Phenothiazine-*N*-carbonyl Chloride, A Specific Inactivator of Chymotrypsin

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Phenothiazine-*N*-carbonyl chloride inactivated chymotrypsin and trypsin by means of a 1:1 stoichiometric reaction. Its reaction with chymotrypsin was 29 times as fast as that with trypsin and was inhibited by indole. The reaction of phenothiazine-*N*-carbonyl chloride with chymotrypsin resembled an enzyme-substrate reaction in which the deacylation step is rate-limiting. Slow deacylation occurred, resulting in complete regeneration of active enzyme in 15 h. The pH-rate profile of the inactivation process had a maximum at pH 7.8. These data and other evidence indicate that the reaction of phenothiazine-*N*-carbonyl chloride with chymotrypsin exhibits 'kinetic specificity'. Therefore any hypothesis that attempts to describe the topography of the active site of chymotrypsin should take into account the reactivity of phenothiazine-*N*-carbonyl chloride. The above findings, as well as recent reports of others, are examined within the context of a hypothesis given in an earlier paper (Erlanger, 1967).

Among the approaches used to obtain information about the topography of the active centre of an enzyme are studies of its reactions with specific structurally constrained reagents such as inhibitors, inactivators and substrates (Hein, McGriff & Niemann, 1960; Wilson & Erlanger, 1960; Hein & Niemann, 1962*a,b*; Silver, 1966; Cohen & Schultz, 1967; Erlanger, 1966, 1967). This approach is based on the assumption that the geometry of the active centre is complementary to the known conformation of the reagent. Unfortunately, the very property that makes a constrained reagent useful, its inability to assume more than one (or a few) conformation(s), makes it a more difficult reagent to design, since its reactivity with the active centre depends on the agreement of its restricted conformation with the specificity requirements of the enzyme.

During a study of the topography of the active

centre of α -chymotrypsin, it was found that PCC,* a structurally constrained molecule, was an inactivator of the enzyme. This finding was an outgrowth of earlier studies with a specific inactivator of chymotrypsin, DPCC (Erlanger & Cohen, 1963; Erlanger, Castleman & Cooper, 1963; Erlanger, Cooper & Cohen, 1966*a*). Structurally, PCC can be considered to be a conformationally constrained DPCC molecule.

Mention of the reaction of PCC with chymotrypsin and with trypsin has already been made (Erlanger, 1966, 1967; Erlanger, Vratsanos & Wassermann, 1967*b*). In the present paper we present a comprehensive account of the phenomenon and discuss the implications with respect to the geometry of the active site of chymotrypsin.

EXPERIMENTAL

The α -chymotrypsin used was three-times-crystallized (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). Trypsin was freeze-dried crystalline trypsin (Worthington Biochemical Corp.).

Chymotrypsin was assayed by using the substrate *N*-glutaryl-L-phenylalanine *p*-nitroanilide (Erlanger, Edel & Cooper, 1966*b*). α -*N*-Benzoyl-DL-arginine *p*-nitro-

* Abbreviations: PCC, phenothiazine-*N*-carbonyl chloride; DPCC, *NN*-diphenylcarbamoyl chloride.

anilide hydrochloride was used to assay for tryptic activity (Erlanger, Kokowsky & Cohen, 1961).

The molar concentration of active chymotrypsin was determined by coulometric-amperometric titrimetry by using DPCC (Erlanger, Buxbaum, Sack & Coper, 1967a) or colorimetrically by using 4-carboxy-2-nitrophenyl *NN*-diphenylcarbamate (Erlanger & Edel, 1964).

