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STRUCTURAL AND STERIC SPECIFICITY OF α-CHLOROKETONES AS INHIBITORS OF α-CHYMOTRYPSIN*

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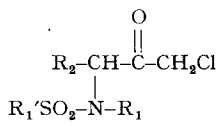
SUMMARY

1. Compounds of the general formula



where $R_2 = C_6H_5CH_2-$, CH_3-, $R_1' = CH_3C_6H_4-$, C_6H_5-, CH_3- and $R_1 = H-$, CH_3-, have been synthesised and evaluated as inhibitors of α-chymotrypsin (EC 3.4.4.5). Results indicate that the structural specificity of the inhibitors parallels that of specific substrates of similar structure. L-1-Tosylamido-2-phenylethyl chloromethyl ketone (L-TPCK) ($R_2 = C_6H_5CH_2-$, $R_1' = CH_3C_6H_4-$ and $R_1 = H-$) reacts at a faster rate than DL-1-methanesulphonylamido-2-phenylethyl chloromethyl ketone (DL-MPCK) ($R_2 = C_6H_5CH_2-$, $R_1' = CH_3-$ and $R_1 = H-$). The rate of inactivation with DL-MPCK at pH 7.15 is approx. 250 times faster than with D- or L-1-benzenesulphonylamidoethyl chloromethyl ketone (D- or L-BECK) ($R_2 = CH_3-$, $R_1' = C_6H_5-$ and $R_1 = H-$). D-TPCK is not an inhibitor, and the *N*-methyl derivative of D- and L-TPCK (D- and L-*N*-methyl-TPCK) ($R_1 = CH_3-$) were too insoluble and were not evaluated as inhibitors.

2. There is evidence for equilibrium complex formation prior to alkylation: the K_I values (in 10% acetonitrile, v/v) for DL-MPCK at pH 6.15 and D- and L-BECK at pH 7.15 are 14.9, 32 and 29 mM, respectively.

3. The nature of the interaction of these inhibitors with the complementary "loci" of the enzyme active site has been explored through their effect on the H_2O_2 oxidation of methionine-192. L-TPCK at concentrations of 0.575 and 0.97 mM at

Abbreviations: TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone; *N*-methyl-TPCK, *N*-methyl derivative of TPCK; MPCK, 1-methanesulphonylamido-2-phenylethyl chloromethyl ketone; BECK, 1-benzenesulphonylamidoethyl chloromethyl ketone. In general discussion these compounds are referred to as chloroketones, and the inactivated enzymes are designated as alkylated enzymes. L-ATEE, *N*-acetyl-L-tyrosine ethyl ester; Diglyme, diethyleneglycol dimethyl ether.

* This work includes material from a thesis submitted by Suriender Kumar to the Graduate School of Boston University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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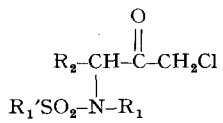
pH 4.45 has no effect either on the rate or extent of oxidation of the methionine. Similar results are obtained with L-BECK at concentrations of 3.5 and 7.0 mM at pH 7.05; though at these concentrations the compound is a competitive inhibitor of the α-chymotrypsin-catalysed hydrolysis of *N*-acetyl-L-tyrosine ethyl ester.

4. It is proposed that L-BECK interacts only with that part of the active site which does not include the hydrophobic region.

INTRODUCTION

The irreversible inactivation of α-chymotrypsin (EC 3.4.4.5) by L-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) has been studied by SCHOELLMANN AND SHAW[1]. They showed that the reaction of the enzyme with TPCK is stereospecific, the L-form being the inhibitor, and that inactivation occurs through the modification of histidine-57 (refs. 2, 3).

In this paper we have further elucidated the steric and structural specificity patterns of the inactivation process, by a study of the interaction of α-chymotrypsin with a series of inhibitors of the general formula



where $R_2 = C_6H_5CH_2-$, CH_3-, $R_1' = CH_3C_6H_4-$, C_6H_5-, CH_3- and $R_1 = CH_3-$, H–.

Evidence for the formation of an equilibrium complex between the enzyme and chloroketones (prior to alkylation) has been obtained; and dissociation constants of some of the complexes have been measured. Specific interactions of some of the inhibitors with the hydrophobic locus of the enzymatic active site for the binding of alkyl side chains[4,5] have also been probed.

