Protection of Chymotrypsin from Inactivation by a Nitrogen Mustard Antitumor Drug by Prior Acylation of the Active Center (35706)

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Chlorambucil {*p*[bis(2-chloroethyl)amino]phenylbutyric acid} (CAB) is a nitrogen mustard derivative which is in current clincal use in the treatment of certain malignancies (1–3). CAB has been reported to react with DNA (4) and with proteins (5–7). Among the proteins it affects is α-chymotrypsin (XT) whose esterolytic activity is inhibited upon preincubation with CAB (8). Since CAB reacts with such functional groups as carboxyl, amino, imidazole, and thiol (9), it could not be categorically stated whether CAB inhibits chymotrypsin by reacting at the active site of the enzyme or at sites other than the active site, thereby causing an alteration in the conformation of the protein with a concomitant loss in enzymic activity. An opportunity to resolve this question presented itself by the use of the unique property of *p*-nitrophenyl trimethyl acetate (TMAc-pNP), a substrate which binds at the active site of XT in a 1:1 combination to form a complex, TMAc-XT, which is remarkably stable at neutral pH values and is reactivated by hydroxylamine (10).

This communication suggests that CAB binds and inactivates XT at the active center in view of the protection from inactivation by CAB afforded by TMAc-XT.

Materials and Methods. TMAc-XT was prepared according to the method of Balls *et al.* (10). To 1 ml each of (1) α-XT (1 mg/ml in 1 m*M* HCl), (2) TMAc-XT (1 mg/ml in water), and (3) TMAc-XT (2 mg/ml in water) was added 0.8 ml of 0.1 *M* $NaHCO_3$. CAB, dissolved in 95% ethanol, was added to the enzyme solutions to a final ethanol concentration of 10% and a final CAB concentration of 5.0×10^{-3} *M* for solutions 1 and 2, and a final CAB concentration of 1.0×10^{-2} *M* for solution 3. These solutions were preincubated at room temperature for 30, 60, and 90 min, at which time aliquots of solutions 1 and 2 were taken for activity measurements with 3.5 ml of 3.87×10^{-4} *M* glutaryl-L-phenylalanine-*p*-nitroanilide in a solution of

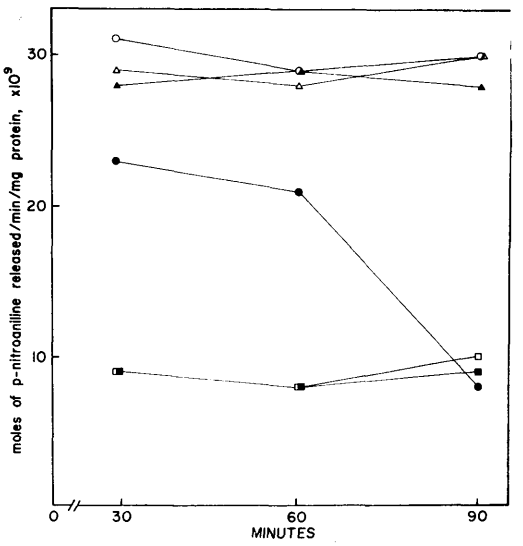

FIG. 1. Reaction of chlorambucil with trimethylacetyl-chymotrypsin. Chymotrypsin, chlorambucil-treated chymotrypsin, and trimethylacetylchymotrypsin were tested for enzymic activity with glutaryl-L-phenylalanine-*p*-nitroanilide. Trimethylacetylchymotrypsin was also tested for activity upon removal of the acyl moiety with hydroxylamine at pH 7.0 (10) before and after treatment with chlorambucil. Aliquots were taken for activity measurements at the indicated times. See text for details. ○, α-chymotrypsin; ●, α-chymotrypsin in 5.0×10^{-3} *M* chlorambucil; □, trimethylacetylchymotrypsin; ■, trimethylacetylchymotrypsin treated with 5×10^{-3} *M* chlorambucil; △, trimethylacetylchymotrypsin, activated with hydroxylamine; ▲, trimethylacetylchymotrypsin, treated with 10^{-2} *M* chlorambucil and subsequently activated with hydroxylamine.

