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Kinetic investigations of horseradish peroxidase in AOT/n-heptane reverse micelles

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Abstract

The oxidation of typical horseradish peroxidase substrate, 2,2′-azino-bis[3-ethyl-benzothiazoline-(6)-sulfonic acid] (ABTS), has been studied in AOT/n-heptane reverse micelles. The rate of product formation has been found to be over an order of magnitude higher in reverse micelles than in homogeneous aqueous solution. The rate constants of all three elementary steps of horseradish peroxidase reaction with ABTS are higher in reverse micelles than in homogeneous aqueous solution. The rate limiting step of compound I formation is the exchange between enzyme filled and hydrogen peroxide filled micelles. The exchange step most probably also influences the rate of ABTS reactions with compound I and compound II, although the changes in their rate constants due to a different water and enzyme structure in reverse micelles cannot be excluded.

Keywords: Horseradish peroxidase; Microemulsion; Reverse micelle; Reaction kinetics; Stopped-flow

1. Introduction

Reverse micelles are spherical aggregates of water and surfactant dispersed in an apolar solvent. The polar heads of surfactant molecules face into the interior water pool of reverse micelle whereas the hydrophobic tails are directed towards the bulk solvent.

It has been well established that enzymes can be incorporated into reverse micelles while retaining their activity. Several theoretical models for enzyme kinetics in reverse micelles, recently reviewed by Bru et al. [1], have been proposed. In the first approximation they can be divided into diffusional and non-diffusional models [1]. Diffusional models consider that diffusion effectively controls the enzymic reactions which occur inside reverse micelles. Three mechanisms are proposed for the exchange of solubilizates between reverse micelles. In the case of hydrophilic molecules, the formation of transient dimer is postulated [2]. For AOT as a surfactant, exchange occurs with a second-order rate constant of 10^6–10^8 M^{-1} s^{-1}, indicating that one collision in 100–10000 results in content exchange [2]. Exchange of amphiphilic or interface-bound molecules may occur during an encounter between droplets without fusion [1]. In

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the case of molecules that are distributed to any great extent in both water and organic solvent, intermicellar transfer may occur through the organic phase [1].

The non-diffusional models assume that the flow of substrate to the enzyme in reverse micelles is not limited by mass transfer, because the enzymic reaction is slow compared with the exchange of solutes between water pools [1].

Most of the kinetic studies of enzymes in reverse micelles has been done under steady-state conditions. In some cases the k_{cat} was found to exceed the aqueous value (so called 'superactivity') [3]. There are, however, few papers reporting the influence of micellar microenvironment on the individual rate constants of enzymatic reaction [4,5].

An outstanding 'superactivity' was found, among others, for heme enzymes, catalase [6] and horseradish peroxidase (HRP) [7–11]. For the latter the dependence of the k_{cat} on w_0 (the molar water to surfactant concentration ratio) has been shown to be bell-shaped and the k_{cat} value obtained at optimum w_0 was about 100 times higher in reverse micelles than in water [7,8].

Peroxidases are enzymes whose primary function is catalyzing the oxidation of variety of substrates, AH_2, by peroxides of a general formula ROOH, where R can be either H or an organic group.

$$\text{Peroxidase} + \text{ROOH} \xrightarrow{k_1} \text{Compound I} + \text{ROH} \quad (1)$$

$$\text{Compound I} + AH_2 \xrightarrow{k_2} \text{Compound II} + AH^{\cdot} \quad (2)$$

$$\text{Compound II} + AH_2 \xrightarrow{k_3} \text{Peroxidase} + AH^{\cdot} + H_2O \quad (3)$$

