Inhibition of Pig Liver Esterase by Trifluoromethyl Ketones: Modulators of the Catalytic Reaction Alter Inhibition Kinetics†

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Received June 23, 1988; Revised Manuscript Received August 19, 1988

ABSTRACT: The kinetics of substrate hydrolysis by pig liver esterase show activation by various substrates as well as activation by organic solvents (both V_{max} and K_m increase) [Barker, D. L., & Jencks, W. P. (1969) *Biochemistry 8*, 3890]. The trifluoromethyl ketones 1,1,1-trifluoro-4-phenylbutan-2-one (TPB) and 1,1,1-trifluoro-4-(*p*-hydroxyphenyl)butan-2-one (OH-TPB) are slow, tight binding inhibitors of pig liver esterase with K_i values of 6.8×10^{-9} M and 6.0×10^{-9} M, respectively. Acetonitrile, TPB, and OH-TPB as well as the substrates pNPA and ethyl lactate caused a 15–130-fold increase in the rate of association (k_{on}), and dissociation (k_{off}), of the enzyme–TPB complex. The value of K_i (k_{off}/k_{on}) did not change. The effect cannot be attributed to half-sites reactivity since an increase in k_{off} of OH-TPB is also observed with enzyme monomers. The results are consistent with a model proposed for the catalytic reaction (Barker & Jencks, 1969) which invokes two binding sites on each esterase subunit, a catalytic site and an effector site. Occupation of the effector site can increase k_{off} and k_{on} for the inhibitors TPB and OH-TPB. Not all compounds which bind at the effector site increase k_{off}. Butanol binds at the effector site but does not effect k_{off} of TPB. The results also indicate that an aromatic or a hydrophobic structure and a carbonyl group are required for optimal interaction with the effector site.

Pig liver carboxylesterase (EC 3.1.1.1) catalyzes the hydrolysis of a variety of uncharged esters including aromatic and aliphatic esters, thioesters, and amides (Ocken, 1967; Krish, 1971). Mammalian liver esterases, in general, are believed to play a role in drug detoxification, although the metabolic function of the pig liver enzyme remains unclear (Heymann, 1982; Mentlein & Heymann, 1984; Ali et al., 1985). Pig liver esterase demonstrates substrate activation, as well as activation by compounds such as methanol and acetonitrile (Alder & Kistiakowsky, 1961; Hofstee, 1954; Barker & Jencks, 1969b). This activation is seen as an increase in V_{max}. K_m is generally also increased. For instance, V_{max} for pNPA[1] increases from 1.7×10^4 mol min^{-1} (mol of enzyme)$^{-1}$ at low concentrations of pNPA (<1 mM) to 3.9×10^4 mol min^{-1} (mol of enzyme)$^{-1}$ at high concentrations of pNPA (>1 mM). K_m for pNPA is increased from 7.8×10^{-5} M to 0.69×10^{-3} M. It has been proposed that each subunit contains an effector site and a catalytic site (Barker & Jencks, 1969b). Compounds which alter V_{max} or K_m bind at the effector site.[2]

Trifluoromethyl ketones inhibit crude mouse and human liver microsomal carboxylesterases as well as purified pig liver esterases (Ashour & Hammock, 1987). In the course of kinetic studies with pig liver esterase and 1,1,1-trifluoro-4-phenylbutan-2-one (TPB), a slow binding inhibitor (Morrison, 1982; Williams & Morrison, 1979), we observed that the rate of dissociation of the enzyme–inhibitor complex was increased in the presence of substrate. This suggested to us that the modulators of the catalytic reaction might also affect the kinetics of the enzyme with a slow binding inhibitor. Experiments described here were performed to investigate the effect of substrates as well as other modulators on the kinetics of slow binding inhibition.

Materials and Methods

Enzymes and Materials. Pig liver esterase was purchased from Sigma Chemical Co. and used without further purification. The substrates *p*-nitrophenyl acetate (pNPA) and ethyl lactate were purchased from Aldrich Chemical Co. All other organic compounds used were of reagent grade or better. The inhibitor TPB was synthesized as described below. The inhibitor 1,1,1-trifluoro-4-(*p*-hydroxyphenyl)butan-2-one (OH-TPB) was a gift from Beckton Dickinson Research Center. The compound (*R,S*)-1,1,1-trifluoro-4-phenylbutan-2-ol was a kind gift from Dr. Tzyy-Chyau Liang.

Synthesis of [4-^{3}H]-1,1,1-Trifluoro-4-phenylbutan-2-one ([^{3}H]TPB). Benzaldehyde (2.5 mmol) was added to 1 mL of ice-cold ethanol containing 25 mCi of NaB^3H_4 (ICN) and reacted for 2 h at 25 °C. The reaction was completed by the addition of 1.25 mmol of $NaBH_4$ and allowed to stir for an additional hour. The reaction mixture was extracted with 3 mL of anhydrous diethyl ether five times, and the combined ether extracts were washed with 3 mL of 1 N HCl and 3 mL of saturated NaCl. The ether layer was dried over $MgSO_4$ and concentrated under N_2 gas. This yielded 0.27 g of [1-^{3}H]benzyl alcohol (99%).

