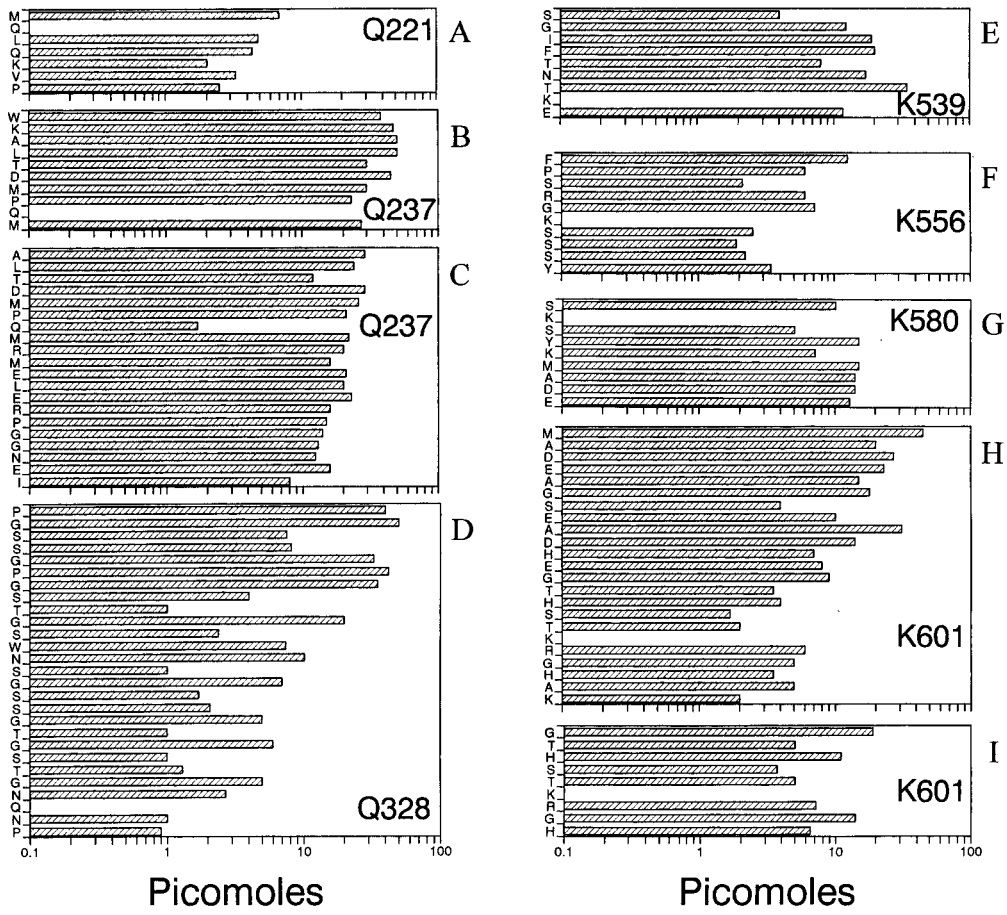

FIGURE 5: Repetitive yields of successive residues during N-terminal sequencing of peptides isolated from proteolytic digests of the polymeric fraction of cross-linked αC45K fragment of fibrinogen. Left-hand panels present data from peptides in which reactive Gln residues were identified, as indicated inside each panel. Right-hand panels correspond to peptides in which reactive Lys residues were identified. The amino acid sequence of each peptide is indicated along the ordinate of each panel.

not depressed. Thus, the panels on the left of Figure 5 identify three different Gln residues at positions 221, 237, and 328. The panels on the right identify four Lys residues at positions 539, 556, 580, and 601.

DISCUSSION

Localization of functionally important regions of fibrinogen αC domains has been hampered by the difficulty of isolating intact homogeneous fragments containing this domain, primarily because of its vulnerability to proteolysis. Previous attempts to prepare full length αC domain from bovine fibrinogen by limited proteolysis resulted in low yields of a 40-kDa αC-fragment which had heterogenous NH_2 termini and was missing a significant portion from its C-terminus (Aα582−610) which is thought to be important for the intramolecular association of these domains in fibrinogen (Veklich et al., 1993; Gorkun et al., 1994). One goal of the present study was to express intact homogeneous recombinant αC fragments in amounts sufficient for their characterization and functional study.

