Evaluation of steady-state kinetic parameters for enzymes solubilized in water-in-oil microemulsion systems

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1. Equations are derived for the steady-state kinetics of substrate conversion by enzymes confined within the water-droplets of water-in-oil microemulsion systems. 2. Water-soluble substrates initially confined within droplets that do not contain enzyme are assumed to be converted into product only after they enter enzyme-containing droplets via the inter-droplet exchange process. 3. Hyperbolic (Michaelis–Menten) kinetics are predicted when the substrate concentration is varied in microemulsions of fixed composition. Both k_{cat} and K_m are predicted to be dependent on the size and concentration of the water-droplets in the microemulsion. 4. The predicted behaviour is shown to be supported by published experimental data. A physical interpretation of the form of the rate equation is presented. 5. The rate equation for an oil-soluble substrate was derived assuming a pseudo-two-phase (oil + water) model for the microemulsion. Both k_{cat} and K_m are shown to be independent of ϕ^{aq} . K_m is larger than the aqueous solution value by a factor approximately equal to the oil/water partition coefficient of the substrate. The validity of the rate equation is confirmed by published data.

INTRODUCTION

Water-in-oil microemulsion systems are colloidal dispersions of water and surfactant in an oil-continuous solvent medium. They are one of the most interesting and physically well-characterized of a group of solvent systems of low water content that have in common the ability to dissolve enzymes with retention of catalytic activity [1–5].

The following is a description of microemulsion physical properties sufficient for an understanding of the concepts introduced in this paper.

Microemulsions are characteristically thermodynamically stable and optically transparent. They are unique among colloidal systems in that they possess a well-defined, highly ordered and easily reproducible structure. In simple microemulsions, formed when the (surfactant + water) volume-fraction is less than about 0.2, the dispersed water-domain is present in the form of tiny (nanometer-diameter) droplets. Each droplet is coated with a close-packed surface monolayer of surfactant molecules, orientated such that the surfactant head-groups are hydrated at the surface of the water-droplet, with the apolar tails protruding out into, and solvated by, the oil. The stability of microemulsions is attributed to the presence of the interfacial surfactant layer, which prevents a thermodynamically unfavourable direct contact between oil and water [6]. The small size of the droplets (approx. one-twentieth of the wavelength of visible light) explains the optical transparency of microemulsions. Droplet sizes have been measured, in a number of different microemulsion systems, by a variety of techniques, including small-angle neutron-scattering [7], proton-n.m.r. spectroscopy [8], fluorescence quenching [9] and dynamic light-scattering [10]. These techniques have also shown that the droplets in a given microemulsion preparation are all of a similar size, i.e. microemulsions are very nearly

monodisperse. To a first approximation, the radius of the water-droplets formed is directly proportional to the compositional parameter, R , where $R = [\text{water}]/[\text{surfactant}]$ (the concentration units are mol/litre of microemulsion). Two surfactants commonly used to stabilize water-in-oil microemulsions are sodium bis-2-ethylhexylsulphosuccinate (anionic) and cetyltrimethylammonium bromide (cationic) [11,12].

At fixed R the droplet concentration is directly proportional to the surfactant concentration [13]. In typical microemulsion preparations the droplet concentration is of the order of 0.1–10 mM, depending on the precise composition. The total (surfactant-covered) interfacial surface area is of the order of 100 m²/cm³ of microemulsion. Algorithms for the calculation of these parameters are given in ref. [14].

The microemulsion droplets are in continuous rapid (Brownian) motion and are free to move randomly and independently throughout the whole volume of the microemulsion. The contents of a given droplet (water, solutes) may be transferred between droplets on collision. The mechanism of this process involves the formation of a ‘transient dimer’ within which intermixing of the contents of the two original droplets occurs. The dimer is short-lived and rapidly breaks down to give two ‘daughter’ droplets structurally identical with the original pair involved in the collision. The second-order rate constant for the exchange of droplet contents, k_{ex} , is of the order of 10⁷ M^{−1}·s^{−1} at 25 °C [9,15–18], and is largely independent of the droplet contents, at least for low-molecular-mass solutes [15,17]. At the droplet concentrations [14] used in experimental systems, therefore, the droplets exchange their contents on the millisecond time scale.

The retention of activity and the stability of enzymes observed in these systems is undoubtedly a consequence of their confinement to the aqueous domain of the microemulsion (i.e.

Abbreviations used: E, enzyme; E·S, the Michaelis complex; k_{cat} , turnover number; k_{ex} , second-order rate constant for the exchange of water-soluble substrates between droplets; K_m^{d} , component with the structure of the Michaelis constant in the expression for K_m^{m} ; K_m^{m} , the hyperbolic constant for an enzyme-catalysed reaction in a microemulsion (eqn. 23); K_m^{w} , the Michaelis constant (eqn. 2); N , Avogadro’s number; P, product of reaction; R , molar ratio of water to surfactant in the microemulsion; V^{d} , volume of a microemulsion droplet; V^{e} , volume of a microemulsion droplet containing E and S; ϕ^{aq} , volume fraction of water in the microemulsion; ϕ^{e} , volume fraction of droplets (E, S); $[\bigcirc]_{\text{T}}$, total microemulsion droplet concentration; $[\bigcirc^{\text{X}}]$, concentration of droplets containing the relevant solute species X (X = E or S); $[\bigcirc^{\text{e}}]$, concentration of empty droplets (containing neither E nor S).

within a water-droplet). Direct information on the structure of proteins in these systems has been obtained by using c.d. and fluorescence techniques [18–22]. These measurements show that proteins retain structural properties characteristic of a primarily aqueous environment.

There is an extensive literature dealing with the use of microemulsions as solvent systems for various enzyme-catalysed reactions. The central interest lies in the ability of microemulsions to dissolve both active enzyme and apolar substrates, thus providing the means for the efficient conversion of substrates into useful products (such as steroids [23,24] and leukotrienes [25]) by use of appropriate enzymes. Much of the published data concerns the measurement of steady-state (Michaelis–Menten) kinetic parameters, usually with comparison of the values obtained with those for the reaction in bulk aqueous solution. Despite this, however, there has been as yet no formal kinetic theory relating the observed kinetics to the molecular kinetics of the reaction, an analysis that surely requires the structural and dynamic properties of the microemulsion to be taken into account.

In order to present a unified theory for the analysis of enzyme kinetics in microemulsion systems, the Briggs–Haldane steady-state formalism has been applied. At this point it is necessary to make a primary distinction between those reactions involving water-soluble substrates and those involving oil-soluble (apolar) substrates. For each case a realistic, and generally applicable, model is proposed for the mechanism of substrate–enzyme encounter and the appropriate rate equation is derived by application of the steady-state approximation.

The present paper is concerned principally with the kinetics of reactions involving water-soluble substrates, the analysis in this case being more involved than for the case of an oil-soluble substrate. The derivation of the appropriate expression for the latter case is given in the Appendix.

