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Enzyme-catalyzed processes in organic solvents

(enzymatic catalysis in nonaqueous solvents/water in enzyme catalysis/lipases/enzymes in organic media/enzymatic transesterification)

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ABSTRACT **Three different lipases (porcine pancreatic, yeast, and mold) can vigorously act as catalysts in a number of nearly anhydrous organic solvents. Various transesterification reactions catalyzed by porcine pancreatic lipase in hexane obey Michaelis–Menten kinetics. The dependence of the catalytic activity of the enzyme in organic media on the pH of the aqueous solution from which it was recovered is bell-shaped, with the maximum coinciding with the pH optimum of the enzymatic activity in water. The catalytic power exhibited by the lipases in organic solvents is comparable to that displayed in water. In addition to transesterification, lipases can catalyze several other processes in organic media including esterification, aminolysis, acyl exchange, thiotransesterification, and oximolysis; some of these reactions proceed to an appreciable extent only in nonaqueous solvents.**

In nature, enzymes function in aqueous solutions. Therefore, it is not surprising that virtually all studies in enzymology thus far have used water as the reaction medium. However, from the biotechnological standpoint there are numerous advantages of conducting enzymatic conversions in organic solvents as opposed to water: (*i*) high solubility of most organic compounds in nonaqueous media; (*ii*) ability to carry out new reactions impossible in water because of kinetic or thermodynamic restrictions; (*iii*) greater stability of enzymes; (*iv*) relative ease of product recovery from organic solvents as compared to water; and (*v*) the insolubility of enzymes in organic media, which permits their easy recovery and reuse and thus eliminates the need for immobilization.

Conventional wisdom dictates that water is required for enzyme action. This conclusion stems from the fact that water participates (directly or indirectly) in all noncovalent interactions maintaining the native, catalytically active enzyme conformation (1–5); hence, removal of water should drastically distort that conformation and inactivate the enzyme. Although this reasoning is undoubtedly correct, the real question is not *whether* water is indeed required but *how much* water. It is hard to imagine that an enzyme molecule can "see" more than just a few monolayers of water around it. As long as this water is localized about enzyme molecules, the rest (i.e., the bulk) of water can probably be replaced with an organic solvent without adversely affecting the enzyme. Since the absolute amount of water contained in those few monolayers is very small, this situation is tantamount to an enzyme functioning in a nearly anhydrous organic medium.

There is some experimental confirmation of the above rationale. Price and co-workers have shown that chymotrypsin (6) and xanthine oxidase (7, 8) are catalytically active when suspended in organic solvents. We recently have found (9) that porcine pancreatic lipase vigorously acts as a catalyst in the 99.98% organic medium; in addition, upon dehydration the enzyme acquires some remarkable new properties—e.g., it becomes extremely thermostable and more selective (9).

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In the present work we examined mechanistically the catalytic behavior of enzymes in organic solvents. The questions concerning the dependence of the enzymatic activity on the water content, the "history" of the enzyme sample and the nature of the solvent, kinetics of enzymatic reactions in organic media, unusual reactions catalyzed by enzymes in organic solvents, and catalytic power of enzymes in organic media vs. that in water have been answered.

MATERIALS AND METHODS

Lipases (EC 3.1.1.3) purchased and their suppliers were: porcine pancreatic lipase and *Candida cylindracea* lipase, from Sigma; and *Mucor* lipase and *Pseudomonas* lipoprotein lipase, from Amano International Enzyme (Troy, Va). The lipases had specific activities of 13, 595, 20, and 827 international units/mg of solid, respectively.

Nearly all chemicals used in this work were obtained commercially and were of the highest purity available. Trichloroethyl butyrate was synthesized from the alcohol and the acid by the general procedure of Allen and Spangler (10). Other esters were synthesized from the corresponding acid chlorides and alcohols by the general method of Morris and Green (11).

