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Biochemistry

Photoaffinity labeling of the primary fibrin polymerization site: Localization of the label to γ-chain Tyr-363

(fibrinogen/fragment D)

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ABSTRACT **Fragment D prepared from human fibrinogen was labeled specifically by photoactivation of the peptide [^{14}C]Gly-Pro-Arg-*N*-(4-azido-2-nitrophenyl)Lys amide. The preparation was freed of excess labeling reagents and then reduced and alkylated. The component α, β, and γ chains were purified by chromatography on carboxymethylcellulose and the radioactivity was found to be restricted to the γ chain. Isolated γ chains were digested with various endopeptidases, both alone and in tandem, and the products were fractionated by gradient HPLC. The amino acid compositions of all labeled peptides led to the conclusion that the modification occurs exclusively on γ-chain Tyr-363.**

The identification of those parts of the fibrinogen molecule that interact during the initial polymerization event leading to formation of the fibrin clot has been a subject of interest for almost 50 years (for a review of early efforts, see ref. 1). The finding that simple peptides patterned on the amino-terminal sequences of fibrin can block polymerization provided convincing evidence that these newly exposed groups formed one part of the interaction (2). Binding studies with these same peptides also implicated the carboxyl-terminal portions of fibrinogen γ chains as the sites of the complementary interaction (3, 4).

Localizing these latter sites has proved difficult and controversial (5–8). In the preceding article (9), photoaffinity labeling was used to demonstrate that the binding site for peptides with the sequence Gly-Pro-Arg must be close to an amino acid in the segment comprising γ-chain residues 337–379. In this article we show that the photolabeled amino acid is Tyr-363 on the γ chain.

MATERIALS AND METHODS

Our initial work on photoaffinity labeling of fibrinogen and its fragments (9) had several technical limitations that hindered localization of the labeled residues more precisely. Accordingly, a number of changes were incorporated into the present study that greatly improved the sensitivity of our analyses. These changes included preparing peptides of significantly greater specific activity, photolabeling under mild conditions aimed at preventing oxidation, using specific proteases in place of CnBr, and using a more sensitive amino acid analyzer. Only those methods and materials that differ significantly from those used in the earlier stages of the study are described here.

Enzymes. Lysyl endopeptidase was obtained from Wako Pure Chemical, Osaka. Digestions were carried out in a 0.05 M Tris buffer (pH 9.0) containing 3 M urea, for 24 hr at 37°C. The ratio of enzyme to substrate was 1:20 (wt/wt). Asparaginyl endopeptidase was obtained from Takara Shuzo, Kyoto. Digestions were conducted in a 0.02 M sodium acetate buffer (pH 5.0) containing 0.01 M dithiothreitol and 0.001 M EDTA, for 16 hr at 37°C. The protease from *Staphylococcus aureus*, strain V8 (*Staphylococcus* V8 protease) was purchased from ICN and used in 0.1 M ammonium bicarbonate (pH 8.0) for 12 hr at 37°C.

Peptide Synthesis. Peptides were synthesized in the same manner as reported by Shimizu *et al.* (9) except that the starting butoxycarbonyl[^{14}C]glycine used for synthesis was of considerably higher specific activity. In the end, the specific activity of the derivatized peptides averaged ≈1000 cpm/nm.

Amino Acid Analysis. Peptides were hydrolyzed as described in our earlier work (9), but analyses for this study were conducted on a Beckman model 6300 amino acid analyzer equipped with an integrator that allowed reproducible analyses at subnanomolar levels.

Large Scale Preparation of Fragment D. To ensure an adequate supply of human fibrinogen fragment D, a large-bed Gly-Pro-Arg affinity column was prepared along the lines first used by Kuyas *et al.* (10). To this end, the peptide *N*-acetyl-Tyr-Gly-Pro-Arg-Pro-Gly-Gly-Glu was coupled to diaminopropylamine-Sepharose (Pierce) with a water-soluble carbodiimide in the presence of the helper molecule *N*-hydroxysuccinimide (11). After thorough washing, the derivatized support was digested with chymotrypsin to remove the *N*-acetyl-tyrosine blocking group. The volume of the final column was ≈20 ml and was routinely used for preparation of 120 mg at a time of fragment D. SDS/polyacrylamide gel electrophoresis showed the affinity-purified material to be homogeneous with an apparent molecular weight of 90,000.

