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Trypsin-SBTI interaction in reverse micelles

A slow intermicellar exchange-dependent binding

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Solubilisate exchange between reverse micelles must take place before any reaction inside reverse micelles occurs if the reactants are confined to the aqueous micellar core. When the interacting species are 2 small molecules or one small molecule and one macromolecule, it has been shown that the exchange is faster than the typical turnover of an enzymatic reaction. The study of the interaction between 2 macromolecules (trypsin and soybean trypsin inhibitor) in reverse micelles carried out in this work reveals that the exchange between these macromolecule-containing reverse micelles slows down by a thousand times and the limiting-step in the exchange, the fusion, by 10^6 times. Both reverse micellar size (ω_0 = [water]/[surfactant]) and temperature affected the rate of the fusion process. A hypothesis for the proposed active role of macromolecules in the exchange process is also given.

Reverse micelle; Trypsin; SBTI; Intermicellar exchange; Macromolecular interaction

1. INTRODUCTION

Nanometer sized water pools solubilized in an apolar solvent using surfactants as dispersing agents, the so-called reverse micelles, constitute a novel environment for enzyme-catalyzed reactions (see [1–3] for reviews). Their radius is directly related to the water to surfactant molar ratio, ω_0 (= [water]/[surfactant]), and so the reverse micelle size is easily controlled experimentally. The encapsulation of the reactants inside reverse micelles adds some new steps to the reaction mechanism we wish to investigate, which involve the intermicellar solubilisate exchange. As demonstrated elsewhere [4], exchange occurs through the formation of a reverse micelle transient dimer which has a sufficiently long lifetime to allow the solubilisates to exchange by diffusion. After this, the reactants interact inside the reverse micelle and then the reaction takes place.

The influence of the exchange process on the kinetics of the reaction is based on the rate-limiting step in the overall turnover, that is, the relative rates of the intermicellar exchange and the reaction [3]: (i) for slow reactions, the dispersed phase can be regarded as a pseudocontinuous phase since solute transport is not the limiting step, (ii) for reactions in the range from milliseconds to microseconds, the reaction rate can be either transport or solute concentration-dependent, and (iii) for fast reactions (in the nanosecond range) the reactants are not redistributed by exchange during the reaction time scale and thus a Poisson distribution of the reactants among reverse micelles has to be assumed; in this case the reaction is transport-dependent.

Enzymatic reactions obeying Michaelis–Menten kinetics in which the rate-limiting step is the first order reaction ES⟶E + P governed by the catalytic constant, fall into the first category as judged by comparison with the rate-limiting step in the exchange process represented by the fusion of reverse micelles: a typical value for the first order fusion rate constant is $k_{fus} \cong 4000\ s^{-1}$ estimated from experiments of small molecule intermicellar exchange at $\omega_0 = 20$ [4], while a typical value for the first order catalytic constant is about a hundred times lower. This has allowed us to apply the concepts of classical in-water enzymology to reverse micelles from a theoretical [5] and practical [6,7] point of view. However, one could argue that the size of the protein, which is in the same range of the reverse micelle size, affects the fusion process and hence the enzymatic reaction becomes a diffusion-limited process in reverse micelles. Experimental evidence suggesting that this is not the case is provided by the work of Vos et al. [8], who showed that the exchange rate constant between protein-containing and small molecule-containing reverse micelles is in the same range as in the case of exchange between small molecule-containing reverse micelles.

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Abbreviations: SBTI, soybean trypsin inhibitor; BAEE, N$^{\alpha}$-benzoyl-L-arginine ethyl ester; AOT, dioctyl sodium sulfosuccinate; CTAB, cetyl trimethylammonium bromide

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The question arises when one asks whether the situation described above still holds in the case of reactions dependent on macromolecular interaction or whether the process turns into a transport-dependent one, i.e. diffusion-limited.

In order to answer this question we present here the kinetics of macromolecular interaction in reverse micelles, and in particular the interaction between trypsin and SBTI in AOT reverse micelles through the loss of hydrolytic activity of trypsin on BAEE in the presence of SBTI.