Water was removed from organic media by shaking them with 3A molecular sieves (Linde, South Plainfield, NJ), followed by storing them in the presence of the adsorbent. The water concentration both in organic media and in the enzymes was measured by the optimized Fischer method (12). Commercial preparations of lipases were found to contain the following amounts of water: porcine pancreatic lipase, 3.6%; *Candida cylindracea* lipase, 6.1%; and *Mucor* lipase, 4.8%.

Enzymatic reactions in organic solvents were initiated by addition of a lipase powder to a mixture of substrates or their solution in organic solvents. The suspension was placed in a stoppered flask and shaken on an orbit shaker at 250 rpm and 20°C. Periodically, 0.5-μl aliquots were withdrawn and assayed by gas chromatography using a 530-μm fused silica capillary column (Hewlett–Packard). Lipase-catalyzed hydrolysis of tributyrin in water was followed potentiometrically with a Radiometer pH-stat.

Unless indicated otherwise, porcine pancreatic lipase was prepared as follows. Commercial sample of the enzyme was dissolved in 0.05 M glycine buffer (pH 8.4) and cooled to 4°C. Then an equal volume of cold (−20°C) acetone was added, and the mixture was stirred at 4°C for 30 min. The precipitated enzyme was recovered by centrifugation, washed with cold acetone, and dried first under air and then under vacuum at room temperature. The resultant powder of the enzyme contained 0.5% water.

When needed, lipases were completely and irreversibly inactivated with the active center-specific reagent diethyl *p*-nitrophenyl phosphate as described (13).

RESULTS AND DISCUSSION

An ideal model for investigation of enzymatic reactions in organic solvents should satisfy the following criteria: (*i*) the

enzyme should be readily available and inexpensive; (*ii*) the enzyme should be cofactor-independent, as common cofactors are insoluble in organic solvents; (*iii*) substrates should be soluble in organic media; and (*iv*) water should be a nonreactant in the enzymatic process. Lipases (EC 3.1.1.3) meet all of these requirements. Although the natural lipolytic reaction is hydrolysis of carboxylic esters (14, 15), lipases also can catalyze transesterifications (which do not involve water as a substrate) (9, 16). Therefore, in this work we investigated transesterification (and other reactions not requiring water) in organic solvents catalyzed by three different lipases—porcine pancreatic, yeast (*Candida cylindracea*), and mold (*Mucor* sp.).

Tributyrin (glyceryl tributyrate) and heptanol were dissolved in 2-pentanone, followed by dehydration of the mixture and addition of a known amount of water. Then a lipase powder was added, and the suspension formed [proteins are insoluble in this as well as in almost all other organic solvents (17)] was shaken at 20°C. Fig. 1 shows the dependence of the rate of the transesterification reaction between tributyrin and heptanol catalyzed by three lipases on the percentage of water in the organic medium. One can see that the enzymatic activities of porcine pancreatic and mold lipases are only slightly dependent on the percentage of water in the range from 0.02% to 1%, whereas that of yeast lipase gradually decreases as the water content is lowered in that range. It should be stressed, however, that despite the quantitative differences, all three lipases exhibit a robust catalytic activity even *at the percentage of water as low as 0.02%*—i.e., in the 99.98% organic medium. This enzymatic activity is sustained over extended periods of time and is sufficient to nearly complete the reaction: for example, under the conditions given for Fig. 1, the degree of conversion in the transesterification reaction catalyzed by porcine pancreatic lipase after 3 days exceeds 95%. Lipase-catalyzed transesterification in 2-pentanone is not merely an artifact due to the presence of protein because, when the lipases were inactivated by the active center-specific reagent diethyl *p*-nitrophenyl phosphate (13), all three exhibited no catalytic activity in the organic medium.