HPLC. All HPLC was conducted on a Beckman HPLC model 110A fitted with a Vydac C_{18} column equilibrated with 0.1% trifluoroacetic acid. Elution was accomplished with linear acetonitrile gradients. Effluents were always measured at 220 nm, and flow rates were always 1.0 ml/min.

EXPERIMENTAL PROCEDURES

Photolysis and Specificity of Labeling. Photolysis was carried out with a Rayonet RPR-3500A reactor (Southern New England Ultraviolet, Branford, CT). A number of control experiments were performed, including photolysis of the modified peptide alone and irradiation of the modified peptide in advance of adding fragment D (in the dark). As expected, labeling was almost completely inhibited by the presence of nonradioactive Gly-Pro-Arg-type peptides. Moreover, an experiment in which the radioactive peptide was photoactivated and fragment D were subsequently added in the dark failed to produce any incorporation of radioactivity.

Irradiation of the modified peptide alone showed that its photolysis was complete in ≈15 min at 4°C. Accordingly, all subsequent studies were conducted for 30 min. Solutions containing fragment D (3 mg/ml) were first purged with nitrogen for 15 min and then incubated with [^{14}C]Gly-Pro-Arg-

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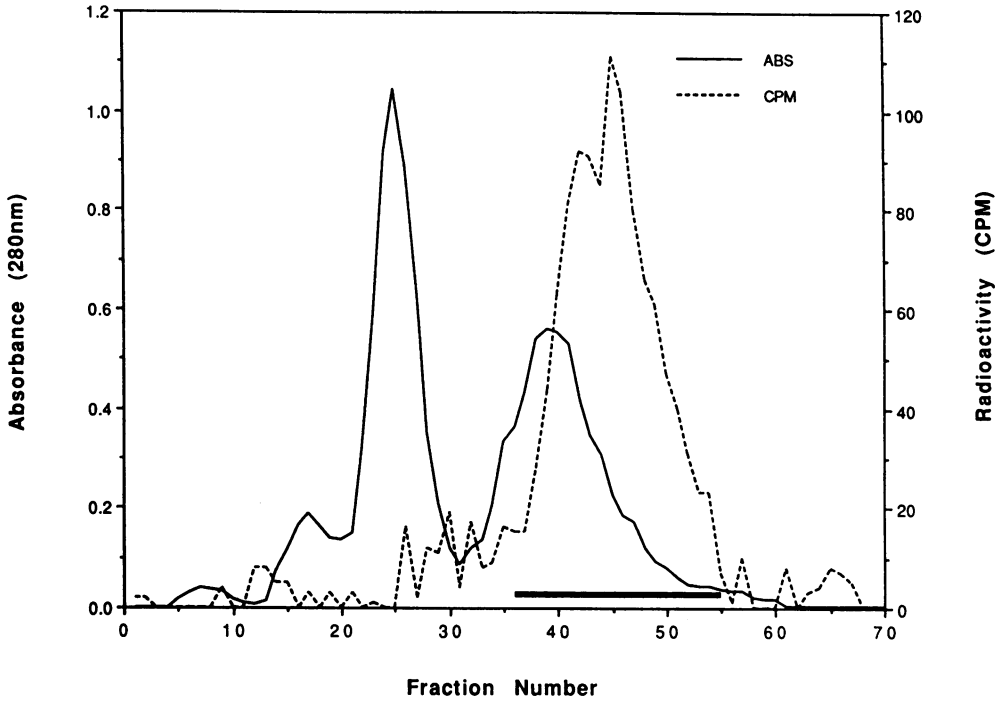

FIG. 1. Chromatography on CM-cellulose of reduced and alkylated fragment D that has been photoaffinity labeled. The column measured 2.5 × 3 cm. The fractions were 3.0 ml; 0.05-ml aliquots were removed for counting. Flow rate, 0.5 ml/min; starting buffer, 0.01 M sodium acetate (pH 5.1). Elution was accomplished with a gradient of increasing ionic strength. Solid bar denotes region pooled for further study.

