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# Labeling of ε-lysine crosslinking sites in proteins with peptide substrates of factor XIIIa and transglutaminase

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**ABSTRACT** **Peptides patterned on the N-terminal sequence of fibronectin were synthesized and tested for amine acceptor qualities in reactions with dansylcadaverine catalyzed either by coagulation factor XIIIa or intracellular transglutaminase (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13). On the basis of inverse half-saturations of the enzymes, the order of acceptor substrate affinity for factor XIIIa was pEAQQIV >> Boc-AQQIV > Boc-QQIV, and for transglutaminase, Boc-QQIV > Boc-AQQIV > pEAQQIV (amino acid residues are shown in one-letter code; pE, pyroglutamic acid; Boc, *tert*-butyloxycarbonyl). Sequence analysis of dansylcadaverine-substituted pEAQQIV indicated that the first of the two adjacent glutamine residues was the target of enzymatic modification. Boc-QIV showed no substrate activity with either enzyme. Crosslinking of crystallins in $Ca^{2+}$-treated rabbit lens homogenate was readily inhibited by Boc-QQIV, Boc-AQQIV, and pEAQQIV, as was the formation of α-chain polymers in human fibrin by pEAQQIV in the presence of human factor XIIIa. SDS/PAGE analysis suggested that the inhibitory peptides selectively blocked the electron donor functionalities in these enzymatic crosslinking reactions.**

Activated fibrin-stabilizing factor (i.e., coagulation factor XIIIa) and the similarly acting transglutaminases (EC 2.3.2.13) bring about the posttranslational crosslinking of protein substrates by promoting the formation of a few $N^{\epsilon}$-(γ-glutamyl)lysine side-chain bridges, with the result of producing a variety of fused homo- and heteropolymeric structures in biological systems (illustrated for dimerization in Scheme 1 on the next page). The kinetic pathway of catalysis by these enzymes, operating with cysteine active centers, is essentially the same as that for proteases such as chymotrypsin, trypsin, or papain, where a Michaelis-type of complexation is followed by the chemical steps of acylation and deacylation. What sets the group of transglutaminases apart from the proteases is a remarkable affinity for the electron-donating second substrate, usually a primary amine, which causes an aminolytic deacylation instead of hydrolysis by water. Unlike with proteases, there is a readily discernible additional complexation of the acylenzyme intermediate with the amine prior to deacylation. This high degree of specificity for the amine made it possible to find synthetic donors [e.g., glycine ethyl ester, *N*-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide (dansylcadaverine) (1)] that could compete effectively against the ε-amino functionalities of lysine in the natural donor protein (marked in the schemes with an oval) that would otherwise become crosslinked to the acceptor protein (marked with a rectangle). Thus, incorporation of the alternative donor ($RNH_2$; Scheme 2) inhibits crosslinking (Scheme 1) and also leads to the enzyme-directed, site-specific labeling of the participating glutaminyl residues in the acceptor protein.

Though amine-type of inhibitors of crosslinking were introduced nearly three decades ago and have been widely used ever since for the specific labeling of the glutaminyl sites of proteins (for a review, see ref. 2) the converse approach of inhibiting crosslinking by blocking of the relevant donor protein had eluded us. However, we are now able to report success in this regard, with the synthesis of suitably small, water-soluble acceptor molecules ($R'CH_2CH_2CONH_2$; Scheme 3) patterned on the N-terminal sequence of fibronectin (3).

## MATERIALS AND METHODS

**Peptide Synthesis.** Reagents and solvents were purchased from Sigma and Aldrich. TLC was performed on Whatman K6F silica-gel glass plates (0.24 mm) with the following solvent systems (vol/vol): (A) ethyl acetate/heptane, 2:1; (B) chloroform/methanol/glacial acetic acid, 10:2:1; (C) 1-butanol/glacial acetic acid/water, 15:6:5; (D) chloroform/methanol/2-propanol, 10:4:4; (E) 1-propanol/water/concentrated ammonium hydroxide/ethanol, 7:4:2:3; (F) 1-propanol/water, 7:3. Plates were viewed under UV light or were developed by ninhydrin (0.25% in 1-butanol for N-deblocked peptides) or by hypochlorite (10%) followed by starch/KI spray for N-blocked peptides (4). Melting points were determined with a Buchi apparatus and are uncorrected. HPLC [Vydac $C_{18}$ column (The Nest Group, Southport, MA), using 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile with a gradient of 0–60% in the latter solvent in 30 min, at a flow rate of 1 ml/min with UV detection at 214 nm] and amino acid analyses (Applied Biosystems, PTC $C_{18}$ column) were performed by K. L. Ngai, Biotechnology Research Laboratory, Northwestern University.

