

The Interaction of Cystine and Cysteine with Beef Liver Catalase*

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A study has been made of the interaction of crystalline beef liver catalase with cystine and with the thiols, cysteine, cysteamine, and penicillamine.

Native beef liver catalase contains 8 SH-groups readily titratable with *p*-chloromercuribenzoate. The catalase SH-groups interact with ³⁵S-cystine to form mixed disulphide groups. A maximum of 4 moles of cysteine residues are bound to the enzyme while concurrently 4 enzyme SH-groups are oxidized to SS-groups.

Incubation of catalase with thiols is associated with the formation of the inactive catalase hydrogen peroxide complex II, confirming the report of Keilin and Nicholls. The inhibition of catalase by thiols is spontaneously reversible. The degree of inhibition obtained under different experimental conditions is proportional to the amount of complex II formed. The data provide evidence that the thiol inhibition of catalase can be quantitatively accounted for by formation of the inactive catalase complex II formed from hydrogen peroxide generated in the oxidation of the thiols.

The inhibition of catalase by cystine is prevented by prior blocking of the enzyme SH-groups with *p*-chloromercuribenzoate. It is spontaneously reversible and, like the cysteine inhibition, it is prevented by ethylenediaminetetraacetate or ethanol. The data indicate that the inhibiting effect of cystine is due to its interaction with catalase SH-groups with the consequent liberation of cysteine, which in turn inhibits the enzyme according to the mechanism outlined above.

It has been known since many years^{1,2} that catalase is inhibited by cysteine and various other thiols. More recently it has been shown that cystine as well exerts a moderate inhibiting effect^{3,4}. Several mechanisms have been suggested for the thiol inhibition of catalase, but none of these has been generally accepted, and no explanation seems to have been offered to account for the inhibition of catalase by cystine.

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The present investigation was prompted by the observation in this laboratory ⁵ that catalase possesses SH-groups which interact with cystamine to form mixed disulphide groups with the concomitant liberation of cysteamine. This finding suggested the possibility that the inhibition by cystine might be due to an interaction with catalase SH-groups with the consequent liberation of the inhibiting thiol, cysteine. The data here presented provide evidence in support of this hypothesis. Furthermore, the results strongly indicate that the inhibition of catalase by cysteine and other thiols is due to the formation of the inactive catalase hydrogen peroxide complex II, as suggested by Keilin and Nicholls ⁶.

EXPERIMENTAL

Materials. Catalase was purchased from Boehringer & Soehne G.m.b.H., Mannheim, Germany (Kat. I). The preparation had a Kat. F (Katalase Fähigkeit) = 80 000 and the ratio $D_{276\text{ m}\mu} / D_{404\text{ m}\mu}$ was found to be 1.14.

³⁵S-cystine (C³⁵S³⁵SC) was obtained from the Radiochemical Centre, Amersham, England.

Hydrogen peroxide as well as the other reagents used were of highest analytical grade. The water used was glass distilled and de-ionized by passage through a mixed bed ion exchange column.

Procedure. Catalase was incubated with ³⁵S-cystine in M/15 phosphate buffer, pH 7.4, containing 0.02 M disodium ethylenediaminetetraacetate (EDTA). The buffer was made from water boiled under a stream of nitrogen and the incubation was carried out in a nitrogen atmosphere. After incubation the labelled components were separated by electrophoresis at pH 2 in paper pretreated with mercuric acetate ⁸. The radioactivity on the strips was located and measured by a strip counter. Three radioactive peaks were obtained corresponding to the location of catalase, cysteine (C³⁵SH), and cystine, respectively. In some experiments the incubation mixture was extensively dialyzed, and the number of labeled residues bound to the catalase was determined by direct plating of the solution and measurement of the radioactivity in a flow-counter. Paper electrophoresis and strip counting established that after dialysis all remaining radioactivity was bound to the catalase.

In the inhibition experiments the solutions were in equilibrium with air. The thiols were brought in solution immediately before they were added to the enzyme solution. The details of the experimental conditions are given below.