NOMENCLATURE

Definition of concentration units

The concentration of a compound dissolved in a microemulsion may be expressed in mol/litre of total microemulsion (M^m), of oil domain (M^{ol}), of aqueous domain (M^{aq}), or of droplet (M^d). The superscripts make the choice of concentration units clear. They also appear in the units of kinetic parameters (rates and second-order rate constants) that contain a concentration dimension, and in equations and reaction schemes, where it is necessary to make clear the frame of reference used to calculate the concentration of the reactant. Aqueous solution parameters containing a concentration dimension are given the superscript 'w'. Parameters such as first-order rate constants, which contain no concentration dimension, require no such distinction.

The choice of reference frame for defining the concentration of a reactant is extremely important when discussing the rates of

bimolecular reactions within the microemulsion. For example, the rate of a reaction occurring within the confines of a single microemulsion droplet is determined by the concentration of the reactants within the droplet and therefore the appropriate units of concentration are mol/litre of droplet. It should also be noted that, with a fixed number of reactant molecules per droplet, their concentrations (and hence the reaction rate) are determined solely by the droplet volume.

THEORY

Derivation of the rate equation for enzymic conversion of water-soluble substrates in water-in-oil microemulsions

The kinetic scheme shown in Scheme 1 is sufficient to describe the steady-state kinetics of the conversion of substrate, S, into product, P, by the majority of enzymes, E, in aqueous solution. E·S is the non-covalent enzyme–substrate (Michaelis) complex. Scheme 1 is strictly applicable under the following conditions [26,27]. (1) The initial substrate concentration is much greater than the enzyme concentration. (2) k_{cat} is a first-order rate constant representing the slowest unimolecular step in the breakdown of E·S to give P and regenerate E. (3) The concentration of product, P, is negligible, so that the rate of the reverse reaction ($P \rightarrow S$) is not significant (i.e. the reaction proceeds under 'initial-rate' conditions). This restriction may be lifted if it can be shown that E is unable to bind P, for the back rate will then be negligible at all concentrations of P.

Application of the steady-state approximation to E and S (i.e. setting $d[E \cdot S]/dt = 0$) yields the Michaelis–Menten equation [26,27]:

$$v^w = \frac{k_{cat}[E_0]^w[S]^w}{K_m^w + [S]^w} \quad (1)$$

where:

$$K_m^w = \frac{k_{-1} + k_{cat}}{k_{+1}} \quad (2)$$

K_m^w is the Michaelis constant, equal to the concentration of S at which $v^w = (k_{cat}[E_0])/2$.

In a water-in-oil microemulsion system both the enzyme and the water-soluble substrate are confined within the water droplets of the microemulsion. The droplet concentration in a typical microemulsion preparation is of the order of millimolar and therefore, at concentrations of enzyme and substrate of this order, there must be at least partial segregation of enzyme and substrate into separate droplets (a kinetically equivalent situation exists in the first moments of mixing a microemulsion containing only S with one containing only E). Scheme 2 is a realistic scheme for the conversion of substrate initially isolated from enzyme in this way, for an enzyme obeying Scheme 1 in aqueous solution. Collision between enzyme-containing and substrate-containing

droplet species, $\textcircled{E}$ and $\textcircled{S}$ respectively, is followed by decomposition of the transient dimer so formed such that both enzyme and substrate molecules are present in just one of the two daughter droplets. This species $\textcircled{E, S}$, may decompose by collision with an empty droplet, $\textcircled{}$, to regenerate $\textcircled{E}$ and $\textcircled{S}$. Alternatively, E and S may combine within the droplet to

Scheme 1. Basic scheme for the reaction between enzyme and substrate in aqueous solution, as described in ref. [26]

Scheme 2. Proposed kinetic scheme for the reaction between enzyme and water-soluble substrate in water-in-oil microemulsions

The scheme refers explicitly to a system of droplets containing no more than one of each reactant species (E or S) per droplet.

form the Michaelis complex $E \cdot S$ (droplet species $(E \cdot S)$). $(E \cdot S)$ may break down either to (E, S) (cf. Scheme 1) or to (E, P) . Since P is formed at a high initial concentration in (E, P) (equal to that of S in (E, S)), the possibility of a reverse reaction, i.e. formation of S from P via $E \cdot S$, should not be ignored, even under 'initial-rate' conditions, where the fraction of S converted into P is negligible. For this reason the $(E, P) \rightarrow (E \cdot S)$ step is included in Scheme 2. In principle, the intermediate species $E \cdot P$ should be introduced to balance the scheme. However, since steady-state kinetics are under consideration, there is no need to distinguish between $E \cdot S$ and $E \cdot P$ [28].

In the absence of direct measurements of the rate of breakdown of the transient dimer, it is not possible to predict *a priori* whether, in a given system, $E \cdot S$ formation takes place within the dimer or within one of the daughter droplets formed on breakdown of the dimer. The form of the final rate equation is, however, critically dependent on this factor. In the current derivation formation of $E \cdot S$ during the lifetime of the dimer is forbidden: the dimer is assumed to break down at a rate much faster than that of $E \cdot S$ binding, so that $E \cdot S$ can form only within a daughter droplet. This approach yields a rate equation that predicts a behaviour that is consistent with all experimental data available at the present time. The derivation of the rate equation obtained given the assumption that $E \cdot S$ may form during the lifetime of the transient dimer is deferred in lieu of appropriate data.

It is assumed that the exchange rate constant, k_{ex} , is independent of the droplet species involved in the reaction. This assumption arises simply because of the practical impossibility of measuring the various exchange rates separately. Measurements of exchange rates for fast irreversible chemical reactions of different types (i.e. with different solutes) all give similar values for k_{ex} . [15–18].

The simplest form of the rate equation is derived by imposing the limitation that enzyme-containing droplets multiply occupied with substrate ($(E, 2S)$, $(E, 3S)$ etc.) are forbidden, so that the immediate precursor of $E \cdot S$ (Scheme 2) is always (E, S) (i.e. $(1E, 1S)$ explicitly). This point is important because the rate of $(E \cdot S)$ formation will be n times faster within the droplet (E, nS) than in (E, S) , and therefore $(E \cdot S)$ formation among a heterogeneous population of droplets, (E, S) , $(E, 2S)$... (E, nS) , cannot be described by a single rate constant at the macroscopic (bulk microemulsion) level, a complication that is best avoided at this stage. Multiple occupation can be avoided in experimental systems by limiting the substrate/droplet ratio, $[S]^m/[E]_T$, to values such that the substrate molecules are present in separate droplets. This ensures that no more than one substrate molecule can be transferred in a single collision between substrate-containing and enzyme-containing droplets. If a normal distribution of substrate molecules over the total droplet population is assumed, the proportion, P_n , of droplets containing n substrate molecules can be estimated by using the Poisson equation, $P_n = \bar{n}^n e^{-\bar{n}} / n!$, where $\bar{n} = [S]^m/[E]_T$ [29]. Solution of this equation shows that multiple occupancy is significant when $\bar{n} \geq 0.1$. When

$\bar{n} = 0.1$ the proportions of the concentrations of the various droplet species, $[(E)]/[S]/[(n, S)]$ ($n \geq 2$), are 0.905:0.0905:0.0045, i.e. only 4.5% of the substrate-containing droplets are multiply occupied. Note that a relatively high percentage of multiply occupied droplets may be tolerated since these still function largely to transfer just one S in a collision. By using the theory of simple (binomial) probabilities, the probability that a $(2S)$ droplet will transfer no, one or two substrate molecules on collision with (E) is 0.25, 0.5 and 0.25 respectively, i.e. there is a 0.67 probability that one, rather than two, substrate molecules will be transferred.

