Protection Afforded to Trypsin and Trypsinogen by Calcium Ion from Inactivation by Chlorambucil (37172)

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The biological alkylating agent, chlorambucil, (*p*[bis(2-chloroethyl)amino]phenylbutyric acid) (CAB) is a nitrogen mustard derivative in current clinical use as an antitumor drug (1). This bifunctional reagent reacts with proteins by alkylation of carboxyl and other groups (2) and also binds to proteins without alkylation (3). CAB has been observed to inhibit the enzymic activity of chymotrypsin (4, 5), while having only a slight effect on the related protease, trypsin (6). CAB also accelerates the activation of chymotrypsinogen by trypsin, although upon prolonged incubation of the zymogen with the drug, complete activation of the zymogen is not attained (6).

Alkylation of trypsinogen by glycinamide results in random modification of the β-carboxyl groups of aspartic acid located in the Val-$(Asp)_4$-Lys hexapeptide which is cleaved upon activation by trypsin (7). Upon such modification of the trypsinogen molecule, an accelerated albeit incomplete activation curve is obtained (7) which is analogous to that reported for the CAB-modified chymotrypsinogen (6).

In view of the marked similarity in structure between trypsin and chymotrypsin and their zymogens, a reexamination of the effect of CAB on trypsin and its effect on trypsinogen was suggested. Owing to the known relationship between calcium ions and the activity of the two enzymes, the possible effect of calcium ions on the inhibition was also investigated.

Materials and Methods. Assays of tryptic activity were performed essentially according to the procedure of Hummel (8) using *p*-toluene-sulfonyl-L-arginine methyl ester (TAMe) as a substrate and measuring the decrease in absorbancy with time at 247 nm in a Turner model 330 spectrophotometer equipped with a Sargent model SLRG recorder attachment. To 2.6 ml of 0.05 *M* Tris–HCl buffer (pH 8.0), containing 12.5 m*M* $CaCl_2$ (to achieve maximum stability of enzyme) were added 0.4 ml of 10 m*M* TAMe and 10 μl of enzyme containing 2.5–2.6 μg of trypsin. Assays were run at room temperature.

Effect of calcium ion on the inactivation of trypsin by chlorambucil. One-tenth milliliter of trypsin (1 mg/ml in 1 m*N* HCl) was added to a mixture of 0.26 ml of 0.05 *M* Tris buffer (pH 8.0), in the presence and absence of 12.5 m*M* Ca^{2+}, and 0.04 ml of ethanol containing 2 μmoles of CAB. The mixture was preincubated at 37° in a shaking water bath and 10 μl aliquots were taken periodically for assay. Control trypsin was similarly run using ethanol in the absence of CAB.

Effect of calcium ion on the inactivation of potential trypsinogen activity by chlorambucil. One-tenth milliliter of trypsinogen (1 mg/ml in 1 m*N* HCl) was added to a mixture of 0.26 ml of 0.05 *M* Tris buffer (pH 8.0), in the presence and absence of 12.5 m*M* $CaCl_2$, and 0.04 ml of ethanol containing 2 μmoles of CAB. The mixture was preincubated at 37° for 2 hr after which time the level of Ca^{2+} in all samples was adjusted to 12.5 m*M* to obtain maximum activation and stability. Five micrograms of trypsin were added to the appropriate zymogen solutions and activation was allowed to proceed at room temperature. Aliquots of 10 μl were taken periodically for assay over a 13 hr time course.

Trypsin, 2× crystalline, and trypsinogen, 1× crystalline, were obtained from Worthington Biochemical Corporation, Freehold, NJ. TAMe was purchased from Mann Re-

search Laboratories, Inc., New York, NY. Other chemicals employed were reagent grade.

Results. CAB (5×10^{-3} *M*) produces slight inhibition of tryptic activity when the enzyme and alkylating agent are preincubated in the presence of 12.5 m*M* Ca^{2+} for 60 min at 37° (Fig. 1). Trypsin, alone, is quite stable in the presence of Ca^{2+}. Although enzymic activity declines rapidly in the absence of calcium ion, there is an appreciably accelerated loss in esterolytic activity upon preincubation with CAB. After 60 min in the absence of Ca^{2+}, trypsin retains 22% of its activity while CAB-treated trypsin exhibits virtually no activity.

When trypsinogen is preincubated for 2 hr with 5×10^{-3} *M* CAB in the absence of Ca^{2+}, activation of the zymogen is almost completely inhibited under conditions of maximal activation by trypsin in the presence of Ca^{2+} (Fig. 2). After similar preincubation of the zymogen–CAB mixture in the presence of Ca^{2+}, more than 90% of the potential activity is retained. Furthermore, CAB-treated trypsinogen is activated to trypsin at an accelerated rate as compared to control trypsinogen when both zymogen preparations have been treated with modifier and trypsin in the presence of Ca^{2+}.

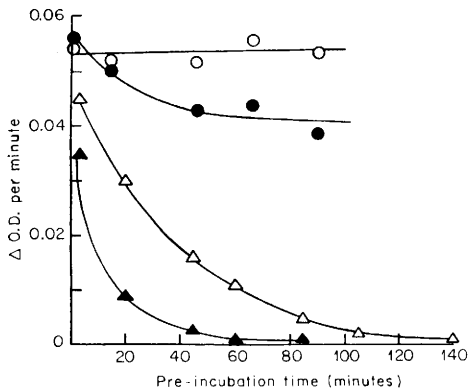

FIG. 1. Effect of calcium ion on the inactivation of trypsin by chlorambucil: (○) trypsin preincubated with 12.5 m*M* Ca^{2+}; (●) trypsin preincubated with 5×10^{-3} *M* chlorambucil in the presence of 12.5 m*M* Ca^{2+}; (△) trypsin preincubated without Ca^{2+}; (▲) trypsin preincubated with 5×10^{-3} *M* chlorambucil. Reaction mixtures were preincubated in 0.05 *M* Tris–HCl buffer (pH 8.0) at 37°. Samples were withdrawn periodically for assay as described in the text.

