Modification of Tryptophan Residues on *Rhizopus delemar* C-Lipase Binding Chlorinated Pesticide by 2-Hydroxy-5-nitrobenzylation

Hiroyuki KANEKI, Miyuki AZETA, Daiji KOHARA, Shigehisa KYOSAKA and Mitsuya TANAKA*

School of Pharmaceutical Sciences, Toho University, Miyama 2-2-1, Funabashi-shi, Chiba 274, Japan. *Received October 14, 1991*

Comparative studies were carried out on the interaction of *Rhizopus delemar* C-lipase with 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), 2,2-bis(4-chlorophenyl)ethane (DIM), dichlorobenzophenone (DCBP) and aldrin by means of 2-hydroxy-5-nitrobenzylation of tryptophan (Trp) residues on the 1:1, 2:1 and 9:1 (7:1 with aldrin) pesticide–lipase complexes.

2-Hydroxy-5-nitrobenzylation was carried out under three conditions: modification with a water-soluble modification reagent, with the same reagent in the presence of olive oil emulsion and with a fat-soluble modification reagent in the presence of emulsion. The Trp residues involved in ligand-binding were specified in terms of their modification patterns.

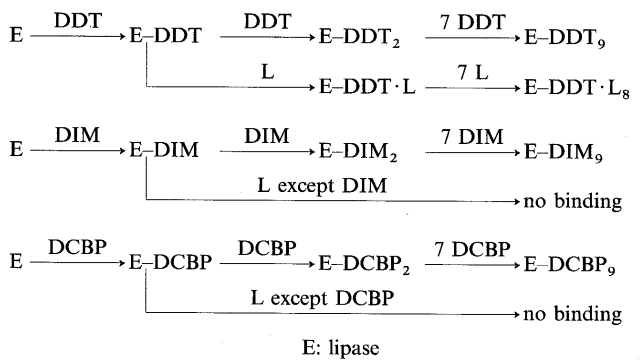
Modification revealed that the binding of pesticide involves five exposed Trp residues. The modification patterns are distinctly different depending on the sort of pesticide. This is consistent with the previous observation that the binding of the above four pesticides affects the binding properties of the lipase quite differently depending on the sort of pesticide. It is suggested for DDT, DIM and DCBP that the binding of the first pesticide molecule, which governs the ensuing complex formation, involves the same Trp residue. This would indicate the presence of three overlapping binding sites for each of three pesticides. On the other hand, firstly binding two aldrin molecules bind to a region not involving a Trp residue.

Keywords *Rhizopus delemar*; lipase; chlorinated pesticide; ligand binding; hydroxynitrobenzylation; modification; tryptophan

The binding of protein and ligand is an interesting subject in regard to substrate–enzyme complex formation, drug–protein interaction and so on. Until recently, much information has been accumulated, especially on the interaction of serum albumin and various drugs, where binding is discussed on the basis of the knowledge of the primary structure of the protein.[1)]

Rh. delemar C-lipase is a triglyceride lipase, a single chain protein with a molecular weight of 41300 and possesses eitht tryptophan (Trp) residues and a high average hydrophobicity of 1270 cal/residue.[2)] Five of the eitht Trp residues are known to be exposed to water on the surface of the lipase molecule and are expected to be concerned with the binding of hydrophobic ligand, such as chlorinated pesticide. Its primary structure has not been reported.

It has previously been reported that chloroethane pesticides and structurally related pesticides, such as 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), 2,2-bis(4-chlorophenyl)ethane (DIM) and dichlorobenzophenone (DCBP) and cyclodiene pesticide such as aldrin, bind so tightly to the lipase in a stepwise manner that 1:1, 2:1 and fully liganded (9:1 for chloroethanes, 7:1 for aldrin) pesticide–lipase complexes are able to exist as a single species in an aqueous solution.[3)] Another distinctive feature of the binding is ligand-specific cooperativity of the binding in the case of DDT, Dimic and DCBP.[3a)] As shown in Chart 1, formation of 1:1-complex determines the sort of pesticide molecule to be bound subsequently and the enzyme activity of fully liganded complex toward tripropionin as well. This concept was presented merely on the basis of titration experiments (plots of enzyme activity *versus* [pesticide]/[enzyme]) and binding studies (determination of unbound and bound ligand by isopropyl ether extraction).