Two Recombinant Fragments Were Expressed with High Yield using the pET-20b Expression Vector. These fragments were αC45K fragment including the sequence Aα221−610 and its truncated version αC30K fragment (Aα368−610). It should be noted that our first attempts to produce recombinant αC45K fragment as a fusion protein with maltose-binding protein using the pMAL-p2 expression vector failed due to extremely high degradation of the final product (results not shown). The use of the pET-20b expression system seems to abolish that problem. We attribute this success at least partially to the strong T7 promotor in the pET expression vector and to the lack of the *Lon* and *ompT* proteases in the BL21 *E. coli* host cell strain which was used for expression of the recombinant αC fragments.

One of the major functions of the C-terminal portions of fibrin(ogen) α chain is to stabilize fibrin clots by formation of fXIIIa cross-linked polymers. Cross-linking of α chains alters the mechanical properties of the clot and enhances a clot's resistance to lysis by plasmin (Henschen & McDonagh, 1986). When recombinant αC45K fragment was incubated in the presence of fXIIIa, the formation of dimers, trimers, tetramers, and higher molecular weight polymers was observed. Such polymerization upon treatment of the αC45K fragment with fXIIIa indicates that COOH-terminal regions of fibrin(ogen) Aα chains are autonomously functioning domains with a tendency to form self-associated type structures. Recently published electron microscopy data indicate that natural αC fragments of bovine fibrinogen have a tendency to self-associate (Veklich et al., 1993). Such a tendency could explain the high efficiency of αC45K cross-linking upon its treatment with fXIIIa. Factor XIIIa introduces covalent isopeptide bonds that stabilize the associated state of the protein and render it detectable by SDS−PAGE.

Treatment of the αC45K fragment with factor XIIIa resulted not only in the formation of polymers but also in a

significant fraction of an internally cross-linked monomeric derivative whose mobility on SDS−PAGE was greater than that of the starting material. Such intramolecular cross-linking occurred also with some oligomeric species, retarding their further polymerization. Intramolecular cross-linking was shown to involve at least Gln^{221} and unidentified Lys residue(s) presumably located in the COOH-terminal portion as presented schematically in Figure 6. The possibility that other Gln residues also participate in this reaction can not be excluded and is in fact suggested by the presence of at least one additional band at a position intermediate between the starting material and the highest-mobility band (Figure 2A). This phenomenon was not detected with the truncated αC30K fragment, which also failed to show any evidence for intermolecular cross-linking.

Reactive Gln residues that can serve as substrates for factor XIIIa and other transglutaminases are often identified through their ability to incorporate low molecular weight amine donors. For example, reactive Gln at positions 237, 328, and 366 in the Aα chain were identified by incorporation of labeled Lys analogues into fibrin (Cottrell et al., 1979; Fretto et al., 1978; Henshen & McDonagh, 1986). More recently, a similar approach has been used to incorporate Gln-containing peptides into several proteins (Parameswaran et al., 1990; Bendixen et al., 1993). However, the specificity of this approach may be compromised by the high (mM) concentrations of the low molecular weight probes that are used. The present study provides the first direct identification of specific Gln and Lys residues in the fibrinogen Aα chain that are actually involved in protein−protein cross-linking as opposed to merely serving as substrates for the incorporation of low molecular weight probes. Two of the three identified Gln residues, 237 and 328, are the same as those previously reported to be reactive; the third at position 221 was not previously identified. The αC45K fragment contains a total of six Gln residues, five of which are located in the N-terminal 25% of the polypeptide chain (Figure 1). Three of the six were identified as reactive in our experiments. We cannot exclude the possible involvement of additional Gln-(s), e.g., Gln^{366} (Cotrell et al., 1979), since we did not sequence every HPLC peak. However, the fact that αC30K, for which Gln^{563} is the only one, did not undergo cross-linking suggests that Gln^{563} is not reactive toward factor XIIIa.