N-(4-azido-2-nitrophenyl)Lys amide (0.33 mM) for 2 hr prior to photolysis at 4°C. The final solution contained 0.15 M NaCl and 0.005 M $CaCl_2$ adjusted to pH 7.4 but otherwise unbuffered. After irradiation, preparations were dialyzed against 0.5% acetic acid in the cold and in the dark in order to remove unincorporated peptide. The amount of labeled peptide incorporated amounted to 0.5–0.6 mol per mol of fragment D.

CM-Cellulose Chromatography. Affinity-labeled fragment D was reduced and alkylated and the component chains were purified by chromatography on CM-cellulose; the conditions were similar to those used in this laboratory (12). Typically, 25 mg of labeled fragment D was applied to a column. The two additional positive charges contributed by the incorporated Gly-Pro-Arg caused the radioactive material to trail the unlabeled peaks of γ chain (Fig. 1). The various peaks were pooled and samples were examined on SDS/polyacrylamide gel. Autoradiography of the gels confirmed that only γ chains were labeled.

HPLC of Enzymatic Digests. Labeled γ chains were digested with lysyl endopeptidase and applied directly to a Vydac C_{18} reverse-phase HPLC column at low pH (0.1% trifluoroacetic acid); elution was accomplished with an acetonitrile gradient

Table 1. Amino acid compositions of selected radioactive pools from HPLC

	Pool I		Pool II		Pool IS		Pool INA		Pool INB		Pool IINA		Pool IINB	
	Obs.	322–338	Obs.	357–373	Obs.	357–373	Obs.	362–365	Obs.	357–365	Obs.	358–365	Obs.	357–365
CM-Cys	+	(1)	–		–		–		–		–		–	
Asx	3.0	(3)	3.5	(3)	3.0	(3)	2.3	(2)	3.0	(3)	3.1	(3)	2.9	(3)
Thr	1.0		1.9	(2)	1.6	(2)	–		1.0	(1)	1.0	(1)	1.0	(1)
Ser	1.4	(1)	1.7	(1)	1.2	(1)	0.3		1.1	(1)	1.1	(1)	1.0	(1)
Glx	2.8	(3)	1.0		0.7		0						0.1	
Pro	0.9		1.4	(2)*	1.6	(2)*	1.0*	(1)*	2.0*	(2)*	2.1*	(2)*	2.0*	(2)*
Gly	3.9	(3)	3.0	(3)*	3.3	(3)*	2.0*	(2)*	2.0*	(2)*	1.7*	(2)*	1.8*	(2)*
Ala	1.4	(1)	1.9	(2)	1.8	(2)	–		1.0	(1)	0.4	(0)	0.9	(1)
Val	TR		–		–		–		–		–		–	
Met	0.8	(1)	–		–		–		–		–		–	
Ile	0.5		1.1†	(2)	0.5†	(2)	–		–		–		–	
Leu	0.2		0.9		–		–		–		–		0.1	
Tyr	0.2		–	(1)	–	(1)	–	(1)	–	(1)	–	(1)	–	(1)
Phe	0.9	(1)	–		0.7		–		–		–		–	
His	0.1		0.3		0.1		–		–		–		–	
Lys	0.6	(1)	1.3	(1)	1.1	(1)	0.3		0.3		0.3		0.3	
Arg	0.5		0.7*	(1)*	0.8*	(1)*	1.0*	(1)*	1.0*	(1)*	1.0*	(1)*	1.0*	(1)*
Total‡		(14)		(18)*		(18)*		(7)*		(12)*		(11)*		(12)*

Consult Figs. 2 and 3 and text for corresponding pool designations. Observed (obs) values are given as calculated ratios based on expected compositions for designated peptides exclusive of tryptophan.

*Values include contributions from Gly-Pro-Arg portion of label.

†Ile-Ile.

‡Totals do not contain tryptophans.

