Enhancing the Enantioselectivity of *Candida* Lipase Catalyzed Ester Hydrolysis via Noncovalent Enzyme Modification

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Abstract: When the lipase of *Candida cylindracea* was treated with deoxycholate and organic solvents (ether–ethanol, 1:1), the enzyme apparently unfolded and refolded to generate a more stable conformer, termed lipase A-form. This lipase A-form was found to be considerably more enantioselective than the native enzyme toward the hydrolysis of a variety of (±)-arylpropionic and (±)-phenoxypropionic esters.

The complexity and diversity of natural products are the result of the remarkable catalytic power of enzymes. Virtually any imaginable organic molecule has been biosynthesized as a consequence of the catalytic activities of enzymes. Hence, it would seem appropriate to make use of these biocatalysts in preparative organic chemistry. Because enzymes catalyze reactions with high efficiency and, more importantly, with unparalleled substrate and stereochemical specificity under mild conditions, it is no surprise that biocatalytic methods are beginning to be widely exploited as a complement to nonenzymatic methods, especially for the preparation of enantiomerically pure compounds.

In recent years, interest in the use of hydrolytic enzymes as synthetic chiral catalysts has risen rapidly.[1] The extracellular microbial lipases (EC 3.1.1.3) are particularly suited for synthetic applications, because they have broad substrate specificites and have no coenzyme requirement for catalysis. Although the natural substrates of lipases are acylglycerols, they can also catalyze the hydrolysis of a wide range of artificial water-insoluble esters with a high degree of enantioselectivity. Lipases are uniquely characterized by their ability to catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and an aqueous phase in which the enzyme is dissolved. Consequently, severe substrate and product inhibition of the enzyme is seldom observed. The synthetic utility of microbial lipases is manifested by the ample number of optically active compounds of varying chemical diversity that have been successfully prepared.[2]

Despite their many promising features, the predictability and reliability of biocatalytic methods must be further refined before they can be construed as standard procedures. There are only a limited number of commercial lipases accessible to synthetic chemists at the present time, and moreover, most biocatalytic reactions are only partially stereoselective. As the discovery of new lipases with well-defined stereochemical properties is a slow, painstaking process, it is necessary to develop methods to extend the usefulness of existing commercial lipases, so that the number required for synthetic applications may be reduced considerably. Several approaches have been used to improve the enantioselectivity of biocatalytic reactions. Besides the conventional optimization of reaction conditions such as temperature[3] and pH,[4] these include the modification of the substrate molecule,[5] the recycling of the product,[6] the use of nonaqueous media for esterification reactions,[7] allosteric activation,[8] and enantioselective inhibition.[9]

Table I. Enantioselective Hydrolysis of (±)-Arylpropionic Esters Catalyzed by Crude *Candida* Lipase

$$(\pm)\text{-}R_1CH(CH_3)CO_2R_2 \rightarrow \underset{\mathbf{1}}{} (S)\text{-}(+)\text{-}R_1CH(CH_3)CO_2H + (R)\text{-}(-)\text{-}R_1CH(CH_3)CO_2R_2$$
$$\mathbf{2} \qquad\qquad \mathbf{3}$$

substrate	reaction time, h	ee_S	ee_P	c	E
1a	120	0.43	0.73	0.37	10
1b	120	0.25	0.52	0.32	4
1c	72	0.13	0.80	0.14	10
1d	48	0.43	0.99	0.31	>100
1e	48	0.70	0.95	0.42	50
1f	96	0.90	0.95	0.48	>100

Table II. Purification of Lipase (A-Form) from *Candida cylindracea*

fraction	protein, mg	total act., units	sp act., units/mg	yield, %
water extraction	107	71 958	670	100
50% $(NH_4)_2SO_4$ precipitation	58	50 546	878	70
SP-Sephadex C-50 chromatography	39	35 382	900	49
deoxycholate and organic solvent treatment	9	14 440	1600	20
second SP-Sephadex C-50 chromatography	5	9950	1990	14

Our continuing interest in developing new strategies for improving the enantioselectivity of biocatalytic reactions led us to the discovery of yet another strategy. In this paper, we describe the experimental details that are important for the noncovalent modification of the protein conformation of the *Candida* lipase leading to a marked enhancement in the enantioselectivity in the resolution of racemic carboxylic esters.

Results

Crude *Candida cylindracea* lipase has been widely used for the kinetic resolution of racemic carboxylic acids and alcohols.[2] Our interest in developing improved methods for the preparation of optically active arylpropionic acids led us to examine the enantioselective hydrolysis of various (±)-2-arylpropionic esters by this crude lipase (Table I). While the enzymatic hydrolyses of **1d–f** were found to be highly enantioselective, with E values ranging from 50 to 100, the hydrolyses of **1a–c** proceeded with low degrees of enantioselectivity, with E values ranging from 4 to 10.