The studies in this communication utilised the rapid kinetic assay method of KUMAR AND HEIN[6], which measures the changes in the steady state kinetic constants, $K_{0(app)}$ and k_0*, as the enzyme is being modified.

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* The kinetic parameters mentioned in this paper refer to the equation

$$v = \frac{k_0 [E]_0 [S]_0}{K_{0(app)} + [S]_0}$$

for the reaction

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2$$

where v = initial velocity, k_0 = catalytic rate constant,

$$K_{0(app)} = K_0 \frac{k_3}{k_2 + k_3}$$

where $K_0 = k_{-1}/k_1$, $[E]_0$ = total enzyme concentration, and $[S]_0$ = initial substrate concentration. For competitive inhibition, K_i denotes the dissociation constant of EI, for the process $E + I \rightleftharpoons EI$.

EXPERIMENTAL PROCEDURES

Materials

α-Chymotrypsin (3 times crystallised, salt-free; Lot CDI 6125-6) purchased from Worthington was used without further purification. *N*-Acetyl-L-tyrosine ethyl ester (L-ATEE) and chromatographically pure D- and L-alanine and D- and L-phenylalanine were products of Mann. Benzene-, *p*-toluene- and methanesulphonyl chlorides were obtained from Matheson, Coleman and Bell, and were not purified further. Diethyleneglycol dimethyl ether (Diglyme) purchased from Fisher was purified of a peroxide impurity according to VOGEL[7]. Spectrograde reagents obtained from Fisher were used in the spectrophotometric assays. All of the other solvents were purified by standard methods. A 40% solution of H_2O_2 was obtained from Mallinckrodt.

Methods

Synthesis of chloroketones

The melting points of the synthesised compounds are uncorrected.

Elemental analyses were performed by Spang Microanalytical Laboratory (Ann Arbor, Michigan). Optical rotation measurements were carried out at room temperature (about 25°).

Synthesis of D- *and* L-*TPCK, and N-methyl derivatives*

The compounds were prepared according to the method of SCHOELLMANN AND SHAW[1]. Their properties are: L-TPCK: m.p. 103–104°, $[\alpha]_D + 9.7 \pm 0.3$ (*c* 5.0 in chloroform); *N*-methyl-L-TPCK: m.p. 106–107°, $[\alpha]_D -255.7 \pm 2.1$ (*c* 4.93 in chloroform); D-TPCK: m.p. 103–104°, $[\alpha]_D - 10.1 \pm 0.9$ (*c* 3.56 in chloroform); *N*-methyl-D-TPCK: m.p. 106–107°, $[\alpha]_D +258.5 \pm 2.1$ (*c* 4.36 in chloroform).

Synthesis of DL-*(1-methanesulphonylamido-2-phenyl)ethyl chloromethyl ketone (MPCK)*

(a) *N*-Methanesulphonyl-DL-phenylalanine was prepared according to the method of HELFERICH AND GRUNERT[8]. Yield, 52%; crystallised from ethyl acetate–*n*-hexane; m.p. 102–104° (reported 104° by HELFERICH AND GRUNERT[8]).

(b) DL-(1-Methanesulphonylamido-2-phenyl) propionyl chloride was prepared by adding 0.041 mole of PCl_5 in five equal portions to an ice-cooled suspension of 0.041 mole of *N*-methanesulphonyl-DL-phenylalanine in 50 ml of dry ether. The mixture was stirred rapidly at 0–4° for 20 min, and at room temperature (about 25°) for 10 min. The acid chloride was obtained in 66% yield as crystals by adding dry light petroleum (b.p. 30–60°) in the cold. Recrystallisation was from ether–light petroleum; m.p. 61–63° (decomp.).

(c) For DL-MPCK the preparation procedure was similar to that for L-TPCK. To avoid decomposition of the diazoketone, HCl gas was passed through the ice-cooled solution. Yield was 39% (0.009 mole of the chloroketone from 0.023 mole of the acid chloride). The compound was crystallised from methanol–isopropyl ether; m.p. 80–82°. ($C_{11}H_{14}ClNO_3S$ requires C, 47.9; H, 5.08; N, 5.08; S, 11.60%. Found: C, 47.68; H, 5.12; N, 4.86; S, 11.46.)

