Application of active-phase plot to the kinetic analysis of lipoxxygenase in reverse micelles

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A new plot for explaining the complex expression of the enzymic activity in reverse micelles has been developed as an extension of the theoretical model described by our group [Bru, Sánchez-Ferrer & García-Carmona (1990) *Biochem. J.* **268**, 679–684]. The plot describes the changes in the relative volume, amount of enzyme (μmoles), enzyme concentration (μM) and substrate concentration (μM) in the phase where the enzyme is active. To illustrate the usefulness of this plot, the complex activity of soya bean lipoxxygenase in reverse micelles acting on its interfacial substrate, octadecadienoic acid, was studied. It showed the key parameters ruling the activity profiles of lipoxxygenase with respect to micelle size (ω_0), micelle concentration (θ) and the substrate/surfactant molar ratio (ρ), which have never been described before.

INTRODUCTION

Enzymology in amphiphilic self-organizing systems has attracted much attention over the last three decades, since the majority of the enzymes *in vivo* function on the surface of biological membranes or inside them, in the so called ‘lipid particles’ [1]. These particles consist mainly of reverse micelles and microheterogeneous aggregates (lamellae, cylinders, etc). The most studied system is that of the reverse micelles because they are macroscopically homogeneous and transparent; they permit the use of all spectroscopic techniques, and enzymes can be entrapped in them with no drastic loss of activity [2,3].

Reverse micelles are spherical water microdroplets surrounded by a monolayer of surfactant molecules dispersed in an apolar solvent. According to El Seoud [4], their physicochemical structure, when the amount of water exceeds the hydration requirements of surfactants, consists of three different domains: free water (similar to bulk water), bound water (a highly structured layer of water molecules) and surfactant tails which penetrate the apolar solvent. Although the term ‘bound water’ usually refers to water molecules bound to the surfactant heads, it should also include the highly structured water bound to the enzyme surface. This implies that free water starts to appear not when the surfactant molecules alone are hydrated but when both surfactant and enzyme molecules are fully hydrated [5]. Thus, the size of the micelle where free water appears depends on the enzyme molecule entrapped in the reverse micelle [6].

These reverse micelles are ruled by a dynamic equilibrium where amphiphilic molecules of surfactant self-assemble spontaneously to form spherical or ellipsoidal aggregates. These modes of aggregation involve the fusion of micelles to give a dimer, which immediately splits into two monomeric micelles giving rise to an exchange of micellar contents. The presence of this dimer explains why the real state of exchange, when measured experimentally, is about $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ instead of the estimated $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ when only the rate of diffusion is involved [2]. Even taking into consideration this value ($10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) the rate of enzymic reaction is clearly several times lower. This allows reverse micelles to be considered as a pseudocontinuous phase [7]. Thus, and from an enzymic point of view, a reverse-micellar medium is equivalent to four phases in equilibrium: free water, bound water, surfactant tails and organic solvent. However,

when the interacting species are two macromolecules (such as trypsin and soya bean trypsin inhibitor), there are diffusional problems, since the exchange between these macromolecule-containing reverse micelles slows down 1000-fold and the limiting step in the exchange, the fusion, decreases 10^6 -fold [5].

As has been described in different theoretical models [7–11], the partition of enzyme and substrate between the interior water-pool phases and the interfacial surfactant region has a strong influence on enzyme activity in reverse micelles. However, the enzymes and substrates described in the literature about reverse micelles are mainly located in free water, and only a few studies have been carried out with interfacial substrates [11], mainly because of the difficulty in understanding the experimental results.

This paper describes a new plot, which uses the equations described in the theoretical model of Bru *et al.* [7,11], to help interpret enzyme kinetics in reverse micelles. It is applied to understanding how the interfacial substrate, octadecadienoic acid, affects the soya bean lipoxxygenase activity in dioctyl sodium sulphosuccinate (AOT) reverse micelles. Lipoxxygenase (EC 1.13.11.12) is a non-haem iron-containing dioxygenase that catalyses the addition of molecular oxygen to fatty acids with a *cis,cis*-1,4-pentadiene system, to give unsaturated fatty-acid hydroperoxide [12]. Reverse micelles are an ideal medium for studying this reaction, since lipoxxygenase is a water-soluble enzyme working with substrates of low solubility (especially at low pH) and with interfacial characteristics. This enzyme has also been shown to retain its catalytic activity in reverse micelles of AOT [13–15], although no detailed study has been carried out on the kinetics of octadecadienoic acid peroxidation catalysed by soya bean lipoxxygenase.