Most peroxidases contain ferric heme in the active site. The heme group in HRP is ferriprotoporphyrin IX with four pyrrole nitrogens bound to the iron (III). The fifth coordination position on the distal side of the heme is vacant in the native enzyme [12]. The initial step in the catalytic cycle is heterolysis of the oxygen–oxygen bond of peroxide and coordination of the oxygen atom to the iron center. Two electrons are transferred from the enzyme to the coordinated oxygen atom, one from the iron and one from the porphyrin. The resulting enzyme intermediate, compound I, is thus described as the oxoferryl [Fe(IV)=O] porphyrin π-cation radical. Reduction of compound I (reaction 2) typically occurs by hydrogen atom transfer. The proton is most probably accepted by a distal histidine residue and electron is accepted by the porphyrin [13]. So formed compound II possesses Fe(IV)=O active group. During the reduction of compound II (reaction 3) hydrogen atom transfer directly to the Fe(IV)=O group is accompanied by proton transfer from the distal histidine to the ferryl oxygen and results in water release and reformation of native enzyme [13].

As in the case of HRP all three reaction steps are irreversible [12] a modified ping-pong mechanism is used to describe enzyme kinetics. Thus, according to Dunford [13] one obtains from Eqs. 1–3 the following:

$$\frac{2[\text{HRP}]_0}{v} = \frac{(k_2 + k_3)}{k_2 k_3} \frac{1}{[AH_2]} + \frac{1}{k_1[H_2O_2]} \quad (4)$$

where $[\text{HRP}]_0$ is the total HRP concentration and $v = -\text{d}[AH_2]/\text{d}t$ is the initial reaction rate.

In a conventional ping-pong kinetics, there is a finite upper limit for the rate of reaction given by k_{cat}. In HRP kinetics described by equation (4) there is no upper limit to the rate of reaction [13]. Thus, neither the Michaelis constant and k_{cat} of HRP can be determined for substrate. In this connection the activity of different HRP samples can be compared only when the rate of product formation is measured under the same H_2O_2 and substrate concentrations.

In this work we examined HRP activity, de-

fined as above, in reverse micelles using 2,2′-azino-di-[3-ethyl-benzothiazoline-(6)-sulfonic acid], commonly known by its registered trade name ABTS, which gives a stable radical cation ($\lambda_{max} = 414$ nm) in reaction with peroxidases [14]. We also investigated the influence of microenvironment of reverse micelles on individual steps in HRP reaction with ABTS. We have already shown, using pulse radiolysis and stopped-flow spectrophotometry, that the observed rate of compound I formation (reaction 1) in reverse micelles depends on w_0 and is almost an order of magnitude higher than in homogeneous aqueous solution [15].

2. Materials and methods

Horseradish peroxidase (HRP) type VI with RZ = 3.0 was purchased from Sigma. Enzyme concentration was determined spectrophotometrically at 403 nm, using $\varepsilon_{403} = 1.02 \times 10^5$ M^{-1} cm^{-1} [16]. Water from the MilliQ Plus (Millipore) system was used throughout. AOT [sodium bis(2-ethylhexyl)sulfosuccinate] from Sigma was dried under vacuum over P_2O_5. AOT reverse micelles were formed by injection of appropriate amounts of aqueous stock solutions either of buffer alone (1 mM phosphate buffer, pH 7.0) or one of reagents (enzyme, hydrogen peroxide, ABTS) in buffer into 0.1 M AOT in n-heptane (Sigma, puriss) to obtain the desired w_0. The mixture was shaken until a completely transparent solution was obtained. One should remember, however, that the introduction of a large hydrophilic enzyme molecule into a reverse micellar system most likely causes the rearrangement of micellar aggregates. Thus, after the newly established equilibrium, w_0 may differ from that of the system without enzyme. In this work we always give w_0 values resulting from the amounts of water and AOT molecules added to the system. All experiments (except those of temperature dependence of k_1) were carried out at 23°C. All concentrations of enzyme and substrate solubilized in reverse micelles were overall concentrations referring to the total volume of the system.

Kinetic measurements were performed on the DX-17 MV (Applied Photophysics) stopped-flow spectrofluorimeter (millisecond and second observations) and on the Hewlett-Packard 8452 diode-array spectrophotometer (conventional time-scale).