pGlu-Ala-Gln-Gln-Ile-Val and its truncated analogues were prepared stepwise by the solution peptide synthesis method from the C terminus. The *N-tert*-butyloxycarbonyl (Boc) amino acids and pyroglutamic acid (pGlu or pE) were coupled via the *N*-hydroxysuccinimide ester procedure (5) or by a modification of the procedure of Konig and Geiger (6) in the presence of 1-hydroxybenzotriazole in *N*,*N*-dimethylformamide, with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride instead of *N*,*N'*-dicyclohexylcarbodiimide (7). Treatment with 50% TFA in anhydrous dichloromethane was used for removing the Boc protecting group after the coupling steps and the TFA salt of the peptide was neutralized with *N*-methylmorpholine just before addition to the solution containing the preactivated ester of the next Boc amino acid. Details of the syntheses and characterization of the blocked peptides by TLC, proton NMR spectroscopy, and elemental analysis will be published elsewhere. Removal of the C-terminal benzyl ester was achieved by catalytic hydrogenation at 50°C (10% Pd/C in *N*,*N*-dimethylformamide/ethanol/water in the approximate volume ratios of 13:5:1). The

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Abbreviations: TFA, trifluoroacetic acid; Boc, *tert*-butyloxycarbonyl; pE or pGlu, pyroglutamic acid.

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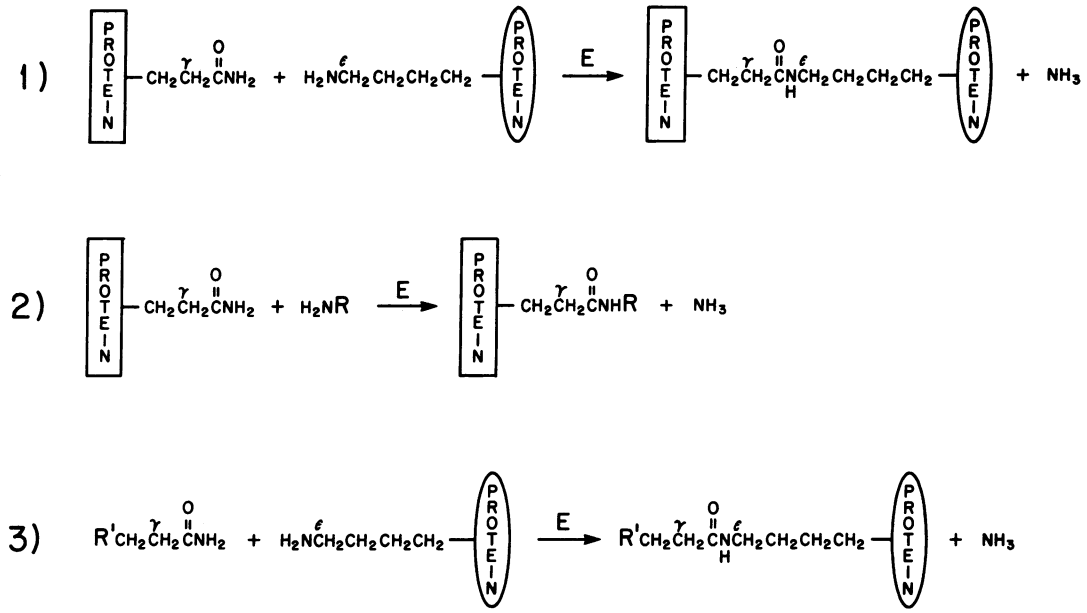

Schemes 1–3. See Introduction for description. E, enzyme.

peptides were homogeneous by TLC and HPLC (with the retention times, $t_R$, given below) and, upon acid hydrolysis, gave satisfactory amino acid analyses (AA) in agreement with the expected molar ratios (in parentheses). Yields of the C-deblocked but still N-blocked peptides were calculated on the basis of the starting C-terminal valine benzyl ester.

