The Protection of Alpha-Chymotrypsin by Hydrocinnamate from Denaturation by Ethanol and Urea (33716)

FELIX FRIEDBERG, J. E. LONG, AND A. S. BRECHER

Department of Biochemistry, Howard University, Medical School, Washington, D. C.; and Department of Biochemistry, The George Washington University School of Medicine, Washington, D. C. 20005

In recent years, several reports have appeared showing that chymotrypsin (CT) can be protected by competitive inhibitors against denaturation. For instance, Hopkins and Spikes (1) showed by measuring rates of denaturation using tryptophan fluorescence that cinnamoyl-chymotrypsin (a covalently bonded enzyme-substrate intermediate produced by reacting CT with cinnamoylimidazole) is more stable in 8 *M* urea than CT. Previously, Martin and Frazer (2) reported that indole-3-propionate, a competitive inhibitor, decreased inactivation of the enzyme by urea. The present study was undertaken to gain information about the protective action of another competitive inhibitor, hydrocinnamic acid (HC) against denaturation of α-CT by urea as well as by ethanol.

Materials and Methods. Determinations of enzyme activity were performed essentially according to the method of Bundy (3). To 2 ml of CT (Worthington Biochemical Corp., 3x crystalline) solution (1 mg/ml in 1 m*M* HCl) was added 0.1 *M* $NaHCO_3$ and varying amounts of 95% ethanol to a final volume of 4 ml. Aliquots of 0.4 ml (200 μg CT) were taken for activity measurements with 3 ml of 3.87×10^{-4} *M* glutaryl-L-phenylalanyl-*p*-nitroanilide solution in 4×10^{-2} *M* Tris buffer, pH 8.0, containing 5×10^{-3} *M* Ca^{2+}. The release of *p*-nitroaniline with time was followed by optical density measurements at 410 mμ.

The HC, dissolved in 95% ethanol, was added to CT solutions to a final ethanol concentration of 10%.

Results and Discussion. Figure 1 shows the effect of various concentrations of ethanol on CT and the protective effect of HC on CT activity at several concentraions of ethanol. Whereas 10% ethanol has essentially no effect on the enzyme activity, 20% ethanol inhibits approximately 50% of the CT activity upon contact with the enzyme for 1 hr. 30% ethanol produces an essentially instantaneous inhibition of CT within 5 min of contact with the enzyme. Sufficiently low concentrations of HC were chosen in order not to inhibit enzymic activity under the test conditions (Fig. 1a). Figure 1b and c shows the protection of the enzyme by HC from

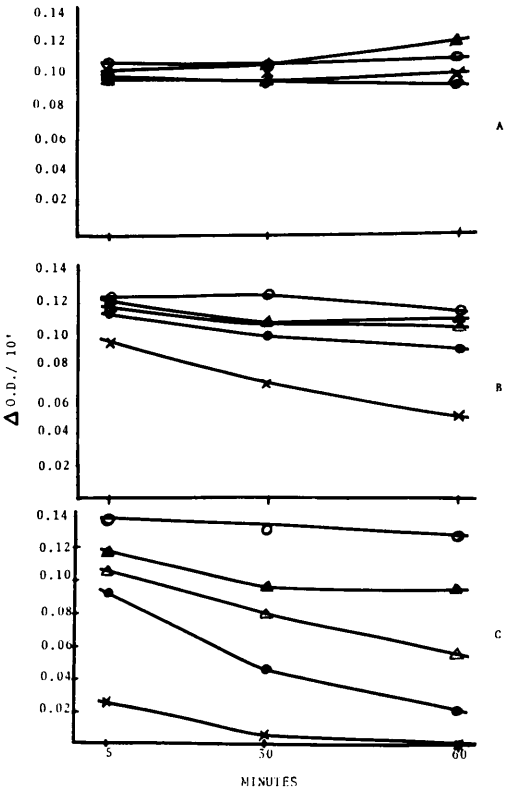

FIG. 1. The protection of chymotryptic activity by hydrocinnamate from inactivation by ethanol: (○) control CT; (X), CT + ethanol; (●), CT + 5×10^{-3}*M* HC; (△), CT + 1×10^{-2}*M* HC; (▲), CT + 2×10^{-2}*M* HC; (A), 10% ethanol; (B), 20% ethanol; (C), 30% ethanol.

denaturation by 20% and 30% ethanol, respectively. The stabilization of CT activity by HC is maintained over the entire period of exposure to alcohol and is dependent upon the concentration of HC which is employed.