3. Results and discussion

The absorption spectra of native HRP in buffer solution and in AOT-reverse micelles are similar (Fig. 1). The position and intensity of the Söret band (403 nm) do not differ. Klyachko et al. [8] have found that at a low degree of surfactant hydration, i.e. at $w_0 < 12$ in an AOT reverse micellar system containing HRP, two absorption peaks appear. The one at 403 nm corresponds to the absorption of heme in HRP and the second at 380 nm is characteristic for the heme located outside the protein. We did not, however, observe the additional band at 380 nm, for HRP in reverse micelles at $w_0 = 10$.

In the earlier works concerning HRP in reverse micelles the k_{cat} has been measured as a function of w_0 and the bell-shaped curve with a maximum at $w_0 = 10$–14 has been observed for

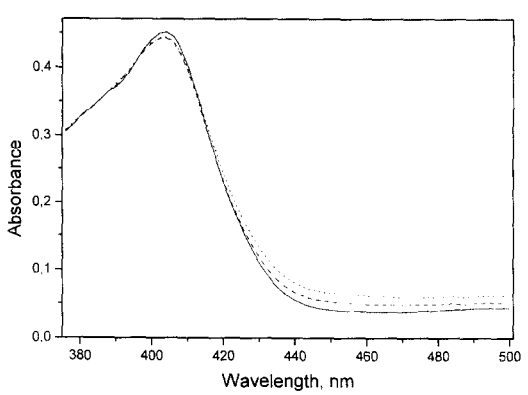

Fig. 1. Absorption spectra of HRP (4.4 μM overall): (solid line) in homogeneous aqueous solution, 1 mM phosphate buffer, pH 7.0; (dashed line) in 0.1 M AOT/n-heptane $w_0 = 20$, in the same buffer; (dotted line) in 0.1 M AOT/n-heptane, $w_0 = 10$, in the same buffer.

AOT reverse micelles [7–11]. In the series of works of Dunford et al. (summarized in [13,14]), it has been shown, that unlike conventional ping-pong kinetics, there is no upper limit for the rate of reaction, given by k_{cat} in peroxidase kinetics. It should be underlined that one obtains different k_{cat} values while seeking saturated conditions by changing substrate concentration at $[H_2O_2]$ = const or changing H_2O_2 concentration keeping substrate concentration constant. In mind of the above restrictions, we determined the rate of product formation in reverse micelles in comparison with homogeneous aqueous solution under the same overall H_2O_2 and substrate concentrations. We measured the initial rates of ABTS oxidation for w_0 values from 10 to 30, keeping overall concentration of the reagents constant: [HRP] = 1.6×10^{-8} M, $[H_2O_2]$ = 10 μM, [ABTS] = 20 μM. The dependence of the changes in absorption due to ABTS oxidation, on the reaction duration time was linear only for short reaction times (< 10 s) for all w_0 studied, and thus the reaction rate could be calculated for the early stage of the reaction. The rate of ABTS oxidation by HRP in homogeneous aqueous solution, at the same reagent concentrations as above, was also measured for comparison. The results are shown in Fig. 2. The initial rate of ABTS oxidation by HRP in reverse micelle for different w_0 is over an order of magnitude higher than in homogeneous aqueous solution. For $w_0 < 17$, the activity appears about 30% higher than for HRP samples solubilized at reverse micelles of $w_0 > 17$. Some authors argue that in case of hydrophilic substrates, the small water fraction inside the reverse micelles represents the actual surrounding in which the reaction takes place and hence the substrate concentration should be defined on the basis of the volume of the water pools [1]:

$$[S_{wp}] = f[S_{ov}] \tag{5}$$

where $[S_{wp}]$ is the substrate concentration expressed with respect to the aqueous volume in reverse micellar solution, f is the ratio between the overall volume and water pool volume, $[S_{ov}]$ is the substrate concentration expressed with respect to the total volume of the reverse micellar solution. For the first-order reactions, the reaction rate measured in the water pool microphase can be expressed as:

$$v_{wp} = k_I[S_{wp}] = k_I f[S_{ov}] \tag{6}$$

But if the reaction is observed in the entire volume, the rate is expressed by:

$$v_{obs} = v_{wp}/f = k_I[S_{ov}] \tag{7}$$

which means that if the chemical step is the rate determining and if the first-order rate constant does not change in the micellar microenvironment, the observed reaction rate should be the same as in homogeneous aqueous solution. In contrast, for the second-order reactions, where the concentrations of reactants S_1 and S_2 are comparable one has:

$$v_{wp} = k_{II}[S_{1wp}][S_{2wp}] = k_{II} f^2 [S_{1ov}] * [S_{2ov}] \tag{8}$$

and

$$v_{obs} = v_{wp}/f = f k_{II}[S_{1ov}][S_{2ov}] \tag{9}$$

This means that the observed reaction rate should be greater than that in homogeneous aqueous solution by a factor f, when such a

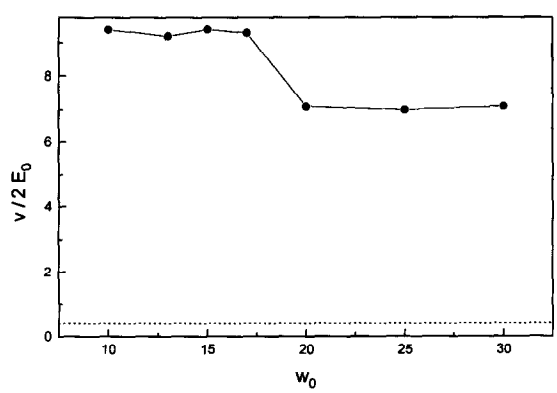

Fig. 2. Initial rate of ABTS oxidation, measured at 414 nm versus w_0. Dashed line denotes the value obtained for homogeneous aqueous solution. [HRP] = 1.6×10^{-8} M, $[H_2O_2]$ = 10 μM, [ABTS] = 20 μM.

reaction is rate-determining and when k_{II} remains unaffected in micellar microenvironment.

If we now assume that chemical reaction is the rate determining step in peroxidase cycle in reverse micelles, we obtain from equation (4) that the reaction rate should be greater in reverse micelles than in water by a factor f, which varies from 55 for $w_0 = 10$ to 18 for $w_0 = 30$. Moreover, Verhaert et al. [17] postulate that the substrate concentration that is relevant to the enzyme is the average intramicellar concentration in substrate-filled micelles. As the overall substrate concentration is low in comparison with micelle concentration [RM] (it varies from $\approx 10^{-3}$ M for $w_0 = 10$ to $\approx 2 \times 10^{-4}$ M for $w_0 = 30$ [18]), the observed reaction rate should be much higher, which is not observed by us. Verhaert et al. [19] have found that the rate of intermicellar exchange very strongly affects the rate of enzymatic reaction even when it is five orders of magnitude higher than the turnover of the enzyme, especially when the $[S_{ov}]$ is low in comparison with micelle concentration. They assumed, however that all the intrinsic rate constants of the enzyme are independent of its environment. On the other hand, Mao et al. [5] have found that one of the individual rate constants in the kinetics of α-chymotrypsin considerably decreases in reverse micelles. Our preliminary, unpublished, circular dichroism studies show that changes in the secondary structure of HRP molecule take place upon its incorporation into reverse micelles. They may influence the mechanism of HRP-catalyzed reaction in reverse micelles. From a practical point of view, however, irrespective of the factor, transport of solubilizates between micelles or chemical reaction is the rate limiting step, ABTS oxidation in reverse micelles is faster than in homogeneous aqueous solution at the same overall H_2O_2 and ABTS concentrations.