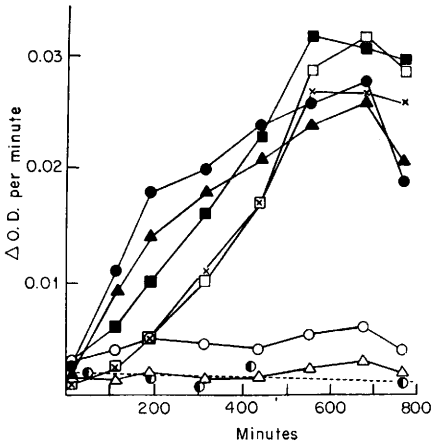

FIG. 2. Effect of calcium on the inactivation of potential trypsinogen activity by chlorambucil. (●) trypsinogen preincubated with 5×10^{-3} *M* chlorambucil in the presence of 12.5 m*M* Ca^{2+}, and then activated with 5 μg trypsin; (○) trypsinogen preincubated with 5×10^{-3} *M* chlorambucil in the absence of Ca^{2+}, and then activated with 5 μg trypsin; (▲) trypsinogen preincubated with 5×10^{-3} *M* chlorambucil in the presence of 12.5 m*M* Ca^{2+} (no activation by added trypsin); (△) trypsinogen preincubated with 5×10^{-3} *M* chlorambucil in the absence of Ca^{2+} (no activation by added trypsin); (■) trypsinogen preincubated with 12.5 m*M* Ca^{2+}, and then activated with 5 μg trypsin; (□) trypsinogen preincubated with 12.5 m*M* Ca^{2+} (no trypsin added); (×) trypsinogen preincubated in the absence of Ca^{2+} (no trypsin added); (◒) 5 μg trypsin in the presence of 12.5 m*M* Ca^{2+}. All reaction mixtures were preincubated in 0.05 *M* Tris–HCl buffer (pH 8.0) at 37°. After preincubation, noncalcium-containing mixtures were adjusted to 12.5 m*M* Ca^{2+}. Trypsin was then added, if required. Activation was allowed to proceed at room temperature and aliquots were taken periodically for assay of trypsin activity.

Discussion. The ability of Ca^{2+} to protect both trypsin and trypsinogen from inactivation by CAB suggests that the inhibiting effect of the drug is due, at least in part, to its interaction with carboxyl groups in the proteins. The effect of Ca^{2+} on trypsin and trypsinogen has been well documented. Ca^{2+} is known to retard trypsin autolysis (9) and to promote the activation of trypsinogen (10). The action of Ca^{2+} on these proteins is believed to be due to the formation of a protein–calcium complex. Two Ca^{2+} binding

sites have been proposed (11). The primary site is common to both trypsin and trypsinogen. The binding of Ca^{2+} to this site results in a conformational change to a more compact structure. The second and less specific site, found only in the trypsinogen molecule, is believed to be on the group of four aspartyl residues on the peptide released during zymogen activation. Sipos and Merkel (12) have proposed that the Ca^{2+} binding to the primary site produces the conformational change by reducing the polar interactions between the charged residues of the molecule. Radhakrishnan, Walsh and Neurath (7) and Delaage and Lazdunski (11) strongly suggest that it is the secondary binding of Ca^{2+} to the group of carboxylates which directs trypsin to the lysine^6–isoleucine^7 bond which is cleaved in the process of trypsinogen activation. The fact that both trypsin and trypsinogen are protected from CAB inactivation by Ca^{2+} suggests that it is the primary site carboxyl groups of the enzyme and the zymogen which are involved in the protection mechanism, and not the secondary tetraaspartyl carboxylate site. Ca^{2+} is more tightly bound to the primary site than to the secondary site (11). Hence, CAB interaction with the primary site may be specifically blocked by Ca^{2+}. Lower binding capacity of Ca^{2+} for the secondary site may account for a proposed higher susceptibility of the secondary site to esterification by CAB. Indeed, alkylation of this site by glycinamide results in an accelerated activation of the zymogen by trypsin without a significantly altered activity. Trypsinogen preincubated with CAB in the presence of Ca^{2+} similarly responds to trypsin by accelerated activation of the zymogen (Fig. 2) to essentially full activity in an identical pattern to the glycinamide studies of Radhakrishnan, Walsh and Neurath (7).

Hence, Ca^{2+} may function not only in the stabilization of the physiologically active trypsin molecule and in the promotion of the activation of trypsinogen to trypsin rather than conversion to inert protein, but in the protection of the enzyme and zymogen from modification by physiologically alien compounds capable of reacting with susceptible carboxy groups.

Summary. Treatment of trypsin with chlorambucil in the absence of calcium ion results in an enzyme incapable of hydrolyzing *p*-toluene-sulfonyl-L-arginine methyl ester. Similar treatment of trypsinogen destroys potential tryptic activity. Calcium ion affects a protective influence on the enzyme and zymogen in the presence of chlorambucil by maintaining the enzymic activity of trypsin, and by preserving much of the potential activity of the zymogen. It is suggested that chlorambucil may affect physiological activity by reacting, in part, with the primary carboxyl binding site in trypsin and trypsinogen.

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