To ascertain that the crude lipase preparation contained no competing enzymes of opposite stereochemical preference and that

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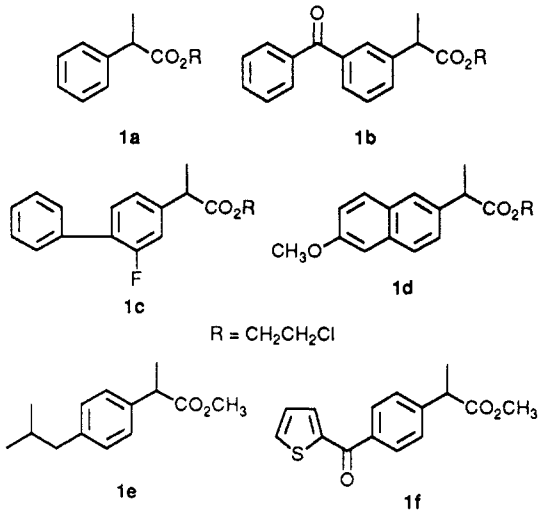

these substrates were all hydrolyzed by a single enzyme protein, the *Candida* lipase was purified to homogeneity. This was accomplished via a five-step procedure (Table II), similar to the published procedure described by Tomizuka et al.[10] Using this purified lipase (A-form), we reexamined substrates **1a–c**; to our surprise, the purified lipase (A-form) was found to be highly enantioselective toward these substrates, with E values of >100 (Table III).

The enantioselective hydrolysis of a series of phenoxypropionic esters catalyzed by the crude lipase and the purified lipase (A-form) ws also compared (Table III).

$$(\pm)\text{-ROCH(CH}_3\text{)CO}_2\text{CH}_3 \xrightarrow[\text{H}_2\text{O}]{\text{lipase}} (R)\text{-(+)-ROCH(CH}_3\text{)CO}_2\text{H} + (S)\text{-(−)-ROCH(CH}_3\text{)CO}_2\text{CH}_3$$

4 **5** **6**

4a, R = 4-ClC_6H_4–; **4b**, R = 2-ClC_6H_4–;
4c, R = 3-ClC_6H_4–; **4d**, R = 2,4-$Cl_2C_6H_3$–;
4e, R = 4-Cl-2-$CH_3C_6H_3$–

It is evident that the purified lipase (A-form) was more enantioselective toward all the substrates **4a–e**. Particularly noteworthy is the high enantioselectively observed for the enzymatic hydrolysis of **4a**; the E value was elevated to a value >100.

To determine at which step of the purification procedure the enzyme preparation became more enantioselective, (±)-**4d** used instead of olive oil as the substrate for enzymatic assay. This compound is a more sensitive indicator for detecting small changes in enantioselectivity, because the crude *Candida* lipase exhibits virtually no enantioselectivity toward **4d** ($E \simeq 1$). Table IV shows that the enantioselectivity changed markedly only after the enzyme had been treated with sodium deoxycholate and the organic solvent mixture consisting of ether–ethanol (1:1); the E value was raised from 1 to 14.

We next examined the elution profile of the lipase on a SP-Sephadex C-50 column before (step 3) and after (step 5) the treatment with deoxycholate and organic solvent. Figure 1 shows that, most of the enzyme activity resided in fractions 2–10 and a small amount of enzyme activity resided in fractions 27–32. In contrast, after treatment with deoxycholate and organic solvent, all of the enzyme activity resided in fractions 27–35 (Figure 2). This indicates that the physical properties of the enzyme have been altered. This is also reflected in the disc-gel electrophoretic pattern of the fractions during purification (Figure 3), which reveals that the lower protein band was gradually converted into the upper protein band (higher isoelectric point) as the purification progressed. Finally, all of the lower protein band was converted into the upper protein band at step 5 of the purification procedure.

While the enantioselectivity of the enzyme preparation toward **4d** at step 3 (SP-Sephadex G-50 column chromatography) of the purification procedure did not differ much from that of crude

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Table III. Comparison of the Enantioselectivity (E Value) of the Crude Lipase and Pure Lipase A-Form

substrate	E: pure lipase A-form	E: crude lipase	substrate	E: pure lipase A-form	E: crude lipase
1a	>100	10	**4b**	14	2
1b	>100	4	**4c**	12	2
1c	>100	10	**4d**	14	1
4a	>100	17	**4e**	37	2

Table IV. Enantioselectivity of the *Candida* Lipase toward (±)-**4d** at Various Stages of Purification

fraction	E
(1) water extraction	1
(2) ammonium sulfate precipitation	2
(3) SP-Sephadex C-50 chromatography	3
(4) sodium deoxycholate and ether–ethanol (1:1 v/v) organic solvent treatment	14
(5) second Sephadex C-50 chromatography	14

Table V. Summary of the Purification Steps Used To Obtain Pure Lipase B-Form

fraction	protein, mg	total act., units	sp act., units/mg	yield, %
(1) water extraction	107	71958	670	100
(2) SP-Sephadex C-50 chromatography	47	44650	950	62
(3) Sephadex G-100 chromatography	20	39900	1995	55

Table VI. Comparison of the Enantiomeric Ratio (E) of Lipase A-Form and Lipase B-Form to That of Crude Lipase

substrate	E: A-form	E: B-form	E: crude
1a	>100	21	10
4a	>100	20	15
4d	13	1	1
4e	37	3	2

Table VII. Enhancement of the Enantioselectivity of Lipase B-Form by Treatment with Deoxycholate and Organic Solvent

	E: **1a**	E: **4e**
(1) pure lipase B-form	21	3
(2) pure lipase B-form treated by deoxycholate and organic solvent	>50	25
(3) pure lipase A-form	>50	37

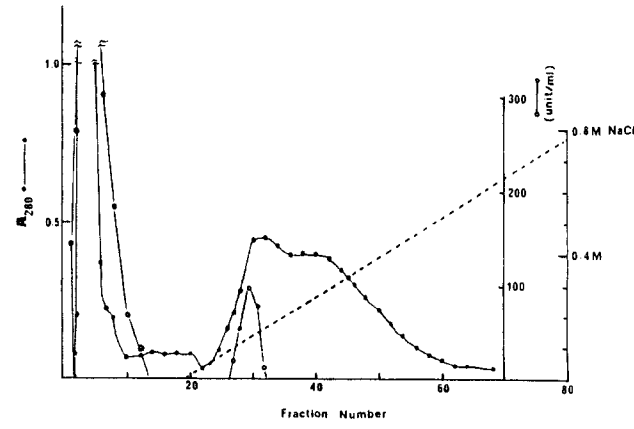

Figure 1. SP-Sephadex C-50 column chromatographic profile (step 3, Table II).

