# LYSINE AS AMINE DONOR IN FIBRIN CROSSLINKING

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The finding that α-(N-p-toluenesulfonyl)-L-lysine methylester was a good inhibitor of fibrin[1] crosslinking (Lorand, 1965) raised the possibility that ε-amino functions of lysine — rather than α-amines of glycine, as previously proposed (Lorand et al., 1962) — of the protein could be involved in the crosslinking reaction. We have gathered several lines of evidence to show the participation of lysine side chains.

Inhibitors of fibrin crosslinking, possessing a 5 - aminopentyl group.

While α-(N-p-toluenesulfonyl)-L-lysine methylester was highly potent, ε-(N-p-toluenesulfonyl)-L-lysine methylester caused no inhibition. This indicates that the amine in the ε position is of critical significance. Nevertheless, drawing conclusions from lysine ester derivatives could still be ambiguous because of the possibility that the ester function, rather than the amine, might have interfered with crosslinking. To obviate this difficulty, N-(5-aminopentyl)-p-toluenesulfonamide ("tosylcadaverine") and N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide("dansylcadaverine") were synthesized. As seen in Fig. 1, both of these are better inhibitors than

[1] The specific experiments described in this paper pertain to bovine fibrin.

glycine methylester; dansylcadaverine, approximately 20-times more potent, is the most effective inhibitor found so far. This then shows that the 5-aminopentyl group satisfies the specificity requirement of the crosslinking enzyme. However, it is clear that substituents attached to this group also play an important role. The dansyl compound is about 5-times more effective than the tosyl; binding to the enzyme is apparently enhanced by increasing the size of the apolar substituent. It is interesting to note in this connection that methyl 6-aminohexanoate is only a very weak inhibitor.

A fluorescent 5-aminopentyl substrate for the crosslinking enzyme.

Several amine inhibitors of fibrin crosslinking have already been shown to actually serve as substrates for the crosslinking enzyme. As such, $^{14}$C-glycine ethylester was covalently incorporated through its amino function; hydroxylamine and hydrazine gave rise to fibrin hydroxamate and hydrazide, respectively (Lorand and Jacobsen, 1964; Lorand, 1965; Lorand and Ong, 1966 a). Chemical degradation of the fibrin hydroxamate revealed that glutamyl side chains of fibrin serve as acceptor groups in the transpeptidation reaction (Lorand and Ong, 1966 b). This was confirmed with the use of the $^{14}$C-glycine ethylester — blocked fibrin (Lorand and Jacobsen, 1964; Lorand, 1965; Lorand and Ong, 1966 a) and enzymatic degradation (Matacic and Loewy, 1966).

As a fluorescent monoamine, dansylcadaverine is particularly well-suited for studying its enzymatic incorporation into proteins. Though less sensitive than fluorescent methods, incorporation can also be conveniently followed by measuring absorbancy, due to naphthalene, near 326 mμ (Massey et al., 1955; Deranleau and Neurath, 1966). As seen in Fig. 2, dansylcadaverine

proved to be a very good donor substrate in conjunction with the crosslinking enzyme. In fact, it promises to be the substrate of choice[2] for detailed kinetic analysis of amine incorporation into fibrin and fibrinogen. As with $^{14}$C-glycine ethylester (Lorand,