The involvement of Lys residues in the C-terminal stretch between residues 518 and 584 was expected on the basis of the suggestion by Doolittle et al. (1977), who identified this peptide (whose only Gln is no. 563) in complex with another in the CNBr digest of fully cross-linked fibrin. Three of the Lys residues shown here to be involved in cross-linking of αC polymers, residues 539, 556, and 580, are located in this region. In addition Lys^{601} situated beyond this region was also involved in cross-linking. Again, the possible involvement of additional Lys residues such as Lys^{508} (Corcoran et al., 1980) cannot be excluded since not all HPLC fractions were analyzed. Nor can it be certain that all of the residues that participate in cross-linking of the αC45K fragment would also participate in the cross-linking of intact fibrin since they might be less accessible in the parent protein. This could be especially true for Gln^{221}, which is located near the NH_2 terminus of the αC45K fragment.

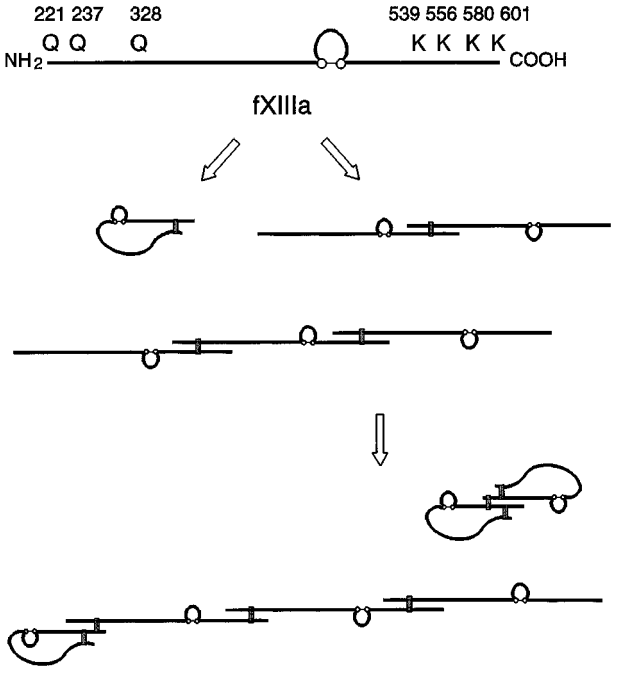

FIGURE 6: Schematic representation of the results of cross-linking of recombinant αC45K fragment of fibrinogen with factor XIIIa. Reactive Gln residues identified in this study are located in the N-terminal portion and Lys residues in the C-terminal portion. Intramolecular cross-linking competes with intermolecular cross-linking and also serves to impede the growth of polymers.

We were unable from our data to make a conclusion about the involvement of a specific Gln-Lys pair in the cross-linking reaction. It is likely that any one of several reactive Gln residues in the N-terminal region could be cross-linked to one of several Lys residues in the C-terminal region and *vice versa*. In any case, the availability of multiple reactive Gln and Lys residues serves to increase the probability that cross-linking occurs between α chains, consistent with the importance of this reaction for stabilizing the fibrin clot.

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REFERENCES

Bale, M. D., Westrick, L. G., & Mosher, D. F. (1985) *J. Biol. Chem. 260*, 7502−7508.
Bendixen, E., Borth, W., & Harpel, P. C. (1993) *J. Biol. Chem. 268*, 21962−21967.
Binnie, C. G., Hettasch, J. M., Strickland, E., & Lord, S. T. (1993) *Biochemistry 32*, 107−113.
Bishop, P. D., Teller, D. C., Smith, R. A., Lasser, G. W., Gilbert, T., & Seale, R. L. (1990) *Biochemistry 29*, 1861−1869.
Cheresh, D. A., Berliner, S. A., Vincente, V., & Ruggeri, Z. M. (1989) *Cell 58*, 945−953.
Corcoran, D. H., Ferguson, E. W., Fretto, L. J., & McKee, P. A. (1980) *Thromb. Res. 19*, 883−888.
Cottrell, B. A., Strong, D. D., Watt, K. W. K., & Doolittle, R. F. (1979) *Biochemistry 18*, 5405−5110.
Credo, R. B., Curtis, C. G., & Lorand, L. (1981) *Biochemistry 20*, 3770−3778.
Doolittle, R. F. (1973) *Adv. Protein Chem. 27*, 1−101.
Doolittle, R. F. (1984) *Annu. Rev. Biochem. 53*, 195−229.
Doolittle, R. F. (1994) in *Molecular Basis of Blood Diseases*, (Stamatoyannopoulos, G., Nienhuis, A. W., Majerus, P. W., & Varmus, H. W. B., Eds.) pp 701−723, Saunders Co., Philadelphia, PA.