Given that the enzyme concentration must be far (usually at least 100-fold) lower than the substrate concentration, in order for Scheme 1 to be valid, the probability of multiple occupation of droplets by enzyme molecules is negligibly small. The conditions under which the rate equation as presently derived is strictly applicable may be summarized thus: $[(E)]_T > [S]^m \gg [E_0]^m$, i.e. $[(E)]_T > [S] \gg [E_0]$, and $\bar{n} < 0.1$ approximately.

In the derivation that follows, formation of $(E \cdot S)$ from (E, S) and (E, P) is regarded, initially, as a unimolecular process, characterized by apparent first-order rate constants, k_{+1}^{obs} and k_{-2}^{obs} respectively. In this way a change in the reference frame for concentration units, from the volume of the whole microemulsion to that of a single droplet, is avoided at this stage, and the concentration units for the rates of each step are uniformly $M^m \cdot s^{-1}$. The necessity to describe processes involving collisions between droplets initially fixes the reference frame for concentration units as the whole volume of the microemulsion.

The rate equation is derived by applying the steady-state assumption to (E, P) , $(E \cdot S)$ and (E, S) . The mass-conservation equations are:

$$[(E_0)] = [(E)] + [(E, S)] + [(E \cdot S)] \quad (3)$$

where $[(E_0)]$ is the total enzyme concentration, and:

$$[(E)]_T = [(E)] + [S] \quad (4)$$

(since $[(E)]_T > [S] \gg [E_0]$). The units of concentration are M^m . Since, in principle, S may exchange into, and react with, E in (E, P) , (E) and (E, P) need not be distinguished. Hence (E, P) does not appear explicitly in eqn. (3). Applying the steady-state approximation to (E, P) , i.e. setting $d[(E, P)]/dt = 0$, yields the steady-state expression:

$$(k_{-2}^{obs} + k_{ex}[(E)]_T)[(E, P)] - k_{cat}[(E \cdot S)] = 0 \quad (5)$$

The $[(E)]_T$ term appears because P may exchange out of (E, P) by collision with either $[(E)]$ or $[S]$ (eqn. 4). Rearranging eqn. (5) for (E, P) gives:

$$[(E, P)] = \frac{k_{cat}[(E \cdot S)]}{k_{-2}^{obs} + k_{ex}[(E)]_T} \quad (6)$$

Similarly, setting $d[(E \cdot S)]/dt = 0$ yields:

$$k_{+1}^{obs}[(E, S)] + k_{-2}^{obs}[(E, P)] - (k_{-1} + k_{cat})[(E \cdot S)] = 0 \quad (7)$$

Substitution of eqn. (6) into eqn. (7), and dividing by $k_{+1}^{\text{obs.}}$, yields, on re-arranging for $[\text{E} \cdot \text{S}]$:

$$[\text{E} \cdot \text{S}] = [\text{E} \cdot \text{S}] \left(\frac{k_{-1} + k_{\text{cat.}}}{k_{+1}^{\text{obs.}}} - \frac{k_{\text{cat.}} k_{-2}^{\text{obs.}}}{k_{+1}^{\text{obs.}} k_{-2}^{\text{obs.}} + k_{+1}^{\text{obs.}} k_{\text{ex.}} [\text{O}]_{\text{T}}} \right) \quad (8)$$

The steady-state expression for $[\text{E} \cdot \text{S}]$, obtained on setting $d[\text{E} \cdot \text{S}]/dt = 0$, is:

$$k_{\text{ex.}} [\text{E}] [\text{S}] - k_{\text{ex.}} [\text{E} \cdot \text{S}] [\text{O}] + k_{-1} [\text{E} \cdot \text{S}] - k_{+1}^{\text{obs.}} [\text{E} \cdot \text{S}] = 0 \quad (9)$$

Substitution of expressions for $[\text{E}]$ (eqn. 3) and $[\text{O}]$ (eqn. 4) into eqn. (9), followed by dividing throughout by $k_{\text{ex.}}$, gives, on making ' $[\text{E}_0][\text{S}]$ ' the subject and cancelling as appropriate:

$$[\text{E}_0][\text{S}] = [\text{E} \cdot \text{S}] \left([\text{S}] - \frac{k_{-1}}{k_{\text{ex.}}} + [\text{E} \cdot \text{S}] \left([\text{O}]_{\text{T}} + \frac{k_{+1}^{\text{obs.}}}{k_{\text{ex.}}} \right) \right) \quad (10)$$

Substitution of the expression for $[\text{E} \cdot \text{S}]$ (eqn. 8) and multiplying out the terms in parentheses gives, on cancelling:

$$[\text{E}_0][\text{S}] = [\text{E} \cdot \text{S}] \left(\frac{k_{-1} + k_{\text{cat.}}}{k_{+1}^{\text{obs.}}} [\text{O}]_{\text{T}} + \frac{k_{\text{cat.}}}{k_{\text{ex.}}} + [\text{S}] - \frac{k_{-2}^{\text{obs.}} k_{\text{cat.}} [\text{O}]_{\text{T}}}{k_{+1}^{\text{obs.}} k_{-2}^{\text{obs.}} + k_{+1}^{\text{obs.}} k_{\text{ex.}} [\text{O}]_{\text{T}}} - \frac{k_{-2}^{\text{obs.}} k_{\text{cat.}}}{k_{\text{ex.}} k_{-2}^{\text{obs.}} + k_{+1}^{\text{obs.}} k_{\text{ex.}} [\text{O}]_{\text{T}}} \right) \quad (11)$$

It is convenient, at this stage, to replace $k_{+1}^{\text{obs.}}$ and $k_{-2}^{\text{obs.}}$ with expressions for the molecular rate constants k_{+1} and k_{-2} for formation of $\text{E} \cdot \text{S}$ and $\text{E} \cdot \text{P}$ respectively.