Synthesis of D- *and* L-*1-benzenesulphonylamidoethyl chloromethyl ketone (BECK)*

(a) D- and L-*N*-benzenesulphonylalanine were prepared by a slightly modified procedure of HEDIN[9]. The reaction between equimolar amounts of benzenesulphonyl chloride and D- and L-alanine was performed at pH 9; and the mixture was stirred at

0° for 1 h, 25° for 1 h and then 35° for 1 h. The D- and L-compounds were obtained in about 70% yield; m.p. 127° (reported 126–127° by GIBSON AND LEVINE[10]).

(b) D- and L-(1-benzenesulphonylamido) propionyl chloride were prepared by a method similar to that for DL-(1-methanesulphonylamido-2-phenyl) propionyl chloride. The compounds, obtained in 69% yield, were crystallised from dry ether–light petroleum (b.p. 30–60°); m.p. 88–90° with decomposition to a red liquid.

(c) D- and L-BECK. The preparation procedure was the same as for DL-MPCK. The compounds crystallised from chloroform–*n*-hexane were obtained in about 54% yield; m.p. 63–65°. For L-BECK $[\alpha]_D$ +19.1 ± 0.8 (*c* 1.2 in chloroform); for D-BECK $[\alpha]_D$ −18.0 ± 0.8 (*c* 1.0 in chloroform). $C_{10}H_{12}ClNO_3S$ requires C, 45.9; H, 4.59; N, 5.35; S, 12.33%. Found for L-BECK: C, 45.8; H, 4.61; N, 5.24; S, 12.10. Found for D-BECK: C, 45.95; H, 4.54; N, 5.58; S, 12.22.

Kinetic measurements

(a) Spectrophotometric method. The rate of inactivation of α-chymotrypsin with the inhibitors in terms of changes in kinetic parameters was followed by the rapid kinetic assay of KUMAR AND HEIN[6].

(b) pH-stat method. For the determination of K_i values for D- and L-BECK, the initial rates of L-ATEE (0.2–10 mM) hydrolysis at pH 7.0, 25°, 0.1 M NaCl and 10% Diglyme were measured in the pH-stat (International Instrument Co., Calif.), in the presence and in the absence of the inhibitors. The concentrations of L-ATEE were much greater than those of the enzyme (20 nM). A flow of wet nitrogen was maintained over the reaction vessel and the acid produced was titrated with 0.05 M NaOH, to constant pH. The inhibitor concentrations used were 3.5 and 7.0 mM L-BECK and 3.1 and 6.2 mM D-BECK. The compounds were observed to be competitive inhibitors of α-chymotrypsin-catalysed hydrolysis of L-ATEE. The K_i values were calculated from the Lineweaver–Burk plots in the presence and absence of the inhibitors.

Inactivation of α-chymotrypsin with the inhibitors

The inactivation of α-chymotrypsin (approx. 40 μM) was studied as a function of varying concentrations of L-TPCK (0.19–0.8 mM) at pH 6.45, of MPCK (1.3–5.2 mM) at pH 6.15, and also with D- or L-BECK (6 mM) at pH 7.15. The reactions were carried out at 30.5° in 0.12 M potassium phosphate, and in 30% Diglyme (for reactions with TPCK) or 10% acetonitrile (for reactions with MPCK or BECK). (The pH values refer to the pH of the solution containing the organic solvents.)

The maximum concentration of organic solvent in the assay mixture was approx. 0.2% and this produced no significant changes in the kinetic parameters.

H_2O_2 oxidation of α-chymotrypsin in the presence and absence of L-TPCK and L-BECK

The H_2O_2 oxidation of the enzyme in the presence or absence of L-TPCK was followed at pH 4.45, 30.5° and in 30% Diglyme. Also, either 0.12 M sodium acetate–acetic acid or 0.12 M potassium phosphate was present. The concentrations of enzyme and H_2O_2 were 43 μM and 6.4 mM, respectively. The L-TPCK concentrations employed were 0 and 0.575 mM for acetate buffer and 0.97 mM for the phosphate buffer. Maximum concentrations of Diglyme, H_2O_2 and TPCK in the kinetic assay mixture were 0.2%, 40 μM and 6 μM, respectively.