E: lipase
L: DCBP, DIM, kelthane, chlorobenzilate

Chart 1

The present investigation was aimed at obtaining evidence for the occurrence of the peculiar ligand-specific complex formation based on a chemical approach. For this purpose, the modification of the hydrophobic side-chain of Trp residues seemed promising. We intended to examine how the bound ligand protects the Trp residue(s) from the modification and how the modified Trp residue affects the ligand binding ability of the lipase.

Fortunately, Chiba *et al.*[2)] studied the modification of Trp residues on the lipase molecule by 2-hydroxy-5-nitrobenzylation using water-soluble modification reagent in the presence and absence of olive oil emulsion and fat-soluble reagent in the presence of the emulsion, and they classified the eight Trp residues into five groups with respect to the state of spatial conformation of the lipase and roles in the lipase activity. Thus, the tentative assignment of Trp residues involved in the pesticide–lipase binding seemed possible by comparison of a modification pattern (a set of numbers of modified residues in three different modifications) without knowledge of the primary structure of the lipase. The tentative assignment is expected to provide an effective guide to assign the modified Trp residues in the primary structure of the lipase for the better understanding of the nature of binding: the presence of eight Trp residues

on the molecule of various pesticide–lipase complexes will straightforwardly require numerous sequencing experiments with Trp-containing peptides.

In this paper, tentative assignment of Trp residues involved in the binding of DDT, Dimic, DCBP and aldrin to the lipase by means of modification of Trp residues is described.

Experimental

Enzyme, Reagent and Pesticide–Lipase Complexes *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.[3a] Chlorinated pesticides were purchased from Wako Pure Chemical Industries Co., Ltd., and dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide (DHNBS-Br) and 2-hydroxy-5-nitrobenzyl bromide (HNB-Br) were from Nacalai Tesque, Inc. The partition constants of HNB-Br (water/olive oil) and DHNBS-Br (olive oil/water) are 0.01 and 0.006, respectively.[2]

Solutions of pesticide–lipase complexes were readily prepared by the rapid addition of an EtOH solution (20 μl) of a stoichiometric amount of pesticide to a 0.1 M sodium acetate buffer (6 ml), pH 6.0, containing 15 to 120 nmol of lipase with stirring at room temperature.

Modification of Trp Residues on Pesticide–Lipase Complexes Trp residues were hydroxynitrobenzylated under three conditions as described by Chiba *et al.* for native *Rh.* lipase as follows.[2]

(1) W condition (W modification; modification of exposed Trp residues with water-soluble reagent, DHNBS-Br). A powder of DHNBS-Br, in a molar ratio of 400:1 to protein, was added to the solution of pesticide–lipase complex in 30 min with stirring at room temperature, and the mixture was stirred continuously for 6 h more. After removal of separated hydroxynitrobenzyl alcohol by centrifugation at 3000 rpm for 10 min, the supernatant was applied to a Sephadex G-25 column (1 × 25 cm) and the column was eluted with the same acetate buffer. Modified pesticide–lipase complex migrated as a fast-moving yellow band and was pooled in *ca.* 10 ml of eluate. The eluate was assayed for enzyme activity toward tripropionin as described previously.[3b] Sufficient trichloroacetic acid solution was added to a part of the eluate to bring the acid concentration to *ca.* 3%, and the yielded precipitate was dissolved in 0.1 N sodium hydroxide. The number of modified Trp residues per mol of lipase was calculated from the absorbance in 0.1 N NaOH at 410 nm based on the molar extinction coefficient of Trp residue treated with HNB-Br, $1.8 \times 10^4\ M^{-1}cm^{-1}$.[2,5]

(2) Ew condition (Ew modification; modification of Trp residues exposed to water in the presence of olive oil emulsion with water-soluble reagent, DHNBS-Br). Olive oil (2 ml) was added to a solution of pesticide–lipase complex and the mixture was emulsified by ultrasonication for 30 s at room temperature. Hydroxynitrobenzylation and assays were carried out in the same manner as in W modification.