*pGlu-Ala-Gln-Gln-Ile-Val (pEAQQIV).* Yield 39%, mp 245–247°C (dec); TLC $R_f$ 0.5 (C), 0.65 (E), 0.58 (F); HPLC $t_R$ 19 min. AA: Glu, 3.0 (3.0); Ala, 1.0 (1.0); Ile, 1.1 (1.0); Val, 0.9 (1.0).

*Boc-Ala-Gln-Gln-Ile-Val (Boc-AQQIV).* Yield 60%, mp 234–237°C (dec); TLC $R_f$ 0.75 (C), 0.68 (E), 0.65 (F); HPLC $t_R$ 22 min. AA: Glu, 2.2 (2.0); Ala, 1.0 (1.0); Ile, 1.1 (1.0); Val, 0.9 (1.0).

*Boc-Gln-Gln-Ile-Val (Boc-QQIV).* Yield 50%, mp 235–237°C (dec); TLC $R_f$ 0.69 (C), 0.05 (D), 0.6 (F); HPLC $t_R$ 21.5 min. AA: Glu, 2.1 (2.0); Ile, 1.0 (1.0); Val, 0.9 (1.0).

*Boc-Gln-Ile-Val (Boc-QIV).* Yield 69%, mp 195–197°C (dec); TLC $R_f$ 0.8 (C), 0.2 (D), 0.59 (F); HPLC $t_R$ 23.8 min. AA: Glu, 1.1 (1.0); Ile, 0.9 (1.0); Val, 0.9 (1.0).

**Inhibition of Crystallin Crosslinking in Lens Homogenate.** Frozen lenses from young rabbits (Pel-Freez Biologicals) were thawed and decapsulated, and the cortex was separated from the nucleus. The cortical portions from three lenses were homogenized by hand in 2 ml of 50 mM Tris·HCl, pH 7.5/100 mM NaCl in a Potter–Elvehjem tissue grinder. Incubations were carried out at 37°C in a total volume of 100 μl that, in addition to homogenate (≈50 mg/ml) and 20% (vol/vol) glycerol, included 0.5–4.0 mM peptide, 2 mM leupeptin (obtained through the U.S.–Japan Cooperative Cancer Research Program), and either 8 mM $CaCl_2$ or 2 mM EDTA. The mixtures were incubated for 15 min at 37°C prior to the addition of EDTA or $CaCl_2$, then incubated for an additional 90 min before the reaction was quenched by the addition of 20 μl of 100 mM EDTA. The samples were centrifuged (15,600 × *g* for 5 min) and 10 μl of supernatant was incubated with 100 μl of 50 mM Tris·HCl, pH 7.1/9 M urea/2% SDS/40 mM dithiothreitol for 60 min. Approximately 150 μg of prepared sample was analyzed by SDS/PAGE using the discontinuous buffer system of Laemmli (8) in 1.5-mm-thick gels in a Protean slab gel apparatus (Bio-Rad). A stacking gel of 3% acrylamide, a resolving gel of 12% acrylamide, and a running buffer of 25 mM Tris, 192 mM glycine, and 0.1% SDS (pH 8.6) were employed. The gel was stained with Coomassie brilliant blue R and was calibrated with molecular mass standards (Bio-Rad); phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

**Inhibition of Fibrin Crosslinking.** Incubation mixtures (50 μl) contained 50 mM Tris·HCl (pH 7.5), 100 mM NaCl, human fibrinogen (2.5 mg/ml; IMCO, American Diagnostica, Greenwich, CT), factor XIII (5 μg/ml; refs. 9 and 10), human α-thrombin (0.32 unit/ml; a gift from J. W. Fenton III, New York State Department of Health, Albany, NY), and, as specified, 10 mM pEAQQIV and 10 mM $CaCl_2$ or 2 mM EDTA. Fibrinogen and factor XIII were preincubated with pEAQQIV for 15 min at 37°C prior to the addition of thrombin and either $CaCl_2$ or EDTA and the cross-linking reaction was stopped after 5, 15, or 60 min by the addition of 100 μl of 50 mM sodium phosphate, pH 7.1/9 M urea/40 mM dithiothreitol/2% SDS. The clots were solubilized at 37°C for 30 min and samples (30 μg) were analyzed by SDS/PAGE (11).