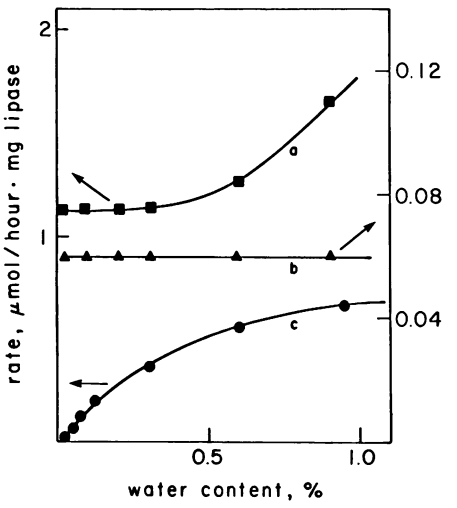

FIG. 1. The dependence of the lipase-catalyzed transesterification reaction between tributyrin and heptanol in 2-pentanone on the percentage of water in the solvent. Curves: a, porcine pancreatic lipase; b, mold (*Mucor* sp.) lipase; c, yeast (*Candida cylindracea*) lipase. Lipase powder (10 mg) was added to 1 ml of 2-pentanone containing 0.3 M tributyrin and heptanol and a given amount of water; the suspension was shaken at 20°C and 250 rpm. The water content of the lipase preparations was 0.5% (after precipitation with acetone and drying), 6.1%, and 4.8% (wt/wt) for porcine pancreatic, yeast, and mold, respectively. In the absence of lipases, no appreciable reaction was observed.

Since all enzymatic reactions in aqueous solutions are strongly pH-dependent, one of the most intriguing aspects of the phenomenon depicted in Fig. 1 is that of the "pH" in a nearly anhydrous organic milieu. To elucidate it, the following experiment was designed. Porcine pancreatic lipase was dissolved in an aqueous buffer, and the pH was adjusted to a given value. Then the enzyme was precipitated with cold acetone, recovered, and dried under vacuum. The enzymatic activity of the resulting lipase preparation was assayed both in water (at pH 8.4) and in a mixture of heptanol and tributyrin (containing only 0.02% water) as a function of the pH of the aqueous solution from which the enzyme was precipitated. One can see in Fig. 2 that the hydrolytic activity of lipase in water (curve a) is independent of the "pH history" of the enzyme sample; this is not surprising because the assay solution was always maintained at pH 8.4 (the optimum for the hydrolytic activity). At the same time, the catalytic activity of lipase in the organic medium very much depends on the pH from which the enzyme was precipitated, with the maximum approximately coinciding with the pH optimum of the enzymatic activity in water. Thus, the enzyme "remembers" the pH of the last aqueous solution that it had been exposed to (i.e., the enzyme's ionogenic groups acquire the corresponding ionization states, which then remain both in the solid state and in organic solvents). When assayed in the organic medium, the specific activity of the commercial sample of porcine pancreatic lipase is one third of that of the enzyme precipitated from pH 8.4; in all subsequent experiments, the porcine pancreatic lipase precipitated from that pH was used.