Table VIII. Kinetic Parameters of the Crude Lipase and Pure Lipase (A-Form) toward (*R*)-(+)-**4a** and (*S*)-(−)-**4a**

kinetic constants	crude lipase		pure lipase A-form	
	R-(+)	*S*-(−)	*R*-(+)	*S*-(−)
k_{cat}, s^{-1}	107 ± 54	5.7 ± 1	33 ± 13	1.1 ± 0.2
K_m, M	0.009 ± 0.002	0.008 ± 0.002	0.0008 ± 0.0005	0.003 ± 0.002
k_{cat}/K_m, M^{-1} s^{-1}	1.2×10^4	7.1×10^2	4×10^4	4×10^2
$E = (k_{cat}/K_m)_R/(k_{cat}/K_m)_S$	17		100	

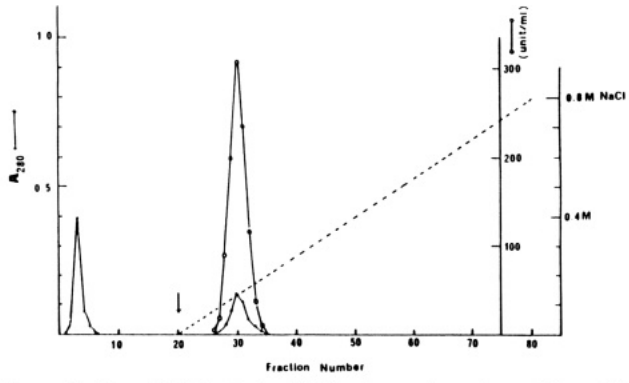

Figure 2. Second SP-Sephadex C-50 column chromatographic profile (step 5, Table II).

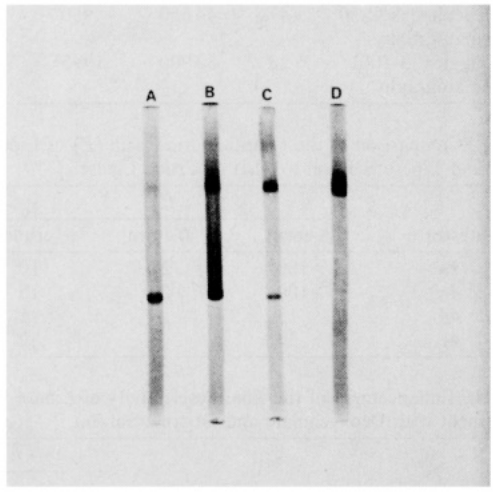

Figure 3. Disc-gel electrophoretic analyses of the fractions during purification of lipase A-form. (a) Step 1, water extraction. (B) Step 3, SP-Sephadex C-50 chromatography. (C) Step 4, sodium deoxycholate and organic solvent treatment. (D) Step 5, second SP-Sephadex C-50 chromatography.

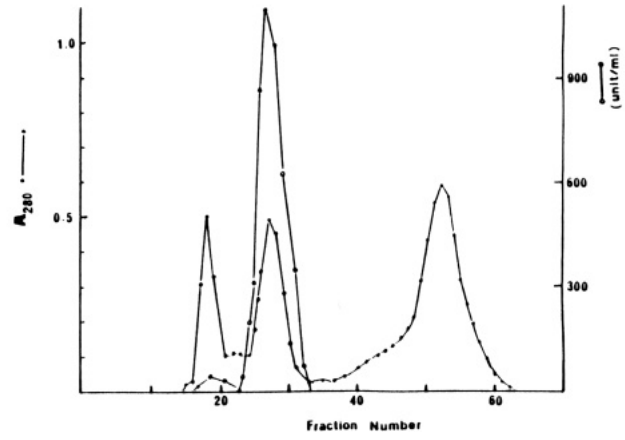

Figure 4. Sephadex G-100 column chromatographic profile (step 3, Table V).

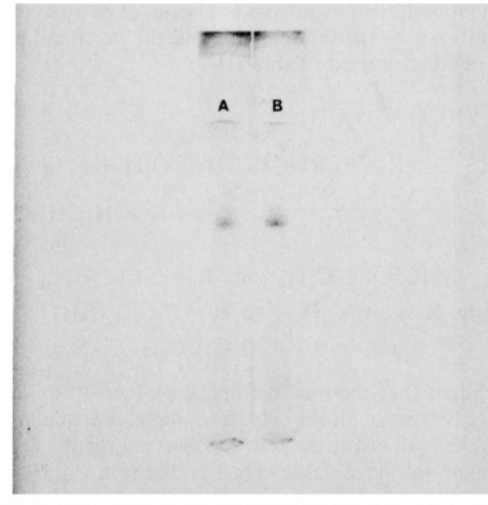

Figure 5. Disc-gel electrophoretic analysis of lipase A- and B-forms. (A) Lipase A-form. (B) Lipase B-form.

enzyme (Table IV), the disc-gel electrophoretic pattern of this preparation revealed the presence of two protein bands (lane B, Figure 3) of equal intensity. This observation suggested that the enantioselectivity of the upper protein band of step 3 and step 5 toward **4d** was different.

