Inactivation of Cathepsin D by Dithiophosgene and by 2,2-Dichloro-1,3-dithiacyclobutanone

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Cathepsin D, purified from bovine spleen, is inactivated by the unstable inhibitors dithiophosgene and 2,2-dichloro-1,3-dithiacyclobutanone. Inhibition constants are identical for both of the compounds tested: K_i 96.1 μM ; k/c 0.406. It appears that the active species is 2,2-dichloro-1,3-dithiacyclobutanone, to which dithiophosgene is hydrolysed before cathepsin D inactivation.

Dithiophosgene (dimeric thiophosgene, 2,2,4,4-tetrachloro-1,3-dithiacyclobutane, $\text{S}-\text{CCl}_2-\text{S}-\text{CCl}_2$) has long been known to react with water to form $\text{S}-\text{CCl}_2-\text{S}-\text{C}=\text{O}$ (2,2-dichloro-1,3-dithiacyclobutanone), with aniline to form $\text{S}-\text{CCl}_2-\text{S}-\text{C}=\text{N}-\text{C}_6\text{H}_5$, and with thiol compounds to form $\text{S}-\text{CCl}_2-\text{S}-\text{C}=\text{S}$ (2,2-dichloro-1,3-dithiacyclobutanethione) (Rathke, 1888; Schönberg & Stephenson, 1933; Delepine *et al.*, 1935; Wortmann *et al.*, 1968, 1970*a,b*; Wortmann & Gattow, 1970). 2,2-Dichloro-1,3-dithiacyclobutanone and 2,2-dichloro-1,3-dithiacyclobutanethione will react with a variety of amines to form the corresponding disubstituted thiuram compounds (Delepine *et al.*, 1935; Wortmann *et al.*, 1968, 1970*a,b*; Wortmann & Gattow, 1970). It appears that no consideration has been given to dithiophosgene and its derivatives as potential protein-alkylating agents. The finding is now reported here that cathepsin D, purified from bovine spleen, is inactivated by dithiophosgene and by 2,2-dichloro-1,3-dithiacyclobutanone.

Materials and Methods

Thiophosgene was obtained from Fluka, Buchs, Switzerland. Dithiophosgene was prepared by the action of u.v. radiation on thiophosgene (Schönberg & Stephenson, 1933). The crystals formed were washed free of thiophosgene by repeatedly dissolving the preparation in hexane and removing the solvent under reduced pressure (yield 70%; m.p. 116°C). 2,2-Dichloro-1,3-dithiacyclobutanone was prepared essentially as described by Schönberg & Stephenson

(1933). Dithiophosgene (0.5 g) was dissolved in 25 ml of 75% (v/v) acetic acid, and the preparation was heated at 58°C for 45 min. The preparation was added to an excess of ice-chilled water, and the precipitate formed was repeatedly washed with water. The precipitate was finally extracted with hexane, and 2,2-dichloro-1,3-dithiacyclobutanone was crystallized by removing the solvent under reduced pressure (yield 16%; m.p. 68°C). Synthesis of dithiophosgene and of 2,2-dichloro-1,3-dithiacyclobutanone was confirmed by taking the mass spectra of the preparations. Cathepsin D was purified from bovine spleen by the method of Smith & Turk (1974). Cathepsin D activity was determined as described previously (Rakitzis, 1974).

Results and Discussion

The time-course of the inactivation of cathepsin D by dithiophosgene is given in Table 1. The rate of cathepsin D inactivation uniformly slopes off after the first 20 min of incubation with dithiophosgene; closely similar results were obtained with 2,2-dichloro-1,3-dithiacyclobutanone as enzyme inhibitor. This behaviour suggests enzyme inactivation by unstable inhibitors (Rakitzis, 1974). That dithiophosgene and 2,2-dichloro-1,3-dithiacyclobutanone are unstable in water was established by adding the cathepsin D moiety in the preincubation enzyme/inhibitor mixture after the inhibitor/Tris/HCl buffer mixture had been incubated at 37°C for 30 min. Under these conditions no inactivation of cathepsin D was observed. The breakdown of dithiophosgene and of 2,2-dichloro-1,3-dithiacyclobutanone was also studied by the determination of the acid produced on hydrolysis. The compound to be tested was dissolved in acetone, and the solution was added to 9 vol. of water. Acid production was determined by automatic titration, with NaOH solutions of appropriate concentration as the titrating agent. To eliminate

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Table 1. Inactivation of cathepsin D by dithiophosgene

Preincubation mixtures of cathepsin D and dithiophosgene consisted of 0.1 M-Tris/HCl buffer, pH 7.50, 10% (v/v) acetone, cathepsin D preparation and dithiophosgene. Dithiophosgene was dissolved in 10% acetone immediately before the start of the experiment. After incubation at 37°C for the time-periods shown, 0.05 ml portions were used for assay of cathepsin D activity.

