Journal of Molecular Catalysis B: Enzymatic 56 (2009) 259–264

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Singlet oxygenation in microemulsion catalysed by vanadium chloroperoxidase

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ARTICLE INFO

Article history:
Received 4 February 2008
Received in revised form 6 May 2008
Accepted 13 May 2008
Available online 24 May 2008

Keywords:
Vanadium chloroperoxidase
Microemulsion
Ethoxylated fatty alcohol
Fish diagram
Singlet oxygen

ABSTRACT

Non-ionic microemulsions compatible with the enzyme vanadium chloroperoxidase were designed to perform singlet oxygenation of apolar substrates. The media were based on mono- and polydisperse ethoxylated fatty alcohols (C_iE_j), octane and aqueous buffer. "Fish" diagrams were determined to identify the Winsor-boundaries and to formulate a monophasic Winsor IV microemulsion with a minimal surfactant concentration, ensuring less singlet oxygen ($^1O_2$) loss than in an aqueous system, thus creating a high oxygenation efficiency. The enzyme was shown to be fully stable in the microemulsion for at least 10 h, converting H_2O_2 into a constant flow of $^1O_2$ in the aqueous microdomains. Part of the $^1O_2$ diffuses into the organic compartments prior to fast physical deactivation of $^1O_2$ by water molecules. In the apolar domains $^1O_2$ quantitatively converts the model substrate 9,10-dimethylanthracene into its corresponding endoperoxide. Near-IR chemiluminescence measurements confirm that the $^1O_2$ signal in the microemulsion is higher than in simple aqueous buffer. In a well-stirred (water/octane) biphasic system endoperoxide formation is also observed but the conversion rate is much lower, most likely due to stronger physical quenching of $^1O_2$.

1. Introduction

Vanadium haloperoxidases are robust enzymes that catalyse the oxidation of halides [1] Eq. (1), the oxidation of sulfides [2,3] and the production of singlet oxygen ($^1O_2$) Eq. (2) [4], reactions of interest in organic synthesis.

$$H_2O_2 + X^- + H^+ \rightarrow HOX + H_2O, \qquad X = Cl, Br, I \tag{1}$$

A key feature of these enzymes for application in synthesis is their stability during storage in mixtures of water and organic solvents up to 60% solvent [5], their stability against elevated temperatures ($T_m \sim 80\,^\circ C$), and, partially due to their vanadate cofactor, towards a high concentration of the substrate H_2O_2, the product HOX and the secondary product $^1O_2$ [4], evoking considerable industrial interest for these enzymes [6,7]. These reactive oxidative compounds quickly inactivate the alternative heme-containing haloperoxidases such as the well-studied chloroperoxidase from *Caldaromyces fumago* (FeCPO) (Fig. 1). An additional disadvantage of the latter enzyme is that it exhibits considerable catalase activity, producing only 'normal' oxygen, thus leading to a waste of H_2O_2. Vanadium haloperoxidases do not show such catalase activity.

After halide oxidation (Cl^- or Br^-) the formed HOX reacts non-catalytically with a second molecule of H_2O_2 to form singlet oxygen ($^1O_2$) [4] Eq. (2). Obviously, high H_2O_2 concentrations favour this reaction.

$$HOX + H_2O_2 \rightarrow {}^1O_2 + X^- + H^+ + H_2O \tag{2}$$

The net reaction of this process is the disproportionation of H_2O_2 into singlet oxygen and water Eq. (3):

$$2H_2O_2 \rightarrow {}^1O_2 + 2H_2O \tag{3}$$

VCPO from *Curvularia inaequalis* is especially interesting because of its ability to oxidise Cl^-, discriminating it from vanadium bromoperoxidases (VBPOs) from seaweeds [1,8]. It was shown that $^1O_2$ produced by VCPO can convert the water soluble anthracene-9,10-bisethanesulphonate to its corresponding endoperoxide [4]. Conversion was only clean by using Cl^- as co-catalyst, whereas with Br^- 20% side products arising from the reaction of the HOBr intermediate with the anthracene was observed thus showing the added value of using the chloroperoxidase. By using a high concentration of H_2O_2 the steady state concentration of the HOCl intermediate can be kept at a minimum, and since VCPO tolerates H_2O_2 concentrations up to at least 0.5 M [4], VCPO is again favoured over FeCPO for this reaction.