The [1-^{3}H]benzyl alcohol (2.5 mmol) was dissolved in 4 mL of methylene chloride, and 2.8 mmol of triphenylphosphine was added. The mixture was cooled over ice while 2.8 mmol of *N*-bromosuccinamide was added slowly. The reaction was allowed to proceed at 25 °C for 2 h. The volume of solvent was reduced to 2 mL and the resulting orange solution exhaustively extracted with *n*-hexane. The pure [1-^{3}H]benzyl bromide was recovered by evaporating the hexane with a slow stream of N_2 gas (Cohen et al., 1976). The yield was 0.28

† This work was supported by National Institutes of Health Grant GM12633-24 (to R.H.A.) and Biochemistry Training Grant 5T32GM07596-11 (to K.N.A.).

[1] Abbreviations: TPB, 1,1,1-trifluoro-4-phenylbutan-2-one; OH-TPB, 1,1,1-trifluoro-4-(*p*-hydroxyphenyl)butan-2-one; pNPA, *p*-nitrophenyl acetate; [^{3}H]TPB, [4-^{3}H]-1,1,1-trifluoro-4-phenylbutan-2-one.

[2] Compounds which affect V_{max} and/or K_m will be called modulators. Substrates can also act as modulators.

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g of slightly yellow liquid (66%).

Sodium ethoxide (3.2 mmol of 2 M in ethanol) was added to a dry, round-bottom flask fitted with a condenser and septum for additions. Ethyl 4,4,4-trifluoroacetoacetate (3.4 mmol) was added and the solution heated to 90 °C. [1-^{3}H]Benzyl bromide (1.6 mmol) dissolved in 1 mL of ethanol was added dropwise over 1 h. The reaction was stirred at 90 °C for 2 h and at 25 °C for 1 h. The reaction mixture was acidified to pH 5 with 1 N HCl and extracted three times with 4-mL portions of diethyl ether. The combined ether extracts were washed with 3 mL of saturated NaCl and dried over $MgSO_4$. The ether was evaporated under N_2 and the crude ester transferred to a flask fitted with a reflux condenser. Concentrated HCl (4 mL) was added, and the solution was refluxed for 24 h at 85 °C. The product was extracted with three 4-mL portions of diethyl ether. The combined ether extracts were washed with 2-mL portions of $NaHCO_3$ and then once with 2 mL of saturated NaCl. The ether layer was dried over $MgSO_4$ and solvent evaporated under a stream of N_2 gas to yield an amber liquid (Fearon, 1981). The product, [^{3}H]TPB, was purified by preparative TLC (chloroform, R_f = 0.5). Radioactive material migrated as a single spot on analytical TLC in chloroform and in 9/1 *n*-hexane/ether (R_f = 0.3) with authentic mass standard. Greater than 90% of the applied radioactive material comigrated with this spot. The final specific activity was 2.6 mCi/mmol with 33% yield: ^{1}H NMR ($CDCl_3$) δ 3.02 (m, 4 H, CH_2), 7.25 (m, 5 H, Ar).

Nonisotopically labeled TPB was synthesized in the identical manner, starting with benzyl bromide. The product migrated as a single spot on analytical TLC with R_f values identical with those of [^{3}H]TPB. The NMR spectrum agreed with that of [^{3}H]TPB. Additionally, a mass spectrum was obtained: M^+ 202.

Analytical Procedures. Enzyme assays were performed as described in the legend to Table I. Protein determination was performed by the method of Bradford (1976). Liquid scintillation counting was performed with a Beckman LS100C scintillation counter using Amersham ACS fluid. All experiments were performed at 25 °C unless stated otherwise.

Kinetic Parameters. K_i values were determined from the final velocities of reaction progress curves of product formation versus time at TPB and OH-TPB concentrations of 10–300 nM as described by Cha (1975). The assays were initiated by the addition of enzyme. Values for k_{on} and k_{off} were also determined from these progress curves (Williams & Morrison, 1979; Cha, 1975).

Additionally, k_{off} was determined by dilution of the enzyme–OH-TPB complex (1×10^{-7} mM), isolation of which is described in a later section, into an assay solution (pNPA as substrate, see legend to Table I) and fitting the progress curve thus obtained to an exponential expression (Williams & Morrison, 1979; Morrison, 1982).