The results obtained provide a better understanding of the complex kinetic behaviour displayed by lipoxxygenase in reverse micelles and, more importantly, illustrate how the balance between enzyme distribution and substrate distribution gives rise to the profiles of activity versus micelle size, micelle concentration and the substrate/surfactant molar ratio, which have not been described before.

MATERIALS AND METHODS

Materials

Soya bean lipoxxygenase IB, octadecadienoic acid and AOT

Abbreviation used: AOT, dioctyl sodium sulphosuccinate (‘Aerosol OT’).

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were obtained from Sigma (Spain) and were used without further purification. Iso-octane (u.v. spectroscopic grade) was from PANREAC (Spain).

Preparation of reverse micelles

Reverse micelles were prepared by injection of equal amounts of 25 mM-borate buffer, pH 9.0, and lipoxygenase solution (20 mg/ml) into 50 mM-AOT/iso-octane stock solution, during constant gentle shaking, until a completely transparent solution was formed.

Enzyme assay

Kinetic studies were performed on a Uvikon 940 spectrophotometer. Lipoxygenase activity was monitored by the appearance of octadecadienoic acid hydroperoxide at its spectral maximum (ϵ_{234} 30000 M⁻¹·cm⁻¹) [13]. To do this spectrophotometrically, the concentration of AOT in the cuvettes was kept below 6 mM to avoid high AOT absorption in the 234 nm region. This enhanced the sensitivity compared with the method used by Kurganov *et al.* [13] (activity recorded at 245 nm), since the ratio A_{234}/A_{245} is 1.6. In addition, recording at 234 nm is also useful for detecting autooxidation of octadecadienoic acid before the enzymic reaction starts.

The reaction medium consisted of octadecadienoic acid at different concentrations, iso-octane and enzyme-containing or empty reverse micelles in such volumes that the AOT concentration never exceeded 6 mM. Lipoxygenase concentration was held constant at 2.7 μ g/ml.

List of parameters

ω_0 : micelle size [H₂O]/[surfactant].

θ : fraction of water (v/v). If ω_0 is held constant this parameter represents the micelle concentration.

ρ : [substrate]/ θ .

Subscripts: _f, free water; _b, bound water; _s, surfactant tails; _{os}, organic solvent.

K_E^1, K_E^2 : enzyme partition coefficients. $K_E^1 = [E]_b/[E]_f$; $K_E^2 = [E]_s/[E]_b$.

k_i : catalytic constant in each domain.

$P_{1,2,3}$: partition coefficients of the substrate. $P_1 = [S]_b/[S]_f$; $P_2 = [S]_s/[S]_b$; $P_3 = [S]_{os}/[S]_s$.

Study of micellar parameters

The effect of micelle size ($\omega_0 = [\text{H}_2\text{O}]/[\text{surfactant}]$) on lipoxygenase activity was studied at a fixed water-volume fraction ($\theta = 0.05\%$) in the reaction medium (2.5 ml) by adding the necessary amount of surfactant to previously formed reverse micelles, to decrease the water/surfactant molar ratio.

The effect of micelle concentration (θ) at a fixed ω_0 value of 30 was studied by adding reverse micelles containing only buffer to the reaction medium until the desired water content was reached. A study of this effect at different substrate concentrations allowed the replotting of the results as a function of the substrate/surfactant molar ratio.

Computer simulations

The experimental results were simulated by inserting the equations of the multiphasic theoretical model described by Bru *et al.* [7,11] into a compiled BASIC program. The values for the unknown parameters (enzyme and substrate partition coefficients and the enzyme kinetic parameters, in each phase) were estimated according to the results previously published for the enzyme in water and in reverse micelles [13].

Soya bean lipoxygenase is assumed to work only in free water since no activity is found in reverse micelles until $\omega_0 = 8$ (see Fig. 1a). It is known that in AOT reverse micelles the bound-water

Fig. 1. (a) Effect of micelle size (ω_0) on lipoxygenase activity and (b) active-phase plot for ω_0 .