Preparation of PCC. Phosgene (15g, 0.3mol) was introduced slowly into a stirred refluxing solution of 40g (0.2mol) of phenothiazine (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) in 300ml of toluene over a period of about 2h. Emerging gases were absorbed in water. After an additional 1h of refluxing and stirring, the excess of phosgene and hydrogen chloride was removed by the application of suction by using a water aspirator. The toluene solution was evaporated to dryness *in vacuo* and the residue was dissolved in 250ml of methanol-toluene (1:1, v/v). The solution was decolorized with charcoal at 60°C for 20min, filtered and allowed to cool. Addition of 500ml of light petroleum and refrigeration overnight gave 42g (79% yield) of almost colourless crystals, m.p. 172–173°C; Dahlbon (1953) reported m.p. 172–173°C. PCC turns green on standing in air, but can be purified by recrystallization from boiling methanol, with charcoal being used for decolorization.

Inactivation of chymotrypsin. Fresh stock solutions of PCC were made up before the experiment, since the solution turned green after several hours. In a typical experiment to determine the kinetics of the inactivation of chymotrypsin, the following procedure was followed. PCC stock solution was prepared by dissolving 9.5mg of PCC in 10ml of methanol and then diluting 1:30 with methanol. Enzyme stock solution was prepared by dissolving 15mg of α -chymotrypsin in 10ml of 1mM-HCl. To 18.5ml of 50mM-tris-HCl buffer, pH7.8, containing 30mM-CaCl₂, was added 1.0ml of the chymotrypsin stock solution. After equilibration at 25°C, 0.5ml of the PCC solution was added and at appropriate intervals 1ml portions were removed and assayed with *N*-glutaryl-L-phenylalanine *p*-nitroanilide (Erlanger *et al.* 1966b) at 37°C (incubation time 1200s). A control in which 0.5ml of methanol replaced the PCC solution was also run.

Inactivation of trypsin. The experiments on the inactivation of trypsin were run in a manner similar to that described for chymotrypsin. Because the reaction is slower, higher concentrations of enzyme and inactivator were used, i.e. $[E] = [I] = 30\mu\text{M}$ for the trypsin experiments compared with $[E] = [I] = 3\mu\text{M}$. The molar concentration of trypsin was calculated on the basis of a molecular weight of 25000. Assay of remaining activity was with α -*N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (Erlanger *et al.* 1961). To perform the assay, 1ml of the inactivation mixture was diluted to 3ml with 1mM-HCl; 0.2ml of solution was assayed.

Determination of kinetics of reactivation of phenothiazine-*N*-carbonyl-chymotrypsin. Since the rate of reactivation was low at 25°C, it was more convenient to run the reaction at 37°C. A stock solution of PCC in methanol was prepared by dissolving 14.3mg in 10ml of methanol and diluting a portion 15-fold with methanol. The chymotrypsin stock solution contained 22.5mg in 5ml of 2mM-HCl. To 27.75ml of 50mM-tris-HCl buffer, pH7.6, containing 30mM-CaCl₂, was added 0.75ml of the PCC solution and 1.5ml of the chymotrypsin solution. Both enzyme

and inactivator were present at a concentration of 9 μM . The reaction proceeded at 37°C, and portions withdrawn at hourly intervals were assayed with *N*-glutaryl-L-phenylalanine *p*-nitroanilide (Erlanger *et al.* 1966b). Over a period of 27h a chymotrypsin control lost about 10% of its activity.

Hydrolysis of PCC and DPCC in the absence of enzyme. The rates of hydrolysis in the absence of enzyme were determined by following the appearance of the products, phenothiazine and diphenylamine respectively, spectrophotometrically. Phenothiazine has a peak absorption at 313nm with $\epsilon 4.05 \times 10^3$ in a 50mM-tris-nitrate buffer, pH7.6, containing 25% (v/v) of acetonitrile. The absorption of PCC at this wavelength is negligible. Diphenylamine, the product of the hydrolysis of DPCC, has a peak at 285nm with $\epsilon 1.6 \times 10^4$. DPCC does not absorb at this wavelength.

Solutions of PCC and DPCC were made up in 50mM-tris-nitrate buffer, pH7.6, containing 25% (v/v) of acetonitrile. The concentration of PCC was 340 μM and that of DPCC 840 μM . The reaction was allowed to proceed in a Beckman model DU spectrophotometer maintained at 32°C.