Abbreviations: VCPO, vanadium chloroperoxidase; VBPO, vanadium bromoperoxidase; FeCPO, heme chloroperoxidase; DMA, 9,10-dimethylanthracene; DMA-O2, 9,10-dimethylanthracene endoperoxide; WI/II/III/IV, Winsor I/II/III/IV.

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1381-1177/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.molcatb.2008.05.014

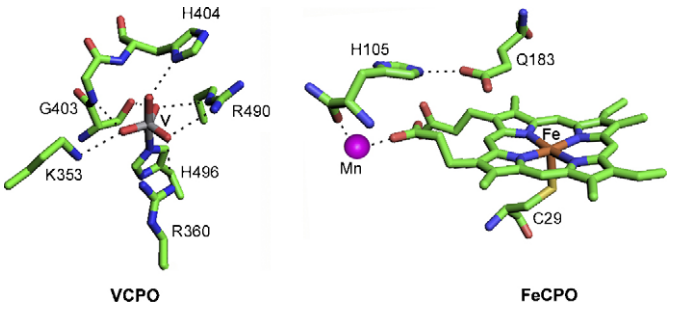


Fig. 1. Cofactor comparison of two singlet oxygen biocatalysts: the vanadate-containing vanadium chloroperoxidase (VCPO) from *Curvularia inaequalis* (left) and the more vulnerable heme-containing chloroperoxidase from *Caldaromyces fumago* (right). The most important active site residues of the active sites are shown. The vanadate in VCPO is directly bound to His496, resulting in a penta-coordinated geometry with an axial hydroxo group trans to His496 and three equatorial oxo ligands. A hydrogen bonding network is formed with several neighbouring residues which are strongly conserved among various vanadium haloperoxidases. The figure has been prepared using PyMol [22] (pdb codes; VCPO: 1idq, FeCPO: 1cpo).

One of the simplest and most effective inorganic singlet oxygen catalysts is sodium molybdate [9], however in comparison with this system there are several arguments in favour of the enzyme system: (i) the enzyme's catalytic rate is 10^3 to 10^4 times higher [4]; (ii) it catalyses $^1O_2$ production at slightly acidic pH at which molybdate is inactive; (iii) the use of the environmentally abundant vanadate is 'greener', which is additional to the green effect of the higher catalytic rate of VCPO.

An important limit for performing singlet oxygen chemistry with VCPO is the low solubility of typical organic substrates in aqueous media. Another disadvantage is the relative short lifetime of singlet oxygen in water ($\tau_\Delta \cong 4\,\mu s$), due to a higher physical quenching of $^1O_2$ in water compared to organic solvents ($\tau_\Delta \cong 10$–$1000\,\mu s$). When co-solvent (e.g. MeOH) is used to solubilize the organic substrate a third potential problem arises; the direct contact between a high concentration of organic substrate with the low steady state concentration of the HOCl intermediate in the same phase may lead to unwanted side reactions. In addition the extra solubilization by using co-solvent is limited. To overcome these problems we tried to design microemulsions in which VCPO remains active.

Microemulsions are thermodynamically stable mixtures of apolar solvents ('oils') and water using high concentrations of surfactants leading to small microstructures (10–100 nm), yielding transparent or translucent solutions. These microstructures are only one order of magnitude larger than an enzyme such as VCPO (8 nm × 5.5 nm) [10], thus providing an extremely large interface between the aqueous and oil domains. Depending on relative amounts of components the microstructures range from oil in water droplets to water in oil droplets with an intermediate 'sponge-like' structure. The benefits of the dramatically increased contact area between the aqueous phase and the organic phase and the decreased average travel distance from the aqueous phase to the organic phase for $^1O_2$ has been used before with molybdate as $^1O_2$-catalyst [11]. Depending on several factors such as composition and temperature, microemulsion systems can exist in four macroscopically distinct forms; Winsor I, II, III and IV (Fig. 2). The Winsor IV system, consisting of a single homogeneous phase, was used for our experiments, ensuring that any observed activity indeed takes place in the microemulsion and facilitating UV–vis spectroscopy analysis. Formation of Winsor IV microemulsions requires a large concentration of surfactant, which for most enzymes leads to irreversible denaturation. However, enzyme activity in microemulsions of various types has been shown several times [12,13]; usually