## RESULTS AND DISCUSSION

A previously published TLC procedure (12) was used to detect the enzyme-mediated coupling of dansylcadaverine to peptides. Fig. 1 demonstrates the factor XIIIa-catalyzed reaction of dansylcadaverine with pEAQQIV (lanes 2–4) and with Boc-AQQIV (lanes 6–8). The fluorescent coupling products of both peptides appeared in greater intensities as the reactions progressed and were well separated from the residual free dansylcadaverine, the amount of which diminished in time.

Such experiments were performed with both enzymes and the four peptides synthesized. The TLC plates were scanned [Turner, Palo Alto, CA; model 111 fluorometer; excitation wavelength, 360 nm; emission wavelength, 540 nm (13)] and by measuring peak heights of reflected fluorescence for the dansylcadaverine-labeled peptide products as well as for the unbound dansylcadaverine, the peptide-bound amine could be calculated as a fraction of the sum of the two. Figs. 2 and 3 illustrate the substrate qualities of the peptides for factor XIIIa and for transglutaminase. Interestingly, the relative order of affinities appears to be quite different for the two enzymes. With factor XIIIa, the largest peptide, pEAQQIV, was the best (Fig. 2); with transglutaminase, however, Boc-

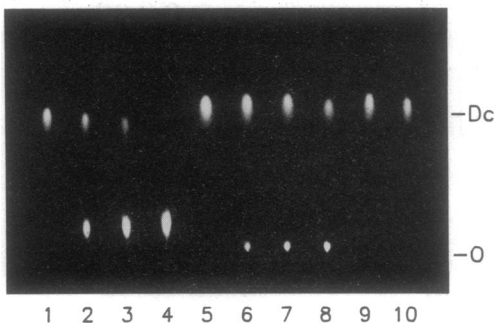


FIG. 1. Factor XIIIa-catalyzed coupling of dansylcadaverine to pEAQQIV and Boc-AQQIV. Reactions were carried out in mixtures of 75 $\mu$l comprising 50 mM Tris·HCl (pH 7.5); 0.2 mM dansylcadaverine; 10 mM dithiothreitol; thrombin-activated factor XIII (20 $\mu$g/ml); 2 mM (lanes 2 and 6), 4 mM (lanes 3 and 7), or 8 mM (lanes 1, 4, 5, and 8) pEAQQIV (lanes 1–4) or Boc-AQQIV (lanes 5–8); and either 10 mM $CaCl_2$ (lanes 2–4, 6–8, and 10) or 2 mM EDTA (lanes 1, 5, and 9). Following incubation at 37°C for 30 min, 2-$\mu$l aliquots were spotted on a TLC plate (Polygram 0.1-mm Polyamide-6, 20 × 20 cm; Macherey & Nagel, Alltech Associates) and the plate was developed for 60 min in aqueous 1% pyridine (pH 5.4). The dried plates were photographed under UV light (366 nm). Dc, dansylcadaverine; O, origin.

QQIV was the preferred substrate, and pEAQQIV itself was even less effective than Boc-AQQIV (Fig. 3). Half-saturating concentrations for transglutaminase could be achieved with 0.1–0.2 mM Boc-QQIV or Boc-AQQIV, whereas nearly an order greater concentration of pEAQQIV was required to obtain the same for factor XIIIa. Coupling of dansylcadaverine to Boc-QQIV could also be monitored by HPLC after the transglutaminase reaction was quenched with EDTA (Beckman Ultrasphere octyl column, 4.6 × 250 mm; isocratic elution with 80% acetonitrile in 0.1% TFA; flow rate, 1.5 ml/min). With fluorescence detection, the labeled tetrapeptide emerged at about 2.76 min and free dansylcadaverine at 5.05 min.

