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The effect of substrate partitioning on the kinetics of enzymes acting in reverse micelles

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A theoretical model for the expression of enzymic activity in reverse micelles previously developed [Bru, Sánchez-Ferrer & García-Carmona (1989) Biochem. J. **259**, 355–361] was extended in the present work. The substrate concentration in each reverse-micelle phase (free water, bound water and surfactant apolar tails) and the organic solvent was expressed as a function of the total substrate concentration, taking into account its partition coefficients, that is, partitioning of the substrate in a multiphasic system. In each phase the enzyme expresses a catalytic constant and a K_m. Thus the whole reaction rate is the addition of the particular rates expressed in each domain. This model was compared with that developed for a biphasic system [Levashov, Klyachko, Pantin, Khmelnitski & Martinek (1980) Bioorg. Khim. **6**, 929–943] by fitting the experimental results obtained with mushroom tyrosinase (working on both 4-t-butylcatechol and 4-methylcatechol) to the two models. The parameters which characterize reverse micelles, ω_0 (water/surfactant molar ratio) and θ (fraction of water) were investigated. The ω_0 profile was shown to be hyperbolic for both substrates. Activity towards 4-t-butylcatechol decreases as θ increases, this observation being attributable to a dilution of the substrate. A K_m of 7.8 M for 4-t-butylcatechol could be calculated on the basis of the biphasic model, whereas it was 13.5 mM when calculating on the basis of our model. A new parameter, ρ (=[substrate]/θ), was defined to characterize those substrates that mainly solubilize in the reverse micelle ('micellar substrates').

INTRODUCTION

Enzyme kinetics in organic solvents pose several questions for classical enzymology. Among the questions raised are: what catalytic properties does an enzyme express in a near-anhydrous medium? Would the enzyme be more active in this medium? How can the Michaelis–Menten constant be expressed? In short, how does an enzyme work in organic solvents?

One experimental approach to resolve these questions uses reverse micelles, which provide a homogeneous medium where the reactants' diffusion does not limit the reaction rate [1] and in which the enzyme is as active as in water [2]. Reverse micelles, apart from displaying these properties, are a physically well characterized system [3–6], and the stability of the entrapped enzymes can be as high as in water [7–8]. There have been several recent reviews on reverse micelles [9–11].

The answer to the questions posed above is fundamental for developing a solidly based enzymology in organic solvents, especially in order to make use of their biotechnological potential [12,13].

In reversed micelles, changes in the substrate specificity for alcohol dehydrogenase [14] and pancreatic lipase [15] and changes in the reaction direction for hydrolases [16] have been described; α-chymotrypsin [17], acid phosphatase [18], laccase [9] and peroxidase [19] have been shown to be superactive enzymes; linear dependence of the K_m on the amount of water has been found for trypsin [2,20] and polyphenol oxidase [7].

These novel aspects of enzymology are not an easy task to include in a general theory, though several approaches have been developed [21,22].

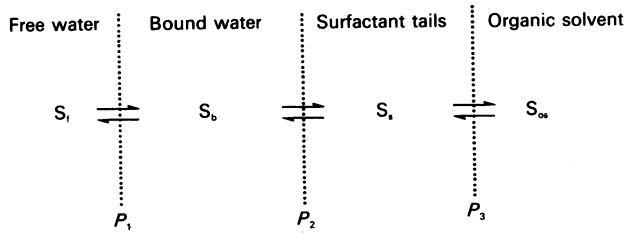
To understand micellar enzymology [23] we must, on the one hand, have a knowledge of the structure [24] and dynamics [1] of reverse micelles and, on the other hand, take this into account in order to express the true kinetic parameters.

In a previous paper [22], we developed a theoretical model based on enzyme partitioning in the reverse micelle. This model explains the dependence of the catalytic constant on both ω_0 (water/surfactant molar ratio), and θ (fraction of water, v/v) (we will refer to it as the 'enzyme-partitioning' model), showing that whereas ω_0 deeply affects the activity, θ has an appreciable effect only at critical ω_0 values. Even the superactivity can be explained through this model.

In the present study we have extended the model to include the effects that the micellar parameters have on enzyme kinetics when the partition of the substrate is taken into account (we will refer to it as the 'multiphasic' model). In addition, we describe a study of mushroom tyrosinase entrapped in Brij 96/cyclohexane reversed micelles and fit the results to the model, comparing it with a model developed for a biphasic system [20].