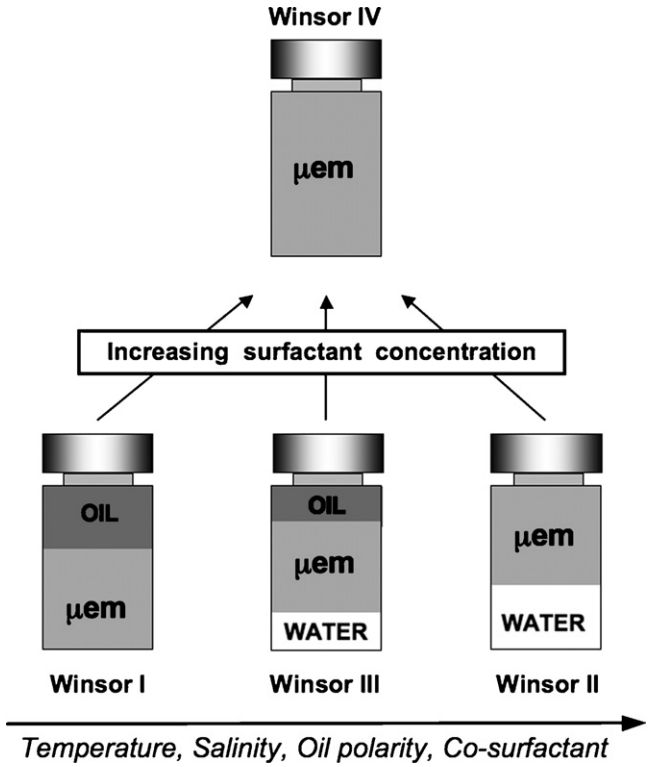


Fig. 2. Modification of the Winsor type of microemulsion system by scanning one of the formulation variables or by increasing surfactant concentration. The system shown is based on a non-ionic surfactant and has equal volumes of water and oil.

tailor-made solutions have to be formulated. As indicated above, VCPO is generally a very stable enzyme, and we have investigated the $^1O_2$ production in microemulsions based on non-ionic ethoxylated alcohols (general formula: C_iH_{2i+1}–$(O$–CH_2–$CH_2)_j$–OH or C_iE_j).

2. Experimental

2.1. Chemicals

Polydisperse surfactant ($C_{13}H_{27}$–$(O$–CH_2–$CH_2)_j$–OH = $C_{13}E_j$) with different average ethylene oxide numbers (i = 4, Merlipal 24/40; i = 6, Merlipal 24/60; i = 7, Merlipal 24/70; i = 9, Merlipal 24/99) were a gift from Sasol Germany GmbH. The "pure" surfactant tetraethylene glycol monodecyl ether ($C_{10}E_4$) was synthesized according to Zhu et al. [14]. 9,10-Dimethylanthracene and monochlorodimedon were purchased from Aldrich. 9,10-Dimethylanthracene endoperoxide used for HPLC calibration curve was photochemically synthesised as described before [15,16]. Millipore water (18.3 MΩ cm^{-1}) was used for all experiments.

2.2. Enzyme

Recombinant VCPO was heterologously expressed and purified as described previously [17], and 1–10 μM stock solutions were routinely stored in 100 mM Tris–SO_4 buffer pH 8.3. For the chemiluminescence experiment a 100 μM stock solution was made by concentration using a Centricon-30 from Millipore. The buffer was exchanged with 100 mM acetic acid/NaOH buffer (pH 5.0), containing an additional 100 μM vanadate thus avoiding cofactor loss due to low pH.