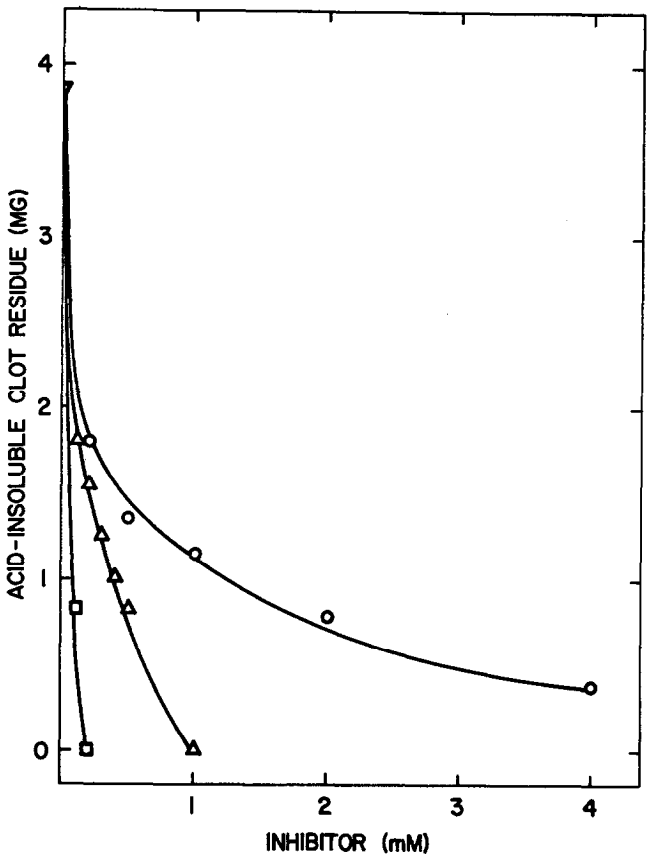


Fig. 1. Inhibitors of Fibrin Crosslinking: (o) glycine methylester; (Δ) tosylcadaverine; (□) dansylcadaverine; (▽) no inhibitor present. Procedure of Lorand et al., (1962).

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[2](a) In preliminary experiments (with fibrin as acceptor), an apparent Michaelis constant for dansylcadaverine of about $2 \times 10^{-4}$M was obtained.

(b) Dansylcadaverine also proved to be a versatile substrate for measuring the activity of other transpeptidating enzymes, e.g. transglutaminase (Lorand et al., 1966).

(c) Such a fluorescent probe serves to visualize directly the amine-acceptor peptide chains of the protein and the immediate environment of the incorporating site. Electrophoresis and chromatography (Clegg and Bailey, 1962) of sulfitolyzed dansylcadaverine-blocked fibrin (ca 3 moles per mole of protein) shows most fluorescence in the tyrosine chain (Lorand and Tokura, unpublished data).

1965; Lorand and Ong, 1966 a), incorporation into fibrin proceeds much faster than into fibrinogen, showing that thrombin opens up acceptor sites in the protein.

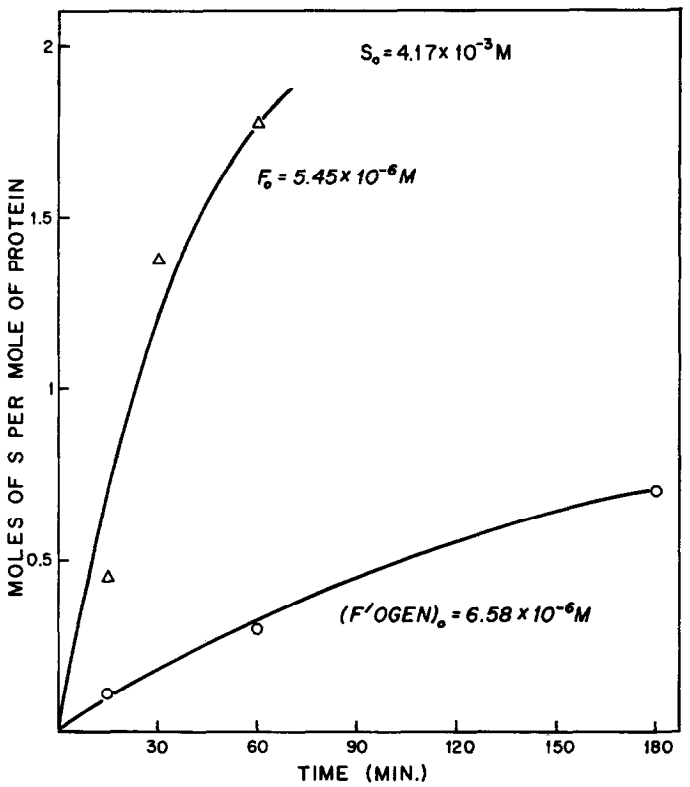


Fig. 2. Incorporation of Dansylcadaverine (S) into Fibrin (F) and Fibrinogen (F'ogen). Method as described for $^{14}C$-glycine ethylester by Lorand and Ong (1966 a), except that the crosslinking enzyme (i.e. thrombin-activated stabilizing factor; 54 µg/ml) was preactivated (Konishi and Lorand, 1966); thus, no thrombin was present during incorporation. Following treatment with trichloroacetic acid, the proteins were dissolved in 5M urea — 0.5% sodiumdodecylsulfate, pH 8. Incorporation values on ordinate were derived from fluorescence ($\lambda_A$ = 360 mµ; $\lambda_F$ = 520 mµ) without correction for quenching. Absorbancy ratios of 326 mµ : 280 mµ gave about 1.6 times higher values.