THEORY

The new aspects of the substrate's presence are (i) its distribution among phases that make up reverse micelles and the continuous oil phase (Scheme 1) and (ii) the expressed K_m of each kind of enzymic form within each phase. Since the exchange



Scheme 1. Substrate distribution in the reversed-micelle system

Schematic representation of reverse micelle as a multiphasic system. The phases can be considered as continuous when the timescale of the process we are dealing with is higher than exchange between micelles [11].

Abbreviations used: TBC, 4-t-butylcatechol; 4MC, 4-methylcatechol; AOT, dioctyl sodium sulphosuccinate ('Aerosol OT').

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between the solutes in the reversed micelles is faster than the enzymic reactions [11], there is no hindrance to the diffusion of reactants.

List of parameters

ω_0: micelle size.
θ: fraction of water. Far from critical ω_0 values it is equivalent to the micellar fraction.
α: fraction of free water.
β: fraction of bound water.
γ: fraction of surfactant tails.
j: α, β or γ.
$P_{1,2,3}$: partition coefficients of the substrate.
Subscripts: $_f$, free water; $_b$, bound water; $_s$, surfactant tails; $_{os}$, organic solvent; $_T$, total; $_f$, $_f$, $_b$ or $_s$; $_{app.}$, apparent; $_{mic.}$, micellar.
k_i: catalytic constant.
n: number of water molecules bound per surfactant polar head.
K^1_E, K^2_E: enzyme partition coefficients.

The equilibrium distribution of the substrate, which is set up at zero time [25], is ruled by the respective partition coefficients (P_x):

$$P_1 = \frac{[S]_b}{[S]_f} \quad (1)$$

$$P_2 = \frac{[S]_s}{[S]_b} \quad (2)$$

$$P_3 = \frac{[S]_{os}}{[S]_s} \quad (3)$$

The material balance will be:

$$[S]_T = [S]_f \cdot \alpha + [S]_b \cdot \beta + [S]_s \cdot \gamma + [S]_{os} \cdot (1-\alpha-\beta-\gamma) \quad (4)$$

$$[E]_T = [E]_f \cdot \alpha + [E]_b \cdot \beta + [E]_s \cdot \gamma \quad (5)$$

where α, β and γ are the volume fractions of free water, bound water and surfactant tails (subscripts $_{f,b}$ and $_s$ respectively). The assumption was made that the active enzyme is present only in the micelles.

Each particular substrate concentration can be expressed as a function of the partition coefficients, of the relative volumes and of the overall substrate concentration by matching equations (1)–(4):

$$[S]_f = \frac{[S]_T}{\alpha + P_1\beta + P_1P_2\gamma + P_1P_2P_3(1-\alpha-\beta-\gamma)} \quad (6)$$

$$[S]_b = P_1[S]_f \quad (7)$$

$$[S]_s = P_1P_2[S]_f \quad (8)$$

$$[S_{os}] = P_1P_2P_3[S]_f \quad (9)$$

According to the 'enzyme-partitioning' model [22] the maximum velocity that each particular enzyme can express is:

$$V_{max.,i} = k_1 \cdot [E]_i \cdot j \quad (10)$$

where j is α, β or γ; thus, the activity expressed in each phase will be:

$$v_i = \frac{V_{max.,i}[S]_T}{K^{app.}_{m_i} + [S]_T} \quad (11)$$

if the enzyme obeys Michaelis–Menten kinetics, where $K^{app.}_{m_i}$ are:

$$K^{app.}_{m_f} = K_{m_f}(\alpha + P_1\beta + P_1P_2\gamma + P_1P_2P_3(1-\alpha-\beta-\gamma)) \quad (12)$$

$$K^{app.}_{m_b} = K_{m_b}\frac{(\alpha + P_1\beta + P_1P_2\gamma + P_1P_2P_3(1-\alpha-\beta-\gamma))}{P_1} \quad (13)$$

$$K^{app.}_{m_s} = K_{m_s}\frac{(\alpha + P_1\beta + P_1P_2\gamma + P_1P_2P_3(1-\alpha-\beta-\gamma))}{P_1P_2} \quad (14)$$