(3) Eo condition (Eo modification; modification of Trp residues exposed to oil with fat-soluble reagent, HNB-Br). The same manipulation as that described in Ew condition was conducted except for the use of HNB-Br (in a molar ratio of 400:1 to protein) dissolved in acetone (100 μl) instead of DHNBS-Br.

Modified Trp residues (mol per mol of lipase) with enzyme activity in parentheses were as follows. Each value represents the mean ± S.D. of 3 to 5 experiments except for mixed complexes binding aldrin. Activity is relative to the activity of unmodified pesticide–lipase complex. N.D. represents activity not detectable.

Native lipase: W, 5.1 ± 0.22 (0.93 ± 0.030); Ew, 4.2 ± 0.12 (0.49 ± 0.053); Eo, 3.8 ± 0.19 (N.D.). E-DCBP: W, 4.0 ± 0.08 (0.98 ± 0.020); Ew, 4.0 ± 0.20 (0.42 ± 0.034); Eo, 3.0 ± 0.25 (0.03 ± 0.020). E–DDT: W, 3.9 ± 0.21 (0.94 ± 0.038); Ew, 3.9 ± 0.20 (0.48 ± 0.021); Eo, 3.1 ± 0.24 (0.06 ± 0.055). E–DIM: W, 3.9 ± 0.12 (0.96 ± 0.024); Ew, 3.8 ± 0.22 (0.48 ± 0.045); Eo, 2.9 ± 0.16 (N.D.). E–$DCBP_2$: W, 3.8 ± 0.23 (0.99 ± 0.040); Ew, 4.1 ± 0.18 (0.41 ± 0.044); Eo, 3.0 ± 0.15 (0.04 ± 0.040). E–DDT_2: W, 2.9 ± 0.11 (0.94 ± 0.015); Ew, 1.0 ± 0.06 (0.49 ± 0.034); Eo, 5.2 ± 0.18 (N.D.). E–DIM_2: W, 2.9 ± 0.12 (0.95 ± 0.033); Ew, 3.8 ± 0.34 (0.49 ± 0.021); Eo, 2.1 ± 0.09 (N.D.). E–$DCBP_9$: W, 1.0 ± 0.05 (0.97 ± 0.024); Ew, 0.95 ± 0.033 (0.46 ± 0.022); Eo, 3.1 ± 0.19 (N.D.). E–DDT_9: W, 1.9 ± 0.09 (0.05 ± 0.038); Ew, 0.94 ± 0.028 (0.54 ± 0.028); Eo, 2.1 ± 0.10 (N.D.). E–DIM_9: W, 0.1 ± 0.13 (0.91 ± 0.015); Ew, 1.0 ± 0.09 (0.49 ± 0.016); Eo, 2.1 ± 0.22 (N.D.). E–aldrin: W, 4.9 ± 0.21 (1.06 ± 0.060); Ew, 3.8 ± 0.16 (0.43 ± 0.019); Eo, 3.6 ± 0.25 (N.D.). E–$aldrin_2$: W, 4.8 ± 0.12 (1.06 ± 0.076); Ew, 3.8 ± 0.26 (0.42 ± 0.046); Eo, 3.8 ± 0.32 (N.D.). E–$aldrin_7$: W, 3.7 ± 0.22 (N.D.); Ew, 1.0 ± 0.12 (0.24 ± 0.053); Eo, 3.7 ± 0.29 (N.D.). E–DDT·DIM: W, 3.0 ± 0.12 (0.93 ± 0.028); Ew, 1.0 ± 0.066 (0.43 ± 0.052); Eo, 4.9 ± 0.15 (N.D.). E–DDT·DCBP: W, 3.2 ± 0.18 (0.95 ± 0.020); Ew, 0.94 ± 0.088 (0.48 ± 0.044); Eo, 5.2 ± 0.22 (N.D.). E–DDT·$aldrin_2$: W, 4.1 (0.98); Ew, 4.0 (0.40); Eo, 2.8 (N.D.). E–DDT_2·$aldrin_2$: W, 2.9 (0.98); Ew, 0.84 (0.38); Eo, 4.7 (N.D.).