2.3. UV–vis

Spectra and traces were measured on a temperature controlled Cary-50 spectrometer.

2.4. Chemiluminescence

IR-luminescence originating from $^1O_2$ at 1268 nm was measured with our in-house built apparatus consisting of a N_2 cooled Ge-diode detector from North Coast Scientific Corp. (Model EO-817L) and an optical fiber connected to a lock-in amplifier from Stanford Research Systems Model SR830 DSP. The detector was powered by a North Coast Scientific Corp. Model Bias Supply Model 823A. Experiments were performed in a rectangular 3 mL quartz cuvet. Conditions H_2O-based microemulsion: 10% polydisperse surfactant $C_{13}E_{6.5}$, 5% 1-pentanol, octane: aqueous solution = 1:1 (v/v), aqueous solution consisting of 5 μM VCPO, 100 mM acetate buffer pH 5, 100 mM H_2O_2, 20 mM Cl^- and 100 μM vanadate. $T = 25 \pm 0.1$ °C. Components were carefully mixed with a Hamilton syringe and allowed to reach the temperature of the thermostated cell holder. The reaction was started by addition of H_2O_2, resulting in a total volume of 2.275 mL (with a 1 mL aqueous phase contribution). In the experiment with only aqueous solution the aqueous phase was 1 mL, thus the total volume in both experiments was different, leading to a small correction when the signals were compared (see Section 3). To determine this factor the $^1O_2$ signal produced by 1 mL and 2.275 mL 0.1 M molybdate standard mixed with 1 M H_2O_2 was measured in our setup, resulting in a correction factor 1.2. In the experiment with >10 h of singlet oxygen production (see Section 3) similar conditions as in Fig. 5 were used, with 0.5 μM VCPO and 250 mM H_2O_2.

2.5. Determination of the average ethylene oxide number (n)

Mixing weight m_j of polydisperse surfactant $C_{13}E_j$ (molecular weight = M_j) with weight m_k of polydisperse surfactant $C_{13}E_k$ (molecular weight = M_k) leads to an equivalent surfactant $C_{13}E_n$ whose average ethylene oxide number n can be estimated by the following formula:

$$n = \frac{m_j.j/M_j + m_k.k/M_k}{m_j/M_j + m_k/M_k}$$

2.6. Singlet oxygenation of 9,10-dimethylanthracene (DMA) in microemulsion with $C_{10}E_4$

Experiments were performed in a 0.3 mL glass reactor in the dark at $T = 23 \pm 0.1$ °C. Components were carefully mixed in the following order: 26 mg $C_{10}E_4$, 100 μL 10 mM 9,10-DMA in octane, 46 μL water, 10 μL 1 M acetic acid/NaOH buffer pH 5.0, 20 μL 100 mM Cl^-, 20 μL 1 M H_2O_2 and 4 μL 9 μM VCPO.

2.7. Singlet oxygenation of 9,10-dimethylanthracene (DMA) in biphasic system

Same protocol as in microemulsion without $C_{10}E_4$.

2.8. Detection of DMA and $DMAO_2$

The formation of the corresponding endoperoxide ($DMAO_2$) was followed by HPLC (Waters 600 chromatograph equipped with Novapak C18 (4-mm) column), using 70% acetonitrile (HPLC-grade). UV detection was performed with a variable-wavelength monitor (Waters 490E). Calibration curves of both DMA and $DMAO_2$ showed a linear response in the range used in our study.