Thus the overall activity is:

$$v = \sum_{i=f,b,s} v_i \quad (15)$$

Levashov *et al.* [20] proposed a biphasic model where the reverse micelles and the organic solvent are the two phases that form the system. They expressed the reaction rate for an entrapped enzyme obeying Michalis–Menten kinetics as:

$$v = \frac{k_{cat.,app.} \cdot [E]_T \cdot [S]_T}{K^{app.}_m + [S]_T} \quad (16)$$

where:

$$k_{cat.,app.} = k_{cat.,mic.} \quad (17)$$

$$[E]_T = [E]_{mic.} \cdot \theta \quad (18)$$

$$K^{app.}_m = K^{mic.}_m \frac{1 + \theta(P-1)}{P} \quad (19)$$

P being defined as follows:

$$P = [S]_{mic.}/[S]_{os} \quad (20)$$

It is obvious that, depending on P, we obtain a linear dependence of $K^{app.}$ on θ whose slope can be positive or nil.

Eqn. (16) can be linearized, and its terms can be arranged in two forms:

$$\frac{1}{v} = \frac{K^{mic.}_m}{V_{max.}}\left[\frac{1+\theta(P-1)}{P}\right]\frac{1}{[S]_T} + \frac{1}{V_{max.}} \quad (21)$$

or

$$\frac{1}{v} = \left[\frac{P-1}{P} \cdot \frac{K^{mic.}_m}{V_{max.} \cdot [S]_T}\right]\theta + \frac{1}{V_{max.}} + \frac{K^{mic.}_m}{V_{max.} \cdot [S]_T \cdot P} \quad (22)$$

A plot of $1/v$ against $1/[S]_T$ at several fixed θ values, using eqn. (21), gives us the $V_{max.}$ and $K^{app.}_m$. The value of P can be calculated by means of a plot of $1/v$ against θ at several fixed $[S]_T$ values by using eqn. (22).

Below we discuss which of the two models is the better at explaining the experimental results obtained with tyrosinase working on t-butylcatechol (TBC). Likewise, the differences between the two models will be discussed.

MATERIALS AND METHODS

Mushroom tyrosinase was purchased from Sigma (Deisenhofen, Germany). An enzyme solution in water (1 mg/ml) was dialysed against twice-distilled water for 24 h and was used throughout the kinetic experiments.

TBC and 4-methylcatechol (4MC) (Aldrich, Steimheim, Germany), Brij 96 (Sigma) and cyclohexane (Panreac, Barcelona, Spain) were used without further purification. All the other chemicals were of the highest purity available.

Preparation of reversed micelles

Reversed micelles were prepared by injection of 20 μl of aqueous solution/ml of Brij 96 in cyclohexane. The Brij 96

concentration depended on the required ω_0. The aqueous volume consisted of 10 μl of 50 mM-acetate buffer, pH 5, and 10 μl of either the dialysed enzyme or water, in order to make either enzyme-containing or empty reversed micelles. After gently shaking for 15 s–1 min the solutions became transparent and the reversed micelles were ready to be used.

Study of micellar parameters: ω_0 and θ

ω_0 was studied at a fixed θ value (0.002) in the reaction medium by varying the Brij 96 concentration. As the surfactant concentration decreased, so the formation of micellar solutions was delayed, and ω_0 values no higher than 20 could be obtained within 1 min.

θ was studied at a fixed ω_0 value (10) by adding empty micelles to the reaction medium.

Enzyme assay

Kinetic studies were performed on a Uvikon 930 spectrophotometer equipped with thermostatically controlled cells. Catecholase activity was monitored by the appearance of either 4-t-butyl-*o*-benzoquinone at 375 nm (ϵ 1667 $M^{-1}\,cm^{-1}$) or 4-methyl-*o*-benzoquinone at 375 nm (ϵ 1591 $M^{-1}\cdot cm^{-1}$) after the addition of tyrosinase entrapped in Brij 96/cyclohexane reversed micelles to TBC or 4MC dissolved in cyclohexane respectively. The standard reaction medium contained 1 μg of tyrosinase/ml in 25 mM-acetate buffer, pH 5, at 25 °C.

Fitting of the data

The partition coefficients and the kinetic parameters (±S.E.M.) were obtained by fitting the experimental data to the multiphasic model equations by means of non-linear regression [26].