Rapid-mix experiments were used to determine k_{on} directly. A 10-mL Pierce Reacti-Vial, fitted with a rubber septum, was held in place over a vortex mixer. Additions of reactants and quenching solution were made with syringes already in place through the septum. Small volumes were added with a spring-loaded Hamilton syringe. Pig liver esterase (0.8×10^{-3} mM) and OH-TPB [$(4.4–88) \times 10^{-3}$ mM] in a total volume of 110 μL were incubated together for various times (4–30 s) before the addition of 2.5 mL of 0.4 mM pNPA and 0.1 M potassium phosphate buffer, pH 7.5. In reactions in the presence of acetonitrile, the acetonitrile was premixed with the inhibitor to a final concentration of 946 mM in the preincubation mixture. The reaction with substrate was allowed to proceed for 10 s before quenching with 5 mL of acetonitrile. Product formation was determined spectrophotometrically from the absorbance at 412 nm. The value of k_{on} was determined directly from the slopes of plots of log (total product) versus time of incubation. Time points were determined in duplicate with a 5% variation between samples. The reaction obeyed pseudo-first-order kinetics.

Isolation of Enzyme–Inhibitor Complex. Pig liver esterase, at 2×10^{-2} mM, was incubated with 10×10^{-2} mM TPB or OH-TPB in a total volume of 1 mL for 30 min at 25 °C and passed through a 1 × 44 cm Sephadex G-25 gel filtration column at 4 °C at a flow rate of 0.5 mL/min. Fractions of 1.5 mL were collected and analyzed for protein. The fractions were also tested for the presence of free inhibitor by assaying with pig liver esterase. Those fractions containing protein were assayed spectrophotometrically for enzyme activity to assure that the initial activity was zero.

Preparation of Monomeric Enzyme–OH-TPB Complex. The dissociated enzyme was prepared by the method of Barker and Jencks (1969a). Pig liver esterase (2×10^{-2} mM) was incubated in 0.01 M acetate buffer, pH 4.5, for 40 min, followed by incubation with 10×10^{-2} mM OH-TPB for 20 min. The solution was neutralized to pH 7.5 by the sequential addition of 2.0 M sodium chloride and 0.4 M potassium phosphate buffer to give a final concentration of 0.1 M potassium phosphate buffer and 0.5 M sodium chloride. The enzyme–inhibitor complex was then isolated as already described with the exception that all buffers and assay solutions contained 0.5 M sodium chloride. Under these conditions, the enzyme remains in the dissociated form during the course of the experiment.

Centrifugation Columns. Columns of Sephadex G-50 find (1-mL total bed volume) were made in 1-mL tuberculin syringes as described by Penefsky (1979) but were not prespun to remove intercolated buffer. Samples of 100 μL were loaded, and the eluent was collected by centrifugation for 2 min in a clinical centrifuge. The volume of eluent collected per column was 0.7 mL.

Results

Inhibition of Esterase by TPB and OH-TPB and Dependence of Dissociation Rate. The trifluoromethyl ketones TPB and OH-TPB were tested as inhibitors of pig liver esterase. Figure 1 shows product formation over time at various TPB concentrations. Both TPB and OH-TPB (data for OH-TPB not shown) are slow binding inhibitors (Morrison, 1982; Williams & Morrison, 1979) as evidenced by the decrease in the rate of product formation over time. The final velocities of these progress curves were used to calculate K_i. Values of 1×10^{-9} M and 8.3×10^{-9} M were obtained for TPB and OH-TPB, respectively.

Plots of k_{obs}, obtained from the progress curves, versus inhibitor concentration were used to calculate k_{on} and k_{off} for TPB and for OH-TPB. These plots are linear over the range of inhibitor concentrations used. The values for K_i, k_{on}, and k_{off} are summarized in Table I. The K_i values calculated from k_{on} and k_{off} are in good agreement with those calculated from the equilibrium velocities of the progress curves. In all experiments with TPB and pNPA, 946 mM (5%) acetonitrile was used to dissolve the inhibitor and substrate. Previously, it has been shown that acetonitrile is a modulator of pig liver esterase (Barker & Jencks, 1969b). Therefore, to avoid the use of acetonitrile, the water-soluble inhibitor OH-TPB and the water-soluble substrate ethyl lactate were also used. The data obtained with these compounds are also shown in Table I.

Table I: Effect of Modulators on the Inhibition of Pig Liver Esterase by TPB and OH-TPB[a]

	K_i (M) from final velocity	K_i (M) from k_{off}/k_{on}	k_{on} (M^{-1} min^{-1})	k_{off} (min^{-1})
OH-TPB				
+PNPA, +CH_3CN	8.3×10^{-9}	6.0×10^{-9}	4.5×10^{7}[b]	0.27[b]
+ethyl lactate (50 mM), +CH_3CN	7.0×10^{-9}	7.5×10^{-9}	1.4×10^{7}[b]	0.11[b]
+ethyl lactate (3.5 mM), -CH_3CN	4.3×10^{-9}	5.5×10^{-9}	0.93×10^{7}[b]	0.052[b]
				0.055[d]
-substrate, -CH_3CN		3.7×10^{-9}	0.06×10^{7}[c]	0.0022[d]
-substrate, +CH_3CN			0.86×10^{7}[c]	
TPB				
+PNPA, +CH_3CN	1×10^{-9}	6.8×10^{-9}	2.9×10^{7}[b]	0.20[b]