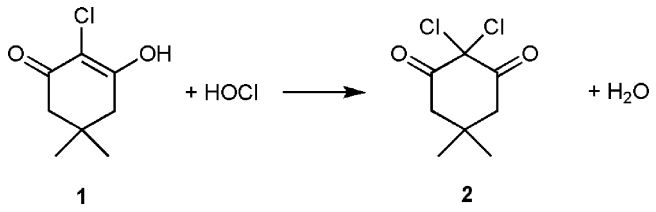
3. Results and discussion

To test VCPO stability and activity in microemulsions we formulated a monophasic Winsor IV system, ensuring that catalysis indeed takes place in the microemulsion. The transparency of this system allows the use of the well-known monochlorodimedone (MCD) assay [18], which requires analysis in the UV. In this assay (Scheme 1) MCD (**1**) reacts with HOCl produced by the enzyme to form dichlorodimedone (**2**), which is accompanied by a loss of UV absorption ($\Delta\varepsilon = 20\,000\ M^{-1}\ cm^{-1}$ at 290 nm).

Initially, we formulated microemulsions based on polydisperse ethoxylated alcohols. The use of these different chain length mixtures has the following advantages: (i) the temperature window in which a monophasic transparent Winsor IV system is found is broader than when monodisperse surfactant is used; (ii) polydisperse ethoxylated alcohols are readily available and inexpensive. Potential drawbacks can be: (i) more cumbersome product isolation from microemulsions containing polydisperse surfactant during eventual application; (ii) more difficult product analysis (e.g. HPLC); (iii) with polydisperse surfactant the use of a co-surfactant (e.g. 1-pentanol) is required to avoid surfactant precipitation and to reduce the viscosity of the microemulsion. For the initial MCD based activity tests these drawbacks were not relevant.

From Fig. 3 it can be concluded that a minimum of 8% surfactant is required to obtain a Winsor IV system. For the MCD analysis we used 10% surfactant with an average ethylene oxide number $n = 6.5$ and the result is shown in Fig. 4.

Fig. 4 shows that the enzyme is active and no inactivation is observed as the reaction proceeds. The catalytic rate observed



Scheme 1. UV based MCD assay for chloroperoxidase activity. $\Delta\varepsilon$ = 20,000 M^{-1} cm^{-1} at 290 nm.

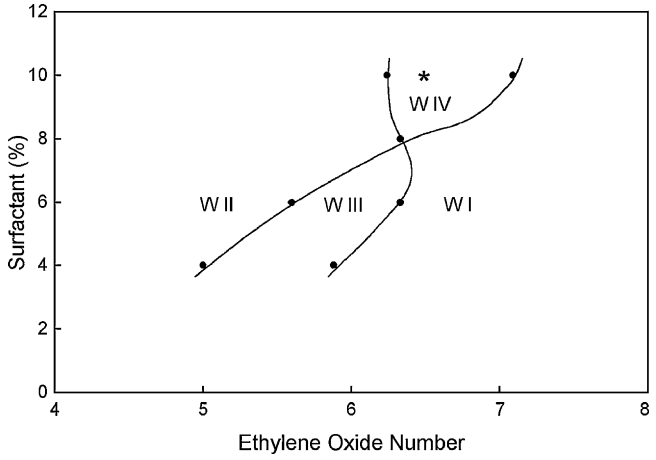


Fig. 3. Phase diagram for determining the optimal formulation of a polydisperse (non-ionic) surfactant based Winsor IV microemulsion for vanadium chloroperoxidase at 25 °C. 5% 1-penthanol was used as cosurfactant. The conditions used for further experiments are indicated by *.

