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Thiolester Substrates for Transamidating Enzymes: Studies on Fibrinoligase

(acyl group transfer/hydrolysis/transglutaminase/Factor XIII)

L. LORAND, C-H. J. CHOU, AND I. SIMPSON

Biochemistry Division, Department of Chemistry, Northwestern University, Evanston, Illinois 60201

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ABSTRACT **Esters of thiocholine were shown to inhibit the crosslinking of fibrin clots by the transamidating enzyme, fibrinoligase (thrombin-activated fibrin-stabilizing factor or activated Factor XIII). Inhibition depended on the nature of the acylating group with the phenylpropionyl, phenylbutyryl, and *trans*-cinnamoyl esters being most effective of the compounds tested so far.**

Use of the thiolesters made it possible for the first time to study the reactions of fibrinoligase in fully synthetic substrate systems. Enzyme-catalyzed acyl-group transfers from the thiol-esters to a fluorescent amine [*N*-(5-aminopentyl) - 5 - dimethylamino - 1 - naphthalenesulfonamide] could be readily demonstrated and measured.

***Trans*-cinnamoylthiocholine reacted with fibrinoligase in a totally calcium-dependent manner in the absence of any added amine, thus providing the first evidence for an esterolytic pathway for this enzyme. Spectral qualities, as well as appreciable extent of solubility in water, would seem to make *trans*-cinnamoylthiocholine a specially suitable substrate for further studies.**

Fibrinoligase, a thiol enzyme similar in many respects to papain or to liver transglutaminase, is formed at the time of coagulation by the limited proteolytic action of thrombin on a zymogen component of blood plasma (called fibrin-stabilizing factor or Factor XIII). Its physiological role is to catalyze the crosslinking of fibrin units by a selective transamidation reaction, producing intermolecular γ-glutamyl-ϵ-lysine bridges that greatly enhance the elasticity of the clot structure. The functioning of fibrinoligase appears to be absolutely essential for normal hemostasis (for a review, see ref. 1).

Previous accounts from this laboratory dealt rather extensively with the amine-donor substrate specificity of the enzyme. This could be studied by inhibition of fibrin crosslinking and by the kinetics of amine incorporation into fibrin or casein. Several synthetic primary amines, especially those containing an aliphatic tetra- or pentamethylamine side chain analogous to the ϵ-NH_2 lysine natural donor groups in fibrin, were particularly effective (2–4).

The present report is directed towards the exploration of acceptor specificity with model substrates in the thiolester series. Apart from acting as inhibitors of the biological crosslinking of fibrin, these compounds now make it possible to study acyl transfers to amines as well as hydrolytic reactions with fibrinoligase in fully synthetic systems. *Trans*-cinnamoylthiocholine is of special interest as a substrate because of the spectral changes that accompany its reactions with the enzyme.

Abbreviation: monodansylcadaverine, *N*-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide.

MATERIALS AND METHODS

The fibrinoligase zymogen (i.e., Factor XIII or fibrin-stabilizing factor) was isolated from 6 liters of bovine plasma by DEAE-cellulose chromatography and elution with a salt gradient (5). The concentrate of active fractions was then filtered through a 3 × 90 cm Agarose A-0.5 (Biorad, Richmond, Calif.) column in Tris–EDTA buffer [0.05 M Tris·HCl (pH 7.5) containing 1 mM EDTA]. Fractions of high activity were pooled and concentrated by passage through a 0.9 × 7 cm DEAE-cellulose column at high ionic strength (0.3 M sodium chloride in Tris–EDTA buffer) and dialyzed against the Tris–EDTA buffer to remove sodium chloride. The purified zymogen was stored as such in ice. Its potency, after thrombin activation, was monitored by the incorporation of [^{14}C]putrescine (54 Ci/mol) into casein with the filter-paper assay recently developed in this laboratory (6). The preparation was stable for at least 6 weeks. Protein concentrations were computed on the basis of an assumed absorbancy (1%, 280 nm) of 15.

Inhibition of fibrin crosslinking was evaluated with only minor modifications of the monochloroacetic acid clot-solubility assay (2). Activation of 0.4 ml of the Factor XIII zymogen (0.3 mg) solution was performed at room temperature (22–24°) by incubation with 0.1 ml of 50 mM calcium chloride and 0.2 ml of thrombin (Parke–Davis, 4 NIH units) for 10 min. Then, 0.5 ml of the inhibitor solution was added, followed 10 min later, by admixture of 0.3 ml of a 0.6% fibrin solution, producing an almost instantaneous gelation. After 30 min, 1.5 ml of 2% monochloroacetic acid was added in an attempt to solubilize the clot overnight. Insoluble or crosslinked clot residues were separated by centrifugation and washed twice with 10 ml of 0.9% sodium chloride and twice with water. The washed proteins were taken up in 2.5 ml of a mixture of 40% urea and 0.2 N sodium hydroxide for measurement of absorbancy at 280 nm.

