INTRODUCTION OF MERCURY INTO THE ACTIVE SITE OF CHYMOTRYPSIN

p-CHLOROMERCURIBENZENESULFONYL-CHYMOTRYPSIN[1]

D. Rizok and J. Kallos

Research Laboratory, Department of Pathology,
St. Mary's Memorial Hospital, Montreal, Canada.

Received December 21, 1964

X-ray diffraction studies of protein structure (Kendrew, 1963) have been facilitated by the preparation of various isomorphous crystalline derivatives of a particular protein containing heavy atoms at specific sites in the crystalline protein. Recently, we have described the introduction of an iodine atom at the active site of CHT[2] using pipsyl chloride or fluoride as an irreversible inhibitor (Kallos and Rizok, 1964). These isomorphous derivatives have proven useful for the location of the active site crystallographically (Sigler et al. 1964). However, to solve the phase problem for three-dimensional structural studies, attachment of a heavy atom such as mercury was required. We wish to describe here the introduction of a single mercury atom into the active site of CHT using CMBF as inhibitor[3] (Fig. 1).

This communication reports (1) the synthesis of the mercury containing inhibitor - CMBF, (2) the preparation of the mercury labeled

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[1]Paper VI, in the series: Studies of the Active Site of Proteolytic Enzymes.

[2]Abbreviations used: CHT, chymotrypsin (experiments carried out using both alpha and gamma CHT, Worthington); pipsyl, p-iodobenzenesulfonyl; CMBF, p-chloromercuribenzenesulfonyl fluoride; CMB-CHT, p-chloromercuribenzene-sulfonyl-chymotrypsin.

[3]While this work was in progress D. Blow and A. Jeffery from Cambridge, E. Surbeck and P. E. Wilcox (1964) from Seattle, and J. Kraut from La Jola (personal communications from D. Davies, P. Sigler and D. Blow) performed similar studies using the mercury containing inhibitor.

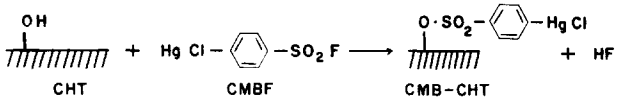

Figure 1. Schematic representation of the reaction of CHT with CMBF.

CHT (CMB - CHT), (3) establishment of the 1:1 stoichiometry of the reaction and (4) chemical evidence that this sulfonylation reaction occurs at the serine residue of the active site of CHT.

The CMBF inhibitor was synthetized in the following manner: the p-chloromercuribenzenesulfonic acid was converted to the corresponding sulfonyl chloride (m.p. 156-158° C; I.R. in KBr 1570, 1480, 1385, 1360, 1085, 1070, 1020 cm^{-1}) by refluxing with triethylamine phosphorous pentachloride and phosphorous oxychloride. The chloride was then transformed to the fluoride by treatment with potassium fluoride. The resulting p-chloromercuribenzenesulfonyl fluoride was recrystallized from toluene (M.P. 260-262° C; I.R. in KBr 1570, 1410, 1395, 1100, 1065, 1020 cm^{-1}); Anal: calcd. C: 18.25; H. 1.01; 8.11; Hg: 50.84; Found: C: 18.15; H: 1.22; S: 7.9; Hg: 51.07).

The inhibition of CHT with CMBF was effected as follows: CHT (50 μmoles) dissolved in phosphate buffer at pH 7.8 at 0° C, was treated with two portions of CMBF (50 μmoles) in ice-cold acetonitrile added successively at 30 minute intervals. The final acetonitrile concentration in the reaction mixture was 15%. The enzyme solution was extensively dialysed at 4° C against 10^{-4} M HCl. The CMB-CHT was isolated by freeze-drying and the residual activity of the lyophilized enzyme was found to be less than 4% as compared to a control, when assayed by the hydrazide method (Niemann and Shine 1955).

Crystalline CMB-CHT was prepared in the solid phase by a procedure similar to the one previously described for solid phase inhibition (Sigler and Skinner 1963, and Kallos, 1964). Crystals of alpha-

CHT were suspended in 80% saturated $(NH_4)_2SO_4$ at pH 6.0 and treated with CMBF for two days, followed by washing with acidic 80% saturated $(NH_4)_2SO_4$. Activity of the crystals was measured by dissolving the crystals in dilute tris buffer. Residual activity was found to be less than 15%.

The 1:1 stoichiometry of the reaction was illustrated by the mercury analysis of the isolated CMB-CHT. Analysis showed the incorporation of one mole of mercury per mole of CHT. (Calcd. for CMB-CHT: mol. Wt. 24,000. Hg: 0.83%; Found Hg: 0.91%). Furthermore, cinnamic acid, a known competive inhibitor of CHT (Neurath and Schwart 1950), protected the enzyme from CMBF inactivation, as shown in Table 1.

TABLE 1

Protection of CHT against CMBF inactivation with Cinnamic Acid

	% Enzymic Activity
CHT + cinnamic acid + CMBF	80
CHT + cinnamic acid	98
CHT + CMBF	4

Chymotrypsin (1 μmole) was incubated with cinnamic acid (10^3 μmoles) in phosphate buffer at pH 7.8. This was further treated with CMBF (total 2 μmoles) under the experimental conditions described in the text.

That the mercury containing inhibitor is attached to the active serine residue was shown by transforming the CMB-CHT through an anhydro-CHT intermediate followed by the transformation of the serine into pyruvic acid by the procedure previously described. (Kallos & Rizok, 1964; Patchornik, 1964.) Pyruvic acid was obtained in a 55% yield as compared to a CHT control. As previously discussed (Kallos & Rizok, 1964), pyruvic acid could only be generated from a modified

serine and therefore its isolation constitutes direct chemical evidence that the reaction with CMBF occurred at the serine residue of the active site of CHT.

The above findings strongly suggest that this mercury containing inhibitor is covalently linked to the serine residue at the active site of CHT.

The fact that the stoichiometric interaction of CMBF with CHT results in the irreversible loss of enzymic activity strongly suggests that the mercury containing inhibitor is covalently linked to the serine residue in the active site of CHT.

The authors wish to thank Drs. P. B. Sigler and D. Davies of the National Institutes of Health, Bethesda, Maryland, and Dr. D. Blow of the Laboratory of Molecular Biology, MRC, Cambridge, England, for the stimulating discussions and exchange of information in connection with this project. We also wish to thank Mrs. K. Avatis for her assistance with some of the early experiments.

This study was supported in part by a grant from the Medical Research Council of Canada.

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