Reinvestigation of the role of α-amines of glycine in fibrin crosslinking.

The demonstration that a substrate (i.e. dansylcadaverine) with a 5-aminopentyl group could be utilized by the crosslinking enzyme (even at pH 7.5), strongly pointed to the participation

of ε-amines of lysine side chains as possible donor groups in crosslinking[3]. Nevertheless, we had to reckon with earlier data (Lorand et al., 1962) which indicated that fewer N-terminal glycines could be found in crosslinked fibrin than in fibrin which was not crosslinked. The apparent reduction of N-termini in the crosslinked protein might, however, have been due to kinetic hindrance, even though urea was present, towards the 1-fluoro-2,4-dinitrobenzene reagent. This would especially be possible in the case of crosslinked fibrin which cannot be dissolved in urea.

Therefore, we now re-examined the amino end groups of the two varieties of fibrin after first degrading them by sulfitolysis (Pechere et al., 1958), a process assumed not to break peptide bonds. When the fluorodinitrobenzene method is applied (Sanger, 1945; Levy, 1954) to the sulfitolyzed proteins, both fibrin and crosslinked fibrin were shown to contain only glycine and tyrosine end groups in identical amounts.

Table I

| N-termini | Weight of Protein* (gm) Containing 1 mole End Group: Fibrin | Crosslinked Fibrin |
|---|---|---|
| Glycine (8 hr. hydrolysis, 68% loss) | 131,000 | 125,000 |
| Tyrosine (24 hr. hydrolysis, 32% loss) | 288,000 | 280,000 |

* Determined from ε-dinitrophenyllysine found in 24 hr. hydrolysates and computed on the basis of known lysine content of fibrin (Mihalyi et al., 1964). The same protein preparations were used for experiments in Tables I and II.

[3] In some experiments, the extent of dansylcadaverine incorporation was greater than 4 per mole of fibrin, indicating that the number of potential amine-acceptor sites may exceed the amount of N-terminal glycines in the protein.

Difference in residual lysine contents of fibrin and crosslinked-fibrin after deamination.

Lysine side chains participating in crosslinking would be resistant to deamination. Therefore, crosslinked fibrin — after deamination and hydrolysis — might show a higher residual lysine content than non-crosslinked fibrin.

70 mg of fibrin and crosslinked fibrin (both sulfitolyzed; Pechere et al., 1958) were treated in a parallel manner (40 ml of 50% acetic acid, 20 ml of 40% sodium nitrate at 0-5°C, 2 hr in nitrogen atmosphere). The precipitated proteins were washed, dialyzed and dried with acetone, ether. Prior to washing, aliquots (half of each protein) were redeaminated, then purified as described. The proteins were hydrolyzed for both 24 and 48 hr. (5.7 N HCl, 105°C, evacuated vials). Hydrolysates were analyzed for residual lysine (emerging at 288 ml on the physiological fluid column, 0.9 x 56 cm; Beckman Spinco Model 120B amino acid analyzer; 0.38 N citrate buffer of pH 4.26; 33°C; 30 ml per hr flow rate) and for amino acids in general (Spackman et al., 1958). The latter were used as reference for computing protein equivalents on basis of data by Mihalyi et al., (1964).

As seen in Table II, there are significant differences between the two types of fibrin in regard to lysine remaining after deamination. The difference, ascribable to crosslinking, amounts to about 0.9 - 1.5 moles of lysine per 100,000 gm of protein.

The data presented in this paper show the participation of lysine side chains as amine donors in crosslinking fibrin. Concerning the nature of acceptor groups, definite evidence has already been presented for the involvement of γ-carbonyls