The calcium chloride, thrombin, and inhibitor solutions were prepared in 0.05 M Tris·HCl (pH 7.5). Control clotting mixtures contained potassium iodide in equal concentrations to the inhibitors used. Fibrin was dissolved in 1 M sodium bromide and the pH of the solution was adjusted to 5.3 with acetic acid (7).

Thiocholine iodide was purchased from Polyscience (Warrington, Pa.); acetylthiocholine chloride and iodide, and butyrylthiocholine chloride and iodide were purchased from Sigma Chemical Co., St. Louis, Mo.; propionylthiocholine iodide was obtained from Mann Res. Lab., New York, N.Y. Phenylpropionyl-, phenylbutyryl-, benzoyl-, trimethylacetyl-,

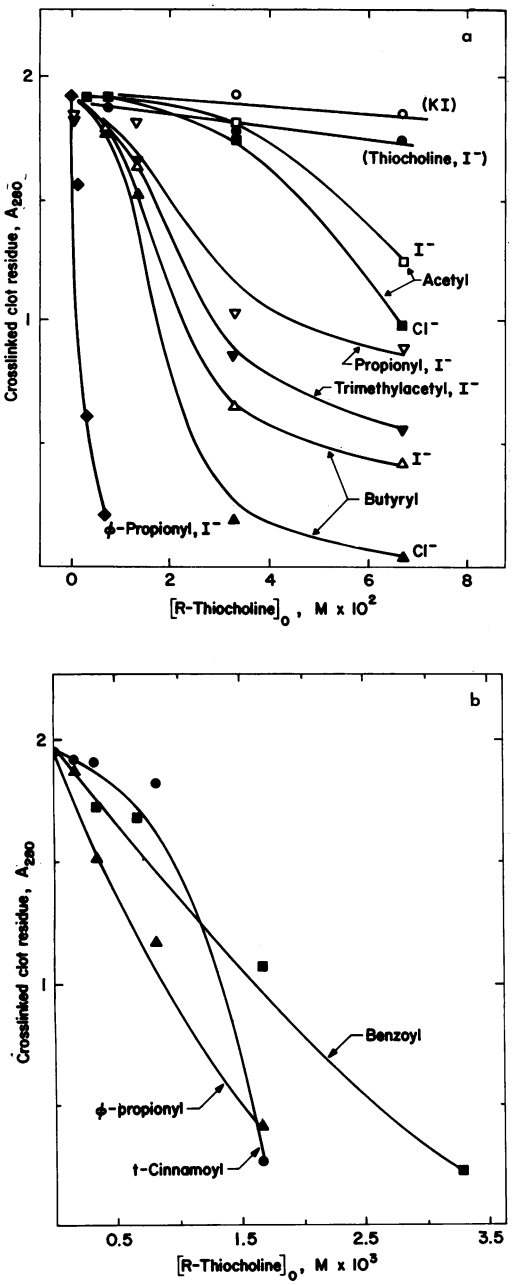

FIG. 1. (*a* and *b*) Specificity of inhibition of the enzymatic crosslinking of fibrin by thiocholine esters containing various acylating (R) groups. For methodology, see text and ref. 2. Potassium iodide, potassium chloride, and thiocholine iodide (<10 mM) showed no inhibition. In Fig. 1*b*, only the iodide salts of thiocholinium esters were used. Inhibition by phenylbutyrylthiocholine (not shown) was indistinguishable from that caused by the phenylpropionyl ester.

and *trans*-cinnamoylthiocholine iodides were synthesized in this laboratory.

N-(5-Aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide or "monodansylcadaverine" (2) (gift from Kabi, Stockholm) was used as donor for the study of enzymatic acyl transfers with thiocholine ester substrates. Reaction mixtures (in 5 × 25 mm Pyrex tubes) were 20 μl of 50 mM calcium chloride, 10 μl of the Factor XIII zymogen solution (15 μg), and 10 μl of thrombin (0.2 NIH units). Activation was allowed

TABLE 1. *Inhibition of the enzymatic crosslinking of fibrin by esters of the thiocholine series* $[RCOSCH_2CH_2\overset{+}{N}(CH_3)_3I^-]$*

R group	Inhibitory potency† (M^{-1})
CH_3	15
CH_3CH_2	29
$(CH_3)_3C$	37
$CH_3CH_2CH_2$	46
C_6H_5	527
C_6H_5CHCH	714
$C_6H_5CH_2CH_2$ *or* $C_6H_5CH_2CH_2CH_2$	910

* (see Fig. 1*a* and *b*).