The catalase activity was determined at 0°C according to the rapid titration technique of Bonnichsen *et al.* ⁹. The catalase stock solution (Kat. I) was diluted to the desired concentration with M/15 phosphate buffer, pH 7.0, and the enzyme concentration was determined spectrophotometrically using the molar absorption coefficient $E_{404} = 6.36 \times 10^5$ (Tauber and Petit ⁷).

The SH-determinations were carried out in M/15 phosphate buffer at pH 7.0, according to the method of Boyer ¹⁰. Because of the high absorption of catalase at 255 m μ , catalase was used in the blank solution.]

The spectrophotometric measurements were carried out in a Zeiss spectrophotometer.

RESULTS AND DISCUSSION

The SH-groups of catalase. The existence of reactive SH-groups in catalase does not seem to have been demonstrated until it was found by Pihl and Eldjarn ⁵ that catalase interacts with cystamine to form mixed disulphides. Later the presence of SH-groups in catalase has been confirmed by Schütte and Nürnberger ¹¹. These authors reported that beef liver catalase contains 6

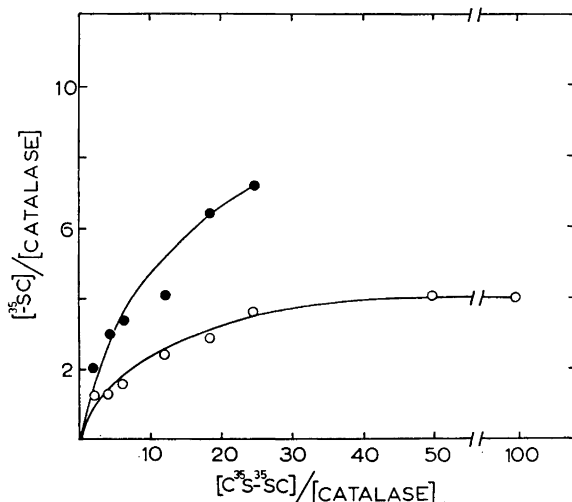


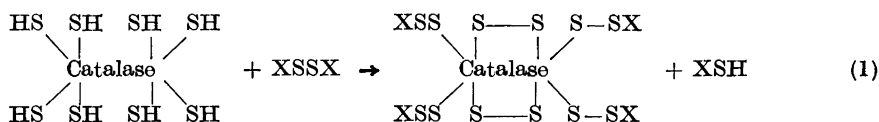
Fig. 1. Binding of cysteine radioactivity to catalase and the concomitant liberation of cysteine as a function of the initial molar ratio of cysteine to catalase. Catalase was incubated in nitrogen atmosphere at 37°C and with ³⁵S-cystine in M/15 phosphate buffer, pH 7.4, containing 1×10^{-2} M EDTA. After 60 min incubation the reaction products were separated by paper-electrophoresis at pH 2, and the radioactivity was measured in a strip-counter.

○—○ Catalase bound ³⁵S; ●—● ³⁵S cysteine liberated.

SH-groups titratable with *p*-chloromercuribenzoate (pCMB). Upon denaturation with 8 M guanidine hydrochloride, 4 additional SH-groups became titratable.

When catalase was incubated with increasing concentrations of ³⁵S-cystine, increasing amounts of radioactivity were bound to the enzyme (Fig. 1). The maximum radioactivity bound corresponded to approximately 4 thiol residues. It can be seen that the radioactivity of the cysteine liberated was approximately twice the radioactivity bound to the protein. The data are in excellent agreement with those previously obtained with labeled cystamine⁵.

On the basis of the previous cystamine data it was suggested⁸ that catalase possesses a total of 8 reactive SH-groups, 4 of which may form stable mixed disulphides, whereas 4 are arranged in pairs with the consequence that the mixed disulphides initially formed react subsequently with adjacent protein SH-groups to form disulphide groups as depicted below:



Quantitative evidence supporting this reaction scheme has been obtained here in experiments where the binding of cysteine residues to catalase was

Recently Keilin and Nicholls⁶ in a preliminary note reported that catalase in the presence of cysteine undergoing auto-oxidation changes its characteristic absorption spectrum to that of catalase hydrogen peroxide complex II, and they concluded that the inhibitory effect of cysteine on the catalytic destruction of hydrogen peroxide may be due to the formation of this inactive complex. They also suggested that the increase in the rate of the auto-oxidation of thiol compounds in the presence of catalase is brought about not by catalase itself but by non-haematin iron and possibly some degradation products of haematin present in catalase preparations.