Binding Experiments by Extraction with Isopropyl Ether An EtOH solution (50 μl) containing 100 nmol of pesticide was added to 50 ml of 0.01 M acetate buffer (pH 6.0) containing 10 nmol of native lipase modified under the W, Ew and Eo conditions, and the mixture was incubated at 30 °C for 30 min. The incubated mixture was extracted three times by shaking for 10 min with each 50 ml of isopropyl ether. For the determination of unbound (or weakly bound) pesticide, the isopropyl ether extract was evaporated to dryness, the residue was dissolved in a small amount of ethanol and the amount of pesticide was determined by gas chromatography. For the determination of lipase activity, the water layer was kept under reduced pressure at 15 °C for 15 min to remove the remaining solvent and the lipase activity was assayed with tripropionin as a substrate. For the determination of bound lipase, the pH of the water layer was adjusted to 4.0 with 0.01 N HCl and incubated at 30 °C for 30 min to release pesticide from lipase, and the liberated pesticide was extracted with isopropyl ether and determined as described for unbound pesticide.

Pesticide bound to modified pesticide–lipase complexes was determined in a similar manner.

Results

Trp residues on the pesticide–lipase complexes were modified by 2-hydroxy-5-nitrobenzylation to give Trp-modified pesticide–lipase complexes. An initial stoichiometric amount of pesticide was recovered when a solution of the modified complex was extracted with isopropyl ether at pH 4.0.

In the present study, the Trp residues are numbered from 1 to 8 and designated as T1, T2, ···, T8 instead of No. 1 to No. 8 as was used by Chiba *et al.*[2] They modified the lipase under three conditions: with water-soluble modification reagent without addition of olive oil (W modification), with the same reagent in olive oil emulsion (Ew modification) and with fat-soluble modification reagent in emulsion (Eo modification), and classified them into five groups. The following brief explanation according to Chiba *et al.* was used here as principal criteria for the assignment of modified residues (the modification pattern for native lipase is shown in Table I). T1 and T2 are modified under the W and Eo conditions, exposed to water and also to oil in the presence of olive oil (presumably located on the hydrophobic surface area of the molecule) and are non-essential to the lipase activity. T3, T4 and T5 are modified under the W and Ew conditions but not under the Eo condition, exposed only to water (presumably on a hydrophilic area) and are non-essential to the activity. T6 is modified under the Ew and Eo conditions, located at the binding site for substrate, exposed both to oil and to water in olive oil emulsion. The modification of T6 decreases the lipase activity to about 50%. T7 is modified only under the Eo condition, exposed only to oil. The modification of T7 completely inactivates the enzyme. T8 is buried in the molecule and modified only when the protein is denatured with urea.

Although Chiba *et al.* modified the lipase at pH 5.0, the modification was carried out at pH 6.0, since a release of pesticide from 9:1 complexes was observed when a solution of 9:1 complex was extracted with isopropyl ether at pH 5.0 (but not at pH 5.5). The same results were

TABLE I. Tentative Assignment of Modified Trp Residues[a] in Lipase Binding Chlorinated Pesticides