The expression for $k_{+1}^{\text{obs.}}$ in terms of k_{+1} is derived by noting firstly that the rate of formation of $\text{E} \cdot \text{S}$ from $[\text{E} \cdot \text{S}]$ must be the same whether it is regarded as a unimolecular or a bimolecular process, when the whole volume of the microemulsion is considered. Thus:

$$k_{+1}^{\text{obs.}} [\text{E} \cdot \text{S}] = k_{+1}^{\text{d}} [\text{E}]^{\text{d}} [\text{S}]^{\text{d}} \phi^{\text{E} \cdot \text{S}} \quad (12)$$

k_{+1}^{d} is the second-order rate constant for the formation of $\text{E} \cdot \text{S}$ within the droplet $[\text{E} \cdot \text{S}]$ (units: $\text{M}^{-1} \text{s}^{-1}$), $[\text{E}]^{\text{d}}$ and $[\text{S}]^{\text{d}}$ are the concentrations of E and S respectively within $[\text{E} \cdot \text{S}]$ and $\phi^{\text{E} \cdot \text{S}}$ is a dimensionless constant, the steady-state volume-fraction of the droplets $[\text{E} \cdot \text{S}]$ in the microemulsion, where:

$$\phi^{\text{E} \cdot \text{S}} = V^{\text{E} \cdot \text{S}} N [\text{E} \cdot \text{S}] \quad (13)$$

$V^{\text{E} \cdot \text{S}}$ is the volume of a single $[\text{E} \cdot \text{S}]$ droplet and N is Avogadro's number. The assumption is now made that the volume of the enzyme molecule is small compared with that of the microemulsion droplets, i.e. $V^{\text{E}} < V^{\text{d}}$, and therefore $V^{\text{E} \cdot \text{S}} = V^{\text{d}}$. In a droplet of volume V^{d} , the concentration of a solute present to the extent of one molecule per droplet is $1/(V^{\text{d}} N)$ (units: M^{d}). Substituting this expression for both $[\text{E}]^{\text{d}}$ and $[\text{S}]^{\text{d}}$, and also the

expression for $\phi^{\text{E} \cdot \text{S}}$ (eqn. 13, noting that $V^{\text{E} \cdot \text{S}} = V^{\text{d}}$) into eqn. (12) gives, on cancelling:

$$k_{+1}^{\text{obs.}} = k_{+1}^{\text{d}} \left(\frac{1}{V^{\text{d}} N} \right) \quad (14)$$

Similarly, in the case of $k_{-2}^{\text{obs.}}$:

$$k_{-2}^{\text{obs.}} = k_{-2}^{\text{d}} \left(\frac{1}{V^{\text{d}} N} \right) \quad (15)$$

The total aqueous phase volume fraction is equal to the total molar concentration of the population of (equal-sized) droplets, multiplied by the volume of a single droplet, i.e.:

$$\phi^{\text{aq}} = V^{\text{d}} M [\text{O}]_{\text{T}} \quad (16)$$

Substitution of eqns. (14) and (15) into eqn. (11), followed by substitution of eqn. (16) for ϕ^{aq} , wherever the $(V^{\text{d}} M [\text{O}]_{\text{T}})$ term appears, yields:

$$[\text{E}_0][\text{S}] = [\text{E} \cdot \text{S}] \left(\frac{k_{-1} + k_{\text{cat.}}}{k_{+1}^{\text{d}}} \phi^{\text{aq}} + \frac{k_{\text{cat.}}}{k_{\text{ex.}}} - \frac{k_{-2}^{\text{d}} k_{\text{cat.}} \phi^{\text{aq}}}{k_{+1}^{\text{d}} k_{-2}^{\text{d}} + k_{+1}^{\text{d}} k_{\text{ex.}} \phi^{\text{aq}}} - \frac{k_{-2}^{\text{d}} k_{\text{cat.}}}{k_{\text{ex.}} k_{-2}^{\text{d}} + k_{+1}^{\text{d}} k_{\text{ex.}} \phi^{\text{aq}}} + [\text{S}] \right) \quad (17)$$

The term $(k_{-1} + k_{\text{cat.}})/k_{+1}^{\text{d}}$ in this equation has the form of the Michaelis constant (cf. eqn. 2), with units of mol/litre of droplets (M^{d}), and is thus referred to subsequently by the symbol K_{m}^{d} .

The net rate of conversion of S into P is $v^{\text{m}} \text{ M}^{\text{m}} \cdot \text{s}^{-1}$, where:

$$v^{\text{m}} = k_{\text{cat.}} [\text{E} \cdot \text{S}] - k_{-2}^{\text{obs.}} [\text{E} \cdot \text{P}] \quad (18)$$

Substituting the expression for $[\text{E} \cdot \text{P}]$ (eqn. 6) and $k_{-2}^{\text{obs.}}$ (eqn. 15), and introducing ϕ^{aq} (eqn. 16), yields:

$$v^{\text{m}} = [\text{E} \cdot \text{S}] \left(k_{\text{cat.}} - \frac{k_{-2}^{\text{d}} k_{\text{cat.}}}{k_{-2}^{\text{d}} + k_{\text{ex.}} \phi^{\text{aq}}} \right) \quad (19)$$

The complete rate equation is obtained by substituting eqn. (17) into eqn. (19) and noting that when each enzyme or substrate molecule is confined within a separate droplet $[\text{E}_0] = [\text{E}_0]^{\text{m}}$ and $[\text{S}] = [\text{S}]^{\text{m}}$:

$$v^{\text{m}} = \frac{\left(k_{\text{cat.}} - \frac{k_{-2}^{\text{d}} k_{\text{cat.}}}{k_{-2}^{\text{d}} + k_{\text{ex.}} \phi^{\text{aq}}} \right) [\text{E}_0]^{\text{m}} [\text{S}]^{\text{m}}}{K_{\text{m}}^{\text{d}} \phi^{\text{aq}} + \frac{k_{\text{cat.}}}{k_{\text{ex.}}} - \frac{k_{-2}^{\text{d}} k_{\text{cat.}} \phi^{\text{aq}}}{k_{+1}^{\text{d}} k_{-2}^{\text{d}} + k_{+1}^{\text{d}} k_{\text{ex.}} \phi^{\text{aq}}} - \frac{k_{-2}^{\text{d}} k_{\text{cat.}}}{k_{\text{ex.}} k_{-2}^{\text{d}} + k_{+1}^{\text{d}} k_{\text{ex.}} \phi^{\text{aq}}} + [\text{S}]^{\text{m}}} \quad (20)$$

$$= \frac{k_{\text{cat.}} (\text{app.}) [\text{E}_0]^{\text{m}} [\text{S}]^{\text{m}}}{K_{\text{m}}^{\text{m}} + [\text{S}]^{\text{m}}} \quad (21)$$

where:

$$k_{\text{cat.}} (\text{app.}) = k_{\text{cat.}} - \frac{k_{-2}^{\text{d}} k_{\text{cat.}}}{k_{-2}^{\text{d}} + k_{\text{ex.}} \phi^{\text{aq}}} \quad (22)$$

with units of s^{-1} , and:

$$K_{\text{m}}^{\text{m}} = K_{\text{m}}^{\text{d}} \phi^{\text{aq}} + \frac{k_{\text{cat.}}}{k_{\text{ex.}}} - \frac{k_{-2}^{\text{d}} k_{\text{cat.}} \phi^{\text{aq}}}{k_{+1}^{\text{d}} k_{-2}^{\text{d}} + k_{+1}^{\text{d}} k_{\text{ex.}} \phi^{\text{aq}}} - \frac{k_{-2}^{\text{d}} k_{\text{cat.}}}{k_{\text{ex.}} k_{-2}^{\text{d}} + k_{+1}^{\text{d}} k_{\text{ex.}} \phi^{\text{aq}}} \quad (23)$$

K_{m}^{m} and each of the terms on the right-hand side have units of M^{m} . In order to see this it is necessary to note that concentrations expressed as mol/litre of droplets may also be expressed as mol/litre of aqueous domain, i.e. $\text{M}^{\text{d}} \equiv \text{M}^{\text{aq}}$, as long as the

droplets are all the same size (eqn. 16, noting that all of the water in the system is confined within these droplets). In principle the 'd' superscript could be replaced by 'aq', but the former is retained in order to emphasize the droplet nature of the aqueous domain.