(Fig. 2A). Aliquots were removed and assayed; a single broad region of radioactive material was pooled and lyophilized. This material was reapplied to the same column, but the elution program was altered so that the gradient was much shallower. In this case, two radioactive peaks were resolved (Fig. 2B). The peaks were hydrolyzed and analyzed. The amino acid composition of the second eluting of these (pool II) was that expected for the peptide spanning residues 353–373, except that tyrosine was absent. The amino acid composition of the earlier-eluting peak (pool I) indicated that more than one peptide was present, one of which was likely residues 322–338 (Table 1). Accordingly, the material was digested further with *Staphylococcus* V8 protease, the target being the glutamates known to exist at γ-chain positions 323 and 328 (γ323 and γ328). The digest was reapplied to the Vydac column and the same shallow gradient was repeated (Fig. 2C). Although several new peaks appeared, the radioactive peak (pool IS) eluted at the same position as before *Staphylococcus* V8 digestion, indicating that the labeled peptide did not contain glutamate and in fact was not residues 322–338. Instead, the amino acid composition of the labeled material corresponded to that of residues 357–373. Once again, tyrosine was absent, and one additional residue each of glycine, proline, and arginine was present (Table 1).

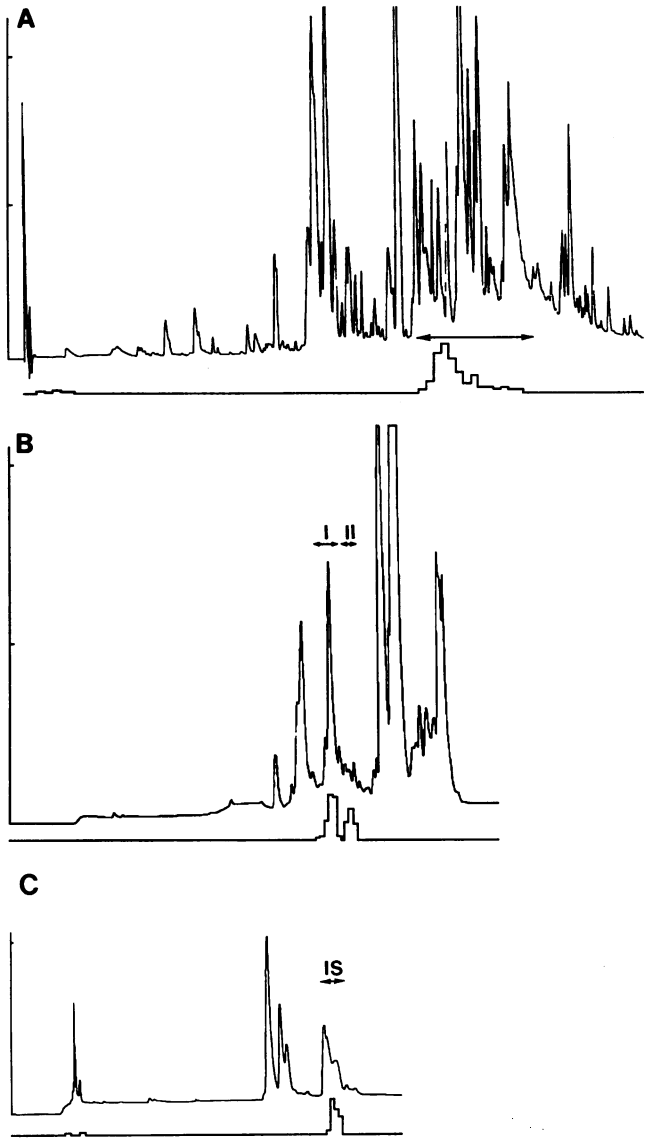

FIG. 2. Chromatograms of various enzymatic digestions of labeled fragment D γ chains separated by HPLC. (A) Lysyl endopeptidase digestion of fragment D γ chains. Fraction size, 2.0 ml; 0.4 ml was removed for counting. Radioactivity is shown as a bar graph under the trace. Solid line denotes pooled fractions. (B) Rechromatography of radiolabeled pools from A with a shallow acetonitrile gradient (fraction size, 1.0 ml; aliquots for counting, 0.25 ml). (C) Digestion of material I in B with *Staphylococcus* V8 protease. Chromatography on HPLC was under the same conditions as in B. Fraction size, 1.0 ml; counting aliquots, 0.1 ml. IS, radioactive peak.