Lipase and pesticide–lipase complexes	W condition								Ew condition								Eo condition							
	N[b]	T1	T2	T3	T4	T5	T6	T7	N[b]	T1	T2	T3	T4	T5	T6	T7	N[b]	T1	T2	T3	T4	T5	T6	T7
Native lipase	5	○	○	○	○	○	—	—	4	—	—	○	○	○	○	—	4	○	○	—	—	—	○	○
Lipase binding chlorinated pesticides																								
1 : 1-Complexes																								
E–DCBP	4	—	○	○	○	○	—	—	4	—	—	○	○	○	○	—	3	—	○	—	—	—	○	○
E–DDT	4	—	○	○	○	○	—	—	4	—	—	○	○	○	○	—	3	—	○	—	—	—	○	○
E–DIM	4	—	○	○	○	○	—	—	4	—	—	○	○	○	○	—	3	—	○	—	—	—	○	○
2 : 1-Complexes																								
E–$DCBP_2$	4	—	○	○	○	○	—	—	4	—	—	○	○	○	○	—	3	—	○	—	—	—	○	○
E–DDT_2	3	—	—	○	○	○	—	—	1	—	—	—	—	—	○	—	5	—	—	○	○	○	○	○
E–DIM_2	3	—	—	○	○	○	—	—	4	—	—	○	○	○	○	—	2	—	—	—	—	—	○	○
9 : 1-Complexes																								
E–$DCBP_9$	1	—	○	—	—	—	—	—	1	—	—	—	—	—	○	—	3	—	○	—	—	—	○	○
E–DDT_9	2	—	—	—	—	—	○	○	1	—	—	—	—	—	○	—	2	—	—	—	—	—	○	○
E–DIM_9	0	—	—	—	—	—	—	—	1	—	—	—	—	—	○	—	2	—	—	—	—	—	○	○
Aldrin–lipase complexes																								
E–aldrin	5	○	○	○	○	○	—	—	4	—	—	○	○	○	○	—	4	○	○	—	—	—	○	○
E–$aldrin_2$	5	○	○	○	○	○	—	—	4	—	—	○	○	○	○	—	4	○	○	—	—	—	○	○
E–$aldrin_7$	4	○	○	—	—	—	○	○	1	—	—	—	—	—	○	—	4	○	○	—	—	—	○	○
Mixed pesticide complexes																								
E–DDT·DIM	3	—	—	○	○	○	—	—	1	—	—	—	—	—	○	—	5	—	—	○	○	○	○	○
E–DDT·DCBP	3	—	—	○	○	○	—	—	1	—	—	—	—	—	○	—	5	—	—	○	○	○	○	○
E–DDT·$aldrin_2$	4	—	○	○	○	○	—	—	4	—	—	○	○	○	○	—	3	—	○	—	—	—	○	○
E–DDT_2·$aldrin_2$	3	—	—	○	○	○	—	—	1	—	—	—	—	—	○	—	5	—	—	○	○	○	○	○

a) Open circles indicate modified Trp residues and horizontal lines indicate unmodified Trp residues. *b*) Number of modified residues rounded to the nearest integer.

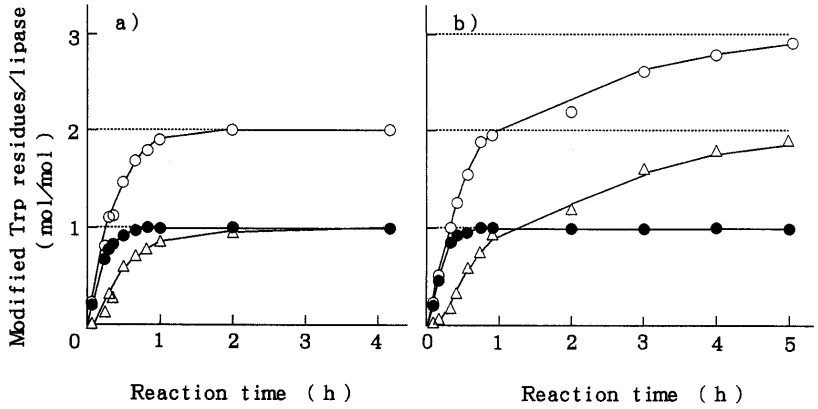


Fig. 1. Eo Modification of 1 : 1 DDT–Lipase Complex and Native Lipase Both Previously Modified under the Ew Condition

Eo modifications were carried out at enzyme concentrations of 12.5 μM at pH 6.0. a) 1 : 1 DDT–lipase complex previously modified under the Ew condition. b) Native lipase previously modified under the Ew condition. ○, total modified Trp residue; ●, modified Trp residue (T7) estimated from the loss of enzyme activity; △, value for total minus value for T7.

obtained at pH 5.0 and 6.0. Tentative assignment of the modified tryptophan residues is presented in Table I.