[a] The enzyme was assayed in 100 mM potassium phosphate, pH 7.5, with 0.4 mM pNPA by following the increase in absorbance at 412 nm. The inhibitor TPB and substrate pNPA were dissolved in acetonitrile such that the final concentration of organic solvent in the assays was 5% (v/v) or 946 mM. Spectrophotometric assays were performed on a Perkin-Elmer 559 UV/vis spectrophotometer using 1-cm quartz cells at 25 °C. Alternately, the enzyme was assayed in 0.5 mM potassium phosphate, pH 7.5, with 3.5 mM ethyl lactate or 50 mM ethyl lactate plus 946 mM acetonitrile, by recording the amount of 10 mM NaOH added over time to maintain a constant pH. The pH was monitored with an Orion Research Model 701 A digital ionalyzer, and NaOH was delivered with a Model SB2 syringe microburet from the Micro-Metric Instrument Co. [b] From reaction progress curves. [c] From rapid-mix experiments. [d] From direct measurement of k_{off} of enzyme–[^{3}H]TPB complex.

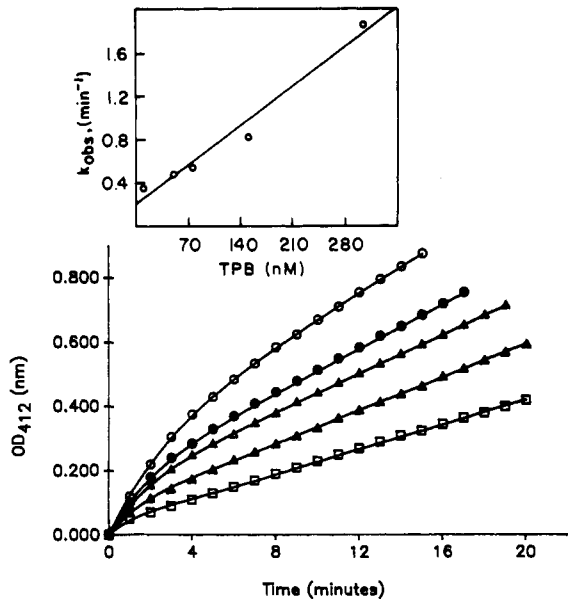


FIGURE 1: pNPA hydrolysis catalyzed by pig liver esterase in the presence of various concentrations of the trifluoromethyl ketone TPB. The reactions contained 0.4 mM pNPA, 9 nM enzyme, 946 mM acetonitrile, and the following inhibitor concentrations: (○) 10, (●) 50, (△) 75, (▲) 150, and (□) 300 nM, in 100 mM potassium phosphate buffer, pH 7.5; total volume 1 mL. Product formation was measured at 412 nm. Reactions were initiated by enzyme addition. The solid lines are generated by fitting the data to an integrated rate equation as described under Materials and Methods. (Insert) Replot of k_{obs} versus inhibitor concentration where k_{obs} is derived from the progress curves.

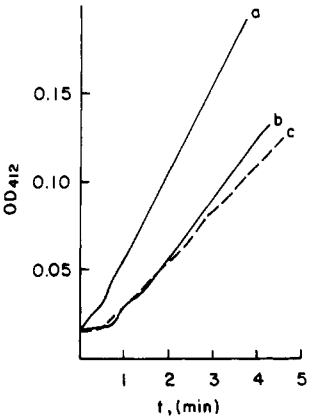


FIGURE 2: Reactivation of the enzyme–TPB complex. Reactions contained 0.4 mM pNPA, 1×10^{-7} mM enzyme or enzyme–OH-TPB complex, and 946 mM acetonitrile in 100 mM potassium phosphate buffer, pH 7.5; total volume was 1 mL. Reactions were initiated by substrate addition. (a) Enzyme was subjected to the conditions for isolation of enzyme–OH-TPB complex but in the absence of OH-TPB; substrate was added at $t = 0$. (b) Conditions identical with those of (a) except enzyme–OH-TPB complex was used. (c) Conditions identical with those of (b) except reaction initiated at $t = 15$ min.

An experiment was done to measure k_{off} for the enzyme–OH-TPB complex by an alternate procedure. The enzyme–inhibitor complex was isolated, as described, except unlabeled OH-TPB was used in place of TPB. The enzyme was diluted into the assay mixture such that the final enzyme concentration was 1×10^{-7} mM. Under these conditions the complex is expected to dissociate completely ($K_i = 6 \times 10^{-9}$ M). The results are shown in Figure 2. In the experiment shown in curve b, pNPA (dissolved in acetonitrile) was added to the reaction mixture at $t = 0$. The initial velocity is essentially zero; within 2 min the velocity reaches 83% of the uninhibited rate (Figure 2, curve a). In another experiment (Figure 2, curve c), enzyme–inhibitor complex was diluted as before and incubated for 15 min before the addition of substrate. If k_{off} were 0.27 min^{-1} as determined from the reaction progress curves, full velocity should be obtained upon addition of the substrate. The initial slope obtained in this experiment was nearly zero, indicating that the enzyme–inhibitor complex had not dissociated to a significant extent in 15 min. This experiment shows that dissociation of enzyme–inhibitor complex is accelerated by substrate and/or acetonitrile. Since after a 15-min incubation the enzyme showed less than 10% of full catalytic activity, k_{off} for OH-TPB in the absence of substrate (in acetonitrile) is <0.01 min. From the rate of recovery of activity after addition of substrate it can be calculated that $k_{off} \simeq 0.67$ min^{-1} (see Materials and Methods). From the reaction progress curves $k_{off} = 0.27$ min^{-1} was determined.