To resolve this question, the enzyme protein of step 3 was further purified to contain only the upper protein band. This was accomplished by chromatographing the step 3 enzyme protein on a Sephadex G-100 column (Table V). Ninety-five percent of the enzyme activity resided in the second peak (fractions 22–33, Figure 4). Disc-gel electrophoretic analysis of this fraction, designated as lipase B-form, revealed that it had the same mobility as lipase A-form (Figure 5).

The enantioselective hydrolyses of **1a**, **4a**, **4d**, and **4e** catalyzed by the crude lipase preparation and by lipase A- and B-forms are shown in Table VI. Although the *E* values for lipase B-form toward both substrates were very similar to thoose of the crude enzyme, the *E* values for lipase A-form were markedly elevated. Table VII shows the results of the treatment of lipase B-form with deoxycholate and organic solvent. It is apparent that the enantioselectivity expressed as *E* values for the hydrolyses of **1a** and **4e** was significantly enhanced upon treatment, indicating that lipase B-form was converted into A-form.

The kinetic constants k_{cat} and K_m of the crude enzyme and lipase A-form for the hydrolyses of both enantiomers of **4a** were determined in a series of competitive experiments using essentially the method described by Hwang et al.[11] The data are recorded in Table VIII. It is noteworthy that the k_{cat} and K_m of lipase A-form for both the *R* and *S* enantiomers of **4a** were lower than those for the crude enzyme. The most notable change is in the K_m for the *R*-(+) enantiomer; the crude enzyme has a K_m of 9 ± 2 mM, whereas the K_m of lipase A-form decreased to a value of 0.8 ± 0.5 mM.

Discussion

The use of deoxycholate and organic solvents for the purification of the *Candida cylindracea* lipase was first described by Tomizuka et al.[10] The physicochemical properties of the purified preparation obtained by this procedure are indistinguishable from those of our lipase A-form. This lipase is a glycoprotein with a molecular weight of 120 000 containing mannose and xylose as the carbohydrate components. It has an abundance of hydrophobic amino

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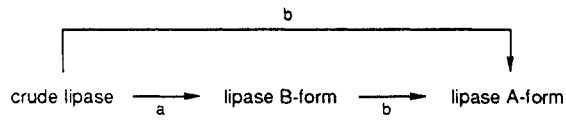
Scheme I[a]



[a] (a) Sephadex G-100 chromatography; (b) sodium deoxycholate and organic solvent (EtOH–ether, 1:1) treatment.

acid residues, especially leucine and isoleucine, and an isoelectric point around pH 4.2.

The enantioselectivity of the hydrolysis of (±)-arylpropionates and (±)-(aryloxy)-propionates was found to be considerably higher with the purified lipase (A-form) than with the crude lipase (Tables I and II). At first, one could surmise that the crude lipase preparation contained other hydrolases of opposite stereochemical preference. However, the disc-gel electrophoretic pattern of the crude preparation (Figure 3) showed that it contained only two protein bands. The major band (lower isoelectric point, P_I) had a greater mobility toward the (+)-electrode than the minor band (higher P_I). The ratio of these two bands varied during the purification procedure. For example, after the enzyme was treated with sodium deoxycholate and organic solvent, the quantity of the lipase with the high P_I increased considerably. These observations were also consistent with the chromatographic behavior of these different preparations in the SP-Sephadex G-50 column. The native lipase did not bind to the SP-Sephadex G-50 column (Figure 1). However, after the lipase was treated with deoxycholate and organic solvent, the lipase activity was adsorbed to the SP-Sephadex G-50 column and was eluted off the column with a NaCl buffer gradient (Figure 2). These apparent changes in the physical properties of the enzyme were also reflected in its enantioselective properties. Most notable was the marked increase in the enantioselectivity of the enzyme after treatment with deoxycholate and organic solvent (Table IV). Prior to the latter treatment, the lipase was in a different form, termed lipase B-form. Amino acid analyses of the A- and B-forms showed no significant differences, indicating that they are chemically identical but conformationally different. The purified lipase (B-form) has the same apparent physical properties as the A-form in terms of mobility in disc-gel and SDS electrophoresis, CD spectra, and UV spectra;[12] the only difference between these two enzyme forms is the difference in their enantiomeric specificity. The E value of lipase B-form is similar to that of the crude lipase (Table VI). When lipase B-form was treated with sodium deoxycholate and organic solvent, the E value of lipase B-form was raised to that of lipase A-form (Table VII). These results indicate that lipase B-form could be converted into lipase A-form possibly by a change in the tertiary structure of the enzyme. Moreover, the difference in the E value of crude lipase and lipase A-form was not due to contamination with other hydrolases.

From the results obtained, we envisage the relationship between the crude lipase and pure lipase A-form and B-form as shown (Scheme I). In the crude powder form, the lipase is probably associated noncovalently with low molecular weight acidic components. This supposition is consistent with the observation that the crude lipase had a greater mobility toward the (+)-electrode in disc-gel electrophoresis and was not adsorbed onto the SP-Sephadex C-50 column. Apparently, the acidic components could be dissociated from the lipase by chromatographing the preparation over a Sephadex G-100 column. This operation generated pure lipase B-form. Upon treatment with deoxycholate–organic solvent, lipase B-form unfolded and refolded to generate the more stable conformer, lipase A-form. If the crude lipase powder was treated directly with sodium deoxycholate and organic solvent, the lipase did not transform completely into lipase A-form; instead the lipase activity was distributed among all three forms. This suggests that the conformational changes induced by this treatment are more difficult when the enzyme is associated with the acidic components.