Modification of Lipases Binding DDT, DIM and DCBP

A. 1 : 1 Pesticide–Lipase Complexes All the modified 1 : 1 complexes of chloroethane pesticides exhibited the same modification patterns. The enzyme activities were the same as in the case of native lipase. The numbers of residues modified by W and Eo modification are one residue each less than those with native lipase and the modified residues overlapping in W and Eo modification of native lipase are T1 and T2. This indicates explicitly the participation of T1 or T2 in the binding.

In Table I, the Trp residue participating in the binding in 1 : 1 complex formation of three pesticides is all arbitrarily designated as T1, since the following experiments on the rates of modification for T1 and T2 suggested that the binding of the first pesticide molecule involves the same Trp residue (T1).

The rates for the modification of T1 and T2 were compared as follows. Ew modified E–DDT was further modified by Eo modification (modification of T2 and T7). As shown in Fig. 1a, the reaction was almost completed within an hour. The Ew modified complex had its 2 Trp residues modified and lost its activity (caused by modification of T7 involved in enzyme activity). The rate of modification of T2, which is estimated by subtracting the rate of loss in enzyme activity from the rate of modification, is only a little lower than the rate for T7. On the other hand, in the Eo modification of Ew modified native lipase (modification of T1, T2 and T7), the rate of modification slowed down after the modification of 2 Trp residues and an additional residue was gradually modified in a period of 5 h, indicating

T1 to be modified much more slowly than T2 (Fig. 1b). Very similar results were obtained with E–DIM and E–DCBP, suggesting that the first molecule of three pesticides binds to the lipase at a site or sites involving T1.

B. 2:1 Pesticide–Lipase Complexes $E–DDT_2$, $E–DIM_2$ and $E–DCBP_2$ gave results which are apparently different from one another in the number of modified residues. The enzyme activities were the same as in the case of native lipase, suggesting that T6 and T7 are not related to the binding. As to $E–DIM_2$, the numbers of modified residues were reduced by 2 each in W and Eo modification compared with the case of native lipase. The modifiable residues of native lipase overlapping in W and Eo modification but not in Ew modification are T1 and T2, indicating the participation of T1 and T2 in the binding. On the other hand, the modification pattern of $E–DCBP_2$ was the same as that of E–DCBP. This indicates that the second DCBP molecule binds to a site which does not involve a Trp residue.

Modification of $E–DDT_2$ presented quite a different situation. In W modification, the decrease by 2 in modified residues compared with the case of native lipase is consistent with 2:1 complex formation. In Ew modification, however, the decrease in modified residues is 3: a decrease by 2 or less residue is expected. Moreover, in Eo modification, an increase in modified residues was observed. These results obtained by Ew and Eo modification can be explained taking a conformational change of the molecule into account. It is conceivable that the property of environment of T3, T4 and T5 residues alters from a hydrophilic to a hydrophobic one on 2:1 complex formation, which permits the access of fat-soluble reagent dissolved in oil.

Modification of 2:1 mixed pesticide–lipase complexes, 1:1:1 E–DDT·DIM and 1:1:1 E–DDT·DCBP, which are prepared starting from E–DDT, gave results essentially the same with those for $E–DDT_2$. This is in accord with the fact that these complexes bind additional ligand molecules to form mixed 1:8:1 complexes with enhanced activities like $E–DDT_9$ (Chart 1).

C. 9:1 Pesticide–Lipase Complexes Modification of $E–DIM_9$ and $E–DCBP_9$ is readily explained as in Table I by comparison with the modification of the corresponding 2:1 complexes. In W and Ew modification of $E–DIM_9$ and $E–DCBP_9$ and Eo modification of $E–DDT_9$, the number of modified residues decreased by 3 when compared with those in the corresponding 2:1 complexes. On W modification $E–DDT_9$ had 2 Trp residues modified and unexpectedly lost its entire activity, indicating the modification of T7 (involved in the enzyme activity) and probably T6 (at the binding site for substrate). These findings imply that T3, T4 and T5 are not modified owing to the protection by the binding of 7 ligand molecules from modification, and that, in $E–DDT_9$, T6 and T7 are exposed on the 9:1 complex formation and modified under the W condition.