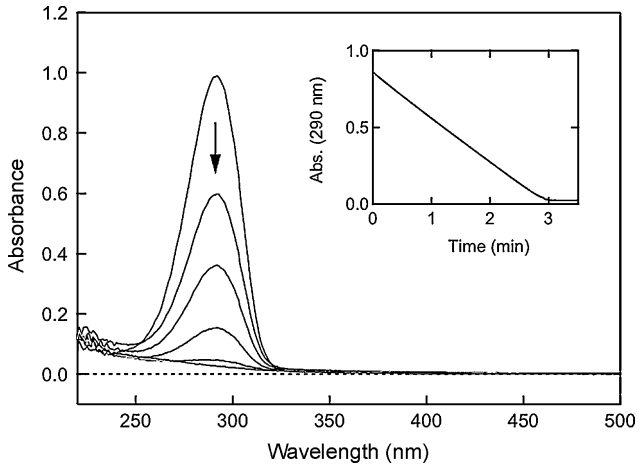


Fig. 4. Vanadium chloroperoxidase activity in a polydisperse non-ionic surfactant based Winsor IV microemulsion as measured by UV–vis absorption using the monochlorodimedone assay [18]. The first spectrum is the spectrum before the start of the reaction. The inset shows an experiment with the same conditions followed continuously at 290 nm, the initial absorption is less than 1.0 due to the mixing time. Conditions: 10% polydisperse surfactant (C_iH_{2i+1}–(O–CH_2–CH_2)$_j$–OH), 5% 1-pentanol, octane: aqueous solution = 1:1 (v/v), aqueous solution consisting of 50 nM VCPO, 100 mM acetate buffer pH 5.0, 1 mM H_2O_2, 20 mM Cl^-, 100 μM vanadate, $T = 25 \pm 0.1$ °C. The overall solution contained 50 μM MCD. The reaction was started by addition of H_2O_2.

($14\,s^{-1}$) is similar to the rate observed in aqueous solution (pH 5, 20 mM Cl^-) [17,19], proving full activity of the enzyme in the microemulsion and confirming its general stability.

As explained in the introduction $^1O_2$ is produced by VCPO in the absence of a strong HOCl scavenger like MCD [4]. To measure $^1O_2$ production in microemulsion its chemiluminescence signal at 1268 nm Eq. (4) was used with similar conditions as in Fig. 4, however, a high concentration of VCPO (5 μM) was used to ensure sufficient signal.

$$^1O_2 \rightarrow {}^3O_2 + h\nu(1268\,\text{nm}) \quad (4)$$

As a consequence also a high H_2O_2 concentration was used to avoid instantaneous consumption of the H_2O_2. Using a monophasic Winsor IV system ensures a minimal quenching loss of $^1O_2$. As a comparison the same experiment was conducted in a simple aqueous solution. The results are shown in Fig. 5.

Fig. 5 confirms that the enzyme is active in a microemulsion and a clear added value of the microemulsion in comparison with the simple aqueous system is observed, since the signal produced in the microemulsion is 10-fold higher. A small part of this difference can be attributed to the difference in volume of the solution (2.275 mL microemulsion vs. 1 mL aqueous solution); in our detector setup this leads to a correction factor of 1.2, as measured by $^1O_2$ production by a molybdate standard in the respective volumes. After correction a factor of about 8 remains. The slow decrease of the signals in time is due to the decreases of the H_2O_2 concentration. A stable luminescence signal corresponding to a constant formation of $^1O_2$ is observed by using only 1 μM VCPO and increasing the H_2O_2 concentration to 250 mM; the enzyme then remains active >10 h corresponding to 125.000 turnovers with respect to $^1O_2$ (not shown), confirming its high stability towards this product [4].

In the actual singlet oxygenation of a hydrophobic substrate in a H_2O-based microemulsion the effect of using a microemulsion is expected to be higher in comparison with only producing $^1O_2$ without adding a substrate, since less back diffusion to the aqueous microdomains will occur due to reaction with the substrate. To test this singlet oxygenation rate and yield in microemulsion and to facilitate product analysis we formulated a microemulsion based on pure surfactant "inside" the polydispersed $C_{13}E_{6.4}$ one. As previously described, $C_{13}E_{6.4}$ is the best adapted surfactant for the octane/water system, since the clear Winsor IV microemulsion is obtained for the minimum concentration of surfactant (8%) [14]. Rather to carry out the difficult synthesis of pure $C_{13}E_6$ or $C_{13}E_7$, we have focussed on an equivalent and shorter surfactant. Shorter equivalent surfactant can be obtained by reducing both hydrophobic (alkyl chain) and hydrophilic (ethylene oxide) parts. Using the Hydrophilic Lipophilic Deviation (HLD) developed by Salager et al. [20,21] it was shown that $C_{10}E_4$ can substitute $C_{13}E_{6.4}$ in the octane/water system. Moreover, the use of this surfactant presents several advantages: the co-surfactant pentanol is not necessary due to the good solubility property of this small size surfactant and the interface is fluid enough since the $C_{10}E_4$ is a liquid at room temperature. For the VCPO enzyme 100 mM acetate buffer and 20 mM Cl^- are necessary and the phase diagram in Fig. 6 shows the small effect of these components in the aqueous phase on the previously published octane–H_2O–$C_{10}E_4$ system.