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It has been shown that lipases derived from *Rhizopus delemar* could be modified by treatment of the enzyme with phospholipids.[13] The complexed enzyme had a different substrate specificity, isoelectric point, and conformation (α-helical content). Also, porcine pancreatic lipase (PPL) was modified by phospholipids to alter its molecular weight.[14] We, therefore, attempted to convert lipase A-form into lipase B-form by the addition of the residue obtained after evaporation of the organic layer (step 4) to lipase A-form and by the addition of phosphatidylcholine. However, the enantiomeric ratio (E value) did not change. These results suggested that the induced conformational change by the deoxycholate–organic solvent mixture may not be reversible.

In the denaturation process, the enzyme activity is generally more easily affected than the overall conformational integrity of the protein, because the active site of the enzyme is usually situated in a limited region that is more flexible than the molecule as a whole.[14] Therefore, it is not surprising that a subtle change in the tertiary structure around the active-site region could not be detected by physical methods such as electrophoresis, circular dichroism, and ultraviolet absorption spectra, but was manifested only by changes in enantioselectivity. Using a competitive method, it was possible to dissect the kinetic parameters k_{cat} and K_m with the substrate **4a** for the crude enzyme and lipase A-form. It is apparent from the results of Table VIII that both the k_{cat} and the K_m decreased after the enzyme had been treated with deoxycholate and organic solvent. The most pronounced change was a 10-fold decrease in K_m for the R enantiomer of **4a**.

The enhancement of enantiomeric specificity of *Candida* lipase by deoxycholate–organic solvent treatment is the result of the non-covalent modification of the enzyme protein. The conformational flexibility of enzymes in solution allows one to further manipulate the tertiary structure by other means such as changing the reaction conditions. These include the addition of organic solvent, detergents, or inorganic salts or changes in the pH of the reaction medium.[16] The observed enhancement of enantioselectivity by use of bile salts, organic solvents, and temperature could also be due to noncovalent modification of the enzyme protein conformation.

Experimental Section

Materials. *Candida cylindracea* lipase powder (L1754, Type VII), SP-Sephadex C-50 powder, olive oil, sodium deoxycholate, RIA grade bovine serum albumin (fraction V powder, 96–99%), sodium dodecyl sulfate, acrylamide, *N,N'*-methylenebis[acrylamide], ammonium persulfate, Tris, TEMED, glycine, ketoprofen, and bromophenol blue were products from Sigma. Sephadex G-100 and the molecular weight calibration kit were products of Pharmacia, Uppsala, Sweden. Coomassie brilliant blue R-250 and Bradford reagent were purchased from Bio-Rad. Diazald, 2-(2,4-dichlorophenoxy)propionic acid, 2-(4-chloro-*o*-tolyloxy)propionic acid, 2-(2-chlorophenoxy)propionic acid, 2-(3-chlorophenoxy)propionic acid, 2-(4-chlorophenoxy)propionic acid, and 2-phenylpropionic acid were purchased from Aldrich Chemical Co. PM-10 ultrafiltration membrane was a product of Amicon.

^{1}H NMR spectra were recorded on a Bruker WM-200 spectrometer in deuteriochloroform with tetramethylsilane as the internal standard. Optical rotations were measured with a Perkin-Elmer Model 241C polarimeter in the indicated solvents. A Model M-6000 pump equipped with a U6K injector and a Model 77 double-beam UV (254-nm) detector (Waters Associates) were used for high-pressure liquid chromatography (HPLC). Thin-layer chromatography (TLC) was performed on plastic sheets coated with a 0.2-mm thickness of silica gel 60F-254 (E. Merck, Darmstadt, West Germany). Flash column chromatography was performed with Baker silica gel (40 μm). All solvents were glass-distilled prior to use.

Concentration of the Enzyme Fractions. The enzyme samples obtained from column chromatography were concentrated by using an Amicon

(13) (a) Iwai, M.; Tsujisaka, Y. *Agric. Biol. Chem.* **1974**, *38*, 1241, 1249. (b) Iwai, M.; Tsujisaka, Y.; Tominaga, Y. *Agric. Biol. Chem.* **1979**, *43*, 893. (c) Iwai, M.; Shimada, Y.; Tsujisaka, Y. *J. Biochem.* (*Tokyo*) **1980**, *88*, 533. (d) Shimada, Y.; Iwai, M.; Tsujisaka, Y. *J. Biochem.* (*Tokyo*) **1981**, *89*, 937.

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ultrafiltration kit fitted with a PM-10 (62 mm) membrane under a stream of nitrogen gas at a pressure of 20–30 psi.

Protein Determination. Protein concentrations were measured by a Coomassie blue dye-binding method[17] (Bio-Rad protein assay) using RIA grade BSA as standard (0.66 OD unit at A_{280} = 1 mg/mL). The protein concentrations of the column chromatogrpahic fractions were determined by measuring the absorbance at 280 nm.