Since the paper of Keilin and Nicholls was not supported by experimental data, experiments have been carried out to study whether or not the inhibition of catalase by thiols can be accounted for by complex II formation. In Fig. 2 the changes in the absorption spectrum occurring when catalase was incubated with a thousand fold molar excess of cysteamine, is shown. The spectrum shows features characteristic of the catalase peroxide complex II¹⁵. Thus, the absorption at the Soret band at 405 $m\mu$ is decreased, while the absorption at 435 $m\mu$ is increased. The absorption band of catalase at 625 $m\mu$ disappeared and an absorption maximum typical of catalase hydrogen peroxide complex II appears at 568 $m\mu$.

At 435 $m\mu$ catalase and catalase complex I have an isosbestic point¹⁵. The absorption at this wave-length can therefore be taken as a measure of the amount of catalase complex II formed. In Table 2 data are given on the enzyme inhibition and the concurrent increase in optical density at 435 $m\mu$ when

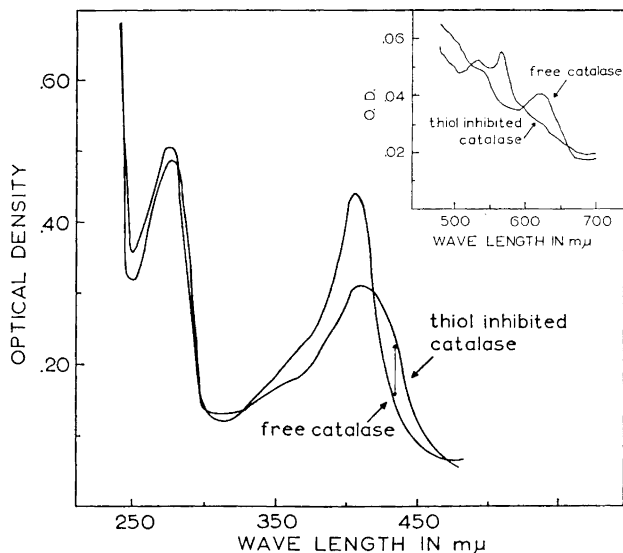


Fig. 2. The spectra of free catalase and thiol inhibited catalase. Catalase (6.92×10^{-7} M) was incubated for 1 h in M/15 phosphate buffer, pH 7.0, at 23°C with a thousand fold molar excess of cysteamine. The spectrum of the thiol inhibited catalase was recorded when the enzyme inhibition was maximal (70 per cent).

Table 2. The inhibition of catalase by various thiols and the concomitant increment in optical density at 435 m μ . Catalase (7×10^{-7} M) was incubated at 20°C in M/15 phosphate buffer, pH 7.0, with the thiols. The solutions were in equilibrium with air.

Conditions		Incubation time in min.	A = inhibition in per cent	B = $\Delta D_{435 \text{ m}\mu}$	$\frac{A}{B} \times 10^2$
	(Thiol) (Catalase)				
Catalase + cysteine	10^2	21	36	0.037	9.7
	10^2	41	32	0.036	8.9
	10^2	380	4.5	0.006	7.5
Catalase + cysteine	10^3	5	0	0	—
	10^3	20	12	0.021	5.7
	10^3	25	18	0.024	7.5
	10^3	30	21	0.028	7.5
	10^3	40	34	0.043	5.9
	10^3	50	39	0.045	6.5
	10^3	60	41	0.060	6.8
	10^3	70	49	0.064	7.7
Catalase + cysteamine	2.5×10^2	25	7.5	0.009	8.3
	2.5×10^2	35	10	0.012	8.3
	2.5×10^2	45	14	0.016	8.7
	2.5×10^2	60	16.5	0.019	8.7
	2.5×10^2	90	21	0.028	7.5
	2.5×10^2	180	13	0.022	5.9
Catalase + cysteamine	10^3	25	13	0.015	8.6
	10^3	35	19	0.022	8.6
	10^3	45	22	0.030	7.4
	10^3	60	23	0.027	8.5
	10^3	70	34	0.049	7.0
	10^3	110	60	0.078	7.7
Catalase + penicillamine	10^3	13	21	0.030	7.0
	10^3	39	48	0.069	7.0
	10^3	170	73	0.101	7.2