RESULTS AND DISCUSSION

Using molecular oxygen, both aqueous tyrosinase [27,28] and tyrosinase entrapped in reversed micelles [7] catalyse the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase activity) and further oxidation of *o*-diphenols to *o*-quinones (catecholase activity).

In the present paper the dependence of catecholase activity on the micellar parameters ω_0 and θ is considered.

Owing to the number of parameters that the complete proposed model bears, it would be difficult to fit the results to one set of parameters. In order to simplify the fittings it is better to study first the parameters independent of substrate concentration and afterwards those in which the substrate is involved.

The steps to follow to obtain a complete set of parameters are as follows: (i) the dependence of $V_{max.}$ on ω_0 is studied; (ii) these experimental results are fitted to the enzyme-partitioning model by non-linear regression. This gives the partition coefficients of the enzyme between the micellar domains and its catalytic constants (one for each micellar phase). Then the numerical values of these parameters are substituted into the equations; (iii) the dependence of the reaction rate on θ is studied; (iv) these experimental results are fitted by non-linear regression to the global model, made up from the enzyme-partitioning model plus eqns. (1)–(15) of the present paper. As a result, values for P_1, P_2, P_3, $K_{m,f}$, $K_{m,b}$ and $K_{m,s}$ are obtained.

Dependence on ω_0: kinetic parameters independent of substrate concentration

By using the previously described model [22], the partition coefficients for the enzyme and the catalytic constants can be determined.

Curve A in Fig. 1 shows the dependence of $V_{max.}$ on ω_0 by using TBC as substrate. $V_{max.}$ was graphically determined by means of double-reciprocal plots. From the pattern observed we can deduce that tyrosine mostly works in the free water of the water pool of the reversed micelles. The previous model predicts that, when we consider the maximal reaction rate, the ω_0 pattern depends neither on the kind of substrate nor its concentration. Thus if we use a hydrophilic substrate such as 4MC instead of TBC, the ω_0 profile is only affected by the rate of breakdown of the enzyme–substrate complex. Curve B in Fig. 1 shows a dependence with respect to ω_0 similar to that obtained with TBC. Thus it seems to be reasonable to believe that mushroom tyrosinase solubilizes mainly in the free water of the reversed micelles.

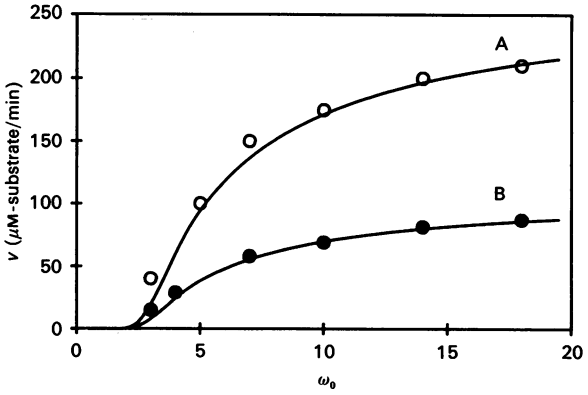

Fig. 1. Effect of ω_0 on $V_{max.}$

This effect was checked for TBC (○) and 4MC (●) at $\theta = 0.002$. The experimental points were fitted by using our previous model [22] and the following sets of parameters (±S.E.M.): curve A: $K^1_E = 0.437 \pm 0.018$, $K^2_E = 0.478 \pm 0.032$, $k_f = 32202 \pm 474$ μM-TBC/(min·μM-E), $k_b = 0$, $k_s = 0$; curve B: $K^1_E = 0.441 \pm 0.017$, $K^2_E = 0.484 \pm 0.030$, $k_f = 13052 \pm 177$ μM-4MC/(min·μM-E), $k_b = 0$, $k_s = 0$. In both curves $n = 3$. Continuous lines are the theoretical curves. For experimental conditions, see the Materials and methods section.

It has been shown that such a profile is bell-shaped for grape polyphenol oxidase when 4MC is used as substrate in Brij 96/cyclohexane reversed micelles [7]. These results seem to indicate that the nature of the ω_0 pattern may be a consequence of the external groups of the enzyme and their interaction with the reverse-micellar medium. Indeed, grape polyphenol oxidase is a thylakoidal enzyme [29] that is extracted by means of detergents [30], whereas mushroom tyrosinase is a soluble enzyme [31].