Modification of Lipase Binding Aldrin In the case of aldrin, the modification patterns of 1:1 and 2:1 aldrin–lipase complexes (E–aldrin and $E–aldrin_2$) were the same as of native lipase as shown in Table I, indicating that Trp residues do not relate to the binding of the first and second aldrin molecules. This coincides with the previous indication that 2 molecules of aldrin could bind to the lipase regardless of whether 2 molecules of DDT were bound to the lipase or not (Chart 2).[3b] This view is also supported by the modification patterns of 1:2:1 and 2:2:1 DDT aldrin–lipase complexes (Table I), which are the same with those for E–DDT and $E–DDT_2$, respectively.

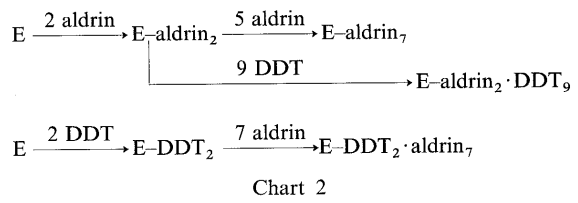


Chart 2

TABLE II. Binding of Pesticides to the W, Ew and Eo Modified Lipases

Pesticide (100 nmol)	Unbound pesticide (nmol)	Relative activity[a]	Bound pesticide (nmol)
W modified lipase (10 nmol)			
DDT	97±4.5	1.03±0.094	N.D.
DIM	98±4.4	0.96±0.055	N.D.
DCBP	98±3.8	0.94±0.021	N.D.
Ew modified lipase (10 nmol)			
DDT	82±3.3	1.03±0.048	18±2.3
DIM	82±4.3	0.96±0.054	17±1.9
DCBP	80±3.5	0.95±0.032	19±2.1
Eo modified lipase (10 nmol)			
DDT	102±1.9	N.D.	N.D.
DIM	101±3.4	N.D.	N.D.
DCBP	100±2.4	N.D.	N.D.

Lipase (10 nmol) was treated with 100 nmol of pesticide at pH 6.0. Each value represents the mean±S.D. of 2 to 5 experiments. N.D., not detectable. *a*) The ratio of activity between before and after treatment of isopropyl ether.

The modification pattern of $E–aldrin_7$ suggested that T3, T4 and T5 participate in the binding of 5 ligand molecules. On W modification of $E–aldrin_7$, it lost its entire enzyme activity, like $E–DDT_9$. The exposure of T6 and T7 both in $E–DDT_9$ and $E–aldrin_7$ seems to be concerned with the mechanism of activation. The change in the environment of T3, T4 and T5, which is observed in the modification of $E–DDT_2$, was not observed.

Chloroethane Pesticide-Binding Ability of Modified Native Lipase The binding ability was examined as follows. A preincubation mixture of lipase and excess pesticide at pH 6.0 was extracted with isopropyl ether to remove the unbound (or weakly bound) pesticide, and then the bound pesticide was recovered by extraction with isopropyl ether at pH 4.0. As shown in Table II, only Ew modified lipase, which has 2 unmodified Trp residues, T1 and T2, binds 2 molecules of each pesticide. On the other hand, Eo modified lipase, though having 3 unmodified Trp residues, T3, T4 and T5, can bind none of pesticides. This suggests that T1 and T2 are of primary importance in the binding and T3, T4 and T5 serve a role in the formation of the subsequent 9:1 complexes from the 2:1 complexes.

Discussion

The binding of some chlorinated pesticides to *Rh.* lipase seems very tight as seen in the binding constant of $E–DDT_9$ which was estimated at larger than $1.6 \times 10^{11}\,M^{-1}$ from titration experiments.[3a] The modified pesticide–lipase complexes were found to retain the initial stoichiometric

amounts of pesticide. This tight binding facilitated the present modification experiments.