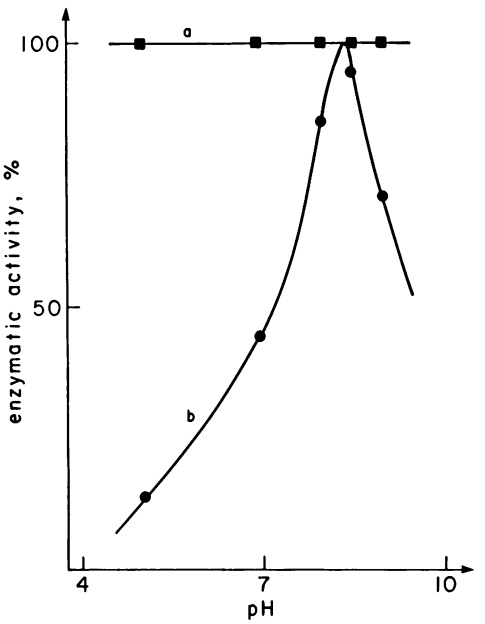

FIG. 2. The dependence of the porcine pancreatic lipase activity on the pH of the aqueous solution from which the enzyme was recovered. Curves: a, lipase-catalyzed hydrolysis of tributyrin in water (100% corresponds to the rate of 1.4 mmol/hr·mg of enzyme); b, lipase-catalyzed transesterification between tributyrin and heptanol in their mixture (2 M heptanol in the ester) containing 0.02% water (100% corresponds to the rate of 5.6 μmol/hr·mg of enzyme). Porcine pancreatic lipase was precipitated from aqueous solutions of different pH with acetone as described; then the recovered enzyme was dried under vacuum. For curve a, 10 mg of the enzyme was added to 15 ml of 0.3 M tributyrin in water containing 0.1 M KCl. The mixture was adjusted to pH 8.4, and the enzymatic hydrolysis was carried out at that pH and 20°C in a pH-stat cuvette. For curve b, 10 mg of the enzyme was added to 1 ml of 2 M heptanol in tributyrin, and the suspension was shaken at 20°C and 250 rpm.

It was of definite interest to examine how enzymatic activity of lipases in nonaqueous media depends on the nature of the solvent. Inspection of Table 1 reveals that all three lipases are catalytically active in a wide variety of organic solvents. The only solvents in which all of the enzymes were completely inactive were dimethyl sulfoxide and formamide. Since these are the only two of the list that can dissolve proteins (17) (in all others the lipases existed exclusively as suspensions), one can hypothesize that enzymes in them change their conformation and consequently inactivate. The lipases are diverse in their sensitivity to the nature of the organic solvent (Table 1). For instance, while the catalytic activity of porcine pancreatic lipase is comparable in different solvents, that of the yeast lipase varies by almost 3 orders of magnitude, being highest in water-immiscible solvents (paraffins, butyl ether, toluene, and carbon tetrachloride) and lowest in water-miscible ones (acetonitrile, tetrahydrofuran, dioxane, pyridine, and acetone). The *Mucor* lipase occupies an intermediate position with respect to its sensitivity to the nature of the solvent.

The data shown in Table 1 can be explained in terms of the model described in the Introduction: there is a certain amount of water molecules required for the enzymatic activity. In the case of porcine pancreatic lipase, this water is tightly bound to the enzyme and even hydrophilic solvents do not strip it. On the other hand, this essential water is apparently bound much more loosely to the yeast lipase; therefore, it is partitioned into water-miscible (but not water-immiscible) solvents. Hence, the main factor in the effect of organic solvents seems to be not their interaction with the enzyme molecule itself but with the enzyme-bound water.

Table 1. Initial rates of the lipase-catalyzed transesterification reaction between tributyrin and heptanol in different organic solvents

Solvent	Reaction rate, μmol/hr·mg of enzyme		
	Porcine pancreatic lipase	Yeast lipase	Mold lipase
Hexane	5.2	4.0	0.31
Dodecane	4.0	5.5	0.34
Hexadecane	2.9	6.3	0.32
Ethyl ether	5.1	0.10	0.12
Isopropyl ether	5.1	0.55	0.20
Butyl ether	4.7	2.5	0.20
Acetonitrile	2.2	0.04	0.04
Tetrahydrofuran	2.0	0.02	0.05
Dioxane	1.4	0.01	0.04
Toluene	2.1	0.95	0.08
Pyridine	1.3	0.01	0.02
Dimethyl sulfoxide	0	0	0
Formamide	0	0	0
Carbon tetrachloride	1.5	0.60	0.12
Acetone	1.2	0.02	0.06
2-Pentanone	1.1	0.03	0.06
2-Heptanone	1.2	0.08	0.10

A lipase powder (10 mg) was added to 1 ml of an organic solvent containing 0.3 M tributyrin and heptanol; the mixture was shaken at 20°C, and the time course of the reaction was followed by gas chromatography. Organic solvents contained 0.02% (wt/wt) water [measured by the Fischer method (12)] except for the paraffins, toluene, and carbon tetrachloride, in which that water concentration is not attainable and, therefore, it was even lower (our method of measurement does not afford an exact determination in that range). The water content of the lipases was 0.5%, 6.1%, and 4.8% for porcine pancreatic (which was precipitated from pH 8.4, see text), yeast and mold, respectively. The lipases inactivated with diethyl *p*-nitrophenyl phosphate exhibited no enzymatic activity in all organic solvents.

