Kinetic Study on the Binding of 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane to *Rhizopus delemar* C-Lipase: Rate Constants and the Effect of Slow Addition of the Ligand on Complex Formation

Hiroyuki KANEKI and Mitsuya TANAKA*

School of Pharmaceutical Sciences, Toho University, Miyama 2-2-1, Funabashi-shi, Chiba 274, Japan. *Received June 1, 1992*

***Rhizopus delemar* C-lipase (E) binds 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (D or DDT) to form stable 1:1, 2:1 and 9:1 DDT–lipase complexes as follows.**

$$E \xrightarrow[\text{fast}]{k_1} ED \xrightarrow[\text{slow}]{k_2} ED_2 \xrightarrow[\text{very slow}]{k_3} E'D_2 \xrightarrow[\text{very fast}]{k_4} E^*D_9$$

Competitive binding experiments with a mixture of E and ED and that of E and $E'D_2$ yielded $k_1:k_2:k_4=80:1:400$. The k_2 value was estimated to be $1.6\times10^8\,M^{-1}s^{-1}$ by following the decrease in [ED] at a low concentration of reactants, ED and DDT, where the reaction was terminated at a given time by scavenging DDT with the addition of excess $E'D_2$. The k_3 value, $0.021\,s^{-1}$, was determined by following the formation of $E'D_2$ from ED_2 in the presence of ED. $E'D_2$ was estimated by converting $E'D_2$ rapidly into E^*D_9 by adding an adequate amount of DDT (ED_2 cannot bind DDT), and the excess free DDT was depleted by the pre-existing ED to stop E^*D_9 formation from newly born $E'D_2$.

The continuous slow addition of a limited amount of DDT to ED made the rate of the second order reaction, ED to ED_2, comparable to the rate of the slow first order reaction, ED_2 to $E'D_2$, thereby favoring the formation of E^*D_9. The estimation of k_2 and k_3 from the variation in the final population of each DDT–lipase species as a function of the rate of ligand addition was described.

Keywords kinetics; diffusion controlled kinetics; ligand binding; multibinding; rate constant; DDT; chlorinated pesticide; *Rhizopus delemar*; lipase

Introduction

Tripropionin-hydrolyzing activity of *Rhizopus delemar* C-lipase (E) is enhanced by a factor of 4.4 by 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT or D). A possible mechanism of the binding of DDT to the lipase was presented as shown in Chart 1 on the basis of titration experiments (plots of enzyme activity *versus* [DDT]/[enzyme]) and binding experiments (determination of unbound and bound ligand by isopropyl ether extraction).[1] The lipase binds DDT molecules to form 9:1 DDT–lipase complex (E^*D_9) with highly elevated activity toward tripropionin, through the stepwise formation of 1:1 and 2:1 DDT–lipase complex (ED and ED_2), a very slow step of conformational change of ED_2 to $E'D_2$ and finally "simultaneous" binding of seven ligand molecules.

Eight tryptophan (Trp) residues, T1 to T8, on the lipase molecule were specified in terms of the amino acid sequence around the Trp residue, and it has been demonstrated that the first DDT molecule binds to a particular site involving T1 and the second to a site involving T2.[2] Although DDT was recovered from the complexes quantitatively by isopropyl ether extraction at pH 4.0, the binding is seemingly irreversible at pH 6.0 and no indication of dissociation was observed. Therefore, it seems reasonable to delineate this consecutive binding system in terms of rate constants for binding, k, rather than equilibrium constant K (or binding constants K_b), although multibinding systems were usually studied in the state of equilibrium.[3]

It has previously been observed that, when equimolar DDT was added to a solution of ED at once under stirring,

$$E \xrightarrow[\text{step 1}]{k_1\,(\text{fast})} ED \xrightarrow[\text{step 2}]{k_2\,(\text{slow})} ED_2 \xrightarrow[\text{step 3}]{k_3(\text{very slow})} E'D_2 \xrightarrow[\text{step 4}]{k_4(\text{very fast})} E^*D_9$$

Chart 1

$E'D_2$ was formed exclusively without any change in tripropionin-hydrolyzing activity, while, when equimolar DDT was added slowly, the enzyme activity increased, indicating the formation of E^*D_9 as well as $E'D_2$.[1b] It was conceivable that the slow addition of ligand disfavored the second order reaction, ED to ED_2, and made its rate comparable to the slow first order reaction, ED_2 to $E'D_2$, which was transformed rapidly to E^*D_9.

Quantitative determination of each DDT–lipase complex by high performance liquid chromatography (HPLC) enabled us to study the binding system in detail. This paper describes the determination of the rate constants for each binding step and substantial demonstration of the effect of slow ligand addition. The unusually large values for the diffusion-controlled rate constants, k_1 and k_4, are discussed.