At this point, the compositions of the purified peptides indicated that all the radiolabel was incorporated in the peptide encompassing residues γ357–373. Because this peptide contains two asparagines, we digested both of the purified peptides (pools I and II; Fig. 2B) further with asparaginyl endopeptidase and applied the digests to the same Vydac column run with the same gradient (Fig. 3). Two radioactive peaks appeared in the digest of pool I. The amino acid composition of one of these (INA) was that expected for the tetrapeptide Gly-Tyr-Asp-Asn (residues 362–365), except the tyrosine was absent and one additional glycine, proline, and arginine were present. The other labeled peptide (INB) corresponded to the nonapeptide spanning residues 357–365, with the same exceptions. Evidently the cleavage at Asn-361 was incomplete, perhaps because of the occurrence of an Asn-Gly linkage, the sequence most often involved in spontaneous deamidations (13, 14). Partial deamidation of that asparagine would explain the incomplete cleavage as well as the two peaks with identical amino acid compositions found after the initial digestion with lysyl endopeptidase (Fig. 2).

The treatment of pool II with asparaginyl endopeptidase also produced two labeled peptides, one of which (IINB) was identical to pool INB (residues 357–365), and the other of which (IINA in Table 1) had an identical composition except that the amino-terminal alanine was absent. These results further support the conclusion that the difference between peptide II and peptide I (Fig. 2B) was the deamidation of Asn-361.

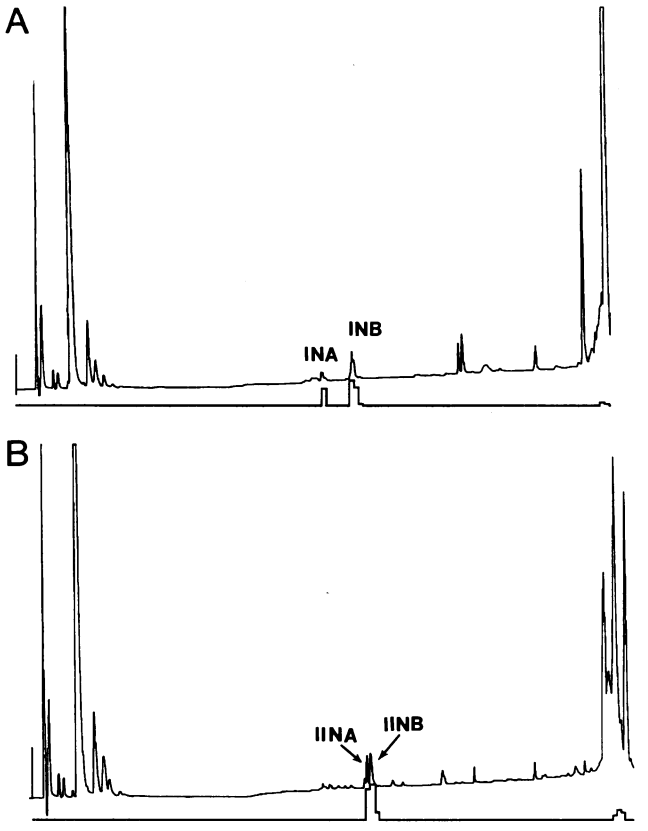

FIG. 3. (A) HPLC chromatograms of the asparaginyl endopeptidase digest of material I in Fig. 2B. (B) Asparaginyl endopeptidase digest of material II in Fig. 2B. Fraction size, 1.0 ml in both cases; 0.1-ml aliquots were removed for counting.

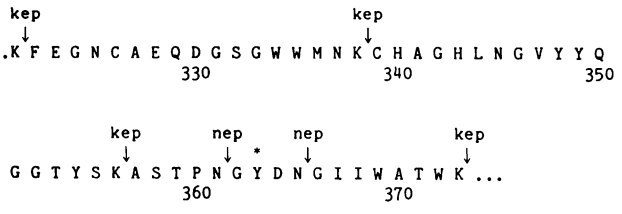

FIG. 4. Amino acid sequence of that part of the human fibrinogen γ chain that is relevant to this study. Key digestion points: kep, lysyl endopeptidase; nep, asparaginyl endopeptidase. Asterisk denotes labeled tyrosine at position 363. See Table 1 for experimentally determined amino acid compositions of expected peptides. The γ-chain sequence and numbering is from Lottspeich and Henschen (15).