One wonders whether enzymes, when placed in such unnatural environments as nearly anhydrous organic media, will still obey conventional Michaelis–Menten kinetics. On the basis of the classical lipolytic hydrolysis reaction (14, 15), the kinetic scheme of the lipase-catalyzed transesterification is likely to involve formation of a noncovalent enzyme–ester complex, which then transforms to an acyl-enzyme intermediate and the alcohol product is released; this is followed by interaction of the acyl enzyme with the nucleophile (the other alcohol) to form another binary complex, which then yields the new ester and the free enzyme. This mechanism [compulsory order with no ternary complexes (18)] affords a set of parallel straight lines when the reciprocal initial rates are plotted against the reciprocal ester concentrations at given concentrations of the alcohol. When the maximal velocities determined by this method are subsequently plotted against the reciprocal concentrations of the alcohol substrate, again a straight line is obtained (18).

Fig. 3 presents the porcine pancreatic lipase-catalyzed reaction between tributyrin and heptanol in hexane subjected to the kinetic analysis described above. One can see that the kinetic behavior shows an excellent agreement with that expected for Michaelis–Menten kinetics. The same patterns were observed for other lipase-catalyzed transesterifications in hexane involving both other esters and other alcohols. The Michaelis constants and maximal velocities determined from

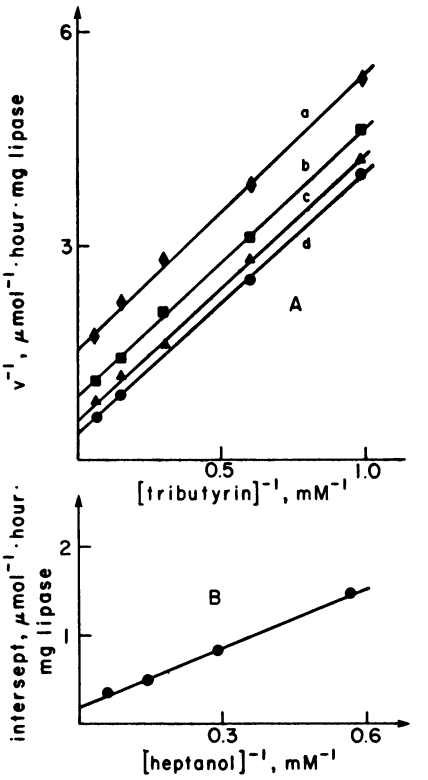

FIG. 3. Initial rate kinetics of the transesterification reaction between tributyrin and heptanol in hexane catalyzed by porcine pancreatic lipase. (*A*) Reciprocal rates (v) vs. reciprocal tributyrin concentrations at different concentrations of heptanol (the primary plot). Concentrations of tributyrin were varied at the following fixed concentrations of heptanol: 1.77 mM (curve a), 3.5 mM (curve b), 7.07 mM (curve c), and 16.6 mM (curve d). (*B*) Intercepts of the straight lines in the primary plot in *A* vs. the reciprocal heptanol concentrations (the secondary plot). Porcine pancreatic lipase (5 mg) (precipitated from pH 8.4 and containing 0.5% water) was added to 1 ml of hexane containing given concentrations of tributyrin and heptanol (water content of the mixture was <0.02%). The suspensions were shaken at 20°C and 250 rpm. The initial rates were determined on the basis of at least five independent measurements.