The dependence of k_{on} and k_{off} for an inhibitor upon the substrate was surprising, and deserved further investigation. Techniques were therefore used which allowed us to determine k_{on} and k_{off} for the inhibitor without a requirement for the presence of substrate.

Association and Dissociation Rates of Enzyme–OH-TPB Complex. Rapid-mix experiments were used to determine the association constant (k_{on}) of OH-TPB for pig liver esterase in the absence of substrate and modulators. Enzyme was mixed rapidly with inhibitor (in 5–100-fold excess) for varying amounts of time and substrate added to measure the amount of free enzyme remaining as described under Materials and Methods. From these experiments $k_{on} = 0.06 \times 10^7$ M^{-1} min^{-1} was obtained for OH-TPB. This rate is 15-fold slower than k_{on} determined in the presence of ethyl lactate and 130-fold slower than k_{on} determined in the presence of pNPA and acetonitrile (Table I). The data indicate that substrate and

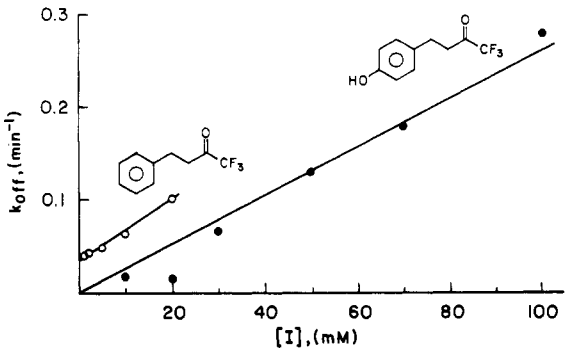


FIGURE 3: Effect of TPB or OH-TPB on the rate of dissociation of enzyme–TPB complex. Enzyme–inhibitor complex was isolated as described under Materials and Methods. Reactions contained 0.01 mM enzyme–TPB, TPB or OH-TPB at the concentrations indicated, and 100 mM potassium phosphate buffer, pH 7.5; total volume was 0.8 mL. Concentration of TPB or OH-TPB (I) was as indicated. Aliquots were removed at various times and placed on centrifugation columns. Eluent from the centrifugation columns was subjected to protein determination and liquid scintillation counting. Values of k_{off} were determined directly from the slopes of first-order plots of log (cpm/μg of protein) versus time. Duplicate determinations gave k_{off} values ± 7%.

possibly acetonitrile significantly increase k_{on} for the inhibitor. The effect of acetonitrile on k_{on} for OH-TPB was therefore tested. Rapid-mix experiments performed in the presence of 5% acetonitrile gave $k_{on} = 0.86 \times 10^7$ M^{-1} min^{-1}, 15-fold faster than in the absence of acetonitrile. Therefore acetonitrile, as well as substrate, increases the rate of formation of the enzyme–inhibitor complex. If K_i remains unchanged, as the data in Table I indicate, then there is a corresponding increase in the dissociation rate (k_{off}).

The rate of dissociation of the enzyme–inhibitor complex was determined by adding the complex formed with isotopically labeled TPB to an excess of unlabeled TPB or OH-TPB and determining the loss of protein-bound radioactivity as a function of time. Loss of radioactivity followed first-order kinetics. A plot of k_{off} versus added unlabeled inhibitor concentration is shown in Figure 3. Over the range of inhibitor concentrations used in these experiments, there is a linear relationship between k_{off} and OH-TPB concentration, with k_{off} values ranging from 0.016 to 0.28 min^{-1}. A similar experiment was done in which unlabeled TPB and 946 mM acetonitrile were used in place of OH-TPB. As the concentration of TPB increases from 1 to 20 mM (the solubility limit of TPB), k_{off} increases in a linear fashion from 0.04 to 0.11 min^{-1}. When the concentrations of TPB and OH-TPB are equal, a larger increase in k_{off} was observed with TPB. This effect is presumably due to acetonitrile. This demonstrates that TPB and OH-TPB increase the off-rate of the enzyme–inhibitor complex.