Walde *et al.* [32] reported a specific substrate effect on the enzymic activity of trypsin in dioctyl sodium sulphosuccinate (AOT)/iso-octane reversed micelles. They observed that, when using N^{α}-benzoyl-L-arginine ethyl ester as substrate, trypsin displays a bell-shaped ω_0 profile with a maximum at $\omega_0 = 10$. However, when they used N^{α}-benzoyl-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide as substrate, an appreciably less hydrophilic substrate, tryptic activity increased from $\omega_0 = 6.6$ to 14.4. This specific substrate effect disagrees with both the predictions of our model and our results. Nevertheless, such an effect can be seen when a non-saturant substrate concentration is assayed; wherever the enzyme dissolves, the partition coefficients of the substrate will play the most important role in the substrate concentration on which the enzyme works. Thus, when the substrate dissolves in the same domain as the enzyme along with ω_0, i.e. bound water, an apparent hyperbola-like shape can be observed.

To fit the results accurately to the model it is necessary to consider only three water molecules being tightly bound per

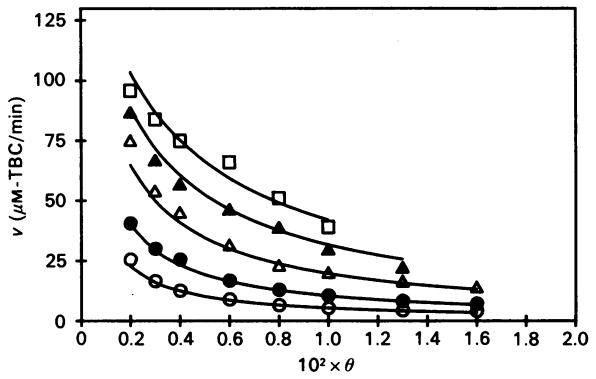

Fig. 2. Effect of θ on the reaction rate

Several TBC concentrations were examined: 2.5 mM (○), 5 mM (●), 10 mM (△), 17.5 mM (▲) and 25 mM (□) at $\omega_0 = 10$. The experimental points were fitted by using the extended model with the following sets of parameters (±S.E.M.): $P_1 = 47.30 \pm 1.04$, $P_2 = 9.67 \pm 0.23$, $P_3 = 0.00017 \pm 0.00004$, $K_{m,t} = 13.56 \pm 0.29$ mM, $K_{m,b}$ and $K_{m,s}$ are not considered because the enzyme is not active in those domains. Continuous lines are the theoretical curves. Other conditions are as in Fig. 1.

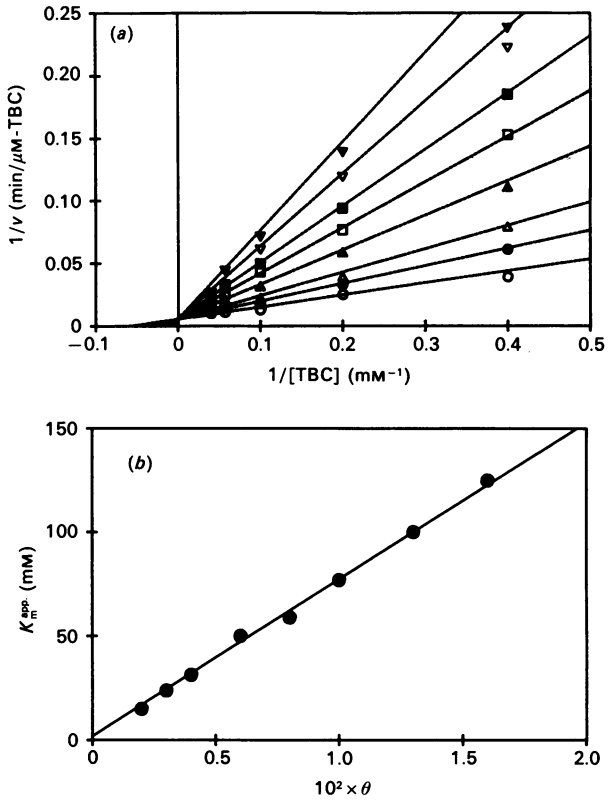

Fig. 3. (*a*) Double-reciprocal plot of the experimental and theoretical data and (*b*) effect of θ on the apparent Michaelis constant