Materials and Methods

Materials *Rhizopus delemar* C-lipase was purified according to the method of Iwai and Tsujisaka[4] from commercial products of *Rh.* lipase (Seikagaku Kogyo Co., Ltd.) as in the previous reports.[1] DDT was purchased from Wako Pure Chemical Industries Co., Ltd.

HPLC of DDT–Lipase Complexes Both purification and quantitative analyses were performed as follows unless otherwise specified. Sample solutions were routinely applied to a column of Asahipak C4P-50 (4.6 × 250 mm, Asahi Chemical Industry Co., Ltd.) equilibrated with 50 mM sodium acetate buffer, pH 6.0, containing 10% acetonitrile. The column was eluted with the same buffer for 5 min and then with a linear gradient from 10 to 40% acetonitrile in the same buffer for 6 min at a flow rate of 0.8 ml per min.

Preparation of DDT–Lipase Complexes Solutions of DDT–lipase complexes, ED and $E'D_2$, were prepared by the rapid addition of an EtOH solution (20 μl) of a stoichiometric amount of DDT with a microsyringe to a 50 mM sodium acetate buffer (5 ml), pH 6.0, containing 2.5 to 51 nmol of lipase with stirring at 30 °C. The solutions were incubated for more than 10 min for the reaction to be completed.[2,5] The complexes were purified by HPLC and used on that day.

Kinetic Measurement The measurement was carried out in 50 mM sodium acetate buffer, pH 6.0, at 30 °C. Generally, in the experiments, an

appropriate amount of DDT in EtOH (20 μl) or DDT–lipase complex in the buffer (50—260 μl) was rapidly added to a reaction solution (5 ml) under mixing with a magnetic stirrer.

Lipase activity toward tripropionin was assayed with a pH-stat as described previously.[1] For the experiment of the effect of the continuous slow addition of DDT, an EtOH solution of DDT (20 μl) was added to a reaction solution (5 ml) with a continuous injector (Micro Feeder JP-S, Furue Science Co., Ltd.) as modified to be suitable for the present study.

Computation Differential equations which could not be transformed to an algebraic form(s) were solved numerically by the Runge–Kutta method. Nonlinear squares analyses were carried out by iterative fitting by the damping Gauss–Newton (or Marquardt) method, which was combined with the Runge–Kutta method when necessary. For experiments on competitive binding and continuous DDT addition, numerical integration was continued until unbound DDT was depleted to less than 0.05% (or 0.1%) of total (bound and unbound) DDT.

Results

General Properties of DDT–Lipase Complexes Previously, the enzyme species, E, ED, $E'D_2$ and E^*D_9, were partially separated with a CM-Sephadex C25 column.[1b] In the present study, they were clearly separated by reversed-phase HPLC as shown in Fig. 1a. Enzyme species were recovered quantitatively (the lowest recovery was 99.1%), and they retained the original enzyme activity and the correct DDT/E ratio when examined after removal of acetonitrile. This finding substantiates the formation of ED, $E'D_2$ and E^*D_9, which has been presented on the basis of the titration and binding experiments[1] and then supported by the chemical modification study.[2] The very slow step (step 3 in Chart 1, ED_2 to $E'D_2$) has been presumed to explain the exclusive formation of $E'D_2$ from equimolar ED and DDT without the formation of E^*D_9.[1] The presumed complex, ED_2, having a half-life of 33 s (described below), could not be distinguished by HPLC from $E'D_2$.

The binding in the complexes was very tight; formation of ED from E and $E'D_2$, $E'D_2$ from ED and E^*D_9, and ED or $E'D_2$ from E and E^*D_9 was not observed when solutions containing two complex species were analyzed for an intermediately-liganded complex after being kept for 2 and 24 h at 30 °C in 50 mM acetate buffer, pH 6.0 (data not shown).

We had believed in the direct transformation of $E'D_2$ to E^*D_9. However, the elution profile of a fresh mixture of $E'D_2$ and 7 mol of DDT exhibits the presence of intermediate peaks between the peaks for $E'D_2$ and E^*D_9 (Fig. 1b), presumably peaks for 9 : 1 DDT–lipase complexes precursory of E^*D_9 (k_4 is extremely large as described below and all DDT molecules bind instantly).