At low concentrations of OH-TPB the dissociation rate of the [^{3}H]TPB–enzyme complex should be the intrinsic dissociation rate of that complex. An experiment identical with that described in the legend to Figure 3 was performed except 4.3×10^{-2} mM OH-TPB was added to the isolated enzyme–[^{3}H]TPB complex (8.6×10^{-3} mM). k_{off} was determined as 0.0022 min^{-1}. The value for K_i in the absence of acetonitrile could then be calculated with this k_{off} and the k_{on} from rapid-mix experiments (0.06×10^7 M^{-1} min^{-1}). This afforded $K_i = 3.7 \times 10^{-9}$ M, a value in close agreement with those obtained from the reaction progress curves (see Table I), confirming that K_i is not changed in the presence of modulators.

The ability of substrate to increase the dissociation of the enzyme–TPB complex was measured directly. The substrate ethyl lactate (50 mM) was added along with 4.3×10^{-2} mM OH-TPB to the enzyme–[^{3}H]TPB complex. A k_{off} of 0.055 min^{-1} was calculated, a dissociation rate 25-fold greater than that determined in the absence of ethyl lactate and in close agreement with that determined for reaction progress curves (0.052 min^{-1}, Table I).

Table II: Modulators of TPB Dissociation[a]

compound added to reaction	modulator concn (mM)	k_{off} (min^{-1})	K_i (M)
OH-TPB	0.043[b]	0.0022	
OH-TPB	35	0.093	6×10^{-9}
acetone	35	0.003	5×10^{-3}
1,1,1-trifluoroacetone	35	0.003	1×10^{-6}
1-butanol	35	0.003	
2-butanone	35	0.036	1×10^{-2}
phenol	35	0.069	
(*R*,*S*)-1,1,1-trifluoro-4-phenylbutan-2-ol	9	0.059 (0.025)[c]	5.6×10^{-5}
benzylacetone	2	0.037 (0.005)[c]	1×10^{-3}
	1	0.037	

[a] Reaction mixture contained enzyme–[^{3}H]TPB complex (8.6×10^{-3} mM), OH-TPB (4.3×10^{-2} mM), and additions as indicated, in 100 mM potassium phosphate buffer, pH 7.5; total volume was 0.1 mL. Modulators and OH-TPB were added to the enzyme–inhibitor complex, incubated for 5 min, and placed on centrifugation columns. Eluent from the centrifugation columns was subjected to protein determination and liquid scintillation counting. The value of k_{off} was determined from the amount of enzyme–[^{3}H]TPB complex remaining. The enzyme–inhibitor complex was isolated as described under Materials and Methods. [b] This concentration of OH-TPB does not significantly accelerate k_{off}. [c] Numbers in parentheses are k_{off} in the presence of equivalent concentrations of OH-TPB.

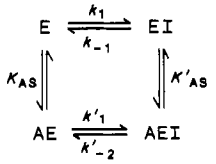
Other Modulators and the Dissociation of Enzyme–TPB. An experiment was performed to determine the effects of several other compounds (other than those listed in Table I) on the off-rate of the enzyme–inhibitor complex. Table II shows the effects of the various compounds of k_{off}. In the presence of 9 mM 1,1,1-trifluoro-4-phenylbutan-2-ol, k_{off} was 2.4-fold faster than in the presence of the same concentration of OH-TPB. The alcohol is more effective than the corresponding ketone (OH-TPB), although the ketone has the lower K_i (9300-fold lower). In the presence of benzylacetone k_{off} was 7.5-fold faster than in the presence of the same concentration of OH-TPB. Butanone and phenol affected the off-rate to a much lesser extent than OH-TPB.

Butanol had no significant effect on k_{off}, nor did acetone and 1,1,1-trifluoroacetone. These compounds either do not bind to the effector site or bind but do not change k_{off}. This point was further investigated for 1-butanol. The enzyme–[^{3}H]TPB complex was incubated with 30 mM OH-TPB and 49 mM butanol. The k_{off} was calculated as described in Table II. The dissociation rate in the presence of butanol was 0.04 min^{-1}, while in its absence $k_{off} = 0.08$ min^{-1}. Butanol causes a 2-fold decrease in the dissociation rate of the enzyme–inhibitor complex; i.e., it modulates the effect of OH-TPB on k_{off}. This finding shows that 1-butanol binds to the effector site but does not change k_{off} and, presumably, k_{on}. It has been shown that 1-butanol prevents substrate activation in the hydrolysis of ethyl butyrate (Levy & Ocken, 1967). Its effect on the catalytic reaction is therefore very similar to its effect on the interactions between enzyme and inhibitor.

Stoichiometry of Enzyme–Inhibitor Complex. Esterase–inhibitor complex was prepared with [^{3}H]TPB as described under Materials and Methods. After gel filtration chromatography 0.9–1.1 mol of inhibitor was bound per mole of enzyme.