Doolittle, R. F., Cassman, K. G., Cottrell, B. A., & Friezner, S. J. (1977) *Biochemistry 16*, 1715−1719.

Doolittle, R. F., Watt, K. W. K., Cottrell, B. A., Strong, D. D., & Riley, M. (1979) *Nature 280*, 464−468.

Edelhoch, H. (1967) *Biochemistry 6*, 1948−1954.

Fretto, L. J., Ferguson, E. W., Steinman, H. M., & McKee, P. A. (1978) *J. Biol. Chem. 253*, 2184−2195.

Gorkun, O. V., Veklich, Y. I., Medved, L. V., Henschen, A. H., & Weisel, J. W. (1994) *Biochemistry 33*, 6986−6997.

Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−326.

Hada, M., Kato, M., Ikematsu, S., Fujimaki, M., & Fukutake, K. (1982) *Thromb. Res. 25*, 163−168.

Henschen, A., & McDonagh, J. (1986) in *Blood Coagulation* (Zwaal, R. F. A., & Hemker, H. C., Eds.) pp 171−241, Elsevier Science Publishers, Amsterdam.

Kirschbaum, N. E., & Budzynski, A. Z. (1990) *J. Biol. Chem. 265*, 13669−13676.

Koopman, J., Haverkate, F., Grimbergen, J., Egbring, R., & Lord, S. T. (1992) *Blood 80*, 1972−1979.

Koopman, J., Haverkate, F., Grimbergen, J., Lord, S. T., Mosesson, M. W., DiOrio, J. P., Siebenlist, K. S., Legrand, C., Soria, J., Soria, C., & Caen, J. P. (1993) *J. Clin. Invest. 91*, 1637−1643.

Lijnen, H. R., Soria, J., Soria, C., Collen, D., & Caen, J. P. (1984) *Thromb. Haemost. 51*, 108−109.

Maekawa, H., Yamazumi, K., Muramatsu, S., Kaneko, M., Hirata, H., Takahashi, N., de Bosch, N. B., Carvajal, Z., Ojeda, A., Arocha-Pinango, C. L., & Matsuda, M. (1991) *J. Biol. Chem. 266*, 11575−11581.

Medved, L. V., Gorkun, O. V., Manyakov, V. F., & Belitser, V. A. (1985) *FEBS Lett. 181*, 109−112.

Mosesson, M. W., Alkjaersig, N., Sweet, B., & Sherry, S. (1967) *Biochemistry 6*, 3279−3287.

Mosher, D. F. (1976) *J. Biol. Chem. 251*, 1639−1645.

Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., & Gray, T. (1995) *Protein Sci. 4*, 2411−2423.

Parameswaran, K. N., Velasco, P. T., Wilson, J., & Lorand, L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8472−8475.

Sakata, Y., & Aoki, N. (1980) *J. Clin. Invest. 65*, 290−297.

Sakata, Y., & Aoki, N. (1982) *J. Clin. Invest. 69*, 536−542.

Soria, J., Soria, C., & Caen, J. P. (1983) *Br. J. Haematol. 53*, 575−586.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1995) *Methods Enzymol. 185*, 60−89.

Tuszynski, G. P., Srivastava, S., Switalska, H. I., Holt, J. C., Cierniewski, C. S., & Niewiarowski, S. (1985) *J. Biol. Chem. 260*, 12240−12245.

Veklich, Y. I., Gorkun, O. V., Medved, L. V., Nieuwenhuizen, W., & Weisel, J. W. (1993) *J. Biol. Chem. 268*, 13577−13585.

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