DISCUSSION

The following discussion assumes implicitly that the model presented here adequately describes the kinetics of the enzyme-catalysed conversion of water-soluble substrates in droplet-type water-in-oil microemulsions.

Properties of the rate equation

The principal features of the rate equation may be summarized in the following points. (1) When k_{-2}^d is zero eqn. (22) reduces to $k_{cat.(app.)} = k_{cat.}$ and eqn. (23) reduces to $K_m^m = K_m^d \phi^{aq} + (k_{cat.}/k_{ex.})$. Thus $k_{cat.}$ is independent of ϕ^{aq} and a plot of K_m^m versus ϕ^{aq} should be linear, with a slope of K_m^d and ordinate intercept $k_{cat.}/k_{ex.}$. (2) When k_{-2}^d is finite $k_{cat.}$ is dependent on ϕ^{aq} . In the limiting case that k_{-2}^d is much greater than $k_{ex.} \phi^{aq}$, $k_{cat.(app.)}$ falls to zero (eqn. 22) and the enzyme-catalysed reaction will not proceed. In the general case a plot of $1/k_{cat.(app.)}$ versus $1/\phi^{aq}$ will be a straight line of slope $k_{-2}^d/(k_{cat.} k_{ex.})$ and ordinate intercept $1/k_{cat.}$. (3) In view of the requirement (point 2) for $k_{-2}^d < k_{ex.} \phi^{aq}$ in order for substrate turnover to proceed, under conditions where the enzyme may bind P, eqn. (23) reduces to $K_m^m = K_m^d \phi^{aq} + (k_{cat.}/k_{ex.}) - (k_{-2}^d k_{cat.}/k_{+1}^d k_{ex.}) - (k_{-2}^d k_{cat.}/k_{ex.}^2 \phi^{aq})$. The form of the dependence of K_m^m on ϕ^{aq} is therefore determined by the relative sizes of the first and fourth terms, as discussed below.

There are presently no published examples of experiments involving suitable reversible reactions, and hence most of these predictions cannot be substantiated without further studies. There are, however, several examples demonstrating a dependence of K_m^m on ϕ^{aq} [30–32], which can be taken as good evidence for the validity of the model. One such example is the study by Martinek *et al.* [30] on trypsin (EC 3.4.21.4) with the synthetic substrate *N*-benzoyl-DL-arginine *p*-nitroanilide in a water-in-oil microemulsion system stabilized by cetyltrimethylammonium bromide. At constant $R = 25$, K_m^m (this nomenclature) was found to be directly proportional to ϕ^{aq} over the range 0–0.4, whereas $k_{cat.}$ remained constant (see Fig. 3 of ref. [30]). This result is consistent with a system for which k_{-2}^d is negligible and $k_{cat.}/k_{ex.} \ll K_m^d \phi^{aq}$. The data show that $k_{cat.}/k_{ex.} = 10^{-10} \text{ M}^m$ and that $K_m^d \phi^{aq}$ is of the order of 10^{-3} M^m . At the present time there are no published studies for high- $k_{cat.}$ systems wherein the $k_{cat.}/k_{ex.}$ term is likely to be significant and consequently no deviations from direct proportionality between K_m^m and ϕ^{aq} have yet been reported. Given that, as published data [30–33] suggest, $K_m^d \phi^{aq}$ will typically be of the order of 10^{-2} – $10^{-3} \phi^{aq} \text{ M}^m$ and given that $k_{ex.} = 10^7 \text{ M}^{-1} \text{ s}^{-1}$, $k_{cat.}/k_{ex.}$ is not expected to be significant unless $k_{cat.}$ is greater than about 10 s^{-1} (given a working lower limit for ϕ^{aq} of 0.01). The reduced form of the third term of eqn. (23) (point 3 above) will be significant (comparable with $k_{cat.}/k_{ex.}$) only when k_{-2}^d is large enough to be comparable with k_{+1}^d . Similarly, the (reduced) fourth term will only be significant when k_{-2}^d is comparable with $k_{ex.}^2 \phi^{aq}$. Note that unless this is so the $K_m^d \phi^{aq}$ term will be much larger and K_m^m will be linearly dependent on ϕ^{aq} . Given that k_{-2}^d is unlikely to exceed $10^6 \text{ M}^{-1} \text{ s}^{-1}$ (as discussed below), the fourth term can reasonably be expected to take a maximum value of $10^{-7}/\phi^{aq} \text{ M}^m$ ($k_{cat.} = 10 \text{ s}^{-1}$). Given that $K_m^d \phi^{aq}$ is of the order of 10^{-3} – $10^{-2} \phi^{aq} \text{ M}^m$, the two terms become comparable only when $\phi^{aq} < 0.01$. As a consequence, a linear dependence of K_m^m on ϕ^{aq} is predicted when $\phi^{aq} > 0.01$, as in a typical experimental system.

The assumption that the enzyme should be smaller than, or equal to, the size of the microemulsion water droplet (eqn. 14) is acceptable, at least to a first approximation, for the enzymes for which data are presently available. For example in the case of α -chymotrypsin, which is equivalent in size to the droplets formed in $R = 12$ cetyltrimethyl bromide or sodium bis-2-ethylhexylsulphosuccinate systems [14], K_m^m is directly proportional to ϕ^{aq} when R is as low as 5.6 [32].

Physical interpretation of the rate equation

The dependence of $k_{cat.(app.)}$ on k_{-2}^d reflects the potential for P to bind to the active site of E in (E, P) (the droplet species formed immediately on release of P from $E \cdot P$), a process favoured by the high instantaneous concentration of P in (E, P) , before P can transfer to another droplet via the exchange process. Since $E \cdot P$ cannot bind S, the effect of P-binding is to lower the time-averaged concentration of functional active sites in the system. The dependence of $k_{cat.(app.)}$ on ϕ^{aq} predicted by eqn. (22) is symptomatic of this behaviour and arises from the dependence of the rate of E–P binding on the size and concentration of the droplets (these parameters being combined in the ϕ^{aq} term, eqn. 16). The rate at which P can exchange out of (E, P) is dependent on the concentration of host droplets for P (the apparent first-order rate constant for the exchange-out process is $k_{ex.}[(\text{droplet})]_T$), and the droplet volume determines the rate of the reaction between E and P. Clearly, a low concentration of small droplets (low overall ϕ^{aq}) will result in a low value for $k_{cat.(app.)}$ relative to $k_{cat.}$, since the rate of binding of E to P is maximized and the opportunity for P to escape from (E, P) by exchange is minimized.