2. EXPERIMENTAL

2.1. *Materials*

Dioctyl sodium sulfosuccinate was purchased from Sigma (Deisenhofen, Germany) and isooctane UV-IR grade from Panreac (Barcelona, Spain). Both were used without further purification. Crystallized trypsin from bovine pancreas was from Boehringer-Mannheim (Barcelona, Spain) and soybean trypsin inhibitor type I-S and N^{α}-benzoyl-L-arginine ethyl ester were from Sigma.

All aqueous solutions were prepared in 0.1 M Tris-HCl buffer, pH 8.5, containing 5 mM $CaCl_2$. In order to improve the reproducibility in the assays, concentrated stock solutions of 1 mM trypsin and 1 mM SBTI were divided into aliquots and frozen. When required, aliquots were thawed and diluted to 40 μM trypsin and 120 μM SBTI and discarded after use. Autolysis [7] and hence experimental errors were thus minimized.

2.2. *Preparation of reverse micelles*

Reverse micelles were prepared by microsyringe injection of the required volumes of aqueous solutions into 1 ml of 0.2 M AOT in isooctane. After vigorous shaking a transparent solution was obtained as judged by visual inspection. Three types of reverse micellar solutions were prepared to carry out the experiments: (i) containing trypsin and buffer, (ii) containing SBTI, BAEE and buffer, and (iii) containing BAEE and buffer.

2.3. *Enzyme activity assay*

Progress curves of the reaction were monitored following the appearance of product at 253 nm (ϵ_{253} = 1600 $M^{-1}\cdot cm^{-1}$, in AOT reverse micelles ω_0= 5–20 [10], ϵ_{253} = 1150 $M^{-1}\cdot cm^{-1}$ in water [11]) in a Kontron Uvikon 940 spectrophotometer equipped with thermostated cells and fed with a Haake D1-G thermostatic bath (± 0.2°C). In reverse micelles the reaction was started by mixing equal volumes of micellar solutions 1 and 2 (with inhibitor) or 1 and 3 (without inhibitor) in a quartz cuvette (1 ml, 1 cm optical path). In buffer, trypsin was added to a BAEE and SBTI solution to start the reaction. Except when stated the temperature was 25°C.

2.4. *Treatment of the product accumulation curves*

Product vs time curves were transformed into velocity vs time curves by deriving the former with respect to time: $v_i = (P_{i+\Delta t} - P_i)/\Delta t$. The resulting curves were fitted by non-linear regression to a monoexponential equation of the type: $v_i = v_0 e^{-kt}$ using the mathematic program BMDP (Statistical Software, Inc., Los Angeles, USA). The parameter k ± SE was obtained after at least 7 iterations.

3. RESULTS AND DISCUSSION

3.1. *Inhibition of trypsin by SBTI in water and in reverse micelles*

The kinetics of trypsin inhibition was investigated by comparing the product accumulation curves with or without SBTI present in both aqueous and reverse micellar systems under similar conditions of pH (the pH of the buffer before solubilization) and temperature.

Fig. 1 shows such curves: in the absence of SBTI the product of BAEE hydrolysis accumulates under steady state conditions both in buffer (curve a) and in AOT reverse micelles ω_0 = 10 (curve b), although more slowly in the latter case. In the presence of SBTI, the difference is apparent since in water the inhibition is instantaneous in both conditions [SBTI] < [trypsin] (curve c) and [SBTI] > [trypsin] (curve d), while in reverse micelles at ω_0 = 10 the inhibition is slow even when [SBTI] > [trypsin] (curve e). However, at ω_0 = 25 (curve f) the inhibition is fast again. Under our experimental conditions, a slow inhibition cannot be originated by a competitive binding of the substrate and the inhibitor to the enzyme since both of them are fast equilibria.

According to a solubilisate exchange mechanism proposed by Fletcher et al. [4], the formation of the inactive complex SBTI-trypsin in reverse micelles would require the previous collision and fusion of one reverse micelle containing trypsin and another containing SBTI, the overall process being characterized by a second order exchange rate constant, k_{ex}, and the fusion by a first order rate constant, k_{fus}. After that, inhibition takes place inside reverse micelle and finally the system rearranges itself as expressed in Scheme 1.