(*a*) $10^2 \times \theta$ values are: 0.2 (○), 0.3 (●), 0.4 (△), 0.6 (▲), 0.8 (□), 1 (■), 1.3 (▽) and 1.6 (▼). The straight lines are the theoretical values. (*b*) $K_m^{app.}$ is the value calculated by means of the double-reciprocal plot in (*a*). The continuous line represents the theoretical dependence calculated by using eqn. (12).

surfactant polar head. No physical studies on hydration requirements of Brij 96 have been reported, but it is clear that the hydration number of this surfactant has to be smaller than that of ionic surfactants, mainly because of the absence of the counterion. For example, AOT, which is the most commonly used surfactant, requires about nine water molecules to hydrate its polar head, of which six belong to the sodium counterion [10]. Indeed, when the weakly hydrated cation Cs^+ is used instead of Na^+, the bound-to-free water transition occurs at $\omega_0 = 4$ [33].

Dependence on θ: kinetic parameters dependent on the substrate concentration

In the enzyme-partitioning model [22], where the maximal reaction rate or the overall catalytic constant was considered, we saw that the most important effect on the catalytic activity was produced by ω_0. On the other hand, θ had little effect, especially when studied far from critical ω_0 values (far from the number of water molecules tightly bound to the surfactant polar head).

Quite the reverse seems to occur with θ if the model is extended by introducing the substrate (and consequently its distribution among the reversed-micelle domains).

A theoretical treatment of the θ effect, from the point of view of the substrate partitioning, was carried out by Levashov *et al.* [20], who considered the reverse-micelle system as biphasic. In the present paper the reverse-micelle system is considered as multiphasic. In order to prove which model is closer to reality, our experimental results were fitted to both models.

Fitting results to the biphasic model

Fig. 2 (symbols) shows the variation of the initial rate with respect to θ at several TBC concentrations. In a biphasic system, the solute concentration in a phase depends on its partition coefficient and the relative volumes of the phases. If the phase where the solute tends to be more concentrated increases with respect to the other, a dilution of the solute occurs in the former. θ represents the relative volumes, since the overall volume does not vary. As the increase of θ leads to a decrease in the reaction rate, it is reasonable to believe that a dilution of the substrate is taking place. If this is so, the substrate could be expected to be dissolved in the micellar phase. On this basis, θ only affects K_m but not $V_{max.}$.

Fig. 3(*a*) (symbols) shows a double-reciprocal plot. The set of lines obtained by linear regression of data have the same intercept, as should be expected from eqn. (21). The maximal rate remains constant, having a value of 175 μM-TBC·min^{-1}, the same as that obtained in Fig. 1 at $\omega_0 = 10$; however, the K_m varies with θ.

As Fig. 3(*b*) shows, the experimental $K_m^{app.}$ has a linear relationship with θ. According to eqn. (19), the slope of this straight line is $K_m(P-1)/P$ and the intercept K_m/P. So, when $P \gg 1$, as seems to occur here, the intercept is very small and the slope is approx. K_m. A value of 7.8 M is obtained. P could be evaluated as well, but being so big, the $K_m^{app.}$-versus-θ plot is not sensitive enough for this purpose.

In the paper by Levashov *et al.* [20], who studied the hydrolysis of N^{α}-benzoyl-DL-arginine *p*-nitroanilide catalysed by trypsin in AOT/octane reverse micelles, this problem was not put forward because they considered the substrate completely micellar, owing to its ionic character; thus P tended to infinity. Our data, replotted according to eqn. (22), are shown in Fig. 4 (symbols). A linear regression fit showed a common intercept whose coordinates are $-1/(P-1)$ and $1/V_{max.}$ in accordance with eqn. (22). An approximate value for P of 8000 was obtained.