In summary, six different radioactive peptides were obtained that should have included tyrosine γ363 (Fig. 4), but none of them did. Instead, each contained one extra residue each of glycine, proline, and arginine, as would be expected for a modified peptide from affinity-labeled material. In addition, small amounts of lysine were found, doubtless from the nitrophenylated lysine in the original material. These data demonstrate that the label in photoaffinity-labeled fragment D is attached to tyrosine γ363.

Computer Graphics. A molecular model of the peptide used for photoaffinity labeling in these studies, Gly-L-Pro-L-Arg-*N*(4-azido-2-nitrophenyl)L-Lys amide, was constructed on a Silicon graphics personal Iris computer with the aid of the SYBYL programming package made available by Tripos Associates (St. Louis). The peptide was examined in both the conformation calculated to have the minimum potential energy and the fully extended form. The photoactive group is located 8–12 Å from the α-amino group and 10–17 Å from the guanidino group of the arginine, depending on the conformation (Fig. 5).

DISCUSSION

Generally speaking, reagents for affinity labeling tend to react with residues near the ligand-binding pocket rather than within the site itself. In the case of peptide ligands, only an approximation can be made of the space swept out by the reactive group when the peptide is bound in the pocket. That the tetrapeptide Gly-Pro-Arg-Lys amide modified with a 4-azido-2-nitrophenyl group exclusively labels a single tyrosine in fibrinogen fragment D indicates that the tyrosine

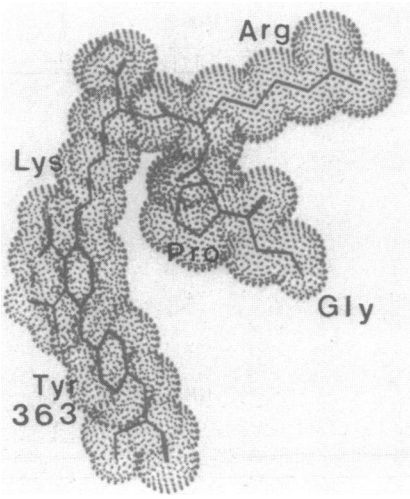

FIG. 5. Molecular model generated by computer graphics of Gly-L-Pro-L-Arg-*N*(4-azido-2-nitrophenyl)L-Lys amide attached to a tyrosine side chain.

hydroxyl is situated within a zone 10–20 Å from the innermost residues of the binding pocket (Fig. 5). As it happens, hydroxyl groups are good targets for the electrophilic attack of rearranged nitrenes (16). It may also be possible to label other nucleophilic side chains in the region by shortening or lengthening the arm onto which the aryl azide was attached.

As for the nature of the side chains within the pocket itself, it is likely that carboxyl groups are needed to bind the positively charged α-amino group and the guanidino group of the critical arginine. On the basis of comparative sequence studies, we have proposed that those carboxyls reside either in the stretch γ291–298 or in the stretch γ316–323 (17). In this regard, it should be noted that all known fibrinogen γ chains (human, rat, bovine, frog, and lamprey) have a tyrosine at position 363; tyrosine does not occur at this position in any known β chain (human, rat, bovine, chicken, and lamprey), nor does it occur at this position in six fibrinogen-related proteins.

Our results are in accord with past studies involving those variant human fibrinogens that exhibit defects in polymerization thought to be associated with the site complementary to Gly-Pro-Arg, all of which involve changes in the carboxyl-terminal third of the γ chain. These include substitutions at positions γ275, -292, -308, -310, -329, -330, and -375 (18). As is apparent from the numbers of these positions, these mutations occur in a region of $\approx$100 amino acids that includes Tyr-363. Recently, Koopman *et al.* (19) reported another variant fibrinogen with impaired polymerization; in this case, two adjacent amino acids are missing as a result of a 6-base-pair deletion. The deleted residues are Asn-319 and Asp-320. A combination of the results from affinity labeling, comparative sequence analysis from diverse species, and polymerization-defective human fibrinogens may yet allow a total reconstruction of the Gly-Pro-Arg binding site.

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