(a) Different octadecadienoic acid concentrations were examined: 1.28 mM ($\square$), 0.4 mM ($\bullet$) and 0.020 mM (∇) at a fixed water content ($\theta = 0.05\%$). The experimental points are the symbols and solid lines are the computer-simulated rates determined using the multiphasic model [7,11] with the following set of parameters: $K_E^1 = 100$, $K_E^2 = 0.001$, $k_i = 6000 \text{ min}^{-1}$, $k_b = 0$, $k_s = 0$, $P_1 = 1$, $P_2 = 10000$, $P_3 = 0.008$, $K_{mr} = 0.05 \text{ mM}$. $K_{m,b}$ and $K_{m,s}$ were not considered because the enzyme is not active in those domains. (b) The figures on the curves represent the percentage change in the volume of free water (1), substrate concentration (2), μ moles of enzyme (3) and enzyme concentration (μM) (4). See the Materials and methods section for an explanation of how the plot is obtained.

region ranges from ω_0 values of 1 to 8 and that free water starts to appear above this value [2]. Thus, the enzyme's catalytic parameters in bound-water and surfactant-tail phases are not taken into consideration.

The enzyme partition coefficients (K_E^1 , K_E^2) are difficult to measure experimentally, and so the location of the enzyme is simulated by using the different activity patterns with respect to K_E^1 , K_E^2 shown by Bru *et al.* [7].

Finally, the substrate partition coefficients were chosen according to the distribution of octadecadienoic acid in a buffer/iso-octane solution [13]. Octadecadienoic acid concentration was 80-fold higher in organic solvent than in buffer. Therefore, the product of P_1 , P_2 and P_3 must be 80 since $[S]_{os} = P_1 \cdot P_2 \cdot P_3 \cdot [S]_f$ (see 'List of parameters'). P_2 is the highest value because octa-

decadienoic acid partitions mainly at the surfactant interface [13].

Active-phase plot

Once the parameters that simulate the dependence of enzyme activity upon ω_0 , θ or ρ are obtained, the active-phase plot is constructed by using the computer to calculate the percentage change in the volume (μl), enzyme concentration (μM), amount of enzyme (μmoles) and substrate concentration (μM) in the phase in which the enzyme is active. These values listed by the computer are introduced into a plotting program to obtain the graph. Because percentages are used instead of real values, this plot is independent of the overall enzyme and substrate concentrations employed.

RESULTS

Effect of micelle size

An important parameter for characterizing a reverse micellar system is the molar ratio of water to surfactant ($\omega_0 = [\text{H}_2\text{O}]/$

[AOT]). This parameter determines most of the structural and physical properties of reverse micelles (for example the size of water pools) and strongly affects the enzymic activity [2]. In the case of lipoxigenase, a linear increase in activity, which did not reach a saturation pattern, was observed (Fig. 1a). This profile is reproduced at different substrate concentrations, which shows that no 'pH artifacts' [16] are produced by the fatty-acid substrate at low ω_0 values, where a lower amount of buffer is present than at the higher substrate concentrations. These 'pH artifacts' consist of different ω_0 profiles according to the substrate concentration used, and are generally produced when acidic substrates are assayed. They were demonstrated recently by Walde *et al.* [16] with acidic substrates of α -chymotrypsin, and the same authors suggested that lipoxigenase acting on octadecadienoic acid might produce the same artifacts [16]. However, the experimental results presented in this paper show that this is not the case. This can be explained by the fact that in octadecadienoic acid and AOT reverse micelles, the substrate is clearly partitioned at the interface [13] and only a low concentration of substrate, always below the buffering capacity of the buffer, is present in the water pool.

The experimental results were simulated using the multiphasic model described by Bru *et al.* [7,11] (Fig. 1a, solid lines). Lipoxigenase was assumed to be active in free water only, because activity is not found when ω_0 values are below 8, and the substrate is clearly located at the interface (see the Materials and methods section).

The complex activity profiles shown by lipoxigenase in reverse micelles would be easier to interpret if the changes in the phase where the enzyme is active could be followed. To solve this problem, we have developed a new plot called the 'active-phase plot'. This consists of plotting the percentage changes in volume (μl), substrate concentration (μM) and enzyme expressed both in concentration (μM) and in amount (μmoles) in the enzyme active phase with respect to the micellar parameter (ω_0 , θ , ρ) under study.