Fitting the data to the biphasic model leads us to a paradoxical conclusion: the substrate seems to undergo a dilution when θ increases, meaning that it dissolves in the micellar phase, but, at the same time, K_m is extremely high; bearing this in mind, one can ask how tyrosinase expresses such a high K_m when the

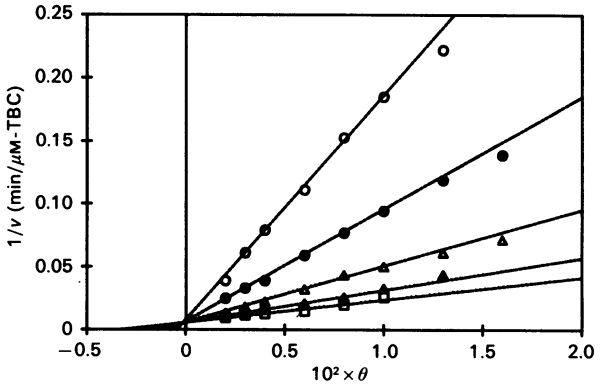

Fig. 4. Dependence on θ of the reciprocal of the reaction rate

The data are replotted from Fig. 2. Symbols represent the same TBC concentrations as in Fig. 2. The straight lines are the theoretical values.

substrate is practically all dissolved in the same reduced volume as the enzyme.

Fitting results to the multiphasic model

This model considers a reverse-micelle system made up of a maximum number of four phases: free water, bound water, surfactant tails and organic solvent. Bardez *et al.* [34], who used fluorescent probes derived from naphthalene of increasing polarity, showed these three solubilization sites in the reverse micelles, the organic solvent being the fourth. As free water only appears when surfactant hydration requirements have been satisfied, the system would have three phases in this case. Even two phases will form the system before the addition of water. The experiments described here were performed with a four-phase system.

The variation of θ involves a parallel variation of free water, bound water and surfactant, the organic-solvent volume varying in a complementary way. That is, if θ increases, the organic-solvent volume decreases, and vice versa.

Data from Fig. 2 were fitted by non-linear regression to the model (curves). As ω_0 experiments show, no activity is expressed when no free water is present. Consequently, we only considered the kinetic parameters in free water in the model fittings.

The set of parameters that best fits the experimental results yielded a $K_{m,f}$ value of 13.5 mM. The partition coefficients obtained indicate that the substrate is mainly solubilized in the surfactant tails and, to a minor extent, in the other reverse-micelle phases and the organic solvent. Two facts support these results: (i) the amount of TBC that the system can support before it gets turbid increases with θ, and (ii) the low solubility of TBC in water. It is known that, with the cationic surfactant cetyltrimethylammonium bromide, the presence of a co-surfactant is necessary to form reversed micelles [35], stabilizing the system within limits. Above a certain concentration of co-surfactant the system leaves the equilibrium and becomes turbid [36,37]. In this way it seems that TBC behaves as a co-surfactant, and can thus be regarded as an interfacial substrate.

When θ varies, the ratio $\alpha/\beta/\gamma$ keeps constant, provided that ω_0 is far from critical values, as occurs here, and the organic-solvent volume is variable. Consequently a dilution of the substrate is taking place as θ increases, which may be the cause of the decrease in activity.

The kinetic behaviour of tyrosinase in Brij 96/cyclohexane reverse micelles agrees with eqn. (11), where only the enzyme dissolved in free water is active; thus i = f and $K_m^{app.}$ then takes the form of eqn. (12).

As Fig. 3(*a*) (broken lines) shows, a Lineweaver–Burk plot of the curves from Fig. 2 gives a set of lines that cross at the intercept. The continuous line in Fig. 3(*b*) was obtained from eqn. (12). As we know θ, ω_0 and n, the values of α, β and γ are readily calculated. Thus we can use eqn. (12) to simulate the dependence of $K_m^{app.}$ on θ. In contrast with the biphasic model, the slope is not the true K_m, but it is involved in a more complex expression.

It can be deduced from these plots that the enzyme is only active in one phase; otherwise the experimental and theoretical results would be expected to deviate from linearity in a double-reciprocal plot.

Another difference with the biphasic model is that the abscissa of the interception point in Fig. 4 (broken lines) is not the result of a unique partition coefficient, but of a more complex expression involving three partition coefficients.

We have seen that, although both models are capable of fitting the experimental results, the true K_m of tyrosinase is quite different, depending on which model is used to calculate it. The simple approach of the biphasic model leads us to a situation in which it was hard to match such a high K_m with the solubilization site of the substrate. On the other hand, the multiphasic model, taking into account the structure of reverse micelles, achieves a set of parameters that satisfactorily describe the kinetics of tyrosinase in reverse micelles.

The concept of micellar substrate: ρ

Wherever the substrate dissolves, provided that it is in a reversed-micelle domain, a definite dependence between the reaction rate and the ratio $\rho = [S]_T/\theta$ can be established.