Preincubation time (min)	% of activity remaining after preincubation with dithiophosgene				
	None	0.217 mM	0.435 mM	0.870 mM	1.74 mM
10	98.0	74.9	67.0	49.4	47.4
20	95.0	66.3	56.6	40.4	36.2
30	93.0	66.3	53.8	34.4	32.0
40	91.0	63.3	54.8	33.6	30.5
50	89.0	63.3	52.8	31.7	27.4

Fig. 1. Effect of preincubation of cathepsin D with dithiophosgene (●) and with 2,2-dichloro-1,3-dithiacyclobutanone (○)

Preincubation time was 30 min, the rest of the conditions being as described in Table 1. The results were obtained from nine independent experiments.

interference by atmospheric CO_2 , a titration end point of pH 6.3 was used. Titration was continued to the apparent end of the reaction, for a period of at least 2 h. With dithiophosgene there was an instantaneous production of 2 mol of acid/mol of compound, followed by an exponential production of 0.71 mol of acid/mol of compound (first-order rate constant was

-0.046 min^{-1} at 37°C). With 2,2-dichloro-1,3-dithiacyclobutanone there was only an exponential production of 0.75 mol of acid/mol of compound (first-order rate constant -0.050 min^{-1} at 37°C). It is not clear why the slow phase of acid production from dithiophosgene, as well as acid production from 2,2-dichloro-1,3-dithiacyclobutanone, consists only of 0.7 mol of acid/mol of compound. A plausible explanation is that there is ring-opening with both compounds, with concomitant H^+ ion uptake. Ring-opening has been shown to take place during the reaction of 2,2-dichloro-1,3-dithiacyclobutanone with a variety of nucleophiles (Delepine *et al.*, 1935; Wortmann *et al.*, 1968). Dithiophosgene and 2,2-dichloro-1,3-dithiacyclobutanone have been reported to be practically insoluble in water (Schönberg & Stephenson, 1933; Wortmann *et al.*, 1970a,b). However, it appears that, when these compounds are first dissolved in acetone and then mixed with water, they remain in solution up to a concentration of 5 mM; at higher concentrations both dithiophosgene and 2,2-dichloro-1,3-dithiacyclobutanone are precipitated out when the acetone solution is added to water or to the Tris/HCl buffer, pH 7.50.

The effect of preincubation of cathepsin D with dithiophosgene or with 2,2-dichloro-1,3-dithiacyclobutanone for enough time to allow complete disappearance of the inhibitor is shown in Fig. 1. Results were plotted according to eqn. (5) of Rakitzis (1974). The constants to be obtained from such a plot are K_i , the dissociation constant of the reversibly formed enzyme-inhibitor complex, whose subsequent first-order reaction leads to irreversible inhibition, and the ratio k/c , where k is the first-order inactivation constant of the reversible enzyme-inhibitor complex and c is the first-order rate constant of inhibitor disappearance. Fig. 1 shows that the dependence of $\log(v/v_i)$ on $\log[I]_0$ is identical for both of the compounds tested. The value for K_i obtained from the graph is $96.1 \mu\text{M}$, and the k/c value is 0.406. The fact that dithiophosgene is not more inhibitory than 2,2-

dichloro-1,3-dithiacyclobutanone suggests that the latter compound is the active species, and that dithiophosgene is hydrolysed to 2,2-dichloro-1,3-dithiacyclobutanone before cathepsin D inactivation. This interpretation is in complete agreement with the automatic titration experiments described above.

Dithiophosgene and its derivatives have received extremely little attention altogether, most of the work on these compounds being restricted to molecular structure and electronic configuration studies (Jones *et al.*, 1957; Busfield *et al.*, 1964; Krebs & Beyer, 1968, 1969; Frenzel *et al.*, 1970; Bock & Wagner, 1972). The inactivation of cathepsin D by dithiophosgene and by 2,2-dichloro-1,3-dithiacyclobutanone most probably occurs through alkylation of a nucleophilic group on the enzyme molecule. Since 2,2-dichloro-1,3-dithiacyclobutanone has been shown to react with two amino groups per molecule of compound, with concomitant ring-opening, it might be possible for these compounds to cross-link adjacent nucleophilic groups on the enzyme molecule. The reactivity of chloro-substituted dithiacyclobutane compounds clearly is to be explained through the principles applying to the highly reactive chloro-substituted sulphides (Ogston *et al.*, 1948; Price, 1958). Chloro-substituted dithiacyclobutane compounds may present additional aspects of specificity in the development of irreversible enzyme inhibitors.

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