Inhibition by indole. Inhibition by indole of the reaction of PCC with chymotrypsin was carried out as follows. Indole stock solution was prepared by dissolving 18.75mg of indole in 5ml of methanol. PCC stock solution was prepared by dissolving 6.28mg of PCC in 5ml of methanol and then diluting 1:30 with methanol. Chymotrypsin stock solution was prepared by dissolving 10.0mg in 5ml of 2mM-HCl. Four reaction mixtures were prepared: (a) chymotrypsin control, 18.0ml of 50mM-tris-HCl buffer, pH7.5, containing 30mM-CaCl₂+1ml of methanol; (b) indole control, 18.0ml of buffer+0.5ml of methanol+0.5ml of indole solution; (c) PCC+chymotrypsin, 18.0ml of buffer+0.5ml of methanol+0.5ml of PCC in methanol; (d) PCC+chymotrypsin+indole, 18.0ml of buffer+0.5ml of indole+0.5ml of PCC. At zero time 1.0ml of chymotrypsin was added and portions were drawn periodically for assay with *N*-glutaryl-L-phenylalanine *p*-nitroanilide as described above. Final concentrations were: indole, 800 μM ; chymotrypsin, 4 μM ; PCC, 4 μM . With respect to the chymotrypsin control, less than 10% of the activity was lost during the experimental run. The indole control revealed only a negligible effect on the assay at the concentrations used.

RESULTS

Course of the reaction of PCC with α -chymotrypsin. Fig. 1 shows the course of the reaction of PCC with an equimolar concentration of α -chymotrypsin at 37°C. There occurred an initial fast inactivation of the enzyme to the extent of about 85%, followed by a slow recovery of activity that was almost complete in about 15h. The almost complete inactivation at equimolar concentrations of enzyme and inhibitors indicates a 1:1 stoichiometry, similar to that found with DPCC (Erlanger *et al.* 1966a). Unlike the reaction with DPCC, however, deacylation occurred at a measurable rate, first-

order kinetics prevailing between 3 and 15h with k_{+3} calculated as $2.53 \times 10^{-3} \text{ min}^{-1}$ (the designation k_{+3} is explained in the Discussion section).

Kinetics of the reaction of PCC with α -chymotrypsin and with trypsin. Table 1 gives the second-order rate constants for the acylation reaction of PCC with chymotrypsin and with trypsin at 25°C at pH 7.8. The details as to the concentration of reactivator and enzyme are also given. For comparative purposes the second-order rate constants for the reaction of DPCC with the two enzymes are also given. Also included are some data from experiments with dimethyl phosphorofluoridate, a relatively non-specific inactivator of chymotrypsin and trypsin.

At first glance it might appear that DPCC is a better inactivator than PCC. That such an interpretation is not tenable can be shown by experiments designed to estimate the intrinsic reactivities of the two compounds in nucleophilic reactions in the absence of enzyme. The third column of Table 1 gives the second-order rate constants of the reactions of PCC and DPCC with OH^- ion in 50mm-tris-nitrate buffer, pH 7.6, at 32°C. The rates of appearance of diphenylamine and of phenothiazine, the products of hydrolysis, were measured by following

Fig. 1. Course of reaction of PCC ($9 \mu\text{M}$) with α -chymotrypsin ($9 \mu\text{M}$; mol.wt. 25000) at 37°C in 50mm-tris-HCl buffer, pH 7.55, containing 30mm- CaCl_2 and 2.5% (v/v) of methanol.

the appearance of absorption maxima at 285 and 313nm respectively. Pseudo-first-order kinetics were followed by both reactions at several pH values (7.4, 7.6, 7.9). DPCC is about 17 times as reactive as PCC in the absence of enzyme. If a correction is made for this difference in reactivity, it can be concluded that PCC is in reality about 4 times as effective as DPCC as a specific inactivator of α -chymotrypsin (fourth column of Table 1).