Table 2. Kinetic parameters of different transesterification reactions catalyzed by porcine pancreatic lipase in hexane

Ester	Alcohol	K_m (ester) $\times 10^{-3}$ M	K_m (alcohol) $\times 10^{-3}$ M	V, μmol/hr mg of enzyme
Tributyrin	Methanol	42	33	10
Tributyrin	Dodecanol	83	5.2	17
Tributyrin	Heptanol	20	13	4.8
Trichloroethyl butyrate	Heptanol	39	4.5	1.7
Methyl butyrate	Heptanol	>2000*	ND	2*

Conditions were as in the legend to Fig. 3; ND, not determined.
*These parameters could not be determined exactly because of a very poor affinity of the ester to the enzyme.

the corresponding plots are listed in Table 2. One can see that the ratio K_m (ester)/V for a given ester is approximately the same for different alcohols (the first three lines) as is the ratio K_m (alcohol)/V for a given alcohol and different esters (the third and the fourth lines). This is in agreement with the assumed "ping-pong" mechanism (19) and, hence, further confirms the fact that lipase-catalyzed transesterifications in hexane obey the Michaelis–Menten kinetics.

Having established that the lipases can function as catalysts in nearly anhydrous organic media, one then wishes to know how high their catalytic activity is in such unnatural environments. To that end, we have determined the acceleration effect afforded by the lipases in the reaction between tributyrin and heptanol in hexadecane. The data obtained are presented in Table 3 in which the acceleration effect is expressed as the ratio of the rate of the lipase-catalyzed transesterification (at an enzyme concentration of 10 mg/ml) to that of the reaction in the absence of the enzyme. For the purpose of comparison, acceleration effects afforded by the lipases in the reaction of hydrolysis of tributyrin in water also were determined and are shown in the last column in Table 3. One can see that the catalytic power of each lipase in the transesterification reaction in hexadecane compared to that in the hydrolysis reaction in water is of the same order of magnitude. That is, the enzymes seem to display their full inherent catalytic power in organic solvents, not just a small fraction of it. This strongly suggests that the enzyme conformations in organic solvents and in water are nearly identical [although in the former it is more rigid (9)].

Thus far, we have examined various transesterification reactions catalyzed by lipases in organic solvents. We have found that several other processes also can be carried out enzymatically in nonaqueous media. For example, porcine pancreatic lipase, in addition to transesterification, also can catalyze reactions of esterification, aminolysis, acyl exchange, thiotransesterification, and oximolysis. The rates of these enzymatic reactions in different organic solvents are shown in Table 4. It should be stressed that some of these processes can be conducted only in nonaqueous solvents—e.g., the esterification reaction between butyric acid (0.3 M) and heptanol (0.3 M) catalyzed by porcine pancreatic lipase at 10 mg/ml stops in water (0.05 M glycine buffer, pH 8.4) at a degree of conversion of <0.1%, whereas the degree of conversion of the same reaction in hexane after 2 hr exceeds 90%.

Table 3. Acceleration effects afforded by lipases in the transesterification reaction between tributyrin and heptanol in hexadecane and in the hydrolysis of tributyrin in water

Lipase	Acceleration effect* $\times 10^{-6}$: Transesterification in hexadecane†	Acceleration effect* $\times 10^{-6}$: Hydrolysis in water‡
Porcine pancreatic	1.4	3.5
Yeast	2.8	0.7
Mold	0.14	0.17

In the transesterification reaction, 0.01 M tributyrin and heptanol in hexadecane were used; in the hydrolysis reaction in aqueous solution (0.1 M KCl, pH 7.5), 0.01 M tributyrin was used. Lipases were used at 10 mg/ml, and other conditions were the same as shown in Table 1.
*Defined as the ratio of the rate of the lipase-catalyzed reaction (enzyme concentration at 10 mg/ml) to that of the nonenzymatic one.
†The rate of the transesterification in the absence of lipases at 20°C was too slow to measure. Therefore, it was measured at 5°C increments in the temperature range from 85°C to 120°C and then was extrapolated to 20°C using the Arrhenius dependence.
‡The rate of the hydrolysis in the absence of lipases at pH 7.5 and 20°C was too slow to measure. Therefore, it was measured at increments of 0.5 pH unit in the pH range from 9.5 to 12.0 and then linearly extrapolated to pH 7.5 (all at 20°C).