Table II

| Reference Amino Acid | | Residual Lysine (moles) per $10^5$ g of Fibrin (1) | Fibrin (2) | Crosslinked Fibrin (1) | Crosslinked Fibrin (2) | Δ Lysine due to Crosslinks moles/$10^5$ g | | | |
|---|---|---|---|---|---|---|---|---|---|
| Asp | a | 0.379 | 0.342 | 1.324 | 1.318 | 1a | 0.945 | b | 1.031 |
| | b | 0.413 | 0.347 | 1.444 | 1.462 | 2a | 0.976 | b | 1.115 |
| Thr | a | 0.411 | 0.376 | 1.428 | 1.462 | 1a | 1.017 | b | 1.161 |
| | b | 0.443 | 0.383 | 1.604 | 1.518 | 2a | 1.086 | b | 1.135 |
| Ser | a | 0.448 | 0.413 | 1.523 | 1.583 | 1a | 1.075 | b | 1.335 |
| | b | 0.490 | 0.427 | 1.825 | 1.804 | 2a | 1.170 | b | 1.377 |
| Glu | a | 0.377 | 0.342 | 1.274 | 1.283 | 1a | 0.897 | b | 0.994 |
| | b | 0.392 | 0.340 | 1.386 | 1.422 | 2a | 0.941 | b | 1.072 |
| Pro | a | 0.535 | 0.493 | 1.968 | 1.894 | 1a | 1.433 | b | 1.411 |
| | b | 0.576 | 0.505 | 1.987 | 2.042 | 2a | 1.401 | b | 1.537 |
| Gly | a | 0.460 | 0.421 | 1.620 | 1.662 | 1a | 1.160 | b | 1.261 |
| | b | 0.467 | 0.420 | 1.728 | 1.800 | 2a | 1.241 | b | 1.381 |
| Ala | a | 0.423 | 0.391 | 1.563 | 1.522 | 1a | 1.140 | b | 1.104 |
| | b | 0.472 | 0.400 | 1.576 | 1.687 | 2a | 1.131 | b | 1.287 |
| Val | a | 0.552 | 0.424 | 1.675 | 1.657 | 1a | 1.123 | b | 1.219 |
| | b | 0.495 | 0.423 | 1.714 | 1.581 | 2a | 1.233 | b | 1.158 |
| Ileu | a | 0.486 | 0.353 | 1.340 | 1.369 | 1a | 0.854 | b | 1.000 |
| | b | 0.408 | 0.349 | 1.408 | 1.445 | 2a | 1.016 | b | 1.096 |
| Leu | a | 0.409 | 0.451 | 1.411 | 1.443 | 1a | 1.002 | b | 1.057 |
| | b | 0.439 | 0.375 | 1.496 | 1.557 | 2a | 0.992 | b | 1.182 |
| His | a | 0.453 | 0.377 | 1.505 | 1.418 | 1a | 1.052 | b | 1.105 |
| | b | 0.470 | 0.375 | 1.575 | 1.520 | 2a | 1.041 | b | 1.145 |

(1) = deaminated once; (2) = deaminated twice
a = 24 hr. hydrolysate; b = 48 hr. hydrolysate

of glutamyl residues in the protein (Lorand and Ong, 1966 b). It will be recalled that fibrin hydroxamate (prepared with the crosslinking enzyme to effect the incorporation of 3.2 moles of hydroxylamine per mole of fibrin), after Lossen – rearrangement and total acid-hydrolysis, gave rise to diaminobutyric acid (derived from the γ-glutamylhydroxamate residues) in 25% yield. With the use of a physiological fluid column (PA-35; 0.9 x 22 cm; Beckman Spinco Model 120B amino acid analyzer; Spackman et al., 1958) and with 0.2N citrate buffer of pH 4.2, it was also established that diaminopropionic acid (emerging at 265 ml effluent; 33°C; 50 ml per hr flow rate) was absent. Hence, there is no evidence to indicate the labelling of β-carbonyls of aspartyl residues by hydroxylamine.

Considering our findings so far, the dipeptide moiety produced by the crosslinking enzyme between fibrin molecules (F and F') is represented as γ-glutamyl-ε-lysine:

$$F\!-\!\underset{-\,CO.}{\overset{-\,NH.}{}}\!CH.(CH_2)_2\ CO.\ NH.\ (CH_2)_4.\ CH\!\underset{.OC\,-}{\overset{.HN\,-}{}}\!F' \ ,$$

shown as a single covalent contact for fibrin dimer.

Guinea pig liver transglutaminase has been known to catalyze the formation of similar crosslinks in other proteins (Waelsch, 1962). It has also been demonstrated (Bruner-Lorand, et al., 1966) that transglutaminase, through its specific enzyme activity, could actually replace the fibrin crosslinking enzyme. Forming of γ-glutamyl-ε-lysine crosslinks by the latter is further in keeping with the extreme similarities between these two enzymes.

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