† Inhibitory potency is defined as the reciprocal of the initial concentration of the compound that causes 50% reduction in the amount of crosslinked (i.e., monochloroacetic acid-insoluble) clot.

to take place for 10 min at room temperature, then 5 μl of 3 mM monodansylcadaverine and 30 μl of thiolester solutions (to yield the concentrations specified in the text) were added, and the reaction mixture was incubated at 37°. At various times, 1-μl aliquots were taken and spotted onto a thin-layer chromatographic plate (Brinkman Polyamid) and dried immediately by exposure to air current. Consecutive samples were applied 1.5-cm apart and the plate was developed by ascending chromatography in 1% pyridine adjusted to pH 5.4 with acetic acid. The positively charged monodansylcadaverine substrate moved with the solvent front (8), whereas the uncharged monodansylcadaverine derivative stayed at the origin. Disappearance of substrate and appearance of the product were quantitated by fluorescence reflectance scanning with a Turner model 111 Fluorimeter, as described (8).

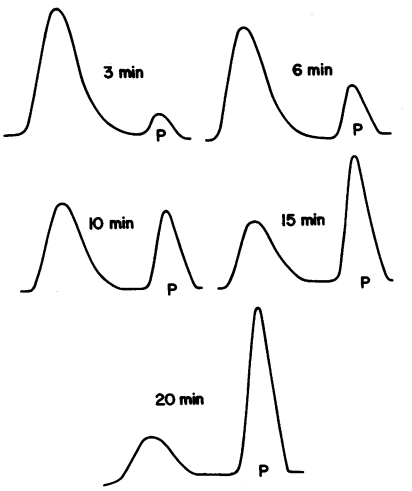

FIG. 2. Fluorescence reflectance scans after analysis by thin-layer chromatography of samples (taken at the times indicated after addition of enzyme) from a reaction mixture initially containing 0.2 mM monodansylcadaverine and 40 mM acetylthiocholinium iodide. *P* (product) denotes the uncharged fluorescent species remaining near the point of application. Complete conversion to product was observed in 45 min. For methodology, see text and ref. 8.

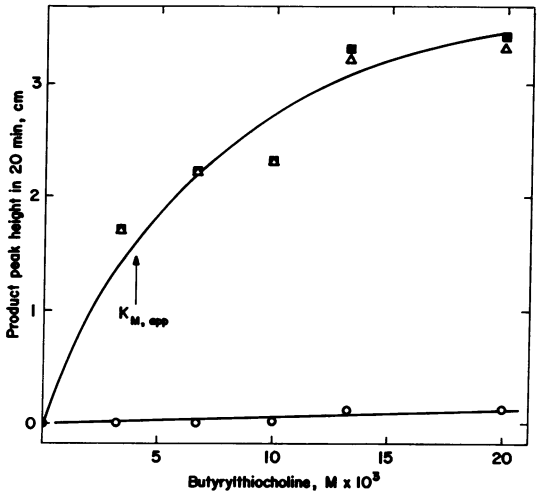

FIG. 3. Reaction between butyrylthiocholinium iodide (initial concentrations on *abscissa*) and monodansylcadaverine (0.2 mM added), as measured by the formation (in 20 min) of the fluorescent product remaining near point of application on thin-layer chromatographic analysis (see Fig. 2). Net enzymatic rates (△) were obtained by substraction of nonenzymatic values (○) from those measured in the presence of fibrinoligase (■).