Although this inhibition is a kinetically irreversible process, it is, in fact, an equilibrium shifted towards the complex form (K_i = 6.8·10^9 M^{-1} at pH 8.3 [12]). In conditions such as [trypsin] ≥ [SBTI], it was demonstrated [10] that in AOT reverse micelles the reaction is as specific as in water and that the equilibrium constant is, as in water, of strong binding

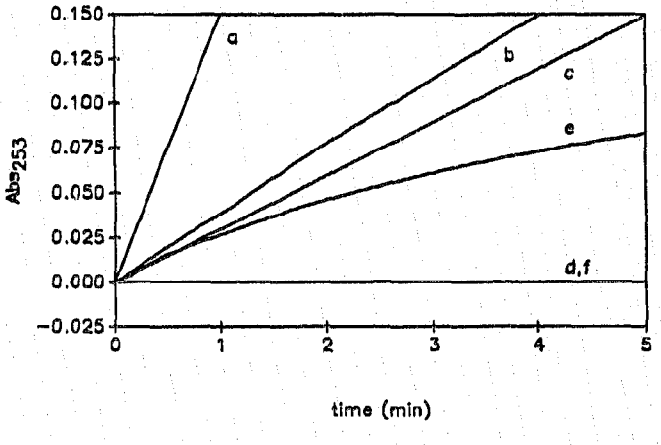
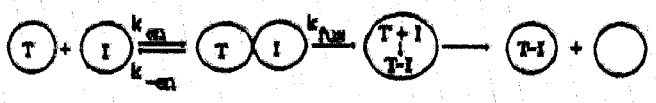


Fig. 1. Experimental recording of BAEE hydrolysis by trypsin with and without SBTI in water and in AOT reverse micelles (see text for explanation). In buffer: (a) no SBTI; (c) [SBTI] = 0.08 μM; (d) [SBTI] = 0.2 μM. In reverse micelles: (b) ω_0 = 10, no SBTI; (e) ω_0 = 10, [SBTI] = 0.9 μM; (f) ω_0 = 25, [SBTI] = 0.9 μM. In all cases [trypsin] = 0.1 μM and [BAEE] = 0.5 mM.



Scheme 1. Mechanism of solubilisate exchange and macromolecular interaction in reverse micelles.

type since at trypsin/SBTI ratio 1:1 trypsin was practically all inhibited. Thus, the most likely hypothesis to explain the time-dependent inhibition in reverse micelles is that the previous process of micellar fusion is being hindered by the presence of macromolecules, i.e. the macromolecular reaction we are carrying out inside reverse micelles is faster than the exchange of protein–protein containing reverse micelles. This leads us to assume that proteins are distributed randomly (Poisson distribution) throughout the droplets at equilibrium, achieving a situation similar to that described by Vos et al. [8] for the quenching of triplet state in reverse micelles, provided that (i) the average occupancy of inhibitor is near zero ([SBTI]/[droplets] ≅ 0), (ii) the inhibition is exchange limited, and (iii) the inhibitor concentration is higher than trypsin concentration ([SBTI] ≅ constant along reaction time). The resulting equation describing the decay of tryptic activity then takes the form:

$$v_t = v_0 e^{-(1/\tau_0 + k_{ex}[SBTI])t} \quad (1)$$

where v_0 and v_t stand for the reaction rate at the times 0 and t respectively, and τ_0 is the lifetime of trypsin in the absence of inhibitor. If we work in conditions where the hydrolytic reaction rate is under steady state in the absence of inhibitor, then the lifetime of trypsin is too long compared to its lifetime in the presence of inhibitor, hence $1/\tau_0$ is negligible against k_{ex}[SBTI].

As the binding of SBTI to trypsin is extremely strong and faster than the micellar fusion, the parameter that characterizes the decay of tryptic activity must be the first-order micellar fusion rate constant, k_{fus}. Hence

$$k_{fus} = k_{ex}[SBTI] \quad (2)$$

3.2. *Optimization of the experimental conditions*

Firstly, trypsin and BAEE concentrations were optimized to achieve a level of accumulated product under steady state conditions high enough to be spectrophotometrically sensitive. In the best conditions assayed, 0.125 mM of product was formed under steady state (25% of substrate consumption) when using 0.1 μM trypsin and 0.5 mM BAEE overall concentrations. As local concentration changes upon variation of the overall water content [6], a fairly high value of ω_0 16 was used to prevent local substrate dilution effects when ω_0 is increased from 7 to 20, which would lead to lower steady state product levels.