CONCLUDING REMARKS

We have shown in this study that three different lipases can catalyze a number of reactions in nearly anhydrous organic solvents. The catalytic efficiency of the enzymes in organic media is similar to that in water, and their action can be described by conventional Michaelis–Menten kinetics. In addition to the lipases, other enzymes have been found to

Table 4. Various reactions catalyzed by porcine pancreatic lipase in different organic solvents

Solvent	Initial reaction rate, μmol/hr·mg of lipase: Transesterification*	Esterification†	Aminolysis‡	Acyl exchange§	Thiotransesterification¶	Oximolysis‖
Hexane	5.2	2.4	0.60	2.0	3.0	3.0
Acetone	1.2	0.30	0.60	0.54	1.1	1.5
Tetrahydrofuran	2.0	0.36	0.54	0.54	1.8	2.1
Ethyl ether	5.1	0.90	0.24	0.72	1.9	2.1
Pyridine	1.3	0.06	0.60	0.12	1.1	1.1
Toluene	2.1	2.4	0.18	1.1	1.7	2.3

All reactions were followed gas chromatographically by measuring the product concentrations. See Table 1 for other conditions.
*Between 0.3 M tributyrin and 0.3 M heptanol.
†Between 0.3 M butyric acid and 0.3 M heptanol.
‡Between 3 M methyl butyrate and 0.3 M butylamine.
§Between 3 M methyl butyrate and 0.3 M heptanoic acid.
¶Between 3 M methyl butyrate and 0.3 M butanethiol.
‖Between 3 M methyl butyrate and 0.3 M acetone oxime.

function in organic solvents: chymotrypsin (6), xanthine oxidase (7, 8), peroxidase (see below), and polyphenol oxidase (unpublished data). Since these enzymes belong to different classes and obviously consist of the same amino acids as all others, it seems that other enzymes should be able to work in organic solvents as well.

Recently, Japanese authors have reported (20–22) that *Pseudomonas* lipoprotein lipase and horseradish peroxidase become soluble and catalytically active in benzene following their modification with a polyethylene glycol derivative. We re-examined their data and make the following assessment. Unmodified lipoprotein lipase (Amano), although insoluble in benzene, has a catalytic activity in that solvent (saturated with water) that is nearly the same as that reported for the modified enzyme (21, 22). In the case of peroxidase, the commercial (Sigma) enzyme is indeed catalytically inactive in benzene in the reaction of oxidation of dianisidine with H_2O_2. However, when horseradish peroxidase was precipitated from pH 7 (optimum of the enzymatic activity) onto glass powder (this affords a thin layer of the enzyme on the glass surface and, consequently, a more efficient utilization of the available enzyme than in a suspension of the free enzyme), its catalytic activity in benzene was comparable with that of the modified enzyme—both as reported (20) and observed by us. Therefore, we conclude that in the case of both enzymes, chemical modification is not required because its only benefit (which may not be worth the trouble of a very laborious and time-consuming modification procedure) is that it yields a soluble enzyme system, which is hence free of intraparticle diffusional limitations.

We hypothesize that perhaps all enzymes can function in organic solvents, and the real question concerns only finding the appropriate conditions for that (recovery of the enzyme from the correct pH, sufficiently fine suspension of the enzyme, optimal organic solvent, etc.). This would create totally new opportunities in the area of applied enzymology (23), in particular the use of enzymes as catalysts in organic chemistry (24, 25), and should result in many novel enzyme-based processes.

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