catalase was incubated for different lengths of time with various thiols. It should be noted that the inhibition was rather variable from experiment to experiment. However, the significant fact is that under experimental conditions giving enzyme inhibition varying from 4.5 to 73 %, the extent of inhibition was, within experimental error, proportional to the amount of complex II formed. These data provide evidence that the catalase inhibition by thiols can be quantitatively accounted for by catalase complex II formation. The fact that the inhibition data were variable and somewhat erratic is consistent with the view⁶ that a spontaneous oxidation is an essential step in the inhibition mechanism inasmuch as such processes are notoriously difficult to reproduce¹⁶.

It can be seen from Table 2 that when a hundred fold molar excess of cysteine was used, the inhibition of catalase was spontaneously reversed. With a greater excess of cysteine (thousand fold) the inhibition was not reversed in the course of the observation time when the reaction was carried out in phosphate

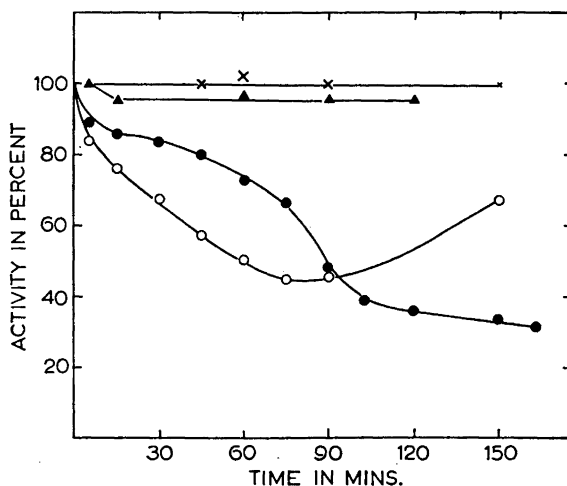


Fig. 3. The reversible inhibition of catalase by cysteine. The effect of EDTA and of ethanol. Catalase (7.0×10^{-7} M) was incubated at pH 7.0 with cysteine (7×10^{-4} M) at 20°C.

- Tris buffer (M/20).
- Phosphate buffer (M/15).
- ×—× » » » containing EDTA (1×10^{-2} M).
- ▲—▲ » » » » ethanol (7×10^{-4} M).

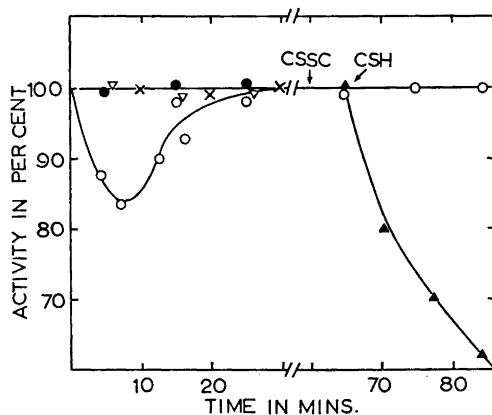
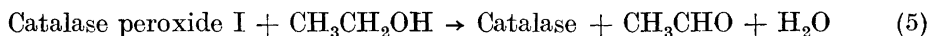


Fig. 4. Inhibition of catalase by cystine (CSSC). Catalase (7.4×10^{-7} M) was incubated at 37°C in M/15 phosphate buffer, pH 7.0, with cystine (3.7×10^{-5} M).