Effect of Subunit Interaction and Dissociation Rate. Pig

Scheme I: Model for Inhibition of an Enzyme with an Effector Binding Site[a]



[a] A = effector; I = inhibitor (TPB or OH-TPB); E = esterase subunit; $k_1 < k'_1$; $k_{-1} < k'_{-1}$; $K_i = k_{-1}/k_1 = k'_{-1}/k'_1$; $K_{AS} = K'_{AS}$.

liver esterase is a trimer in solution. It was previously shown that the kinetics of pNPA hydrolysis, including substrate activation, are not changed when the enzyme is present as a monomer. We wished to determine whether k_{off} of the enzyme–inhibitor complex is increased by pNPA plus acetonitrile when the enzyme is in the monomeric form. The experiment shown in Figure 2 was repeated with monomeric enzyme (Barker & Jencks, 1969a). The results were identical with those of the trimeric enzyme. Therefore, the acceleration of the dissociation rate of inhibitor for the enzyme–inhibitor complex does not involve subunit interaction.

Discussion

TPB and OH-TPB are slow binding inhibitors of pig liver esterase with $K_i = 6.8 \times 10^{-9}$ M and 6×10^{-9} M, respectively. A number of compounds (modulators) (Table I and II) cause an increase in the rate of association and the rate of dissociation of the enzyme–inhibitor complex (Table II), but K_i (k_{off}/k_{on}) is not changed. Many of the compounds which effect k_{on} and k_{off} are also modulators of the catalytic reaction. No compounds have been found which effect the catalytic reaction but do not effect k_{on} and k_{off} for the inhibitor. The action of modulators can be best explained in terms of the two-site model (Scheme I) proposed for the action of modulators on the catalytic reaction (Botts & Morales, 1953). According to that model each subunit contains a catalytic site and an effector site. Occupation of the effector site by a modulator brings about changes in the catalytic site which alter the kinetics of the enzyme–inhibitor interaction, i.e., k_{on} and k_{off} are increased. Possibly occupation of the effector site results in a more open form of the enzyme so that entry to the active site and egress from the active site is facilitated. This conformational change may have additional consequences for the catalytic reaction, i.e., alter rate constants other than those for binding and dissociation. Other modes of action of the modulation may be considered. The action of the modulators could involve subunit interaction (cooperative effects); i.e., occupation of the active site by a modulator exerts an effect on the active site on another subunit. This explanation has been ruled out for both activation of the catalytic reaction (Barker & Jencks, 1969b) and the increase in k_{on} and k_{off} for TPB and OH-TPB in the presence of substrate, since both phenomena are observed with monomeric esterase. A model can also be considered in which the modulator increases k_{off} by displacing the inhibitor from the active site (Jenkins, 1982) and binding of the inhibitors to the active site proceeds more rapidly when a modulator is bound to the active site. This model involves displacement reactions and does not require an effector binding site. The fact that butanol can prevent binding of a modulator but is not itself a modulator is difficult to understand in terms of the displacement mechanism but can readily be explained if an effector binding site is invoked.

The efficacy of a modulator, i.e., the extent to which it increases k_{on} and k_{off}, depends upon K_{AS}, k'_1, and k'_{-1} (Scheme I). It is apparent from the data (Figure 3) that K_{AS} for OH-TPB must be greater than 100 mM, since k_{off} still increases linearly up to 100 mM OH-TPB. It should be noted that for OH-TPB $K_i = 6 \times 10^{-9}$ M. Thus, the affinity of OH-TPB for the effector site is much lower than that for the active site. In the presence of 1 and 2 mM benzylacetone, k_{off} for TPB is the same; therefore, for benzylacetone $K_{AS} < 1$ mM. Since K_i for benzylacetone is 1 mM, the affinity for the effector site is greater than that for the active site. The large discrepancy between K_i and K_{AS} for TPB suggests that the mechanisms of binding at the modulator site and active site are different. Most likely binding at the active site involves addition of serine-OH to the carbonyl group of the inhibitor. This addition probably does not occur at the effector site. However, a carbonyl group is probably optimal for binding at that site.

The requirement for a carbonyl group is apparent from a comparison of the relatively low efficacy of phenol with the high efficacy of benzylacetone in accelerating the dissociation of the enzyme–TPB complex and from a comparison of butanol and butanone. The relative low efficacy of OH-TPB as a modulator is presumably the result of essentially complete hydration of the carbonyl group. The need for a carbonyl group also accounts for the lower efficacy of 1,1,1-trifluoro-4-phenylbutan-2-ol compared to benzylacetone (Table II). The data in Table II also suggest that in addition to a carbonyl group an aromatic or hydrophobic structure is required. The requirement for an aromatic or hydrophobic group becomes apparent from a comparison of benzylacetone with acetone and from the efficacy of phenol in increasing the dissociation rate of TPB.

Acknowledgments

We thank Dr. Patrick Mize for the suggestion to test TPB as an inhibitor of liver esterase.