In the same way the dependence of K_m^m on ϕ^{aq} can be explained. The rate of binding of S to E will be determined by the droplet volume, as in the case of E–P binding, and the dependence on the droplet concentration arises because increasing the total droplet concentration, at constant overall substrate concentration, leads to an increase in the concentration of empty droplets and hence in the rate of the reverse decomposition of (E, S) to (E) and (S) (Scheme 2).

The presence of P in the droplets has no significant competitively inhibitory effect on the reaction because the E–S and E–P binding steps are temporally separated: since P in (E, P) is derived from S in the parent (E, S) droplet, P and S cannot be present simultaneously. Hence P cannot compete with S for binding to E. Indeed, the negative contributions of the third and fourth terms of eqn. (23) predict a decrease in K_m^m under conditions where $k_{-2}^d > 0$. The reduced third term of eqn. (23) can be rewritten as $(k_{-2}^d/k_{+1}^d)(k_{ex.}/k_{cat.})$, showing clearly that as the k_{-2}^d/k_{+1}^d ratio increases the $(E, S)/(E, P)$ ratio increases as a result of the increased rate of $P \rightarrow S$ conversion. Similarly the fourth term can be rewritten as $(k_{-2}^d/k_{ex.} \phi^{aq})(k_{cat.}/k_{ex.})$, showing that as the $k_{-2}^d/k_{ex.} \phi^{aq}$ ratio increases P is more likely to bind to E (and thus to be converted back into S) than to be removed by exchange. Substrate molecules regenerated from product thus contribute to the local (droplet) substrate concentration experienced by the enzyme, and the coefficient $k_{cat.}/k_{ex.}$ has the effect of normalizing these contributions to the overall (mol/litre of microemulsion) concentration scale to which the units of K_m^m refer.

The presence of $k_{\text{cat.}}/k_{\text{ex.}}$ as an independent term in the expression for K_m^{m} normalizes the kinetics by specifically preventing eqn. (20) from predicting the kinetically impossible situation that substrate can be converted into product, by the enzyme, at a rate faster than that at which it enters the droplet. Likewise, the $k_{\text{cat.}}/k_{+1}^{\text{d}}$ term in the expression for K_m^{a} prevents the equation from predicting that substrate can be converted at a rate faster than that at which substrate within the droplet can bind to the enzyme (cf. the $k_{\text{cat.}}/k_{+1}^{\text{w}}$ term in the expression for K_m^{w} , eqn. 2).

Interpretation of K_m^{m}

The kinetics of enzyme-catalysed reactions are those of saturation (Michaelis-Menten) kinetics, a characteristic pattern of behaviour that can reasonably be expected to be independent of solvent type. It is therefore not surprising that eqn. (21) has the form of the Michaelis-Menten equation. However, in a solvent system of this type, where several alternative non-equivalent reference frames are available for defining concentration units, the appropriate units must be chosen carefully if meaningful comparisons are to be made of the behaviour of enzymes in this system with that in aqueous solution. It must be remembered that a K_m is an (apparent) equilibrium constant relating the (apparent) equilibrium concentrations of E, S and E·S, a dynamic situation in which the rates of formation and breakdown of E·S are equal. The breakdown of E·S (either to E and S or to E and P) is a first-order process and the rate is therefore independent of the volume in which E·S is contained, but the formation of E·S is a function of the rate of collision between E and S, and this rate is a function of the volume mutually accessible to the two species, moving randomly and independently, i.e. to their concentrations, with this volume as the frame of reference for defining units of concentration. Thus the concentration units of k_{+1}^{w} , the rate constant for binding of E to S, and therefore of K_m^{w} , are correctly assigned by using this concentration scale.

Eqn. (20) shows unequivocally that the appropriate reference frame is that of the total microemulsion volume (the 'overall' concentration scale). This result is consistent with consideration *a priori* of the dynamic behaviour of enzyme and substrate molecules in microemulsions. Whereas in aqueous solution the formal reactant species are E and S (Scheme 1), those in microemulsions are $\textcircled{\text{E}}$ and $\textcircled{\text{S}}$ (Scheme 2). In both cases both species are free to move, randomly and independently, throughout the total volume of the system. The fact that in microemulsions E and S are enclosed within water-droplets does not alter the overall pattern of dynamic behaviour compared with that in aqueous solution. Since the total volume of the microemulsion is accessible to $\textcircled{\text{E}}$ and $\textcircled{\text{S}}$, the rate of E·S formation is appropriately expressed in units of mol/litre of microemulsion per s. Although the formation of E·S occurs within the intermediate droplet $\textcircled{\text{E, S}}$ such that the actual binding of E to S proceeds at a rate determined by their concentrations within the droplet, the overall kinetics are consistent with this dynamic interpretation, since the initial $\textcircled{\text{E, S}}$ -forming step (collision between $\textcircled{\text{E}}$ and $\textcircled{\text{S}}$) is always rate-limiting. This can be shown by comparing the half-lives of the reactant species (the rate-limiting step is that for which the half-lives of the reactants are longest). The half-life of $\textcircled{\text{E}}$ with respect to $\textcircled{\text{E, S}}$ formation is $1/k_{\text{ex.}}[\textcircled{\text{S}}]$ s and a typical value, based on $k_{\text{ex.}} =$

$10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $[\textcircled{\text{S}}] = 25 \mu\text{M}$, is 4 ms. In contrast, the half-life of $\textcircled{\text{E, S}}$ with respect to $\textcircled{\text{E}\cdot\text{S}}$ formation is $1/k_{+1}[\textcircled{\text{S}}]$ s and a typical value is $15 \mu\text{s}$, assuming a spherical droplet of radius 4 nm and that k_{+1}^{d} is similar in magnitude to k_{+1}^{w} ($= 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) [3]. Thus the rate of $\textcircled{\text{E, S}}$ formation is of the order of 100-fold slower than the subsequent conversion of $\textcircled{\text{E, S}}$ into $\textcircled{\text{E}\cdot\text{S}}$, and therefore the overall concentrations of E ($[\textcircled{\text{E}}] = [\text{E}]^{\text{m}}$) and S ($[\textcircled{\text{S}}] = [\text{S}]^{\text{m}}$) of this step ultimately determine the rate of substrate conversion.