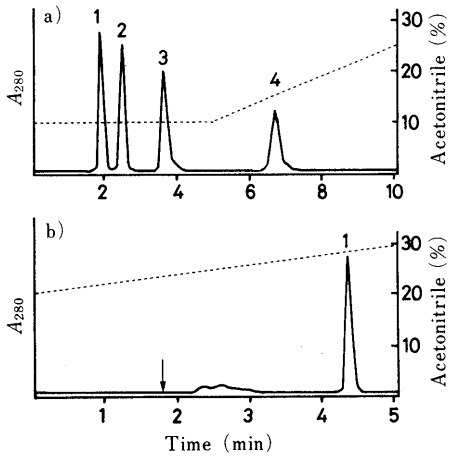

Fig. 1. HPLC of Lipase and DDT–Lipase Complexes

a) 1, native lipase; 2, ED; 3, $E'D_2$; 4, E^*D_9. b) 1, E^*D_9; ↓, retention time of $E'D_2$. A mixture containing $E'D_2$ (50 nM) and DDT (350 nM) was incubated for 20 s and applied to a column equilibrated with 50 mM sodium acetate buffer (pH 6.0) containing 20% acetonitrile. The column was eluted with a linear gradient from 20 to 40% acetonitrile in the same buffer for 10 min.

Overall Rate Equations for the System The rate equations for the system in Chart 1 are,

$$\frac{d[E]}{dt} = -k_1[E][D] \tag{1}$$

$$\frac{d[ED]}{dt} = k_1[E][D] - k_2[ED][D] \tag{2}$$

$$\frac{d[ED_2]}{dt} = k_2[ED][D] - k_3[ED_2] \tag{3}$$

$$\frac{d[E'D_2]}{dt} = k_3[ED_2] - k_4[E'D_2][D] \tag{4}$$

$$\frac{d[E^*D_9]}{dt} = k_4[E'D_2][D] \tag{5}$$

$$\frac{d[D]}{dt} = -\left(\frac{d[ED]}{dt} + 2\frac{d[ED_2]}{dt} + 2\frac{d[E'D_2]}{dt} + 9\frac{d[E^*D_9]}{dt}\right) \tag{6}$$

where t is time and each k_i represents the second order rate constant for the binding at step i except for k_3 which is the first order rate constant. In step 4, the reaction is assumed to be of the second order in spite of the binding of seven ligand molecules at this step, since a simultaneous binding of two or more ligand molecules is unlikely.

Comparison of k_1 and k_2 That k_1 is much larger than k_2 has previously been proposed based on the titration experiments with a mixed solution of E and ED.[1] The k_1/k_2 ratio was estimated by experiments on the competition of E with ED for a limited concentration of DDT, using a solution containing a fixed amount of E and an increasing amount of ED. The formation of E^*D_9 was undetected in the experiments. Since the influence of ED arising from E should be negligible when [ED] is much greater than [E], the rate equations are reduced to Eqs. 1 and 2′.

$$\frac{d[ED]}{dt} = -k_2[ED][D] \tag{2'}$$

Solution of Eqs. 1 and 2′ gives

$$\frac{k_1}{k_2} = \frac{\ln[E] - \ln[E]_0}{\ln([ED]_0 - [E'D_2]) - \ln[ED]_0} \tag{7}$$

where $[E]_0$ and $[ED]_0$ are initial concentrations of E and ED, respectively. Naturally, Eq. 7 is obeyed at any time, t at any [D].

An ethanol solution of DDT which gave a final total (free plus bound) DDT concentration of 50 nM (or 200 nM) was added to a solution containing 50 nM (or 200 nM) of lipase and various concentrations of ED. After incubation with stirring for 10 min to complete the binding reaction,[5] the mixture was analyzed for [E], [ED] and [$E'D_2$]. The k_1/k_2 ratios calculated from Eq. 7 are shown in Table I. The values at $[ED]_0/[E]_0$ larger than 10 seem to represent a proper k_1/k_2 ratio, *ca.* 80. The use of intermediate reactant (ED) more than 30 times the initial reactant (E) is said to be desirable in this kind of study. Nonlinear least squares

TABLE I. Comparison of k_1 and k_2 Values[a]

$[ED]_0/[E]_0$	[E] (nM)	[ED] (nM)	[E'D$_2$] (nM)	k_1/k_2[b]
	$[E]_0=50$ nM			
5	6.65±0.083	284±3.7	6.58±0.077	76±1.3
10	10.1 ±0.14	529±0.8	10.0 ±0.12	79±1.7
15	12.8 ±0.09	773±2.6	12.7 ±0.09	79±1.0
20	14.9 ±0.08	1020±2	14.8 ±0.07	81±0.7
30	18.5 ±0.13	1510±3	18.4 ±0.13	80±1.1
40	21.3 ±0.17	2010±9	21.2 ±0.15	80±1.3
50	23.4 ±0.28	2500±8	23.2 ±0.13	81±1.2
	$[E]_0=200$ nM			
5	27.2 ±0.20	1120± 9	25.7 ±1.01	77±3.2
10	39.6 ±0.46	2110±19	39.4 ±0.25	81±1.0
15	50.9 ±0.17	3040±23	50.0 ±0.99	81±1.8
20	60.1 ±0.57	4058±11	58.5 ±0.57	82±1.1

a) Each value represents the mean±S.D. of 4 runs. b) Calculated from $k_1/k_2=\ln([E]/[E]_0)/\ln(([ED]_0-[E'D_2])/[ED]_0)$.