10% DMSO and 4.2×10^{-2} *M* sodium barbital buffer, pH 8.0, containing 5.0×10^{-3} *M* $CaCl_2$. Aliquots of 0.5 ml of solution 3 were added to equal volumes of 2 *M* hydroxylamine, pH 7.0. The latter solution was permitted to stand at room temperature for 15 min to insure complete reactivation of the enzyme. Aliquots of 0.2 ml were than taken for activity measurements as described above. The release of *p*-nitroaniline with time was followed spectrophotometrically at 410 mμ. Control enzymes with ethanol substituting for ethanolic CAB were treated in an analogous manner.

Results and Discussion. In the course of a 90-min preincubation period with CAB, XT loses 73% of its enzymic activity (Fig. 1). Some 70% of the enzymic activity is inactivated in the formation of TMAc-XT. Upon reactivation of the TMAc-XT with hydroxylamine at pH 7.0 with the subsequent removal of the acyl group, approximately 95% of the activity is recovered. TMAc-XT, treated with CAB, also exhibits 30% of its original activity. However, the latter enzyme preparation, after treatment with CAB and subsequent exposure to hydroxylamine to deacylate the enzyme at the active site, presents 90–97% of its original activity. Significantly, CAB-inactivated XT is not reactivated upon treatment with hydroxylamine (Table I).

The recovery of full hydrolytic activity from deacylation of TMAc-XT which has been treated with CAB indicates that reactions of CAB with acylated protein at sites other than the active site are not reactions of consequence in that major conformation changes leading to an alteration in conformation about the active site of the enzyme do not occur. On the other hand, reaction of XT with CAB inactivates the enzyme. Since the difference between XT and TMAc-XT each treated with CAB and then hydroxylamine, is the alkylation of sterically available sites on the protein molecule other than the active site, and since both preparations retain full activity, it is concluded that the inactivation of XT by CAB occurs by reaction with the enzyme at its active center, and not at a possible allosteric or nonspecific site.

The active center of XT is known to include Ser-195 and His-57 (11). It is not likely that the alkylating agent reacts with the serine residue. It is more likely that CAB reacts with the imidazole group of His-57 or, conceivably, the β-carboxyl of Asp-194 which is adjacent to Ser-195 which has been implicated in the active site (11), provided that the carboxyl group is in a sterically available position. Reaction of CAB with Met-192 also cannot be ruled out as a primary reaction site causing inactivation since the sulfur atom of Met-192 is susceptible to electrophilic attack (12).

Summary. The inactivation of α-chymotrypsin by chlorambucil has been blocked by prior acylation of the active site of the enzyme with *p*-nitrophenyl trimethylacetate. Trimethylacetyl-chymotrypsin which has been deacylated with hydroxylamine at pH 7.0 after exposure to chlorambucil exhibits full enzymic activity in contrast to the loss in activity shown by chlorambucil-treated chymotrypsin. These data suggest that inactiva-

TABLE I. Response of Chlorambucil-Treated Chymotrypsin to Hydroxylamine.

	Moles of *p*-nitroaniline released/min/ml ($\times 10^6$)		Percentage of control activity	
	Preincubation time[a]		Preincubation time[a]	
Addition to α-chymotrypsin	90 min	140 min	90 min	140 min
None	38.9	37.2	100	100
Chlorambucil	12.4	7.0	31.6	18.4
Chlorambucil, followed by hydroxylamine	9.3	4.0	24.1	10.5

[a] After the indicated preincubating times, the enzymes were tested for activity as described in the text.

tion of the enzyme occurs by reaction of the antitumor drug at the active site of the enzyme rather than at a nonspecific or allosteric site.

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