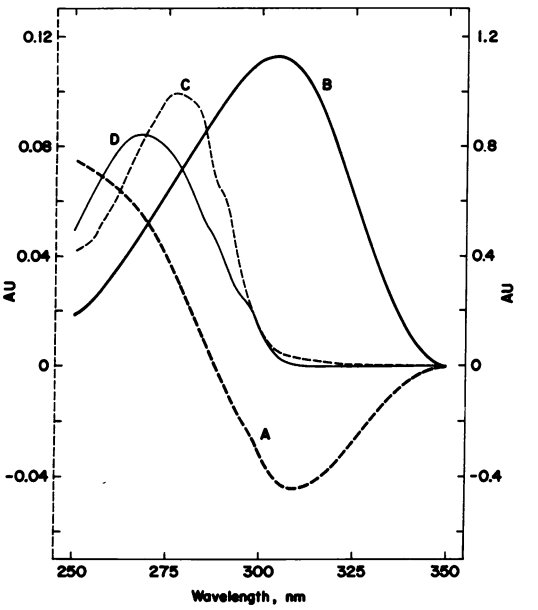

FIG. 4. Spectral analysis of the *trans*-cinnamolythiocholine–fibrinoligase system. *A*. Spectrum of *trans*-cinnamoylthiocholine (44 μM) and fibrinoligase (65 μg/ml) incubated for 85 min in the presence of calcium chloride (4 mM), taken against a mixture of *trans*-cinnamoylthiocholine without any calcium chloride added. (For absorbancy scale, see ordinate on *left*). *B. Trans*-cinnamoylthiocholine (44 μM) in 0.05 M Tris buffer (pH 7.5). (*Right ordinate*). *C*. Fibrinoligase (65 μg/ml) in the 0.05 M Tris buffer (pH 7.5) with either 1 mM EDTA or 4 mM calcium chloride added. (*Left ordinate*). *D. Trans*-cinnamic acid (42 μM) in 0.05 M Tris buffer (pH 7.5). (*Right ordinate*). *AU*, absorbance units.

Spectroscopic experiments with *trans*-cinnamoyl thiocholine iodide were performed in a Cary 16 Spectrophotometer at 25° in semi-microcuvettes with a 10-mm light path.

RESULTS AND DISCUSSION

Preliminary experiments with the intracellular calcium-dependent transamidase, guinea pig liver transglutaminase (9, 10), revealed that various thiolesters—including acetyl- and butyrylcoenzyme A—acted as rather good substrates (unpublished data). Esters of the thiocholine series yielded particularly favorable apparent Michaelis constants; this group of compounds was selected for the study of fibrinoligase.

Thiocholine esters are relatively easy to prepare; quite a few of them are appreciably soluble in water and are relatively stable at pH values near neutrality. The availability of methods for measurement of the liberation of the thiol residue in acyl transfer reactions with these substrates was also considered of potential value.

For examination of the specific requirement of fibrinoligase for the acyl substituent, several thiocholine esters that inhibit the biological crosslinking of fibrin were tested (2). As seen in Fig. 1*a* and *b* and also in Table 1, the benzoyl, phenylpropionyl, and *trans*-cinnamoyl esters were most effective; about 1 mM concentrations of these esters caused a 50% reduction in the size of monochloroacetic acid-resistant clots.

Use of the thiocholine esters enabled us to demonstrate fibrinoligase-catalyzed acyl transfers to an amine [$RCOSCH_2CH_2\overset{+}{N}(CH_3)_3X^- + H_2NR' \rightarrow RCONHR' + HSCH_2CH_2\overset{+}{N}(CH_3)_3X^-$] for the first time with a fully synthetic substrate. Fluorescent(*f*) monodansylcadaverine was chosen as the amine donor. It will be recalled that our earlier studies dealt extensively with the enzymatic incorporation of this amine into proteins (2, 3, 8, 11); in the present work thiocholine esters were used instead of the proteins. Otherwise the thin-layer chromatographic analytical procedures for the study of the disappearance of the free fluorescent amine substrate (H_2NR') and the appearance of the derivatized fluorescent product ($RCONHR'$) were identical to those used (8).

Fig. 2 shows the fluorescence-reflectance scans obtained for the fibrinoligase-catalyzed reaction of acetylthiocholine chloride with monodansylcadaverine. Product formation (as judged by the height of the peak due to the uncharged species remaining near the point of application on polyamide at pH 5.4) was linear during the first 30 min of reaction. By fixing the concentration of monodansylcadaverine and varying that of the thiocholine esters, we obtained apparent Michaelis kinetics as shown in Fig. 3 for butyrylthiocholine.

The derived apparent Michaelis constants (measured at 0.2 mM initial concentrations of monodansylcadaverine) were 16 mM for acetyl-, 4 mM for butyryl-, and 0.3 mM for the phenylpropionyl- or *trans*-cinnamoylthiocholine iodides. It should be noted that the relative order of the reciprocals of these values approximately corresponds to the relative potencies of these compounds for inhibiting the enzymatic crosslinking of fibrin (see Table 1).

The measured initial velocities for the enzymatic transfer of acyl groups to monodansylcadaverine from the thiocholine esters of acetate, butyrate, and trimethylacetate gave the approximate ratios of 30:2.3:1. Reactions in the phenylpropionyl-, *trans*-cinnamoyl-, and benzoylthiocholine series yielded relative rates of 45:9:1.