This discussion has necessarily centred on the way in which the droplet size and concentration determine the steady-state concentration of the species $\textcircled{\text{E}\cdot\text{S}}$, and hence the rate of substrate conversion. Another approach would be to consider the enzyme-containing droplets as a solution into which substrate molecules pass at a certain rate. It would then be possible to discuss the steady-state rate of substrate conversion in terms of a time-averaged substrate concentration experienced by the enzyme molecules. The steady-state kinetic analysis does not, however, give the value of this average concentration, showing only that it is a function of ϕ^{aq} , at constant overall concentration, so that, although it is clear that only one value of ϕ^{aq} gives the same average substrate concentration as the average (= nominal) concentration experienced by the enzyme in aqueous solution, the corresponding K_m^{m} being directly comparable with K_m^{w} , this ϕ^{aq} remains unknown. The observation [30–32] that K_m^{m} can be numerically comparable with K_m^{w} indicates that this ϕ^{aq} lies within the experimentally accessible range of ϕ^{aq} values, with the consequence that, depending exactly on the value of ϕ^{aq} selected, rates higher or lower than those in aqueous solution may be expected when rates are expressed in terms of overall concentration units.

The form of the expression for K_m^{m} (eqn. 23) requires further comment. It has already been shown that K_m^{m} is dominated by the $K_m^{\text{a}}\phi^{\text{aq}}$ term, most especially in the limiting case when $\phi^{\text{aq}} = 1$, when its value, K_m^{a} numerically, is 10^2 – 10^3 times larger than those of the other three terms. The physical interpretation of K_m^{a} can be understood from a consideration of the properties of the $\phi^{\text{aq}} = 1$ system. In this case the volume of the microemulsion is accounted for entirely by water droplets, a (hypothetical) situation that is achieved conceptually by increasing the concentration and/or volume of the droplets until all the oil has been displaced. This system of close-packed aqueous droplets is not equivalent to a simple aqueous solution because the droplet boundaries have not been removed. The rate of E·S formation is lowered to a minimum because $[\textcircled{\text{E}}]_{\text{e}}$, and thus the rate of exchange of S out of $\textcircled{\text{E, S}}$ ($= k_{\text{ex.}}[\textcircled{\text{E}}]_{\text{e}}[\textcircled{\text{E, S}}]$), is maximized. Introduction of larger droplets, although necessarily decreasing $[\textcircled{\text{E}}]_{\text{e}}$, leads to an increase in the lifetime of $\textcircled{\text{E, S}}$ with respect to $\textcircled{\text{E}\cdot\text{S}}$ formation. Therefore the exchange-out rate remains high, as the longer-lived $\textcircled{\text{E, S}}$ species is more likely to participate in an exchange event. In this $\phi^{\text{aq}} = 1$ microemulsion, therefore, E·S formation is hindered as compartmentalization of the aqueous material, in combination with the exchange process, serves to isolate S from E. As a consequence, decreases in k_{+1}^{d} [numerically, the rate of formation of E·S in a $\phi^{\text{aq}} = 1$ system, where E and S are present at a concentration of 1 M^{a} ($\equiv 1 \text{ M}^{\text{m}}$)]

relative to k_{+1}^w , are expected, and thus an explanation is provided for the increased values of K_m^d relative to K_m^w , observed without exception for enzymes with water-soluble substrates, including trypsin [30], α -chymotrypsin [31,32] alcohol dehydrogenase [33] and phospholipase C (C. Oldfield & R. B. Freedman, unpublished work). Increases are typically of the order of 10–100-fold.

In the earlier estimation of the relative magnitudes of the component terms of eqn. (23), a value for k_{-2}^d of $10^6 \text{ M}^{-1} \text{ s}^{-1}$ was used. This was obtained by assuming that k_{-2}^w would at maximum be similar to k_{+1}^w (both rate constants refer to the same process: the binding of substrate, S or P, to E), i.e. about $10^7 \text{ M}^{-1} \text{ s}^{-1}$, and that k_{-2}^d is a minimum of 10-fold lower. This estimation was itself obtained by assuming a 10-fold reduction in K_m^d compared with K_m^w , due entirely to a reduction in k_{-2}^d compared with k_{-2}^w .

It should be noted (i) that, on the basis of the current argument, for any microemulsion system the effective second-

order rate constant for the overall process $\text{E} + \text{S} \rightarrow \text{E} \cdot \text{S}$

will be $k_{+1}^d / \phi^{aq} \text{ M}^{-1} \text{ s}^{-1}$ and (ii) that in an isolated $\text{E} \cdot \text{S}$ droplet

the rate constant for E·S formation can reasonably be expected to be equal to that observed in a much larger volume of aqueous solution, which is why k_{+1}^w could be used in the previous calculation of the half-life of $\text{E} \cdot \text{S}$ with respect to $\text{E} \cdot \text{S}$ formation.

One further aspect of the rate equation should be explained. It has previously been shown that the kinetics of the hydrolysis of water-soluble substrates by trypsin [30] and α -chymotrypsin [31,32] can be fitted to the empirical expression:

$$v^m = \frac{k_{cat} [E_0]^m [S]^{aq}}{K_m^{aq} + [S]^{aq}} \quad (24)$$

and has led to the conclusion that the microemulsion can be regarded, for these purposes, as a pseudo-two-phase (oil + water) system with the enzyme-catalysed reaction confined within the aqueous pseudo-phase. This equation can in fact be generated from eqn. (20), for an irreversible reaction ($k_{cat, (app.)} = k_{cat.}$), by dividing throughout by ϕ^{aq} . For reactions in which $k_{cat.}$ is small, as is so for these data, all constant terms in the denominator except for K_m^d can be ignored, to yield an equation equivalent to eqn. (24) with K_m^d in place of the empirical constant K_m^{aq} . The concept of the microemulsion as a two-phase system, on the strength of eqn. (24), further invites numerical comparison of K_m^d ($= K_m^d$) with K_m^w , and the large differences observed have led to the incorrect conclusion that these enzymes are inhibited in the microemulsion [30,31].

CONCLUSIONS

The present derivation defines clearly the form of the rate equation appropriate to the kinetics of enzyme-catalysed conversions of water-soluble substrates in water-in-oil microemulsion systems, the physical interpretation that should be ascribed to K_m^m and K_m^d , the irrelevance of comparing these parameters with K_m^w and the inadequacy of the pseudo-two-phase approach (it is shown in the Appendix that this approach is adequate when discussing the kinetics of reactions involving apolar substrates).

The present derivation has been confined to the limitation that a droplet can contain only one substrate molecule, in order to avoid the complexities accompanying the analysis of systems containing droplets with various numbers of substrate molecules. In order to take into account higher orders of droplet occupation

it is simply necessary to note that the rate of formation of E·S from $\text{E} \cdot \text{S}$ or $\text{E} \cdot \text{P}$ in a droplet containing nS (or nP) molecules (i.e. in droplet species $\text{E} \cdot nS$ or $\text{E} \cdot nP$) is n times the rate in the droplet $\text{E} \cdot \text{S}$ or $\text{E} \cdot \text{P}$. Thus the rate equations must be modified accordingly, with introduction of expressions for the proportion of each species present in the system for a given overall substrate concentration. Note that in the case of multiple occupancy a simple plot of rate versus overall substrate concentration at constant ϕ^{aq} will not be a rectangular hyperbola (i.e. the enzyme will apparently not obey Michaelis–Menten kinetics, since each of the $\text{E} \cdot \text{S}$, $\text{E} \cdot 2S$, ..., $\text{E} \cdot nS$ droplet species can be ascribed a unique (incrementally decreasing) value of K_m^d .