TABLE II. Comparison of k_1 and k_4 Values[a]

$[DDT]_{add}$[b]	[E] (nM)	[ED] (nM)	[E'D$_2$] (nM)	[E*D$_9$] (nM)	k_4/k_1[c]
	$[E]_0=50$ nM				
50	48.5±0.08	1.46±0.005	43.0 ±0.07	6.92±0.003	4.9±0.28
100	46.9±0.06	3.05±0.073	36.1 ±0.05	13.8 ±0.04	5.1±0.11
150	44.8±0.20	4.86±0.219	29.2 ±0.11	20.6 ±0.35	4.9±0.17
200	42.6±0.12	7.37±0.108	22.4 ±0.07	27.4 ±0.22	4.9±0.12
250	40.2±0.28	9.85±0.318	15.7 ±0.06	34.2 ±0.06	5.2±0.19
300	36.1±0.24	13.8 ±0.21	9.17±0.017	40.8 ±0.01	5.2±0.11
350	28.7±0.66	21.1 ±0.69	3.10±0.007	46.9 ±0.03	5.0±0.20
	$[E]_0=200$ nM				
200	194±0.6	5.81±0.488	170 ±0.8	28.9 ±0.59	5.0±0.44
400	188±0.6	11.6 ±0.36	143 ±0.6	55.4 ±0.70	5.1±0.33
600	179±0.9	19.0 ±0.56	115 ±1.0	84.2 ±0.94	5.0±0.24
800	169±0.7	29.0 ±0.07	88.5 ±1.06	114 ±4.5	5.1±0.35
1000	160±1.7	39.0 ±1.21	62.3 ±0.20	136 ±0.4	5.1±0.26
1200	144±3.4	54.2 ±2.77	35.9 ±0.53	162 ±0.6	5.0±0.36
1400	119±1.9	80.0 ±1.99	11.1 ±0.40	188 ±1.0	5.3±0.25

a) Each value represents the mean±S.D. of 3 to 10 runs. b) DDT was added to a solution of equimolar E and E'D$_2$ to give a final concentration of added DDT, $[DDT]_{add}$. c) Calculated from $k_4/k_1=\ln((E'D_2]_0-[E^*D_9])/[E'D_2]_0)/\ln([E]/[E]_0)$.

analysis with Eqs. 1 and 2 taking the formation of ED from E into consideration, yielded values 80.0±0.16 (S.D.) at 50 nM of $[E]_0$ and 81.1±0.28 at 200 nM.

Comparison of k_1 and k_4 Since $k_4>k_1\gg k_2$, an approximate k_4/k_1 ratio could be obtained from simplified rate equations, assuming a simple competition of E with E'D$_2$ for ligands of limited concentration, as in the case of k_1 and k_2 as follows.

$$\frac{d[E]}{dt}=-k_1[E][D] \quad (1)$$

$$\frac{d[E'D_2]}{dt}=-k_4[E'D_2][D] \quad (4')$$

Solution of Eqs. 1 and 4' gives

$$\frac{k_4}{k_1}=\frac{\ln([E'D_2]_0-[E^*D_9])-\ln[E'D_2]_0}{\ln[E]-\ln[E]_0} \quad (8)$$

where $[E'D_2]_0$ is the initial concentration of E'D$_2$. Equation 8 is obeyed again at any t and [D]. Various amounts of DDT were added to a solution of equimolar E and E'D$_2$. After 10 min the mixture was analyzed for [E], [ED], [E'D$_2$] and [E*D$_9$]. The variation in ligand concentration did not affect the k_4/k_1 value, *ca.* 5, indicating that the binding in step 4 conforms to second order reaction kinetics. Simulation with equations for the overall system (Eq. 1 to 6) using estimated values of rate constants, gave information that, with an initial concentration of 100 nM both for E and E'D$_2$, the formation of ED$_2$ from E is negligible ($[E]_0-([E]+[ED])$ is less than 0.1 nM) and the binding reaction is completed within 2 ms, thereby disallowing the transformation of ED$_2$ into E'D$_2$. Similar results were obtained with 50 and 200 nM of reactants.

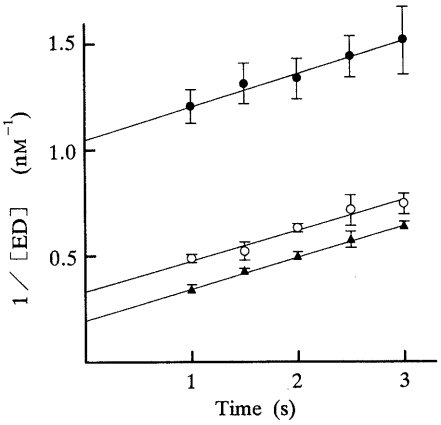

Fig. 2. Estimation of k_2 by Quenching Method
$[ED]_0$: ●, 1 nM; ○, 3 nM; ▲, 5 nM.