Effect of pH. Fig. 2 shows the effect of pH on the rate of inactivation of α -chymotrypsin by PCC. The curve is typical for the acylation of this enzyme and identical with that found with DPCC (Erlanger *et al.* 1966a). It is 'bell-shaped' with an optimum

Fig. 2. Effect of pH on rate of reaction at 25°C of α -chymotrypsin ($4 \mu\text{M}$) with PCC ($5 \mu\text{M}$). Buffers: ○, 240mm-veronal-140mm-acetate (Michaelis, 1931); ●, 50mm-tris-HCl. All solutions contained 30mm- CaCl_2 and 2.5% (v/v) of methanol.

Table 1. Rate constants ($\text{M}^{-1} \text{s}^{-1}$) for the reaction of various inactivators with α -chymotrypsin, trypsin and OH^- ions

Inhibitor	Chymotrypsin	OH^- ions	Relative rate*	Trypsin	Chymotrypsin/ trypsin ratio
PCC	105†	0.6‡	4.15	3.6§	29
DPCC	480†	11.3‡	1	8.2§	59
O $(\text{CH}_3\text{O})_2\text{PF}$	28.3	—	—	7.0	4

* $k_{\text{PCC-E}} \times k_{\text{DPCC-OH}} / k_{\text{DPCC-E}} \times k_{\text{PCC-OH}}$.

† $[\text{E}] = [\text{I}] = 3 \mu\text{M}$ in 50mm-tris-HCl buffer, pH 7.8, containing 30mm- CaCl_2 and 2.5% (v/v) of methanol at 25°C.

‡ $[\text{DPCC}] = [\text{PCC}] = 340 \mu\text{M}$ in 50mm-tris-nitrate buffer, pH 7.6, containing 25% (v/v) of acetonitrile at 32°C.

§ $[\text{E}] = [\text{I}] = 30 \mu\text{M}$, other conditions as in footnote†.

|| Ooms & Van Dijk (1966).

near 7.8, as one would expect if the acylation process were controlled by a basic group of pK_a 6.8 and a conjugate acid with a pK_a near 9.0.

Effect of indole. Preliminary studies showed that indole inhibited the reaction of PCC with α -chymotrypsin. For competitive inhibitors, Metzger & Wilson (1964) have shown that $k/k' = 1 + ([I]/K_I)$ in which k is the second-order rate constant of inactivation in the absence of the inhibitor, k' is the second-order rate constant in the presence of inhibitor, $[I]$ is the concentration of competitive inhibitor, and K_I is the dissociation constant of the chymotrypsin-competitive inhibitor complex, which for the α -chymotrypsin-indole complex is $0.7 \times 10^{-4} M$ (Huang & Niemann, 1953). When $[I] = K_I$, the equation simplifies to $k/k' = 2$. Therefore the rate of inactivation should fall by 50% in the presence of $70 \mu M$ -indole if indole is a competitive inhibitor. The results of such an experiment gave a value for k of $85.2 M^{-1} s^{-1}$ and for k' of $45.5 M^{-1} s^{-1}$ at pH 7.5, with $[E] = [PCC] = 4 \mu M$.

DISCUSSION

The reaction of chymotrypsin with a substrate or an inactivator can be represented as follows:

where $E \cdot S$ is the Michaelis complex and $E-S$ is the acylated enzyme. In the case of the reaction of chymotrypsin with the specific inactivator, DPCC, deacylation of the enzyme proceeded at a negligible rate, k_{+3} being too small to measure. With PCC, however, k_{+3} was measurable. Thus the PCC-chymotrypsin reaction has the characteristics of an enzyme-substrate reaction in which the deacylation step is rate-limiting.