The oxidation process in the presence of L-BECK was carried out at pH 7.05,

30.5° and in the presence of 0.12 M potassium phosphate and 10% Diglyme. The enzyme and H_2O_2 concentrations were 39 μM and 2.14 mM, respectively; the inhibitor concentrations used were 3.5 and 7.0 mM. The concentrations of organic solvent, H_2O_2 and L-BECK in the kinetic assay mixture were approx. 0.07%, 13.4 μM and approx. 23 or 46 μM, respectively.

Kinetics of formation of alkylated enzyme

The reaction of the chloroketones with α-chymotrypsin can be described by a simplified reaction mechanism of the type

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \overset{k_2}{\rightarrow} E' + P \tag{1}$$

where EI is a function of all of the non-covalent enzyme–inhibitor complexes and E' the alkylated enzyme. At any time during the reaction, the conservation equation for the enzyme requires that

$$E_0 = E + EI + E' \tag{2}$$

Since the reaction mixture containing enzyme and inhibitor is diluted by a factor of $> 10^2$ in the assay medium, it is necessary to consider that the quantity measured in our assay is ε, the sum of $E + EI$, and not simply E from the original reaction mixture. Eqn. 2 can then be represented as

$$E_0 = \varepsilon + E' \tag{3}$$

where $\varepsilon = E + EI$. Then, since $K_i = [E]\,[I]/[EI]$, $\varepsilon = [EI]\cdot K_i/[I] + [EI] = [EI]\,(1 + K_i/[I])$, or

$$[EI] = \frac{[\varepsilon]}{1 + K_i/[I]} \tag{4}$$

On differentiating Eqn. 3, for experimental conditions where $I \gg E_0$, one obtains: $0 = dE_0/dt = d\varepsilon/dt + dE'/dt$, or

$$0 = \frac{d\varepsilon}{dt} + k_2\,[EI] \tag{5}$$

By substituting Eqn. 4 for $[EI]$, the steady-state kinetics of enzyme inactivation may be written as

$$-\frac{d\varepsilon}{dt} = \frac{k_2\,[\varepsilon]\,[I]}{K_i + [I]} = k_{obs}\,[\varepsilon] \tag{6}$$

where

$$k_{obs} = \frac{k_2\,[I]}{K_i + [I]} \tag{7}$$

Although pseudo first order kinetics should be observed for any single concentration of I when $I \gg E_0$, the rate of inactivation is not a linear function of $[I]$ (see Eqn. 7) unless $[I]$ is much greater or much smaller than K_i. In the former case, when saturating conditions are obtainable, $k_{obs} = k_2$, while in the latter, Eqn. 7 reduces to $k_{obs} = k_2[I]/K_i$ and $k_{obs}/[I]$ will correspond to k_2/K_i. If a series of inhibitor concentrations in the range of the K_i value are employed, then the different pseudo first

order rate constants obtained can be utilised for calculating the K_i value: Eqn. 7 may be rearranged to give

$$(k_{obs})^{-1} = \frac{K_i}{k_2}[I]^{-1} + 1/k_2 \quad (8)$$

The values of K_i and k_2 are then found from the double reciprocal graphic plots.

FAHRNEY AND GOLD[11] used a similar equation, but they considered only the active enzyme concentration, $[E]$.

RESULTS

Reaction of α-chymotrypsin with L-*TPCK*

The rate of inactivation of α-chymotrypsin (approx. 40 μM) with 0.575 mM L-TPCK in 30% Diglyme at pH 6.45 is shown in Fig. 1. During the course of inactivation, a continuous decrease in ε is observed, while $K_{o(app)}$ remains unchanged. Since the $K_{o(app)}$ value for L-ATEE is not altered even when 80% of the enzyme is present in the alkylated form, the alkylated enzyme must bind the substrate very poorly, if at all. The values of ε can thus be directly related to the sum of the concentrations of catalytically active enzyme and the EI complexes. Control experiments show (Fig. 1) that 30% Diglyme does not produce irreversibly inactive enzyme for the duration of the experiments.