We tried to measure the rate constants of the elementary steps in the reaction of horseradish peroxidase with ABTS. Stopped-flow determinations of the rate constant of compound I formation (k_{1obs}) at different w_0 were performed under second-order conditions, where the initial concentrations of HRP and hydrogen peroxide were equal. The reaction, observed at 405 nm, resulted in a decrease of absorbance, because the extinction coefficient at 405 nm for compound I is about half that of native HRP [13]. Keeping the overall enzyme and hydrogen peroxide concentrations constant, k_{1obs} has been found to increase with w_0 up to $w_0 = 20$–25 achieving a value of $(15.8 \pm 0.2) \times 10^7$ M^{-1} s^{-1} for $w_0 = 20$ [15]. A slightly lower value of k_{1obs} has been found for $w_0 = 30$. The k_1 value measured under the same reaction conditions in homogeneous aqueous solution has been found to be $2.3 \pm 0.3 \times 10^7$ M^{-1} s^{-1}.

The k_{1obs} values obtained are comparable with the rates constant of the exchange process in reverse micelles ($k_{ex} = 10^6 - 10^8$ M^{-1} s^{-1}) [2] suggesting the communication process to be rate limiting step. Assuming chemical reaction to be rate determining step of process (1) in reverse micelles and having in mind that local reactant concentration in water pools of filled micelles is very high (≈ 5600 times higher than overall concentration for $w_0 = 20$), the k_1 value would be ca. three orders of magnitude lower than in the aqueous system.

We investigated the temperature dependence of the rate of reaction (1). From the Arrhenius plots for reaction (1) taking place in homogeneous aqueous solution and in reverse micelles at $w_0 = 20$ the activation energy E_a has been derived. Taking into account that the value of standard molar enthalpy of activation, $\Delta H^{\neq}$ can be obtained from the equation: $\Delta H^{\neq} = E_a - RT$, a standard molar entropy of activation, $\Delta S^{\neq}$ has been derived from Eyring equation.

Table 1
Thermodynamic functions of activation for compound I formation (reaction 1)

Investigated system	$\Delta H^{\neq}$ (kJ/mol)	$\Delta S^{\neq}$ (J/K mol)
homogeneous aqueous solution	10.0	−71.0
$w_0 = 20$	72.0	168.0

The obtained values of the thermodynamic functions of activation, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for reaction (1) are presented in Table 1.

Standard molar entropy and enthalpy of activation for HRP reaction with H_2O_2 obtained by us in homogeneous aqueous solution agree well with literature data [20]. Standard molar enthalpy of activation for the reaction (1) taking place in reverse micelles of AOT in n-heptane is higher than that in water (Table 1). Standard molar entropy of activation is positive contrary to the negative value observed in water solution. The values of standard molar entropy and enthalpy of activation for the reaction (1) taking place in reverse micelles of AOT in n-heptane are nearly identical with those obtained by Fletcher et al. [2,21] for the droplet exchange process in AOT/n-heptane reverse micelles. This supports our suggestion that the rate determining step for the reaction (1) changes from a chemical to a transport one after incorporation of HRP and H_2O_2 into reverse micelles.

At this moment we should correct our earlier suggestions [15] that observed increase of k_{1obs} is most likely due to conformational changes of the enzyme induced by the reverse micelle environment. The same results obtained using the stopped-flow technique and pulse radiolysis, where H_2O_2 is partially formed in organic phase, may indicate that in the latter case H_2O_2 rapidly diffuses to water pools of empty micelles (the concentration of which is two orders of magnitude higher than enzyme-filled micelles) and compound I formation is limited by the exchange between H_2O_2-filled and enzyme-filled micelles.