The finding that neither enzyme could utilize Boc-QIV (Figs. 2 and 3) suggested that, of the two adjacent glutaminyl residues in the reactive peptides, the one closer to the N terminus served as the susceptible target site. Though this would accord with the observations reported for the mode of incorporation of putrescine into fibronectin itself by factor XIIIa (14), it was still necessary to prove the point by sequencing a dansylcadaverine-substituted synthetic peptide

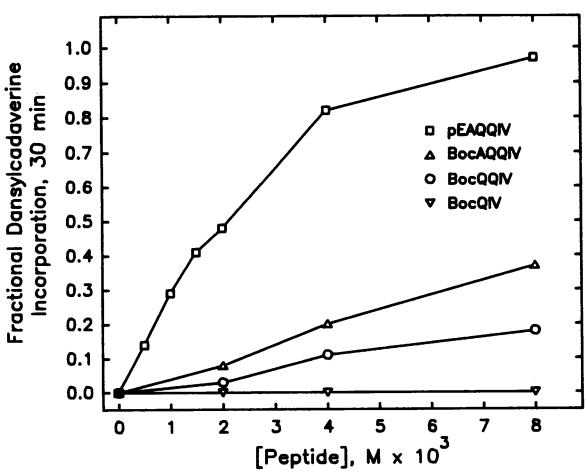


FIG. 2. Factor XIIIa-catalyzed coupling of dansylcadaverine to peptides. Incubations were carried out using the four peptides as described in Fig. 1. The TLC plates were scanned as described in the text and the peptide-bound amine (ordinate) was calculated as a fraction of the total.

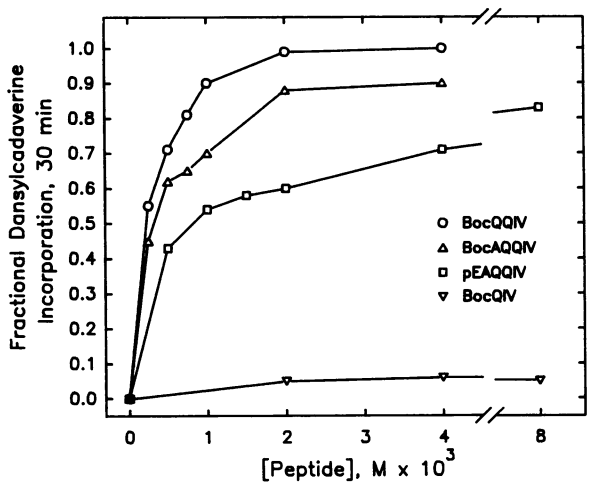


FIG. 3. Transglutaminase-catalyzed coupling of dansylcadaverine to peptides. Incubations were carried out with the four peptides as decribed in Fig. 1, but with transglutaminase (12 $\mu$g/ml; purified by a procedure developed by Paul Turner in this laboratory) in place of thrombin-activated factor XIII. TLC plates were analyzed as for Fig. 2.

substrate. We attached dansylcadaverine to pEAQQIV by factor XIIIa and also by transglutaminase under reaction conditions similar to those described for Figs. 2 and 3. The dansylcadaverine-labeled hexapeptide was purified by HPLC (Beckman Ultrasphere ODS column, 4.6 × 250 mm; 30% acetonitrile in 0.1% TFA; flow rate 1.5 ml/min) and was eluted at about 6.7 min. This material was dried and was treated with pyroglutamyl aminopeptidase (EC 3.4.19.3; Boehringer Mannheim, 2.5 $\mu$g per 100 $\mu$l of sample in 0.1 M sodium phosphate, pH 8/10 mM dithiothreitol/5% glycerol) for 6 hr at 50°C. The deblocked, dansylcadaverine-labeled peptide was purified by HPLC as before (but now eluted at ≈8 min) and was sequenced (Applied Biosystems 477A protein sequencer). The factor XIIIa and the transglutaminase products gave similar results. No usual amino acid was obtained in the second sequencing cycle, but a clear sequence of AXQIV was established. We take this to mean that the derivative removed in the second cycle in sequencing the pyroglutamate-deblocked material represents the glutaminyl residue with the dansylcadaverine substitution.