Optimization of SBTI concentration was achieved when the product amount formed at the end point of the reaction in the presence of SBTI corresponded to a level of product accumulated under steady state conditions in the absence of inhibitor. When using 0.9 μM SBTI, 69 μM of product was formed after 30 min while without inhibitor, 148.8 μM of product was formed in 5 min under steady state conditions. In this experiment an intermediate value of ω_0 12 was used to be able to observe changes in the accumulated product at the end point exclusively caused by inhibition and not by other phenomena such as substrate consumption in the hypothetical case of non-inhibition at low ω_0 values.

3.3. *Evaluation of k_{ex} for macromolecule-containing reverse micelles*

According to equation (2) a plot of k_{fus} vs [SBTI] is a straight line whose slope is the second order exchange rate constant, k_{ex}, between protein containing reverse micelles. Fig. 2 shows the result of such an experiment yielding a k_{ex} value of $3.63 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ at $\omega_0 = 12$.

A comparison of our result for macromolecule–macromolecule exchange, the results for small molecule–small molecule exchange and the results for small molecule–macromolecule exchange with the diffusion-controlled rate constant (k_{ex}) of reverse micelles is given in Table I. As can be inferred from this table, about 1 in 1000 collisions results in solubilisate exchange when the exchange is of type I or II, but only 1 in about 1 000 000 when the exchange is of type III. In fact, the exchange is about a thousand times slower when 2 macromolecules are involved than in the other 2 cases.

For $k_{ex} = 10^7$ $M^{-1} \cdot s^{-1}$, Fletcher et al. [4] estimated for an $\omega_0 = 20$ a k_{fus} value of $4 \cdot 10^3$ s^{-1}. As seen in Fig. 2, such a value is about one million times higher than those found for the fusion of macromolecule-containing reverse micelles, indicating that the energy barrier for the fusion process is dramatically affected by the presence of macromolecules, in particular proteins.

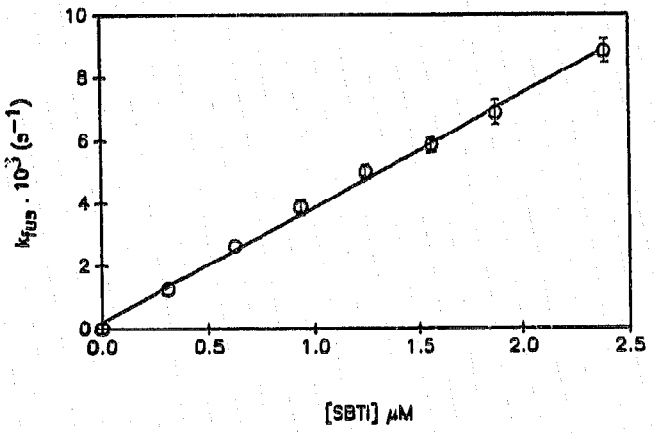


Fig. 2. Dependence of the first order fusion rate constant on the overall SBTI concentration. [Trypsin] = 0.1 μM and [BAEE] = 0.5 mM.

Table I

Comparison between the exchange types and the frequency of collision of reverse micelles

System	ω_0	$k_{ex}/10^6$ ($M^{-1}\cdot s^{-1}$)	Exchange type	Ref.
			I	
AOT/heptane	10	14 ± 4	small molecule–	[4]
	20	14 ± 2	small molecule	[4]
			II	
CTAB/hexanol:	10	33	small molecule–	[8]
isooctane (12:88)	40	10	macromolecule	[8]
			III	
AOT/isooctane	12	0.0036	macromolecule– macromolecule	this work
AOT/heptane		$k_{DC} = 1.7\cdot10^{10}$ $M^{-1}\cdot s^{-1}$		[4]

3.4. *The effect of reverse micelle size and temperature on the fusion of macromolecule-containing reverse micelles*