Note added in proof (received 21 September 1990)

In a recent paper Verhaert *et al.* [35], using a different approach, have considered the effect of microemulsion dynamic properties on the rates of enzyme-catalysed reactions.

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APPENDIX

Derivation of the Michaelis–Menten equation for the case of enzyme plus oil-soluble substrate in water-in-oil microemulsions

In this case substrate is converted into product by enzyme molecules, located within water-droplets, as the substrate partitions between the continuous oil-domain and the water of the droplets. The main difference between this type of kinetics and that described in the main paper is that there is a pool of substrate molecules in the oil-domain. In this case, therefore, transfer of substrate molecules from substrate-containing droplets is not an essential pre-requisite for enzyme–substrate interaction. Appendix Scheme 1 describes this situation in terms of the kinetic steps involved.

An expression for the dependence of v^{aq} on $[S]^m$ is obtained by applying the steady-state approximation to the species $E \cdot S^{aq}$ and to S^{aq} . Considering first $E \cdot S^{aq}$: when $d[E \cdot S^{aq}]/dt = 0$:

$$[S]^{aq} = \frac{K_m^{aq}[E \cdot S^{aq}]}{([E_0]^{aq} - [E \cdot S^{aq}]})} \quad (A1)$$

{given $[E_0]^{aq} = [E]^{aq} + [E \cdot S^{aq}]$ and $K_m^{aq} = (k_{-2} + k_{cat})/k_{+2}$ }. This Michaelis constant K_m^{aq} , has units of mol/litre of aqueous domain.

When $d[S]^{aq}/dt = 0$:

$$k_{+1}[S]^{oil} - k_{-1}[S]^{aq} - k_{+2}[E]^{aq}[S]^{aq} + k_{-2}[E \cdot S^{aq}] = 0 \quad (A2)$$

It is easily shown that:

$$[S]^{oil} = \frac{[S]^m - [S]^{aq}\phi^{aq}}{\phi^{oil}} \quad (A3)$$

Substituting this expression for $[S]^{oil}$ and also the expression for $[S]^{aq}$ (eqn. A1) into eqn. (A2) gives:

$$\begin{aligned} \frac{k_{+1}[S]^m}{\phi^{oil}} - \frac{k_{+1}K_m^{aq}[E \cdot S^{aq}]\phi^{aq}}{([E_0]^{aq} - [E \cdot S^{aq}])\phi^{oil}} - \frac{k_{+1}K_m^{aq}[E \cdot S^{aq}]}{([E_0]^{aq} - [E \cdot S^{aq}])} \\ + \frac{k_{+2}[E_0]^{aq}K_m^{aq}[E \cdot S^{aq}]}{([E_0]^{aq} - [E \cdot S^{aq}])} + \frac{(k_{+2}[E \cdot S^{aq}])^2 K_m^{aq}}{([E_0]^{aq} - [E \cdot S^{aq}])} + \frac{(k_{+2}[E \cdot S^{aq}])^2}{([E_0]^{aq} - [E \cdot S^{aq}])} \\ + k_{-2}[E \cdot S^{aq}] = 0 \quad (A4) \end{aligned}$$

Multiplying eqn. (A4) by $([E_0]^{aq} - [E \cdot S^{aq}])\phi^{oil}/k_{+1}$ and ignoring terms that are squared in $[E \cdot S^{aq}]$ gives, on making ' $[E_0]^{aq}[S]^m$ ' the subject:

$$[E_0]^{aq}[S]^m = [E \cdot S^{aq}] \left([S]^m + K_m^{aq}\phi^{aq} + \frac{k_{-1}}{k_{+1}}K_m^{aq}\phi^{oil} + \frac{k_{+2}}{k_{+1}}\phi^{oil}K_m^{aq}[E_0]^{aq} - \frac{k_{-2}}{k_{+1}}\phi^{oil}[E_0]^{aq} \right) \quad (A5)$$

Given $v^{aq} = k_{cat}[E \cdot S^{aq}]$:

Scheme 1. Proposed kinetic scheme for the reaction between enzyme and oil-soluble substrate in water-in-oil microemulsions

$$v^{aq} = \frac{k_{cat}[E_0]^{aq}[S]^m}{K_m^{aq}\phi^{aq} + \frac{k_{-1}}{k_{+1}}K_m^{aq}\phi^{oil} + \frac{k_{+2}}{k_{+1}}K_m^{aq}\phi^{oil}[E_0]^{aq} - \frac{k_{-2}}{k_{+1}}\phi^{oil}[E_0]^{aq} + [S]^m} \quad (A6)$$

Making the assumptions that $k_{+1} \gg k_{+2}$ and $k_{+1} \gg k_{-2}$, i.e. that the oil/water-phase transfer process is not rate-limiting in the formation of $E \cdot S$, gives:

$$v^{aq} = \frac{k_{cat}[E_0]^{aq}[S]^m}{K_m^{aq}(K_p\phi^{oil} + \phi^{aq}) + [S]^m} \quad (A7)$$

where $K_p = k_{-1}/k_{+1} = [S]^{oil}/[S]^{aq}$, the equilibrium partition coefficient of S between the microemulsion oil-domain and water-domain. These assumptions are justifiable given the extremely large interfacial surface area (up to 100 m²/cm³ of microemulsion) and high surface/volume ratio of the droplets (approx. 10⁷ cm⁻¹). The units of v^{aq} are M^{aq}·s⁻¹ and the reaction can be perceived as proceeding within the aqueous part of a two-phase system. In this context the water-in-oil microemulsion differs from a conventional two-phase system only in that the aqueous 'phase' is dispersed within the volume of the continuous oil phase. From eqn. (A7):

$$K_m^m = K_m^{aq}(K_p\phi^{oil} + \phi^{aq}) \quad (A8)$$

Since the concept of the aqueous domain as a droplet dispersion does not form an integral part of the model for which eqn. (A7) was derived, the k_{cat}/k_{ex} term does not appear (cf. eqn. 23 of the main paper). Similarly, a dependence of k_{cat} on ϕ^{aq} is not expected, since rapid partitioning of apolar reaction products out of the aqueous domain eliminates any potential inhibitory effect. For enzymes with the same catalytic properties in both aqueous solution and the the microemulsion, $K_m^{aq} = K_m^w$. The prediction (eqn. A7) that K_m^m should then be bigger than K_m^w by a factor of $(K_p\phi^{oil} + \phi^{aq})$ is consistent with the observed kinetics ([1–5] and C. Oldfield, B. H. Robinson & R. B. Freedman, unpublished work).

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