The active-phase plot for ω_0 (Fig. 1b) shows an increase in free water as ω_0 increases. The substrate concentration (μM) in free water does not change drastically, although the enzyme concentration increases. This is due to the displacement of lipoxigenase from the bound-water phase to the free-water phase, since the volume of free water increases as bound water decreases, thus maintaining the water-volume fraction (θ) constant throughout the ω_0 experiment. The key parameter for understanding the ω_0 profile in Fig. 1(a) is the amount (μmoles) of lipoxigenase in free water, which shows the same trend as the activity expressed experimentally (Fig. 1b, line 3).

In fact, only two parameters of the active-phase plot were found to be essential to explain the enzymic activity expressed: these were the amount (μmoles) of enzyme and the substrate concentration (μM) in the active phase. This is because of the physical methods used to follow the enzymic activity (mainly spectrophotometric). This raises the question why is enzyme in μmoles important and not μM ? Perhaps because the equipment cannot follow the activity in the volume where the enzyme is active, but can only follow the total volume of the cuvette. Then, why must substrate concentration be in μM and not in μmoles ? This may be because the enzyme can only work with the substrate molecules in the volume of the phase where it is active. The other two parameters [volume and enzyme concentration (μM)] help to explain the movement of substrate and enzyme from other phases to the active phase.

The ω_0 profile obtained in Fig. 1(a) is different to the one obtained by Kurganov *et al.* [13] since this group used different experimental conditions: (i) 50 mM-AOT compared with 6 mM; (ii) activity was followed at 250 nm compared with 234 nm,

Fig. 2. (a) Effect of micelle concentration (θ) on the reaction rate and (b) active-phase plot for θ

The effect of θ was checked at different octadecadienoic acid concentrations: 0.010 mM ($\blacksquare$), 0.020 mM (∇), 0.040 mM ($\blacklozenge$), 0.080 mM ($\triangle$) and 1.28 mM ($\square$) at a fixed micelle size ($\omega_0 = 30$). The continuous lines represent the computer-simulated curves obtained with the same estimated parameters as in Fig. 1. (b) The curve figures represent the same percentage changes as described in Fig. 1.

Fig. 3. Dependence of $V_{\max}$ and K_m on θ

$V_{\max}$ (∇) and K_m ($\circ$) were calculated by means of the double-reciprocal plot obtained from Fig. 2(a). The continuous lines have been derived from a second-order regression.

where hydroperoxide absorption is maximal; (iii) ω_0 profile was developed by changing the water content at a fixed surfactant concentration as opposed to our ω_0 profile at a fixed water content, and finally (iv) the use of the 'injection technique' compared with our 'micelle-mix technique', the latter being more reliable [17].

Effect of micelle concentration

Micelle concentration at a fixed ω_0 (called θ or water-volume fraction) is another important parameter that affects enzyme activity in reverse micelles. This parameter is even more important when the substrate is interfacial, as is the case with octadecadienoic acid [13]. However, θ has never been taken into consideration in the studies of lipoxygenase activity in reverse micelles.

Fig. 2(a) shows how θ affects lipoxygenase at different substrate concentrations. The increase in micelle concentration leads to a dilution of substrate at the interface, thus decreasing the whole activity. This response is similar to that displayed by polyphenol oxidase with its interfacial substrate tert-butylcatechol [11]. At high substrate concentrations the activity increased with θ . This behaviour is not easy to interpret, although an active-phase plot similar to Fig. 1(b) provides a clue.

When θ increases, the volume of free water increases proportionally (Fig. 2b), and so the substrate and enzyme concentrations in free water clearly decrease. However, this increase in the volume of free water displaces the enzyme equilibrium between bound and free water, giving rise to a slight increase in the amount (μ moles) of lipoxygenase in free water. Thus, at a low overall substrate concentration, the dilution of substrate is more important than the increase expressed in μ moles of lipoxygenase, as the substrate concentration in free water is below the K_m . This results in a continuous decrease in activity. At a high overall substrate concentration, the substrate concentration in free water is above the K_m and so the increment in μ moles of lipoxygenase in free water increases the whole activity.