Determination of Lipase Activity. The lipase assay was the same as that used by Tomizuka et al.[10] with slight modifications. A suspension consisting of 75 mL of 2% poly(vinyl alcohol) (PVA) aqueous solution and 25 mL of olive oil was stirred at room temperature for 30 min. To 5 mL of this mixture was added 5 mL of 0.1 M phosphate buffer, pH 7.1. The mixture was preincubated at 37 °C for 10 min with stirring and then 50 or 100 μL of enzyme solution was added. The reaction was kept for 10 or 20 min with stirring at 37 °C and stopped by adding 20 mL of an acetone and ethanol (1:1) mixture. The mixture was titrated with 0.05 N aqueous NaOH, with phenolphthalein as an indicator. One unit of lipase activity was defined as the amount of enzyme that was able to liberate 1 μequiv of acid/min.

Purification of Lipase A-Form. Step 1: Water Extraction. Twenty grams of the crude powder (Sigma) was suspended in 400 mL of distilled water at room temperature, and the suspension was stirred gently with a magnetic stirrer for 30 min. The solution was centrifuged at 15000*g* for 20 min and the supernatant was collected.

Step 2: Ammonium Sulfate Precipitation. To the supernatant was slowly added solid $(NH_4)_2SO_4$ (~140 g) with stirring at 5 °C for 2 h until the solution reached 50% saturation. The mixture was then centrifuged at 15000*g* for 20 min and the supernatant was discarded. The precipitate was dissolved in 30 mL of distilled water, and the solution was dialyzed against distilled water overnight at 4 °C. The dialyzed solution (30 mL) was centrifuged at 15000*g* for 20 min to remove the precipitate.

Step 3: SP-Sephadex C-50 Column Chromatography. The dialyzed enzyme solution was applied onto a SP-Sephadex C-50 column 1.5 × 12 cm) equilibrated with McIlvaine buffer (pH 3.7). The column was first eluted with 160 mL of the same buffer and then eluted with a 500-mL linear gradient that consisted of 250 mL of McIlvaine buffer to 250 mL of the same buffer containing 0.8 M NaCl. The column has a flow rate of 40 mL/h at 5 °C, and 8-mL fractions were collected by a fraction collector. Two peaks were found to have lipase activity (Figure 1). The major peak of lipase activity passed through the column without absorption by simply washing it with the McIlvaine buffer (pH 3.7). The minor peak was eluted by using a NaCl gradient (Figure 1). Fractions 4–8, which contained the major peak, were pooled and concentrated by ultrafiltration through an Amicon PM-10 membrane.

Step 4: Sodium Deoxycholate Treatment and Ethanol–Ether Precipitation. To the concentrated enzyme solution (30 mL) was added 0.5% (w/v) sodium deoxycholate, and the mixture was stirred for 30 min at room temperature. The precipitate was removed by centrifugation at 15000*g* for 20 min. The supernatant (30 mL) was placed in a bath below −10 °C, and a cold solution (90 mL, 3 times the volume of enzyme supernatant) of equal volumes of ethanol and diethyl ether was slowly added with stirring. After being stirred for 30 min, the solution was centrifuged at 3000*g* for 10 min. The supernatant was collected for further study (discussed later). The precipitate was dissolved in 10 mL of McIlvaine buffer (pH 3.7) (made with 0.01 M citric acid and 0.02 M sodium phosphate, dibasic). This solution was dialyzed overnight against the same buffer with stirring at 5 °C. The dialyzed solution was centrifuged at 15000*g* for 20 min to remove the precipitate.

Step 5: Second SP-Sephadex C-50 Column Chromatography. The precipitate obtained by organic solvent treatment was collected and dissolved in a small amount of McIlvaine buffer (pH 3.7). The enzyme solution was again applied onto a SP-Sephadex C-50 column (1.5 × 12 cm), which was eluted with the same solvent system as described in step 3. Only one peak was found to have lipase acitvity. It was eluted by the same NaCl gradient as described in step 3. Its position of elution was found to be the same as that of the minor peak of step 3 (Figure 2).

A summary of the purification procedure is given in Table II. The enantiomeric ratio (*E* value) of the enzyme toward (±)-**4d** was measured at each stage of the purification procedure, and these values are shown in Table IV.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed according to the procedure described by Davis.[18] Gels approximately 10 cm in length were polmerized in cylindrical glass tubes with an inner diameter of 0.5 cm. The running gel (9 cm) consisted of 7.5% acrylamide, 0.18% bisacrylamide, 0.38 M Tris-HCl, pH 8.9, and 0.017% TEMED, and polymerization was initiated by addition of 0.1% ammonium persulfate. The stacking gel (1 cm) was composed of 2.5% acrylamide, 0.62% bisacrylamide, 0.06 M Tris-HCl, pH 6.7, 20% sucrose, and 0.034% TEMED, and polymerization was initiated by light in the riboflavin–TEMED-catalyzed system. The electrophoresis buffer was 0.05 M Tris–glycine, pH 8.3.

Prior to electrophoresis, protein samples (50 μg) were mixed with 1 drop of glycerol and bromophenol blue tracking dye. The gels were subjected to electrophoresis at room temperature using a current of 3 mA/gel in a Pharmacia GE-2/4 electrophoresis apparatus.

Gels were stained for 2 h in 1% Coomassie brilliant blue R in 10% acetic acid solution and destained in a solution containing 7.5% (v/v) acetic acid.