In the expression of $K_{m_i}^{app.}$ [see eqns. 12–14], two kinds of contribution can be distinguished: on the one hand, the con-

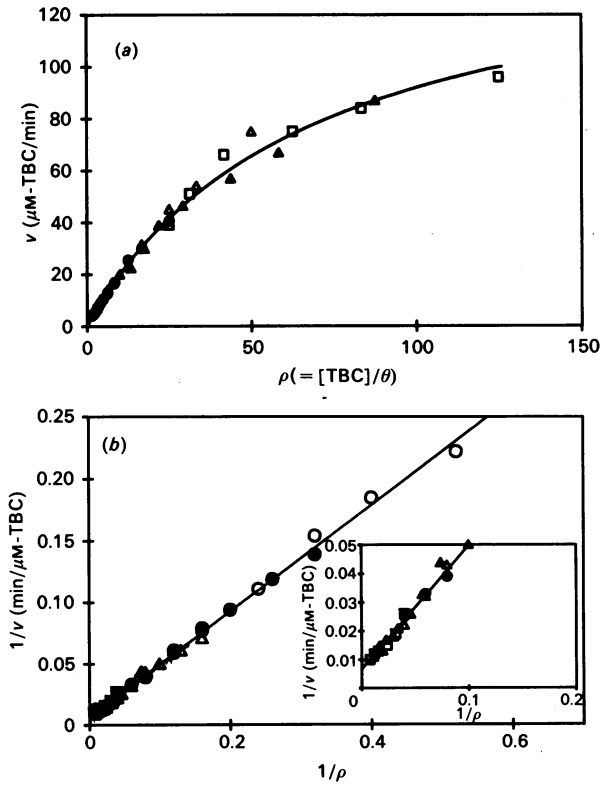

Fig. 5. (*a*) Effect of substrate concentration/θ ratio on the reaction rate and (*b*) double-reciprocal plot

Symbols are the same as in Fig. 2. See the text for an explanation.

tribution of the micellar phases, expressed by the term $\alpha+P_1\beta+P_1P_2\gamma$, and on the other hand, the contribution of the continuous oil, expressed by the term $P_1P_2P_3(1-\alpha-\beta-\gamma)$. The former can be considered as an homogeneous block if ω_0 is far from the critical values, in which case the ratio $\alpha/\beta/\gamma$ remains practically constant. In such conditions, the relationship between $K_m^{app.}$ and θ will depend only on the partition coefficients of the substrate. The distinction between micellar and non-micellar substrates will be governed mainly by P_3. If P_3 is sufficiently small, as in the present case, the contribution of the continuous oil compared with that of the micellar domains should be neglected and thus $K_{m_i}^{app.}/\theta$ is a constant along with θ.

As shown in Fig. 5(*a*), the reaction rate depends on ρ in a hyperbolic manner, in accordance with the equation:

$$v = \sum_{i=f,b,s} \frac{V_{max.,i}\rho}{K_{m_i}^{app.}/\theta+\rho} \qquad (23)$$

which is derived from eqn. (11) by introducing the parameter ρ.

Eqn. (23) describes the sum of three hyperbolae; thus, a double-reciprocal plot of $1/v$ versus $1/\rho$ should show a non-linear dependence. A deviation from linearity should be expected as $1/\rho$ decreases.

Replotting the data from Fig. 5(*a*) in such a way, a linear dependence is observed (Fig. 5*b*); a detail of this plot is shown in the inset. This result is in accordance with our hypothesis that the enzyme is only active in the free-water phase, thus expressing a unique catalytic constant and a unique K_m, as demonstrated by the set of parameters that best fit the experimental data from Figs. 1 and 2. For mushroom tyrosinase working on TBC in Brij 96/cyclohexane reverse micelles, eqn. (23) is reduced to a simple rectangular hyperbola.

It should be noted that, in a pure biphasic system, i.e. water/water-immiscible organic solvent, the partition of TBC will probably be towards the organic solvent. In reversed micelles, we have seen that TBC behaves as an interfacial substrate. This difference in the behaviour of TBC between the two systems makes reversed micelles the more appropriate for the transformation of a range of poorly-water-soluble substrates which, as is the case with TBC, have a somewhat amphiphilic character.

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