Table 2
Dependence of the k_1 rate constant on AOT concentration measured in reverse micelles at $w_0 = 20$

[AOT] (M)	$k_1 \times 10^{-7}$ (M^{-1} s^{-1})	Standard deviation $\sigma \times 10^{-7}$ (M^{-1} s^{-1})
0.05	20.8	2.2
0.1	13.9	0.5
0.2	9.5	0.5
0.25	10.7	2.1

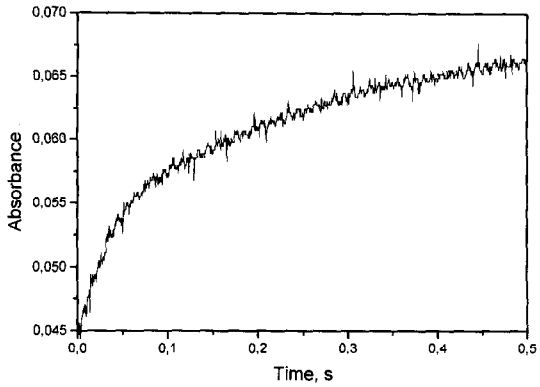

Fig. 3. Kinetic curve taken at 452 nm in homogeneous aqueous solution; [HRP] = [H_2O_2] = 2.0 μM, [ABTS] = 35 μM.

The rate constant, k_{1obs}, decreases on increasing surfactant concentration, while keeping w_0 constant, i.e. with increase in the concentration of reverse micelles (Table 2). The decrease of k_{cat} with increasing surfactant concentration has already been observed for HRP in reverse micelles [1,8,11]. It has been shown that the k_{ex} values measured using reactions of small hydrophilic reactants such as ions, only weakly depends on w_0 in n-heptane [2]. It has been reported, however, that the presence of protein may enhance attractive interactions between micelles [22] and hence may cause the increase of k_{ex}. Micelle concentration decreases and the fraction of enzyme-filled micelles (at constant overall enzyme concentration) increases with increasing w_0 or with decreasing surfactant concentration at constant w_0. This may be responsible for the observed increase of $k_{1obs} = k_{ex}$, with increasing w_0 and with lowering surfactant concentration. However, we cannot explain the low value of k_{1obs} at $w_0 = 30$, i.e. at the highest water loading we could obtain under our experimental conditions.

The rate constants k_2 and k_3 of the HRP reactions with ABTS as a substrate were determined in stopped-flow experiments at equimolar HRP and hydrogen peroxide concentrations and under at least ten-fold excess of ABTS. Under these reaction conditions reactions (2) and (3) show pseudo-first order kinetics. In homoge-

neous aqueous solution reactions with ABTS were monitored at 452 nm, i.e. at the wavelength where HRP as well as its compounds I and II show equal extinction coefficients. Thus, the observed increase in absorption (Fig. 3) should solely be due to $ABTS^{+\cdot}$ radical cation formation. The curve presented in Fig. 3 can be described neither by first nor second order kinetics, but is fairly well described by two consecutive first order processes, most likely representing reactions (2) and (3), respectively. The pseudo-first order rate constant of reaction (2), k'_2, has been calculated from the faster part of the curve. The k_2 value has been calculated from the slope of the linear dependence of k'_2 on ABTS concentration. Pseudo-first order rate constant of reaction (3), k'_3 has been found from the slower part of the curve presented on Fig. 3. The value of k_3 has been calculated in the same manner as k_2. The values of k_2 and k_3 obtained in homogeneous aqueous solution are $(2.5 \pm 0.2) \times 10^5$ and $5.0 \pm 1.2 \times 10^4$ M^{-1} s^{-1}, respectively.

We were unable to separate kinetically two steps of $ABTS^{+\cdot}$ formation at 452 nm in reverse micelles, which may suggest that the rates of ABTS reactions with compounds I and II are comparable. Thus we measured the rate constant k_{3obs} in stopped-flow experiments by the following procedure. Reverse micellar solution containing H_2O_2 was mixed with equal amount