The inactivation of CT by 8 *M* urea is rapid and essentially complete (Fig. 2). The HC (2×10^{-2} *M*) allows for retention of some enzymatic activity (39%) during the prolonged exposure to urea. The capacity of HC to protect against the action of urea, is however, less pronounced than that to protect against the action of ethanol. Conformation changes in CT induced by urea and by ethanol may differ markedly, and the ability of HC to stabilize the protein at the active site may be related to the nature and extent of unfolding of CT by the specific denaturing agents.

A protection constant, π, analogous to a Michaelis-Menten constant K_m, has been defined empirically by Burton (4) as that concentration of substrate which provides half-maximal protection against spontaneous inactivation at a given pH and temperature. Instead of substrate, a competitive inhibitor may be used. Since denaturation of CT is instantaneous and complete in the presence of 30% ethanol, π can be calculated from the data on the protection of CT by HC against 30% ethanol as 1.4×10^{-2} at 25° (Fig. 3). This compares with a K_i for HC of 0.6×10^{-2} (at pH 7.8 and 25°) as reported by Neurath and Gladner (5) and with a K_i of

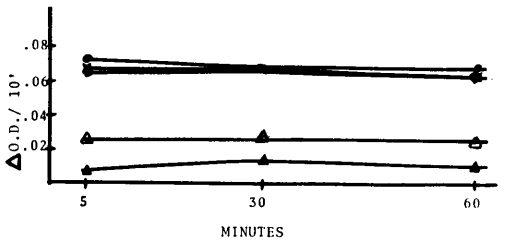

FIG. 2. The effect of hydrocinnamate on the inactivation of chymotrypsin by urea: (●) control CT; (○), CT + 10% ethanol; (X) CT + 2×10^{-2}*M* HC in 10% ethanol; (▲), CT + 8 *M* urea in 10% ethanol; (△), CT + 2×10^{-2}*M* HC + 8 *M* Urea in 10% ethanol.

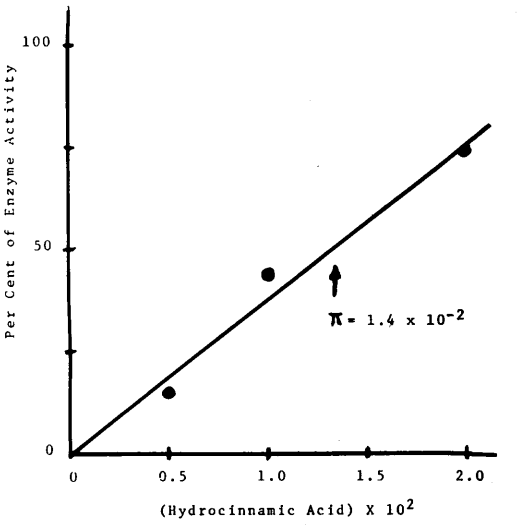

FIG. 3. The protection of chymotryptic activity by hydrocinnamate from inactivation by 30% ethanol.

2.8×10^{-2} (at pH 7.9 and 25°) as reported by Huang and Niemann (6). The differences in K_i may be attributed to differences in buffer species employed, *e.g.*, phosphate buffer (5) and Tris buffer (6), as well as pH and media utilized.

Summary. The protection of α-chymotrypsin from denaturation by ethanol and urea by hydrocinnamic acid is reported. The protection constant, π, for hydrocinnamic acid was determined to be 1.4×10^{-2} in the presence of 30% ethanol.

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