In an effort to obtain the K_i values for L-TPCK, inactivation studies were performed at different initial inhibitor concentrations (0.19–0.8 mM). As expected from Eqn. 6, pseudo first order kinetics were observed for each concentration, since $I \gg E_0$ for all of them. The pseudo first order rate constants determined at pH 6.45 are summarised in Table I: the results (Expts. 1–4) show that $k_{obs}/[I]$ is constant

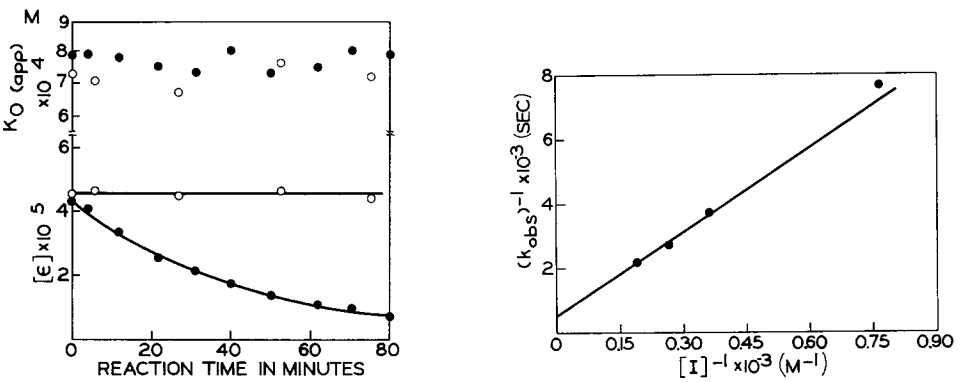

Fig. 1. Reaction of L-TPCK with α-chymotrypsin at pH 6.45, 30.5°, in 0.12 M potassium phosphate, 30% (v/v) Diglyme. Enzyme and inhibitor concentrations were 42 μM and 0.575 mM, respectively. The values of $K_{o\,(app)}$ and $[\varepsilon]$ were determined according to the procedure given in *Methods*. (●) and (○) represent, respectively, the values for enzyme being inactivated with L-TPCK, and for the control from which the inhibitor has been excluded.

Fig. 2. Determination of K_i value for reaction of DL-MPCK with α-chymotrypsin at pH 6.15, 30.5°, in 0.12 M potassium phosphate, 10% (v/v) acetonitrile. Plot of $(k_{obs})^{-1}$ *vs.* $[I]^{-1}$ according to Eqn. 8 in *Methods*. Values of $[I]$ used are for the DL-isomer. Values of k_{obs} (Expts. 5–8, Table I) were obtained from pseudo first order plots, where inhibitor concentration (1.3–5.2 mM) was much greater than enzyme concentration (approx. 40 μM).

TABLE I

REACTION OF α-CHYMOTRYPSIN WITH L-TPCK, DL-MPCK AND D- AND L-BECK

Enzyme and inhibitor mixture was incubated at 30.5°, and at various time intervals 20 μl was removed and assayed according to the procedure given in *Methods*. k_{obs} values were obtained from pseudo first order plots of log [ε] *vs.* incubation time. Expts. 1–4 in 30% (v/v) diglyme. Expts. 5–11 in 10% (v/v) acetonitrile; $k_{obs}/[I]$ values were obtained by using the concentration of the DL-isomer.

Expt. No.	*Inhibitor*	*pH*	$[E_0] \times 10^5$ *(M)*	$[I] \times 10^3$ *(M)*	$k_{obs} \times 10^4$ *(sec^{-1})*	$k_{obs}/[I]$ *(M^{-1}·sec^{-1})*	$K_i \times 10^3$ *(M)*	$k_2 \times 10^3$ *(sec^{-1})*
1	L-TPCK	6.45	4.5	0.19	1.25	0.66		
2	L-TPCK	6.45	4.3	0.391	2.45	0.63		
3	L-TPCK	6.45	4.2	0.575	3.68	0.64	>4	>2.64†
4	L-TPCK	6.45	4.4	0.805	5.27	0.65		
5	DL-MPCK	6.15	4.1	1.30	1.30	0.10		
6	DL-MPCK	6.15	4.0	2.74	2.67	0.097		
7	DL-MPCK	6.15	4.1	3.80	3.65	0.095	14.9*	1.82
8	DL-MPCK	6.15	4.1	5.20	4.56	0.087		
9	DL-MPCK	6.00	4.1	5.20	3.25	0.062		
10	DL-MPCK	7.15	4.1	2.74	8.98	0.33		
11	DL-MPCK	7.65	4.1	1.48	6.75	0.456		
12	D-BECK	7.15	4.0	5.96	0.068	0.0012**	32***	
13	L-BECK	7.15	4.0	6.06	0.068	0.0012**	29***	approx. 0.04†