Determination of the Molecular Weight by SDS–Polyacrylamide Slab Gel Electrophoresis. This determination was performed according to a modified procedure of Laemmli[19] as described in the Pharmacia electrophoresis manual. The running gels (2.5 × 80 × 140 mm) were composed of 7.5% acrylamide (acrylamide:bisacrylamide = 30:0.8), 0.38 M Tris-HCl (pH 8.8), 0.1% SDS, 0.04% TEMED, and 1% ammonium persulfate. The electrophoresis buffer was 0.05 M Tris–0.38 M glycine (pH 8.3). Protein samples (50 μg) were prepared by incubation for 3 min at 100 °C in electrophoresis buffer containing 1% SDS, 5% mercaptoethanol, 50% glycerol, and bromophenol blue tracking dye. Electrophoresis was carried out in a Pharmacia GE-2/4 apparatus at room temperature with the circulation of cooling water at a constant voltage of 60 V until the sample had stacked, at which time the voltage was increased to 120 V until the dye front moved to 1.5 cm from the bottom of gel. Gels were stained for 2 h with 1% Coomassie brilliant blue R-250 in 10% acetic acid and 50% ethanol and then destained with a solution containing 7% acetic acid and 10% methanol. The proteins (5 μg) used as molecular weight standards were chymotrypsinogen (25 000), ovalbumin (43 000), serum albumin (67 000), and aldolase (158 000).

Treatment of Pure Lipase A-Form with Phospholipid. Phosphatidylcholine, used for the treatment of pure lipase, was obtained from egg lecithin. Phospholipid treatment of pure lipase A-form was carried out as follows: A mixture containing 0.6 mg/mL lipase and 1 mg/mL phosphatidylcholine in 5 mL of 0.2 M phosphate buffer (pH 7.0) was stirred at room temperature for 5 h. The control sample was prepared as described above except no phosphatidylcholine was added to the mixture. The mixture was used directly for the determination of the enantiomeric ratios of (±)-**4a** and (±)-**4d**.

Enantiomeric Ratio (*E*). The racemic ester substrate (200 mg) was suspended in 2 mL of 0.2 M phosphate buffer (pH 7.0), and crude enzyme powder (50 mg) or pure enzyme (0.5 to ~1 mg) was added. The suspension was stirred at room temperature. The extent of conversion was analyzed by thin-layer chromatography using hexane–EtOAc (3:1 or 5:1) as the developing solvent. The compounds were detected by the spray reagent [3.5% $Ce(SO_4)_2$ in 2 N H_2SO_4] or by UV light. When the conversion was near 50%, the reaction was terminated by adjusting the pH of the solution to 2 with 1 N HCl. The product (acid) and remaining substrate (ester) were extracted with ether (3 × 4 mL). The ether layer was collected and dried. Then 2 mL of 0.25 M $NaHCO_3$ and 2 mL of hexane were added and the mixture was stirred violently to separate ester and acid. The ester was collected in the hexane layer; the acid was isolated after the aqueous phase was acidified and extracted into ether.

The recovered remaining substrate (ester) was used to measure the enantiomeric excess directly by ^1H NMR spectroscopy with CCl_4 as the solvent in the presence of 0.1 to ~0.5 equiv of the chiral shift reagent $Eu(hfc)_3$. The product (acid) was treated with an ethereal solution of diazomethane to convert it into the methyl ester, and the ee was measured by the same method.

The enantiomeric ratio (*E* value) was calculated from

$$E = \frac{\ln [(1 - c)(1 - ee_S)]}{\ln [(1 - c)(1 + ee_S)]}$$

where $c = ee_S/(ee_S + ee_P)$. See ref 6.

Determination of Kinetic Parameters (K_m and V_{max}) from Competitive Measurements. The kinetic constants K_a and V_a for the fast-reacting enantiomer A and K_b and V_b for the slow-reacting enantiomer B may be obtained directly from competitive measurements[7,11] by using the equations

$$t = \frac{A_0 - A}{V_a} + \frac{B_0 - (B_0/A_0^{1/E})A^{1/E}}{V_b} + \frac{K_a}{V_a} \ln \frac{A_0}{A} \quad (1)$$

$$E = \frac{V_a K_b}{V_b K_a} = \frac{\ln (A/A_0)}{\ln (B/B_0)} \quad (2)$$

where *E* is the enantiomeric ratio; A_0 and B_0 denote initial concentrations;

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t = time; and K_a, K_b and V_a, V_b are the Michaelis constants and maximal velocities for A and B, respectively. By insertion of the E value and three sets of experimental data $[(t_1, A_1), (t_2, A_2), (t_3, A_3)]$ into eq 1, the values of V_a, V_b, and K_a may be determined; K_b may be calculated from eq 2. To obtain more reliable kinetic constants, additional sets of data are required.

For this competitive experiment, methyl (±)-2-(4-chlorophenoxy)-propionate [(±)-**4a**] was used as the substrate. The E values for the crude lipase and the purified lipase (A-form) have been determined previously to be 17 and >100, respectively. These experiments were conducted at a substrate concentration of 0.1 M under identical reaction conditions. For each sample, (±)-**4a** was dissolved in hexane and appropriate aliquots were distributed to reaction vials. The hexane was then removed by evaporation using a stream of nitrogen. Crude lipase or lipase A-form ([Et] = 5×10^{-7} M, 22.5 units), dissolved in 1 mL of 0.2 M phosphate buffer, pH 7.0, was prewarmed in a 25 °C water bath for 10 min. It was then carefully added to each vial containing the substrate. The reaction mixture was stirred vigorously at 24 °C with a Corning PC-351 magnetic stirrer. The reaction was terminated by acidification (1 N HCl) to pH 2.0 and the time was recorded. The reaction mixture was then extracted with four 1-mL portions of ethyl acetate. The organic layer was dried over Na_2SO_4, filtered, and then evaporated to dryness. The residue was dissolved carefully in 1 mL of ethyl acetate and an aliquot was used for the subsequent HPLC analysis. The extent of conversion was determined by HPLC analysis using a 4.6 × 500 mm (Alltech porasil, 10 μm) column, which was eluted with a solvent system consisting of hexane–ethyl acetate–acetic acid (500:100:6) at a rate of 4 mL/min. The absorbance at 254 nm was monitored with a Waters Model 440 detector and a Hewlett-Packard 3390A integrator. A standard mixture of (±)-**4a** and its carboxylic acid at varying ratios gave equal molar responses of integral areas. The concentration of the fast-reacting enantiomer, (*R*)-(+)-**4a**, can be calculated from the value of conversion, c, by solving eq 3 when E is known. The data were fitted to