The modification patterns (a set of the numbers of Trp residues modified under the W, Ew and Eo conditions) obviously differ from each other according to the sort of ligand used. This coincides well with the peculiar activity of the pesticides as effectors as shown in Chart 1. It is known that ligand binding not only protects amino acid residues located at the binding sites from modification but also often induces conformational changes which affect the accessibility of the modification reagent to a side-chain of amino acid residue near or distant from the occupied binding site.[6] The activation of pancreatic lipase caused by the binding of triglyceride has recently been reported to accompany a flap of a loop covering the active site and a subsequent conformational change, which permits the access of triglyceride to the catalytic site.[7]

In the modification of native *Rh.* lipase, the Trp residue T6, which is concealed in the absence of olive oil emulsion, was modified under the Ew and Eo conditions (in the presence of triglyceride), indicating that a conformational change occurs in the presence of triglyceride. Therefore the assignment of modified Trp residues was done, taking this point of view into consideration. In the present studies, generally, the number of modified Trp residues decreased with an increasing number of bound ligands, though with some exceptions. This suggests that generally ligand binding exhibited protection here. In the modification of E–DDT_2, however, the number of modified residues was increased by ligand binding in Eo modification and excessively decreased in Ew modification, clearly indicating the occurrence of conformational change. In addition, unexpected modification of the Trp residue at the catalytic site was found in the W modification of E–DDT_9 and E–$aldrin_7$, which are the fully liganded lipase with enhanced activity toward tripropionin. In this situation, it is conceivable that an important confomational change occurs linked with the stepwise formation of activated complexes with DDT and aldrin but not with DIM and DCBP.

This postulate leads to the following outline of pesticide–lipase binding. As to DDT, DIM and DCBP, the first ligand molecule binds to a site involving one of two Trp residues, T1 and T2, located on a hydrophobic region. Binding of the first ligand molecule of three pesticides seems to protect the same Trp residue (T1) from modification, as judged by comparison of the modification velocities for T1 and T2, assuming that the effect on the modification velocity of bound ligand and modified Trp residues is negligible. It is likely that the binding of the first molecule affects the properties of lipase in three different directions (as shown in Chart 1) by binding to their respective binding sites overlapping each other rather than by binding to the same site. The modification patterns for the three pesticides were the same although subtle structural change may occur corresponding to the different effects of the pesticides. On the other hand, a remarkable difference was

$$E \xrightarrow[\text{fast}]{DDT} E\text{–}DDT \xrightarrow[\text{slow}]{DDT} E\text{–}DDT_2 \xrightarrow[\text{very slow}]{} E'\text{–}DDT_2 \xrightarrow[\text{very fast}]{7\ DDT} E^*\text{–}DDT_9$$

asterisk represents the lipase with 4.4-fold activity toward tripropionin

Chart 3

observed in the modification of 2:1 complexes. Modification suggests that a second molecule of DDT and DIM binds to a site involving T2 and a second molecule of DCBP to a site not involving Trp residues, and that, in E–DDT_2, the Trp residues, T3, T4 and T5, which have been exposed only to water in a 1:1 complex, come to be exposed to olive oil as well.

A sequential mechanism has been presented for the binding of DDT by the lipase as shown in Chart 3.[3a,8] The findings that the first DDT molecule exclusively binds to a site involving T1 support the sequential mechanism. It is also evident that the very slow step, E–DDT_2 to E′–DDT_2, involves an important conformational change.

Additional ligand molecules of DCBP, DDT or DIM seem to bind to 2:1 complexes at the same binding sites (an area?) involving the exposed Trp residues, T3, T4 and T5, although it is not ruled out that some of the additional pesticide molecules bind at other sites not involving Trp residues.

The presence of common binding sites for DDT and aldrin (T3, T4 and T5) and binding sites for the firstly binding two aldrin molecules different from the site for DDT was predicted by the previous observation that mixed used of DDT and aldrin as ligands gave E–$DDT_9 \cdot aldrin_2$ or E–$DDT_2 \cdot aldrin_7$ but not a more highly liganded complex, E–$DDT_9 \cdot aldrin_7$.

Direct evidence for the assignment of Trp residues has to await the study on Trp-containing petpides obtained from modified complexes.

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8) In Chart 3, the previous designation of enzyme species, E′D_2→ED_2 is revised as ED_2→E′D_2 in the light of the present study.