* From a plot of k_{obs}^{-1} *vs.* $[I]^{-1}$ (Fig. 2).

** In 10% (v/v) acetonitrile.

*** Evaluated from competitive inhibition of α-chymotrypsin catalysed hydrolysis of L-ATEE at pH 7.05, 25°, 0.1 M NaCl and 10% (v/v) diglyme.

† Calculated from the relationship $k_{obs}/[I] = k_2/K_i$. The K_i values used were those obtained in the 3rd footnote of this table.

(0.65 ± 0.01 $M^{-1} \cdot sec^{-1}$) at all inhibitor concentrations used in the present studies. A plot of $1/k_{obs}$ *vs.* $1/[I]$ (Eqn. 8) was found to pass through the origin, also indicating that the concentrations of I used are considerably less than the value of K_i. Any changes in the ratio $k_{obs}/[I]$ corresponding to $[I] = (1/5)K_i$ can be detected. But, since $k_{obs}/[I]$ was found to be constant up to $[I] = 0.8$ mM, the value of K_i must be considerably greater than 4 mM.

Values of K_i (> 4 mM) for the L-TPCK–α-chymotrypsin system are comparable with $K_{o(app)}$ values of structurally similar compounds[12,13] if the effect of organic solvent is taken into account.

The combination of an increase in the K_i and a decrease in the k_2 values in 30% Diglyme presumably accounts for the low k_2/K_i value (0.65 $M^{-1} \cdot sec^{-1}$) obtained in these experiments, in contrast to the value of 4.7 $M^{-1} \cdot sec^{-1}$ obtained by KEZDY *et al.*[14] at pH 6.49 in 2.3% (v/v) methanol.

The competing inhibitory effect of L-TPCK in the assay mixture at pH 7.0 is negligible because: (a) the relative rates of inactivation and substrate hydrolysis would depend upon the relative values of $[I]/K_i$ and $[S_0]/K_{o(app)}$ in the assay mixture; and, in the experiments, $[I]/K_i$ (< 10^{-3}) for the highest $[I]$ (6.0 μM) in the assay is much less than $[S_0]/K_{o(app)}$ (approx. 3.0). The substrate concentration would have to be depleted by at least a factor of 10^3 before inactivation would become significant, and (b) the rate of inactivation by L-TPCK is much slower (Fig. 1) than the hydrolysis rate of L-ATEE (ref. 6).

It was not possible to obtain a sufficiently high concentration of L-TPCK in a lower concentration of organic solvents for determination of the K_i value.

Reaction of α-chymotrypsin with DL-*MPCK*

The inhibitor concentration was varied between 1.3 and 5.2 mM. At all inhibitor concentrations, pseudo first order kinetics were observed.

In the assay, again there was a continuous decrease in the value of v_{max}, with no change in the value of $K_{o(app)}$. (When 80% of the enzyme had been inactivated, the $K_{o(app)}$ value for L-ATEE remained the same as for the native enzyme.) This suggests that, in the reaction of α-chymotrypsin with DL-MPCK, no enzyme with modified kinetic parameters is produced.

The values of $k_{obs}/[I]$ obtained at pH 6.15 are summarised in Table I. A plot of $1/k_{obs}$ *vs.* $1/[I]$ is shown in Fig. 2, where the values of $[I]$ used are those for the DL-isomer. A straight line is obtained. The values of the kinetic constants k_2 and K_i obtained from the intercept and slope are $1.82 \cdot 10^{-3}$ sec^{-1} and 14.9 mM, respectively.