$$E = \frac{\ln (A/A_0)}{\ln \{[(A_0 + B_0)(1 - c) - A]/B_0\}} \quad (3)$$

eq 1 on an IBM PC using the BASIC program of Duggleby,[20] prepared by Dr. Dexter B. Northrop, to obtain the kinetic constants listed in Table VIII.

Registry No. **1a**, 122623-82-9; **1b**, 114315-58-1; **1c**, 114315-57-0; **1d**, 105052-64-0; **1e**, 124604-52-0; **1f**, 64382-51-0; **2a**, 7782-24-3; **2b**, 22161-81-5; **2c**, 51543-39-6; **2d**, 22204-53-1; **2e**, 95976-43-5; **2f**, 52780-12-8; **3a**, 122674-97-9; **3b**, 122674-99-1; **3c**, 122674-98-0; **3d**, 124649-62-3; **3e**, 124604-53-1; **3f**, 81601-94-7; **4a**, 95262-84-3; **4b**, 122623-80-7; **4c**, 122623-81-8; **4d**, 23844-57-7; **4e**, 23844-56-6; **5a**, 20421-34-5; **5b**, 31460-41-0; **5c**, 31460-39-6; **5d**, 15165-67-0; **5e**, 16484-77-8; **6a**, 95342-42-0; **6b**, 122674-93-5; **6c**, 122674-94-6; **6d**, 122674-95-7; **6e**, 115304-96-6; lipase, 9001-62-1.

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Communications to the Editor

$Os_2(CO)_8$: Transient Existence in Solution As Observed by Time-Resolved Infrared Spectroscopy upon Flash Photolysis of Octacarbonyl-1,2-diosmacyclobutane

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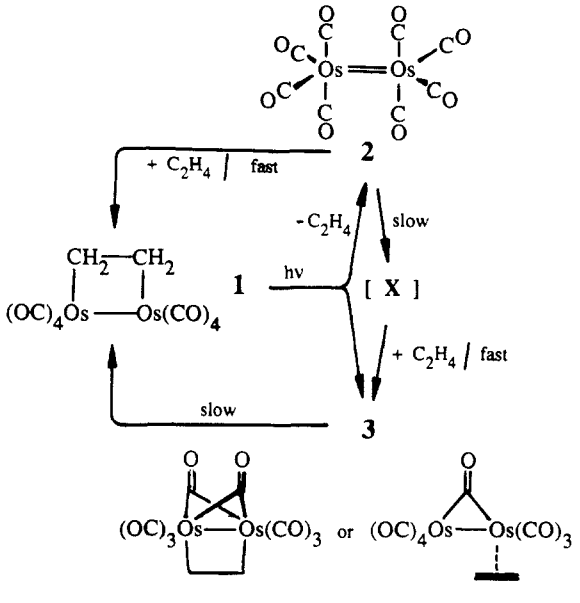
The dinuclear $Os_2(CO)_8$ unit has been invoked as a key intermediate in the photochemical synthesis of octacarbonyl-1,2-diosmacyclobutane complexes from $Os_3(CO)_{12}$[1] and in reactions involving exchange of the bridging organic moiety for other olefins[2,3] or alkyne[3,4] ligands.

Herein we report on the first observation of this species in solution at ambient temperature by means of fast time-resolved infrared spectroscopy[5] in the CO stretching vibrational region. Flash photolysis (λ = 308 nm, XeCl excimer laser) of $Os_2(CO)_8(\mu\text{-}\eta^1,\eta^1\text{-}H_2CCH_2)$ (**1**, 8×10^{-4} M in cyclohexane) at ambient temperature generates $Os_2(CO)_8$ (**2**) as a short-lived species (microsecond time domain) together with a second transient (**3**), apparently a CO-bridged isomer of **1**, which decays much more slowly (millisecond time domain), Scheme I. The ν_{CO} bands

Scheme I



associated with **2** (2058, 2018, 2003 cm^{-1}) and **3** (2068, 2027, 2016, 2000, 1992, 1780 cm^{-1}) have been extracted from series of decay curves monitored at different wavenumbers and in the presence of varying amounts of ethene in the solution.

In detail, the initially observed (≤3 μs after the flash) transient spectrum under neat argon atmosphere is dominated by the ν_{CO} bands of **2** (Figure 1A), but the weak band in the bridging CO region indicates that **3** is also present. Under these conditions, the decay of **2** ($\tau_{1/2} \leq 40$ μs), apart from minor side reactions, involves re-formation of **1** as well as secondary formation of **3**. The former process (but apparently not the latter one) is greatly accelerated by added ethene (ca. 10^{-2} M) such that **2** decays much more rapidly ($\tau_{1/2} \leq 5$ μs) and cleanly with re-formation of **1**, leaving behind the ν_{CO} pattern of **3** (Figure 1B). In ethene-saturated cyclohexane solution, the spectrum of **3** is observed im-

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