Registry No. TPB, 86571-26-8; OH-TPB, 117896-99-8; pNPA, 830-03-5; [^{3}H]TPB, 117897-00-4; CH_3CN, 75-05-8; ethyl lactate, 97-64-3; acetone, 67-64-1; 1,1,1-trifluoroacetone, 421-50-1; 2-butanone, 78-93-3; (*RS*)-1,1,1-trifluoro-4-phenylbutan-2-ol, 14786-43-7; benzylacetone, 2550-26-7; 1-butanol, 71-36-3; phenol, 108-95-2; carboxylesterase, 9016-18-6; benzaldehyde, 100-52-7; [1-^{3}H]benzyl alcohol, 42006-86-0; [1-^{3}H]benzyl bromide, 71778-22-8; ethyl 4,4,4-trifluoroacetoacetate, 372-31-6; benzyl bromide, 100-39-0.

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3β-Hydroxy-Δ⁵-steroid Dehydrogenase/3-Keto-Δ⁵-steroid Isomerase from Bovine Adrenals: Mechanism of Inhibition by 3-Oxo-4-aza Steroids and Kinetic Mechanism of the Dehydrogenase

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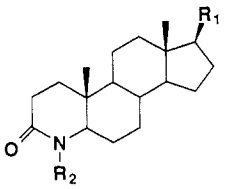
ABSTRACT: Several 3-oxo-4-aza steroids (**1**) have been identified as inhibitors of the 3β-hydroxy-Δ⁵-steroid dehydrogenase/3-keto-Δ⁵-steroid isomerase catalyzed conversion of pregnenolone to progesterone. By kinetically decoupling the two enzyme activities isolated from bovine adrenal cortex, it has been demonstrated that inhibition by **1** occurs through interference of both activities. A preferred ordered association of substrates to the 3β-hydroxy-Δ⁵-steroid dehydrogenase in which the cofactor binds prior to steroid was determined by isotope exchange at equilibrium. With this result, the dead-end inhibition patterns of **1** with the dehydrogenase were interpreted to originate from a preferred association of inhibitor within an enzyme ternate containing NADH; this proposal is supported by data from multiple inhibition analysis indicating synergistic binding of NADH and **1**. Similarly, inhibition of the 3-keto-Δ⁵-steroid isomerase by the 3-oxo-4-aza steroids was enhanced in the presence of the positive effector NADH. On the basis of pH profiles upon V_m, V_m/K_m, and $1/K_i$ for both enzyme activities, inhibition is proposed to result from the structural similarity of **1** to intermediate states formed upon enzyme catalysis.

Over the last several years, members of a 3-oxo-4-aza series of steroids, compound **1**, have been described as potent re-



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versible inhibitors of hepatic and prostatic steroid 5α-reductases (Rasmusson et al., 1986, 1984). It has been proposed that such inhibitors, if selective for steroid 5α-reductase, could be employed as therapeutic agents for the treatment of benign prostatic hypertrophy, acne, and male pattern baldness through blockade of 5α-dihydrotestosterone biosynthesis—the androgen responsible for progression of these disorders (Brooks et al., 1986a). Members of the 4-aza series of steroids currently are being evaluated toward this end (Brooks et al., 1986a,b).

Recent reports have suggested that one of these 4-aza steroids might not demonstrate unique specificity for steroid 5α-reductase. For example, Cooke and Robaire (1986) have reported that *N*,*N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (4-MA)[1] blocks the conversion of pregnenolone to progesterone in testis microsomes. In addition, Chan et al. (1987) have observed inhibition of progesterone synthesis by 4-MA in porcine granulosa cells. These results are consistent with interference by 4-MA of one or both of the reactions catalyzed by the enzymes 3β-hydroxy-Δ⁵-steroid dehydrogenase and 3-keto-Δ⁵-steroid isomerase.

The sequential enzyme activities of 3β-hydroxy-Δ⁵-steroid dehydrogenase (EC 1.1.1.145) and 3-keto-Δ⁵-steroid isomerase (EC 5.3.3.1) catalyze requisite steps in the biogenesis of androgenic, progestogenic, and estrogenic steroidal hormones and, among other metabolites, the corticosteroids. While proteins which catalyze these reactions can be uniquely isolated from bacterial sources (Talalay & Wang, 1955; Batzold et al., 1976), the microsomal-derived activities from mammalian tissues such as ovine adrenals (Ford & Engel, 1974), bovine ovaries, (Cheatum & Warren, 1966), rat testes (Ishii-Ohba et al., 1986a), and rat adrenals (Ishii-Ohba et al., 1986b, 1987) appear to reside within a single protein, possibly utilizing the same steroid binding site for both activities. In this regard, the 3-keto-Δ⁵-steroid isomerase from these sources requires NAD(H) as a positive allosteric effector (Oleinick & Koritz, 1966; Neville & Engel, 1968; Ishii-Ohba et al., 1986a). Oxidation by the 3β-hydroxy-Δ⁵-steroid dehydrogenase is

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[1] Abbreviations: 4-MA, *N*,*N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid: DHEA, dehydroepiandrosterone; ADIOL, 5α-androstane-3β,17β-diol; DHT, 5α-dihydrotestosterone.

0006-2960/89/0428-0140$01.50/0