The effect of ω_0 and temperature was studied under optimized experimental conditions as explained above. Fig. 3 shows the results of such experiments. k_{fus} was also evaluated at 37°C but even at $\omega_0 = 7$ the reaction was completely inhibited from the very beginning. The increase of temperature has a positive effect on the fusion, probably because, besides an increase in the frequency of micellar collision it affects the physical state of the surfactant shell and/or the micellar water. However, the effect of temperature has not the same intensity throughout the range of ω_0 investigated: the increase of k_{fus} is stronger as micelle size increases, suggesting that the interaction between reverse micelles is unequally affected by the presence of macromolecules as a function of the micellar size, as the exponential type growth of k_{fus} seems to indicate. On the contrary, in the exchange type I, k_{ex} was shown to be practically independent of ω_0 and of the nature

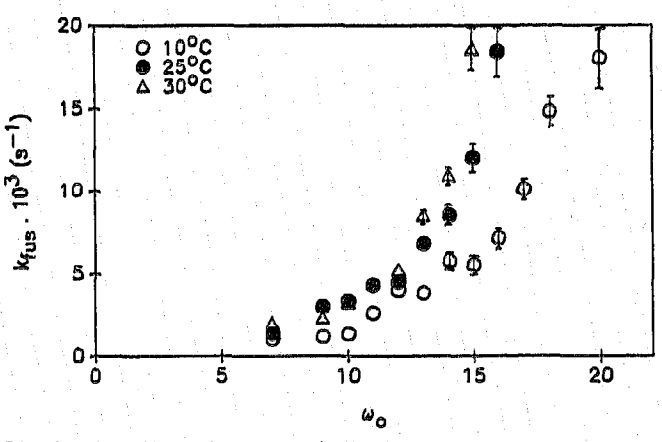
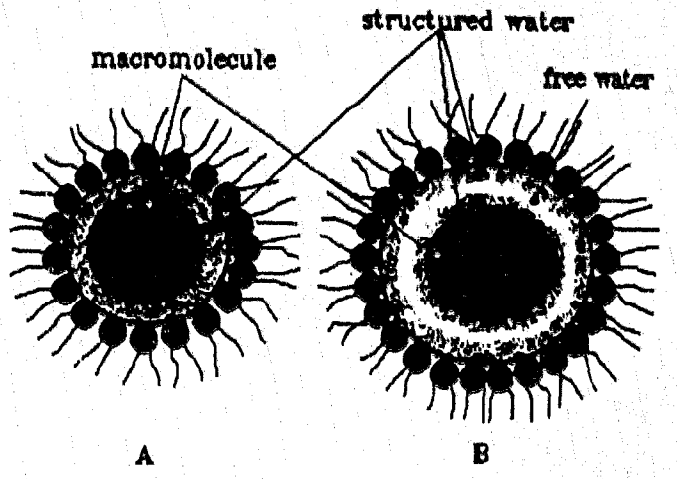


Fig. 3. The effect of reverse micelle size and temperature on the fusion rate constant. [Trypsin] = 0.1 μM, [SBTI] = 0.9 μM and [BAEE] = 0.5 mM.



Scheme 2. Proposed role of the macromolecule in the exchange. (A) At small micelle sizes, all water is structured by the presence of both the surfactant heads and the protein. (B) When hydration requirements have been fulfilled at higher micellar sizes, free water is present inside reverse micelles and disappears the 'structural cohesion' protein-structured water-surfactant thus facilitating the inversion of the surfactant curvature during the fusion process.

and charge of the transferred species, thus indicating a passive role of the solubilisate. In our case, the macromolecules seem to have an active role on the fusion process since we have observed a dependence on the micellar size. The fact that such a dependence is not linear but exponential may be related to the presence of both a layer of structured water associated to the surfactant polar head [13] and a crown of structured water associated to the protein surface. Using the quasi elastic neutron scattering technique [14] it has been shown that in reverse micelles at $\omega_0 = 20$ both AOT and α-quimotrypsin have satisfied their hydration requirements, hence the free water in the protein-containing reverse micelle. Scheme 2 illustrates what probably occurs at low and high reverse micelle sizes in the presence of macromolecules. The lack of free water in a small protein-containing reverse micelle may play a role in the hindrance of the fusion between protein-containing reverse micelles. Note that in exchange type II (see Table I) free water may be present in the small molecule-containing reverse micelle at low ω_0 values.

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