The θ profile shown in Fig. 2(a) has never been described before for any enzyme in reverse micelles. In addition, a previously undescribed hyperbolic increase in overall K_m and $V_{\max}$ was observed in the case of lipoxygenase when surfactant concentration increased (Fig. 3). The normal pattern described is linear for K_m , with no changes being observed for $V_{\max}$, as in the case of polyphenol oxidase [11,18] and trypsin [19]. The difference

Fig. 4. (a) Effect of substrate concentration/ θ molar ratio on the reaction rate and (b) the active-phase plot for ρ

(a) Symbols represent the same octadecadienoic acid (OA) concentrations as given in Fig. 2(a). The continuous lines are the computer-simulated values. (b) The figures on the curves represent the same percentage changes as in Fig. 1(b).

between the above enzymes and lipoxygenase is that in the former the interfacial substrate solubilizes in the water pool at a concentration in the millimolar range, whereas octadecadienoic acid is only soluble in water in the micromolar range. Therefore this fatty acid is a highly interfacial substrate compared with the polyphenol oxidase and trypsin interfacial substrates.

Effect of substrate/surfactant molar ratio

This molar ratio, $\rho = [\text{substrate}]/\theta$, was first described by Bru *et al.* [11] and is very useful for interfacial substrates such as octadecadienoic acid. Fig. 4(a) shows the ρ profile of lipoxygenase at different substrate concentrations. As ρ increases (Fig. 4b), the volume of free water decreases and the enzyme and substrate concentrations (μ M) increase, whereas the effective amount (μ moles) of lipoxygenase decreases. At low overall substrate concentrations, the increase in substrate accessibility as ρ increases gives an initial burst in activity until the decrease in the amount of enzyme neutralizes this substrate effect. At a high overall substrate concentration, the substrate concentration in

free water is several times higher than K_m . Thus, changes in substrate concentration do not affect enzyme activity and only decrease the amount of enzyme (μmoles) seen.

This ρ profile has never been described and differs from that previously described in polyphenol oxidase [11], where the enzyme displayed the same activity at the same ρ , independently of the substrate concentration used. In the case of lipoxygenase, the 'same ρ -different activity' effect is explained by the presence of a higher substrate concentration in free water when the overall substrate concentration is higher, whereas in the case of polyphenol oxidase [11] the substrate concentration in free water is independent of the overall substrate concentration used to reach a particular ρ .

DISCUSSION

The presence of four different physicochemical pseudophases in dynamic equilibrium strongly affects the expression of enzymic activity in reverse micelles, in terms of catalytic efficiency (k_{cat}) [20,21], specificity and substrate affinity [22]. The last effect is more obvious when the substrate is amphiphilic, since it can partition among several or all of the phases of the micellar system. The combination of these enzyme and substrate effects, makes the interpretation of enzyme activity in reverse micelles quite complex.

To understand better the behaviour of the enzyme, three different mathematical models have been described [7–11]. Even though these models represent a good approach to simulating the enzyme behaviour in reverse micelles, they do not permit a simple identification of the key parameter that controls the activity expressed.

The active-phase plot developed in this paper represents a clear step forward in the practical application of theoretical models to explain the complex behaviour of enzymes in reverse micelles. It is particularly useful in cases where the substrate partitions among different phases, as in the case of lipoxygenase.

This plot has the following characteristics: (i) it can be calculated easily from the equations described by Bru *et al.* [11]; (ii) it can be used with all the micelle parameters related to enzymic activity, such as micelle size (ω_0), micelle concentration (θ) and the substrate/surfactant molar ratio (ρ); (iii) it is independent of the substrate concentration used to measure the activity, although it gives the experimentalist a clear picture of how substrate concentration changes in the enzyme active phase; (iv) it also clarifies the movement of enzyme inside the water pool from bound to free water, as a consequence of the appearance of free water. At ω_0 values < 8 , the few water molecules are used to solvate the head of the surfactant molecules and to hydrate the surface of enzyme molecules. When the amount of water is sufficient for both processes, water molecules with physicochemical characteristics similar to those of bulk water start to

appear, and the conformational flexibility of the protein is sufficient to catalyse the reaction. The number of water molecules needed to obtain this free water depends on the protein used [6].

In conclusion, the characteristics described above for the active-phase plot make it a useful tool for enzymologists studying micellar systems. Not only can the enzymic activity in reverse micelles now be understood but also new experiments based on simulated active-phase plots can be developed.

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