Estimation of k_2 by Quenching Method If the formation of E'D$_2$ arising from ED (step 3) is negligible, that is, $k_3\ll k_2[D]$, Eq. 2 is reduced to

$$\frac{d[ED]}{dt}=-k_2[ED][D] \quad (2')$$

therefore, when reactants are equimolar,

$$\frac{1}{[ED]}=k_2t+\frac{1}{[ED]_0} \quad (2'')$$

On the other hand, the comparison of k_1, k_2 and k_4 revealed that the k_4 value is *ca.* 400 times the k_2 value. This enabled us to follow the reaction at step 2, ED to ED$_2$ with time, by stopping the reaction with E'D$_2$ as a scavenger of DDT. The reaction was initiated by the addition of equimolar DDT to a solution of ED, and terminated by the addition of excess E'D$_2$ at an E'D$_2$/ED molar ratio of 10. The reaction mixtures were analyzed for residual [ED]. There was an apparent indication of a second order reaction profile in the 1/[ED] *versus* t plots as shown in Fig. 2. The k_2 value was estimated at 1.6×10^8 M^{-1} s^{-1}.

Estimation of k_3 by Following E'D$_2$ Formation The first order rate constant k_3 for the slow reaction, ED$_2$ to E'D$_2$, was estimated by following the formation of E'D$_2$ with time. E'D$_2$ was determined by converting it rapidly to E*D$_9$ by the addition of DDT in the presence of ED. ED serves as a scavenger of excess DDT and prevents the formation of E*D$_9$ arising from newly born E'D$_2$.

A one-eighth mol of DDT was added to a solution of ED. The rapid formation of ED$_2$ should occur to give a mixture of 7/8 mol of ED and 1/8 mol of ED$_2$. The latter changes to E'D$_2$ slowly. After time, t, 7/8 mol of DDT, necessary for the transformation of 1/8 mol of E'D$_2$ to E*D$_9$, was added. After being kept for 10 min, the reaction

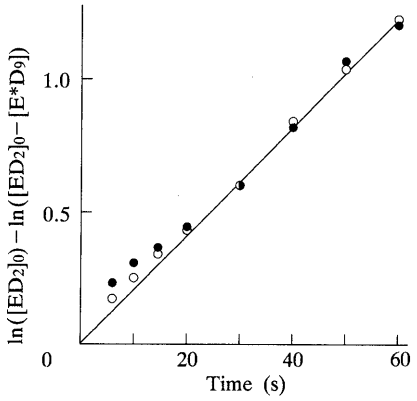

Fig. 3. Estimation of k_3 by Following $E'D_2$ Formation
$[ED_2]_0$: ●, 50/8 nM; ○, 200/8 nM.

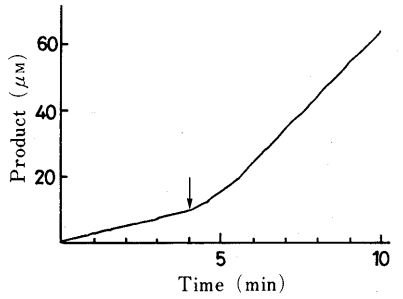

Fig. 4. Time Lag in Activation
DDT (final concentration, 112.5 nM) was added to a reaction mixture containing the enzyme (12.5 nM) and tripropionin (2.93 mM) as a substrate after 4 min incubation (↓).

mixture was analyzed for $[E^*D_9]$. The experiments with 50 and 200 nM of ED are shown in Fig. 3. Systematic deviation of points were observed in a time range of 6—20 s. In this range, $[E'D_2]$ is low and the second addition of DDT would be utilized mainly to yield more ED_2 (and therefore undesired E^*D_9 from ED) than in a time range more than 20 s. In a control experiment with a 175 nM solution of ED to which equimolar DDT was added, the formation of 1.1 nM of E^*D_9 was observed (the results with 50, 200 and 1000 nM ED are shown in Fig. 5c, $T=0$). The data in the time range of more than 20 s indicate that the reaction obeys first order reaction kinetics, independent of reactant concentration. The k_3 value was found to be $0.021\ s^{-1}$.