It was of interest, also, to study the pH dependence of the inactivation of α-chymotrypsin with MPCK, to determine whether the basic group with a pK_{app} of 7.0 is involved, as it is with L-TPCK (ref. 14). In Table I are given the values of $k_{obs}/[I]$ for pH values of 6–7.65 (only four of the pH values are shown in the table). Except at pH 6.0, $[I]$ is less than K_i and the values of $k_{obs}/[I]$ are equivalent to k_2/K_i. At pH 6.0, the value of k_2/K_i was obtained by utilising the calculated K_i value for MPCK at pH 6.15. A plot of k_2/K_i *vs.* pH (not shown here) gave a sigmoid curve in the pH range studied, with a pK_{app} of about 7.0. Thus it appears that, as in the case of L-TPCK, a basic form of histidine-57 is involved in the inactivation with DL-MPCK.

Reaction of α-chymotrypsin with D- *and* L-*BECK*

D- and L-BECK at concentrations of 6 mM, at pH 7.15, caused no change in the $K_{o(app)}$ and very little change in the v_{max} values in the assay over a period of 12 h. About 15% of the inhibitors was autohydrolysed in this time period. From the k_{obs} values of $6.8 \cdot 10^{-6}$ sec^{-1} for both D- and L-compounds, values of $k_{obs}/[I]$ ($1.2 \cdot 10^{-3}$ $M^{-1} \cdot sec^{-1}$, Table I) could be calculated.

Calculation of K_i values for BECK by the method utilised for MPCK proved impracticable. However, D- and L-BECK were found to be competitive inhibitors for

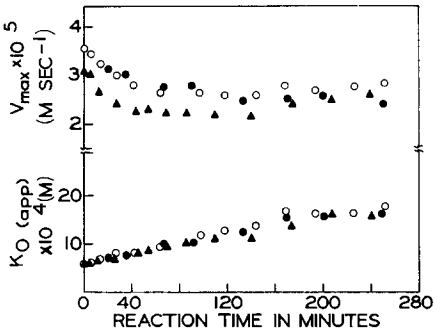

Fig. 3. Effect of L-BECK on the H_2O_2 oxidation of α-chymotrypsin at pH 7.05, 30.5°, in 0.12 M potassium phosphate, 10% (v/v) Diglyme. Enzyme was incubated with inhibitor and the reaction started with the addition of H_2O_2. Final concentrations of enzyme and H_2O_2 were 30–40 μM and 2.1 mM, respectively. $K_{o\,(app)}$ and v_{max} values are represented by (●) in the absence of inhibitor, and by (○) and (▲) at 3.5 mM and 7.0 mM L-BECK, respectively.

the α-chymotrypsin-catalysed hydrolysis of L-ATEE. K_i values of 29 and 32 mM at pH 7.05 and 25° were obtained for L- and D-BECK, respectively (Table I).

H_2O_2 oxidation of α-chymotrypsin in the presence and absence of L-TPCK and L-BECK

The rate of oxidation of the enzyme with H_2O_2 has previously been measured by monitoring changes in the kinetic constants of the L-ATEE–α-chymotrypsin system[6]. The present studies were carried out in the presence of L-TPCK (0.57 and 0.97 mM) at pH 4.45, and of L-BECK (3.5 and 7.0 mM) at pH 7.05 (Fig. 3). $K_{o(app)}$ for L-ATEE increased about 2.5-fold over a period of 200 min, and v_{max} values decreased by 20–25%. The rate of oxidation was unaffected by the presence of either of the concentrations of L-TPCK or L-BECK (Fig. 3).

From these results it appears that these compounds do not inhibit or modify the H_2O_2 oxidation reaction even if they are present, as BECK was, at concentrations which competitively inhibit the α-chymotrypsin-catalysed hydrolysis of L-ATEE.