Next we examined whether the peptides displaying good acceptor substrate qualities toward transglutaminase (i.e., Boc-QQIV, Boc-AQQIV, and pEAQQIV) would inhibit some of the biological reactions catalyzed by these enzymes, together with the simultaneous blocking of participating donors according to the outlines of Scheme 3 presented in the Introduction. Results with the two test systems described below clearly indicate that inhibition of crosslinking and enzymatic attachment of the synthetic peptides to donor functionalities go hand-in-hand.

Inhibition of intracellular transglutaminase was assessed with the use of rabbit lens homogenate where, following the admixture of $Ca^{2+}$, formation of the ≈55-kDa $\beta$-crystallin dimers (designated $X\beta_2$ in Fig. 4) is a sensitive index of the crosslinking activity of the intrinsic enzyme (15–17). The potencies of the peptides in blocking the formation of the $X\beta_2$ crosslinked product diminished in the order predicted from the results of Fig. 3; i.e., Boc-QQIV > Boc-AQQIV > pEAQQIV. As little as 0.5 mM Boc-QQIV caused a significant reduction of $X\beta_2$ (Fig. 4, lane 3). Examination of the SDS/PAGE profile also allowed identification of the crystallin subunits (≈32 kDa, arrow in Fig. 4) serving as donors in what seems to be a heterologous crosslinking reaction. Previously, we were able to label the acceptor subunits with

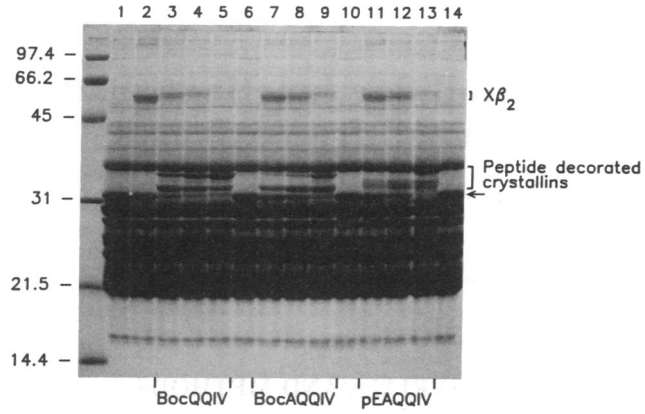


FIG. 4. Synthetic acceptors inhibit crosslinking of crystallins in $Ca^{2+}$-treated lens by blocking the donor partners. Method for activating the intrinsic transglutaminase in rabbit lens homogenate is in *Materials and Methods*. Samples contained either no peptide (lanes 1 and 2) or 0.5 mM (lanes 3, 7, and 11), 1 mM (lanes 4, 8, and 12) or 4 mM (lanes 5, 6, 9, 10, 13, and 14) Boc-QQIV (lanes 3–6), Boc-AQQIV (lanes 7–10), or pEAQQIV (lanes 11–14) and either EDTA (lanes 1, 6, 10, and 14) or $CaCl_2$ (lanes 2–5, 7–9, and 11–13).

dansylcadaverine (16), which were found to be different from those discussed here. Another remarkable feature of the experiments presented in Fig. 4 was the obvious covalent attachment of inhibitory peptides to the donor crystallin subunits under the influence of transglutaminase, i.e., requiring $Ca^{2+}$. As the intensity of the ≈32-kDa donor band diminished with increasing concentrations of substitute acceptors added, unique bands of somewhat larger molecular size appeared in the zone designated as "peptide-decorated crystallins" (Fig. 4). The intensities and upward displacement of such bands increased with higher peptide concentrations; they clearly represent single and multiple peptide substitutions into the donor protein.

Since pEAQQIV was the best of the synthetic acceptors for human factor XIIIa (Fig. 2), this hexapeptide was chosen to test inhibition of the crosslinking of human fibrin by this enzyme. Fig. 5 shows the reduced chain patterns for fibrin exposed to factor XIIIa for various times. In the absence of added peptide (Fig. 5*A*), the disappearance of monomeric $\gamma$ chains and production of $\gamma$–$\gamma'$ dimers is followed by a depletion of monomeric $\alpha$ chains, with formation of $\alpha_n$ polymers, which is essentially completed by 60 min. The latter crosslinking event could be prevented with 10 mM hexapeptide (Fig. 5*B*). At the same time, the position of peptide-substituted $\alpha$ chains shifted upwards (asterisk) in a manner quite analogous to that observed with the peptide-decorated donor crystallin subunits, as illustrated in Fig. 4.