The use of the term substrate to describe PCC and DPCC is fully justified, since both compounds, unlike carboxylic acid chlorides, are rather unreactive in the absence of enzyme (Table 1). Indeed, they can be recrystallized in high yield from boiling methanol; in contrast, benzoyl chloride, for example, has a half-life of only 2.7 min in methanol at $25^\circ C$ (calculated from Norris & Young, 1935). The hydrolysis of PCC by OH^- ions (Table 1) is also considerably slower than that of *p*-nitrophenyl acetate, the second-order rate constant of which was found to be $15.7 M^{-1} s^{-1}$ (compared with $0.6 M^{-1} s^{-1}$ for PCC) under the conditions of pH, buffer and temperature described in footnote† of Table 1. [Jencks & Carriuolo (1960) reported $14.8 M^{-1} s^{-1}$ at $25^\circ C$ under somewhat different conditions.] The reactivity of PCC towards OH^- ions most closely resembles that of esters such as

methyl hydrocinnamate, whose rate of saponification is $0.13 M^{-1} s^{-1}$ at $25^\circ C$ as calculated (Hammett, 1940) from data on its ethyl ester (Bender, Kezdy & Gunter, 1964). On the other hand, the rate of reaction of PCC with chymotrypsin is more than an order of magnitude faster [$105 M^{-1} s^{-1}$ compared with $4.6 M^{-1} s^{-1}$, the latter calculated from k_{cat}/K_m data of Bender (1962) and Bender *et al.* (1964)]. Data on the reaction of chymotrypsin with D-3-carbomethoxy-1,2,3,4-tetrahydro-1-oxisoquinoline, in which k_{cat}/K_m is normalized to correct for the substrate's rate of reaction with OH^- ion (M. S. Silver, M. Stoddard, T. Sone & M. S. Matta, personal communication), allow us to calculate that the above substrate is only about an order of magnitude faster than PCC in the acylation of the enzyme. We therefore conclude that the acylation of chymotrypsin by PCC shows 'kinetic specificity' (Bender *et al.* 1964). Its deacylation also reflects this specificity, especially when one takes into consideration the inherent low reactivity of carbamate esters compared with carboxylic esters. [A discussion of the identity of k_{cat}/K_m with the second-order rates of acylation at low substrate concentrations can be found in Metzger & Wilson (1964).] Further evidence for the kinetic specificity of the aromatic carbamoyl derivatives is their application as chymotrypsin titrants (Erlanger, *et al.* 1967a; Erlanger & Edel, 1964). The concentration of active site can be determined under conditions of reagent excess since the 1:1 stoichiometry of the reaction is undisturbed.

It is therefore our opinion that any hypothesis on the topography of the active site should take into account the activity of PCC. An attempt to do this and also to explain the earlier reported inversion of stereospecificity in the chymotryptic hydrolysis of D-3-carbomethoxy-1,2,3,4-tetrahydro-1-oxisoquinoline (Hein *et al.* 1960) was described by Erlanger (1967). It was proposed that the active centre of chymotrypsin has two sites capable of binding aromatic groups. PCC and the aromatic portions of substrates containing phenylalanine, tyrosine and tryptophan were bound 'productively' at one of these sites whereas the substrate of Hein and his colleagues was bound at the other. The geometric arrangement of the two sites allowed for the proper placement of the susceptible carbonyl group of both classes of substrate.

Crystallographic studies (Steitz, Henderson & Blow, 1969; Birktoft, Blow, Henderson & Steitz, 1970) on α -chymotrypsin have revealed a cleft (or 'hole') which is responsible for the binding of the aromatic portions of toluene-*p*-sulphonyl chloride, formyl L-tryptophan and indole. Although no studies have been made with D-3-carbomethoxy-1,2,3,4-tetrahydro-1-oxisoquinoline, the geometry of the 'pocket' and the location of the catalytic

groups therein are consistent with the striking inversion of stereospecificity observed in the hydrolysis of this compound. Thus it is not necessary to propose a second binding site. However, the reactivities of DPCC and PCC, as well as the reactivation studies with *N*-phenylbenzohydroxamic acid (Cohen & Erlanger, 1960), do not seem to be explicable solely on the basis of the location and geometry of this 'pocket'.

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