A time lag in activation was observed when DDT was added to a reaction solution of hydrolysis of tripropionin by the lipase (Fig. 4). Assuming that the transformation of ED_2 to $E'D_2$ is the rate-determining step for the overall reaction, E to E^*D_9, the product formation is expressed as,

$$\frac{d[P]}{dt} = v(e^{-k_3 t} + 4.4(1 - e^{-k_3 t})) \quad (9)$$

therefore,

$$[P] = v(4.4t + 3.4/k_3(e^{-k_3 t} - 1)) \quad (9')$$

where [P] represents an increase in product concentration at time t after the addition of DDT, v is reaction velocity in the absence of DDT, the value 4.4 is a factor of activation for E^*D_9, and the reaction is assumed to be in the range of initial velocity. The data on the curves were fitted with a nonlinear least squares analysis. The k_3 was found to be $0.021\ s^{-1}$ independent of reactant concentrations and coincident with the above value.

Table III. The Values of Rate Constant

Rate constant	Value of rate constant $M^{-1}s^{-1}$ (or s^{-1})[a]	Method
k_1	$1.24\times10^{10}\pm2.5\times10^{8}$ [f]	Comparison
	$1.26\times10^{10}\pm2.6\times10^{8}$ [g]	
k_2	$1.69\times10^{8}\pm7.4\times10^{6}$ [b]	Time course
	$1.49\times10^{8}\pm4.4\times10^{6}$ [c]	
	$1.47\times10^{8}\pm3.2\times10^{6}$ [d]	
k_3	$2.06\times10^{-2}\pm1.5\times10^{-4}$ [f]	Time course
	$2.07\times10^{-2}\pm3.1\times10^{-4}$ [g]	
	$2.05\times10^{-2}\pm3.1\times10^{-4}$ [e]	Time lag
	$2.11\times10^{-2}\pm1.9\times10^{-4}$ [f]	
k_4	$6.2\times10^{10}\pm3.6\times10^{9}$ [f]	Comparison
	$6.4\times10^{10}\pm5.2\times10^{9}$ [g]	
k_4'	9.0×10^{9}	(Site)

a) Means ± S.D. The k_3 is the first-order rate constant. Initial concentration (nM) of complex species were b) 1, c) 3, d) 5, e) 12.5, f) 50, g) 200.

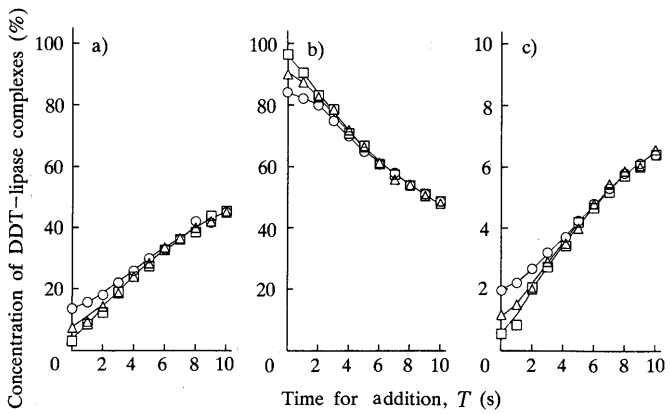

Fig. 5. Continuous Addition of DDT to a Solution of ED
a) ED, b) $E'D_2$, c) E^*D_9. $[ED]_0$: ○, 50 nM; △, 200 nM; □, 1000 nM. Concentration of DDT–lipase complexes are expressed as percentages of $[ED]_0$.

The rate constants obtained were summarized in Table III with the rate constant for site at step 4, k_4', which was calculated from k_4.

Continuous Addition of DDT to a Solution of ED The rate of ligand additon influenced the final population of DDT–lipase complex species. Equimolar DDT was added continuously to a solution of ED in a given time, T, and the reaction was completed by keeping the mixture for 10 min. The populations of DDT–lipase species in the reaction mixtures were found to be dependent on T as shown in Fig. 5. The formation of E^*D_9 and unreacted ED increased with increasing time for addition, while the formation of $E'D_2$ decreased.

If the magnitude of k_4 is large enough compared with k_2 and k_3 to allow supposition that $E'D_2$ is transformed to E^*D_9 in a moment, $d[E^*D_9]/dt = d[E'D_2]/dt$, the rate equations are