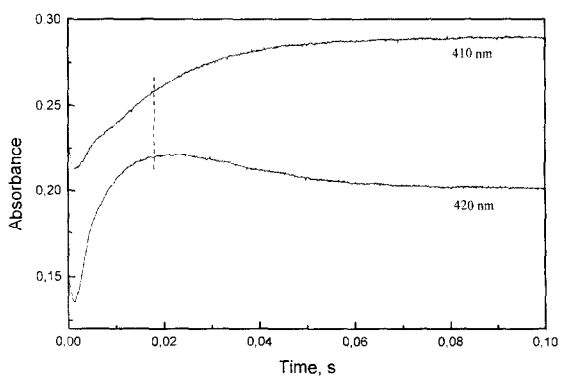

Fig. 4. Kinetic curves taken at 410 nm and at 420 nm in 0.1 M AOT/n-heptane reverse micellar solution, $w_0 = 30$; [HRP] = $[H_2O_2] = 2.6$ μM, [ABTS] = 95 μM.

Table 3
Kinetic constants measured in homogeneous aqueous solution and in AOT/n-heptane reverse micelles at different w_0. k_1 values are taken from [15]

w_0	k_1 (M^{-1} s^{-1}) $\times 10^{-7}$	k_2 (M^{-1} s^{-1}) $\times 10^{-6}$	k_3 (M^{-1} s^{-1}) $\times 10^{-6}$
homogeneous aqueous solution	2.3	0.25	0.051
10	8.2	–	1.3
13	–	–	1.2
15	10.0	–	1.4
17	–	–	1.2
20	15.8	–	0.8
25	15.3	–	0.8
30	12.5	–	0.7

of reverse micellar solution containing HRP and ABTS. The overall concentrations of HRP and H_2O_2 were equal and the concentration of ABTS was at least ten-fold higher in the obtained mixture. The formation of the radical cation, $ABTS^{+\cdot}$, was observed at 410 nm, i.e. at the isosbestic point of HRP and compound II. As, at this wavelength, the absorption changes connected with compound II formation could disturb the reaction kinetics ($\varepsilon_{\text{compound I}} < \varepsilon_{\text{compound II}} = \varepsilon_{\text{HRP}}$ at 410 nm), additional kinetic measurement at 420 nm, i.e. at the compound II maximum, was carried out to find out the range of reaction (3) domination. At this wavelength, where $\varepsilon_{\text{HRP}} < \varepsilon_{\text{compound II}}$, the decrease of absorption indicates that reaction (3) takes place in the main. From the part of the kinetic curve at 410 nm where the reaction (3) dominates (see Fig. 4), the pseudo-first order rate constant k'_{3obs}, in reverse micelle has been calculated. The k_{3obs} value of ABTS oxidation by compound II in reverse micelles has been obtained from the slope of the plot of k'_{3obs} versus $[ABTS]_{ov}$.

The k_{3obs} values for ABTS oxidation measured in reverse micelles above and below $w_0 = 17$ are about 15 times and 25 times higher, respectively, than the k_3 value obtained in homogeneous aqueous solution (Table 3). If the increase of k_{3obs} is connected only with the concentration effect in the water pool, the val-

ues of k_{3obs} would decrease monotonously with increasing w_0. We calculated the initial reaction rate in filled water pools, by using the k_3 value obtained in homogeneous aqueous solution and we found that the rates of exchange and of reaction (3) in reverse micelles were comparable. This means that the exchange could influence the rate of compound II reaction with ABTS. On the other hand the presence of bound water, the fraction of which is high at low w_0 and/or changes in enzyme structure upon incorporation into reverse micelles could also influence the rate of reaction (3). One may suppose that the same factors control reaction (2) in the reverse micelle.

In conclusion, we have shown that activity of horseradish peroxidase in AOT/n-heptane reverse micelles towards ABTS is higher by over one order of magnitude in comparison with homogeneous aqueous solution. The rate constants of all three elementary steps of the HRP reaction with ABTS are higher in reverse micelles than in homogeneous aqueous solution. The rate limiting step of compound I formation is the exchange between the enzyme and the hydrogen peroxide-filled micelles. The exchange step most probably also influences the rate of ABTS reactions with compound I and compound II, although the changes in k_2 and k_3 values due to different water and enzyme structure in reverse micelles cannot be excluded.

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