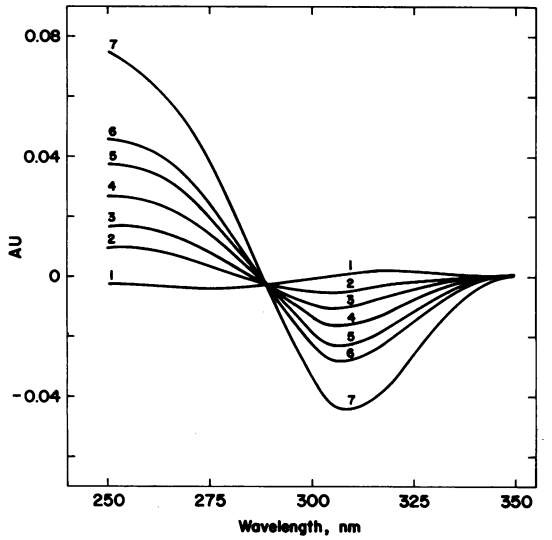

FIG. 5. Time-dependent absorbancy changes in a mixture of *trans*-cinnamoylthiocholine (44 μM), fibrinoligase (65 μg/ml), and calcium chloride (4 mM). The reference cell contained no calcium chloride. Spectral scans (marked 1–7) were obtained at 0, 9, 16, 25, 36, 46, and 85 min, respectively, after the addition of calcium chloride.

On account of the spectral changes that accompany its reactions, *trans*-cinnamoylthiocholine proved to be of special importance. We have been able to demonstrate a totally calcium-dependent reaction of fibrinoligase with this compound in the absence of an amine-donor substrate (Figs. 4 and 5), thus providing the first evidence for an esterolytic (as opposed to transamidase) pathway for the enzyme. In fact, all our data are in accord with the earlier suggestion (2) that fibrinoligase-catalyzed reactions proceed through an acyl-enzyme (a thiolester) intermediate that may be deacylated after a nucleophilic attack by either an amine or water.

The enzymatic rate of hydrolysis of *trans*-cinnamoylthiocholine could be readily measured by following the negative change in absorbancy, due to the disappearance of the thiolester substrate, at 307 nm (see Fig. 5). A Lineweaver–Burk plot, obtained at various initial concentrations of the ester, yielded an apparent Michaelis constant of 0.56 mM.

We are, of course, mindful of the fact that *trans*-cinnamoylthiocholine (and related thiolesters) might become useful as an active-center titrant for fibrinoligase and for transglutaminase, just as *trans*-cinnamoylimidazole (12) could be used as such for various enzymes. The possibility that transamidases may be able to catalyze an acyl group transfer from thiolesters to specific lysine residues (3) in protein substrates is also being explored.

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1. Lorand, L. (1970) Thromb. Diath. Haemorrh. Suppl. **39**, 75–102.
2. Lorand, L., Rule, N. G., Ong, H. H., Furlanetto, R., Jacobsen, A., Downey, J., Oner, N. & Bruner-Lorand, J. (1968) *Biochemistry* **7**, 1214–1223.
3. Lorand, L., Ong, H. H., Lipinski, B., Rule, N. G., Downey J. & Jacobsen, A. (1966) *Biochem. Biophys. Res. Commun.* **25**, 629–637.
4. Lorand, L., Downey, J., Gotoh, T., Jacobsen, A. & Tokura, S. (1968) *Biochem. Biophys. Res. Commun.* **31**, 222–230.
5. Lorand, L. & Gotoh, T. (1970) *Methods Enzymol.* **19**, 770–782.
6. Lorand, L., Campbell-Wilkes L. K., & Cooperstein, L. (1972) *Anal. Biochem.* in press.
7. Donnelly, T. H., Laskowski, M., Jr., Notley, N. & Scheraga, H. A. (1955) *Arch. Biochem. Biophys.* **56**, 369–387.
8. Lorand, L. & Campbell, L. K. (1971) *Anal. Biochem.* **44**, 207–220.
9. Clarke, D. D., Mycek, M. J., Neidle, A. & Waelsch, H. (1959) *Arch. Biochem. Biophys.* **79**, 338–354.
10. Folk, J. E. & Cole, P. W. (1966) *J. Biol. Chem.* **241**, 5518–5525.
11. Lorand, L., Lockridge, O. M., Campbell, L. K., Myhrman, R. & Bruner-Lorand, J. (1971) *Anal. Biochem.* **44**, 221–231.
12. Bender, M. L. & Brubacher, L. J. (1964) *J. Amer. Chem. Soc.* **86**, 5333–5334.