$$\frac{d[ED]}{dt} = -k_2[ED][D] \quad (2')$$

$$\frac{d[ED_2]}{dt} = k_2[ED][D] - k_3[ED_2] \quad (3)$$

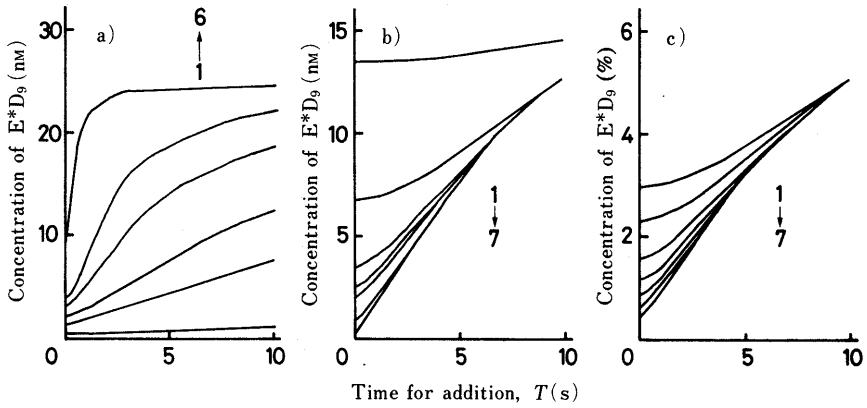

Fig. 6. Computer Simulation of Continuous Addition of DDT to a Solution of ED

a) k_3 dependence. $[ED]_0$ was 200 nM. The k_2 value was fixed at $1.5\times10^8\,M^{-1}s^{-1}$. The k_3 values (s^{-1}) were: 1, 10^{-3}; 2, 10^{-2}; 3, 2×10^{-2}; 4, 5×10^{-2}; 5, 10^{-1}; 6, 1. b) k_2 dependence. $[ED]_0$ was 200 nM. The k_3 value was fixed at $2\times10^{-2}\,s^{-1}$. The k_2 values ($M^{-1}s^{-1}$) were: 1, 10^6; 2, 10^7; 3, 5×10^7; 4, 10^8; 5, 1.5×10^8; 6, 10^9; 7, 10^{10}. c) $[ED]_0$ dependence. The values of k_2 and k_3 were fixed at $1.5\times10^8\,M^{-1}s^{-1}$ and $2\times10^{-2}\,s^{-1}$, respectively. $[ED]_0$ (nM) were: 1, 10; 2, 20; 3, 50; 4, 100; 5, 200; 6, 400; 7, 1000. Concentration of DDT–lipase complexes are expressed as percentages of $[ED]_0$.

$$\frac{d[E'D_2]}{dt}=k_3[ED_2] \tag{4'}$$

$$\frac{d[D]}{dt}=Q-\left(\frac{d[ED]}{dt}+2\frac{d[ED_2]}{dt}+9\frac{d[E'D_2]}{dt}\right) \tag{6'}$$

$$=Q-\left(\frac{d[ED_2]}{dt}+8\frac{d[E'D_2]}{dt}\right)$$

where Q is the rate of ligand addition, or the rate of increase in total (free plus bound) ligand concentration per second.

Figure 6 shows simulation with fixed values for k_2 and k_3. It is seen from Fig. 6c (at $T=0$) that the effect of dilution is also to increase the E^*D_9 formation. Simulation with more complex equations involving k_4 gave practically the same values for the concentrations of each species when k_4 values larger than $5.5\times10^9\,M^{-1}s^{-1}$ were used. Thus, simulation revealed that variation in the concentrations of each complex species in the final reaction mixtures as a function of time of addition, T, yields k_2 and k_3 values (rather than a k_2/k_3 ratio).

Data obtained with three different $[ED]_0$ as shown in Fig. 5 were used in a nonlinear least squares fit to the equations (Fig. 5). The k_2 ($M^{-1}s^{-1}$) and k_3 (s^{-1}) were estimated at $1.59\times10^8\pm2.8\times10^6$ and $2.15\times10^{-2}\pm1.5\times10^{-4}$ at 50 nM of $[ED]_0$, $1.59\times10^8\pm1.7\times10^6$ and $2.15\times10^{-2}\pm1.7\times10^{-4}$ at 200 nM, and $1.82\times10^8\pm2.1\times10^6$ and $2.16\times10^{-2}\pm1.5\times10^{-4}$ at 1000 nM, respectively. The values are coincident with the values obtained above.

Discussion

Comparison of k_1, k_2 and k_4 by competitive binding experiments and estimation of k_2 and k_3 by following the reaction with time provided the values of rate constant at each step, as shown in Table III. The k_3 value, $0.021\,s^{-1}$, obtained from the time course of reaction of ED_2 to $E'D_2$ coincided with the value obtained from the time lag of activation by DDT in the overall reaction, E to E^*D_9, assuming that step 3, ED_2 to $E'D_2$ is a rate-limiting step. Simulation with the rate constants obtained here gave the same titration curves as in the previous reports.[1] Although we have no substantial evidence, the present kinetic study supports the existence of the precursory complex, ED_2.

The effect of slow addition of DDT on the DDT–lipase complex formation was successfully explained and thereby a k_2 value was first obtained together with the k_3 value. Step 3 involving k_3 has been considered to accompany a conformation change of the lipase.[1b] The k_3 value seemed to be acceptable, as the values for conformation changes are known to be in a wide range and the limitation is that the maximum value rarely exceeds $10^4\,s^{-1}$.[6] As to the k_2 value, however, the large value $1.6\times10^8\,M^{-1}s^{-1}$ was not expected when considering that step 2 including k_2 is a slow step in the overall system: the values for k_1 and k_4 calculated on the basis of the k_2 value exceeds those for usually accepted maximum values for the second order rate constant of association (k_{ass}) for enzyme–substrate. So, the k_2 value was directly estimated by following the reaction at low concentrations of reactants. The resultant value coincided well with the above value.