The success with blocking donors in a transglutaminase- or factor XIIIa-directed manner has prompted us to prepare acceptor derivatives that would carry some readily recognizable tracer, such as dansyl or biotin. Just as dansylcadaverine had many uses for labeling $\gamma$-glutaminyl crosslinking sites (18), a dansyl-containing synthetic acceptor probe, for example, would greatly aid in identifying the $\varepsilon$-lysine-donating partner in heterologous crosslinking reactions and in exploring the sequence around the labeled donor residue.

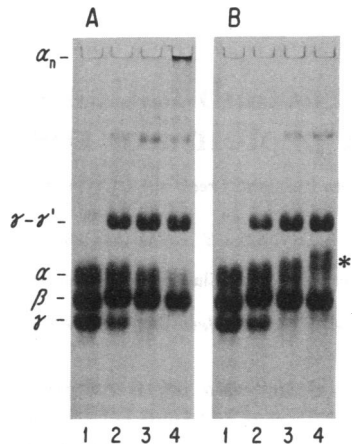


FIG. 5. The hexapeptide pEAQQIV completely prevents formation of $\alpha_n$ polymers in fibrin, during reaction with factor XIIIa, by simultaneously derivatizing the monomeric $\alpha$ chains. Lanes 1, EDTA control; lanes 2–4, with $Ca^{2+}$ for 5, 15, and 60 min. (*A*) No peptide. (*B*) With 10 mM pEAQQIV.

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1. Lorand, L., Rule, N. G., Ong, H. H., Furlanetto, R., Jacobsen, A., Downey, J., Oner, N. & Bruner-Lorand, J. (1968) *Biochemistry* **7**, 1214-1223.
2. Lorand, L. & Conrad, S. (1984) *Mol. Cell. Biochem.* **58**, 9–35.
3. Petersen, T. E., Thogersen, H. C., Skorstengaard, K., Vibe-Pedersen, K., Sahl, P., Sottrup-Jensen, L. & Magnusson, S. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 137–141.
4. Stewart, J. M. & Young, J. D. (1969) *Solid Phase Peptide Synthesis* (Freeman, San Francisco), pp. 62–63.
5. Anderson, G. W., Zimmerman, J. E. & Callahan, F. M. (1964) *J. Am. Chem. Soc.* **86**, 1839–1842.
6. Konig, W. & Geiger, R. (1970) *Chem. Ber.* **103**, 788–798.
7. Sheehan, J. C. & Hlavka, J. J. (1956) *J. Org. Chem.* **21**, 439–441.
8. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
9. Curtis, C. G. & Lorand, L. (1976) *Methods Enzymol.* **45**, 177–191.
10. Lorand, L., Credo, R. B. & Janus, T. J. (1981) *Methods Enzymol.* **80**, 333–341.
11. Schwartz, M. L., Pizzo, S. V., Hill, R. L. & McKee, P. A. (1973) *J. Biol. Chem.* **248**, 1395–1407.
12. Lorand, L. & Campbell, L. K. (1971) *Anal. Biochem.* **44**, 207–220.
13. Chen, K. H. (1975) Ph.D. dissertation (Northwestern University, Evanston, IL), University Microfilms no. 75–7887.
14. McDonagh, R. P., McDonagh, J., Petersen, T. E., Thogersen, H. C., Skorstengaard, K., Sottrup-Jensen, L., Magnusson, S., Dell, A. & Morris, H. R. (1981) *FEBS Lett.* **127**, 174–178.
15. Lorand, L., Conrad, S. M. & Velasco, P. T. (1985) *Biochemistry* **24**, 1525–1531.
16. Velasco, P. T. & Lorand, L. (1987) *Biochemistry* **26**, 4629–4634.
17. Velasco, P. T., Murthy, P., Goll, D. E. & Lorand, L. (1990) *Biochim. Biophys. Acta* **1040**, 187–191.
18. Cariello, L., Velasco, P. T., Wilson, J., Parameswaran, K. N., Karush, F. & Lorand, L. (1990) *Biochemistry* **29**, 5103–5108.