- An additional amount of CSSC (3.7×10^{-5} M) added at the time indicated by the arrow.
- ▲—▲ CSH (7×10^{-4} M) added at the time indicated.
- Catalase pretreated with 10 equiv. of pCMB.
- ×—× EDTA (1×10^{-2} M) present.
- △—△ Ethanol (7×10^{-4} M) present.

buffer, but the reversibility was readily demonstrated when tris-(hydroxymethyl)aminomethane (Tris) buffer was used (Fig. 3). The data are clearly consistent with the inhibition mechanism represented in eqns. 2–4. Appreciable amounts of complex II are only formed and maintained when H_2O_2 is continuously generated¹⁴, and if the H_2O_2 production ceases, reaction 4 is reversed. The spontaneous reversal of the inhibition may therefore be ascribed to the eventual exhaustion of the supply of thiols in solution with consequent decline in the H_2O_2 production. The finding that the inhibition of catalase by cysteine is completely abolished in the presence of EDTA (Fig. 3) supports the above reaction mechanism as EDTA blocks the oxidation of thiols in the absence as well as in the presence⁶ of catalase. The fact that the cysteine inhibition of catalase is virtually abolished in the presence of ethanol (Fig. 3) in agreement with an early observation of Waldschmidt-Leitz *et al.*², is readily explained in terms of the above mechanism, as catalase complex I reacts with ethanol according to the following equation¹⁴:



Accordingly, in the presence of ethanol the steady state concentration of catalase complex I will be kept low and the formation of the inactive complex II will be negligible.

The inhibition of catalase by cystine. The fact that cystine interacts with catalase SH-groups with liberation of cysteine opened the possibility that the inhibition of catalase by cystine might be due to the sequence of reactions 1 to 4. In that event, the cystine inhibition would be expected to be reversible, and it should be influenced by the same factors which influence the thiol inhibition. The data in Fig. 4 demonstrate that the inhibition of catalase by cystine is indeed reversible, and that it is abolished by EDTA and by ethanol. These findings seem to provide strong evidence in support of the mechanism proposed. It was therefore highly unexpected that the cystine inhibition could not be shown to be associated with an increase in the optical density at 435 $m\mu$ (Table 3). However, when the catalase was preincubated for 5 min with a hundred-

Table 3. The inhibition of catalase by cystine. Catalase (7×10^{-7} M) was incubated at 20°C with cystine (7×10^{-5} M) in M/15 phosphate buffer, pH 7.0.

Conditions	Incubation time in min.	A = inhibition in per cent	B = $\Delta D_{435 \text{ m}\mu}$	$\frac{A}{B} \times 10^2$
Catalase + cystine	6	11	0	—
	16	8	0	—
	25	1	0	—
Catalase } * + hydrogen } + cystine peroxide }	20	12	0.021	6.3
	25	15	0.019	8.3
	30	11	0.017	6.5

* The catalase was preincubated for 5 min with a 100 fold excess of hydrogen peroxide.

red fold excess of hydrogen peroxide prior to the addition of cystine, the expected optical density increase at 435 m μ did occur. It should be clear that preincubation with hydrogen peroxide without subsequent addition of cystine gave no enzyme inhibition and no optical density change at this wavelength. No adequate explanation can be offered for the effect of the preincubation, but the fact that the optical density increase showed the same quantitative relationship to the inhibition as in the experiments with cysteine, is hardly fortuitous.

Further evidence in support of the suggested mechanism was obtained in experiments where the effect of blocking the catalase SH-groups was tested. When catalase was pretreated with 10 equivalents of pCMB, no cystine inhibition was observed (Fig. 4). In agreement with this fact it was found that when catalase had been reversibly inhibited by incubation with excess cystine, a procedure which presumably removed all free protein SH-groups (Table 1), the addition of a second portion of cystine gave no inhibition. On the other hand, the addition of cysteine resulted in a prompt inhibition, as expected. Blocking of the enzyme SH-groups had no effect on the enzyme activity in the absence of cystine. The above data establish that the SH-groups of catalase play an essential role in the inhibition of catalase by cystine.

Altogether the results presented in this paper provide strong evidence that the inhibition of catalase by cystine is caused by cysteine liberated in the interaction of the disulphide with the enzyme SH-groups.

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