The upper limit of rate constant of encounter for bimolecular reaction (k_e) is dependent on diffusion constants (D) and radii (r) of reactant molecules and is expressed by the Smoluchowski equation.[7] For enzyme–substrate association, theoretical values for the upper limit of k_e are about $10^{10}\,M^{-1}s^{-1}$.[8] As a case similar to ours, Hiromi[6b,c] described that k_e for spherical reactant molecules, protein with D, $8\times10^{-7}\,cm^2\,s^{-1}$ and r, 23 Å (molecular weight of *ca.* 45000) and ligand with D, $52\times10^{-7}\,cm^2\,s^{-1}$ and r, 8 Å (molecular weight, *ca.* 400) is $1.4\times10^{10}\,M^{-1}s^{-1}$ (the molecular weights of the lipase and DDT are 45000 and 354.5, respectively). In addition, for the rate constant of effective collision, the ratio of effective area (site area) to total molecular surface area (1/10 to 1/100) is to be considered as a steric factor.[6b] Reported values for larger k_{ass} among those for enzyme–substrate association are in the range of 10^8 to 10^7 (or 10^6),[6,8,9] although $2\times10^9\,M^{-1}s^{-1}$ was reported for superoxide dismutase.[10] In this context, the values, $1.2\times10^{10}\,M^{-1}s^{-1}$ for k_1 and $9\times10^9\,M^{-1}s^{-1}$ for k_4 are apparently unusual, although the comparison with the values for enzyme–substrate complex seems to be severe, since, in the binding of substrate, the binding force is utilized to bring the substrate molecule close to its transition state of the reaction.[11] Apart from enzyme–substrate association, k_{ass} values of $2.4\times10^9\,M^{-1}\,s^{-1}$ for iodide ion to Rabbit liver aldolase bound etheno-AMP[12] and 7×10^9 (or near $10^{11}\,M^{-1}s^{-1}$ at

vanishing ion concentration) for the association of *E. coli lac* repressor with its operator[13] were reported.

The binding of DDT to the lipase is very tight. The dissociation constant of E^*D_9 is smaller than 6×10^{-12} M as estimated by the titration experiment[1] while the usual values for enzyme–substrate systems are in a range of 10^{-3} to 10^{-7} M. It is conceivable that strong hydrophobic interaction is operative between the hydrophobic portions of DDT and the lipase with an average hydrophobicity of 1270 cal/residue (the usual values lie between 1000 and 1100 cal/residue).[14] Hydrophobic forces are considered to provide the most important driving force for noncovalent intermolecular interactions in aqueous solution.[11] Although it is accepted that an encounter of reactant molecules is facilitated when the reactant molecules are charged oppositely,[6b,9,10] the effect of hydrophobic interaction on diffusion-controlled reaction rate constants is unknown, as far as we are aware of. It is likely that the hydrophobic interaction between the hydrophobic lipase and DDT molecules largely contributes to the enhancement of the rate constant, like electrostatic interaction. It seems also possible that the hydrophobic ligand molecule is temporally captured by the lipase molecule (or DDT–lipase complex) at its unspecific hydrophobic surface occupying a large area and then the ligand molecule slides to bind to the particular binding site. Different approaches are necessary for this explanation to be plausible, but the idea that the unusually high k_{ass} of *E. coli lac* repressor with its operator is not essentially due to electrostatic attraction but rather to the unspecific binding of the repressor to nonoperator DNA with subsequent diffusion along the chain is suggestive.[13,9c]

It was inexplicable that $E'D_2$ simultaneously binds seven DDT molecules to form E^*D_9. The kinetic study showed that the reaction is of the first order with respect to DDT and experiments with HPLC suggested the possible formation of precursory 9 : 1 DDT–lipase complexes in the freshly mixed solution of $E'D_2$ and DDT. The ligand molecule may bind to the seven particular binding sites at random or sequentially or may bind to unspecific hydrophobic site(s) to produce the precursory complexes with enhanced activity toward tripropionin, which are transformed to stable E^*D_9. The sequential binding of the ligand to specified sites is less likely, as a rate constant of at least 6.3×10^{10} $M^{-1}s^{-1}$ is required for each step.

As shown in the present study, the slow addition of ligand seems to provide a method of analyzing a sequential binding system in which two rapid steps of second order binding reaction are linked by a slow step of first order conversion of a ligand–protein complex without binding ability into an active complex. It is noteworthy that, as shown in Fig. 6a, a reproducible result is difficult to obtain when DDT is manually added to the ED at once, if k_3 is much larger.

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