DISCUSSION

The structural specificity of L-TPCK, DL-MPCK and D- and L-BECK can be estimated from their relative reactivities. It can be seen from a comparison of the k_2/K_i values (Table I) that DL-MPCK is about 250 times more reactive than D- or L-BECK under comparable conditions. If the K_i value for DL-MPCK is assumed to be relatively invariant over the pH range of 6–8, as is known in the case of specific substrates[15], then the K_i value for L-BECK (about 29 mM) at pH 7.0 exceeds that of DL-MPCK by a factor of 2. If it is assumed that only L-MPCK is the reactive isomer and that $K_{iL} = K_{iD}$, then L-MPCK has about a 500-fold higher k_2/K_i value and a 4-fold lower K_i value than L-BECK. A comparison of the k_o and $K_{o(app)}$ values for *N*-acetyl-L-phenylalanine methyl ester ($K_{o(app)} = 1.8$ mM, $k_o = 918$ mM/min per mg protein-nitrogen per ml) and *N*-benzoyl-L-alanine methyl ester[4] ($K_{o(app)} = 9.8$ mM, $k_o = 3.8$ mM/min per mg protein-nitrogen per ml) shows that with respect to the first compound, the k_o value is greater by a factor of about 250, and the $K_{o(app)}$ value is less by a factor of 5. When the k_2 and K_i values in Table I for TPCK, MPCK and BECK are compared, it is apparent that the replacement of a large substituent (phenylmethyl) from the inhibitor by a smaller group (methyl) has the same effect as on the substrates: a drastic change in k_2 and a smaller change in K_i. Thus the structural specificity of inhibitors parallels that of the specific substrates[4,5].

Comparison of the second order rate constants (Table I) for L-TPCK, in 30% Diglyme, and DL-MPCK, in 10% acetonitrile (conditions which favour lower rates for L-TPCK), show that L-TPCK is a more effective irreversible inhibitor than DL-MPCK ($k_{obs}/[I]$ for L-TPCK at pH 6.45 being twice that of DL-MPCK at pH 7.15). The rates of inactivation with D- and L-BECK are slow but similar, and the ratio $(k_2/K_i)_L/(k_2/K_i)_D$ is about equal to 1.0. The k_2/K_i ratio for L- and D-TPCK is greater than 10^2. It is significant that the ratio $(k_o/K_{o(app)})_L/(k_o/K_{o(app)})_D$ for the D- and L-isomers of methylbenzoyl alaninates hydrolysed by α-chymotrypsin is 8.2; but that for the phenylalanine derivatives it is 10^4 (ref. 16). These comparisons suggest, again, similar structural specificities for substrates and chloroketone inhibitors.

Since the chloroketones do not appear to influence the rate of oxidation of methionine-192, it is probable that either (1) the concentration of inhibitors used are

much less than their K_i values, or, (2) interactions of chloroketones do not involve the same enzyme "loci" in complex formation, as with substrates. With L-TPCK, the first alternative is possible. However, L-BECK at concentrations of 3.5 and 7.0 mM acts as a competitive inhibitor for α-chymotrypsin-catalysed hydrolysis of L-ATEE; but it does not protect measurably against oxidation at a concentration of about 7.0 mM. Therefore, the first possibility above does not appear likely, and L-BECK must only interact with that part of the active site which does not include the hydrophobic region.

It follows from this discussion that relatively few of the $R_2\varrho_2$ interactions discussed by HEIN AND NIEMANN[5], in which the benzenesulphonylamido group of L-BECK should interact with the ϱ_2 locus, are involved with this chloroketone. As occurs with substrates, it is possible that $R_2\varrho_2$ and $-CO-CH_2Cl:\varrho_3$ interactions of inhibitors lead to "productive" complexes. However, in the absence of large favourable $R_2\varrho_2$ interactions, the equilibrium between the enzyme and L-BECK is probably dominated by non-productive complexes. It is tempting to suggest, then, that the important difference between productive and non-productive complexes is due to different orientations of substrates and inhibitors at the active site. The anomalously low reactivity of methanesulphonyl fluoride compared with phenylmethanesulphonyl fluoride[11] could be related to these observations.

The work of KNOWLES[17] with substrates leads to conclusions similar to those with chloroketones. A study by DIXON AND SCHACHTER[18] of the protective effect of esters of L-phenylalanine and tyrosine on the H_2O_2 oxidation of chymotrypsin showed enhanced inactivation of the enzyme in the presence of these compounds. However, these authors postulated the formation of a peracid from the H_2O_2 and the "acyl-enzyme" intermediate. Such a situation is not likely to occur in the case of the chloroketones.

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