The critical temperature T^* of the so-called "Fish-tail" point (T^*, C^*) is lowered from 25 to 23 °C, which is in line with the increased salinity (Fig. 2). The critical concentration C^* of this point is about 11%; only above this point a monophasic Winsor IV system is observed. Using 23 °C and 14% $C_{10}E_4$ we tested the singlet oxygenation of the hydrophobic model substrate 9,10-dimethylanthracene (**3**) (Scheme 2).

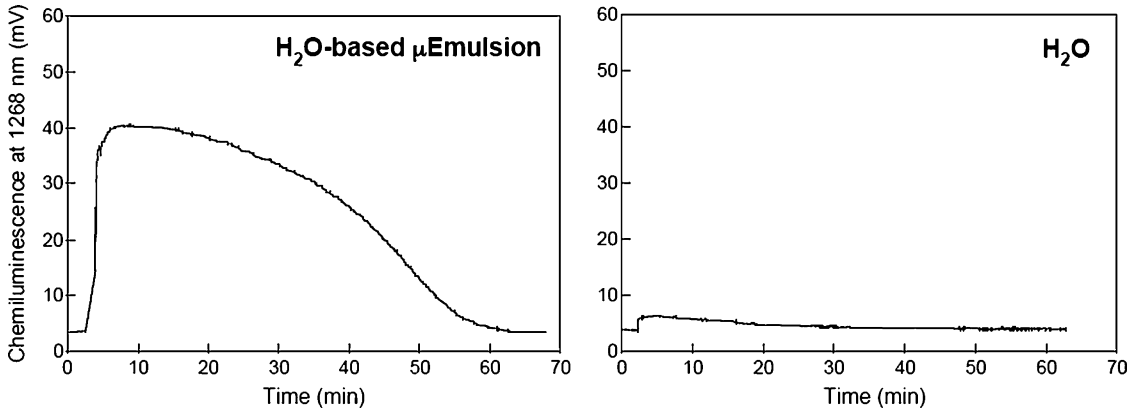


Fig. 5. $^1O_2$ production by vanadium chloroperoxidase in a microemulsion based on H_2O. Conditions: 10% polydisperse surfactant (C_iH_{2i+1}–(O–CH_2–CH_2)$_j$–OH), 5% 1-pentanol, octane: aqueous solution = 1:1 (v/v), aqueous solution consisting of 5 μM VCPO, 100 mM acetate buffer pH 5.0, 100 mM H_2O_2, 20 mM Cl^- and 100 μM vanadate. $T = 25 \pm 0.1$ °C. The reaction was started by addition of H_2O_2. The amount of aqueous phase in both experiments is kept constant (1 mL), for details see Section 2.

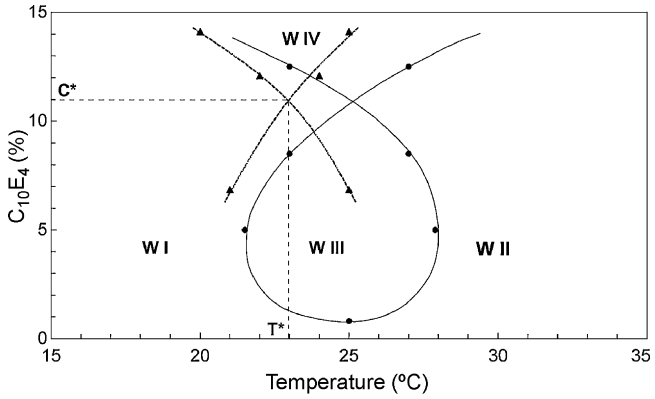
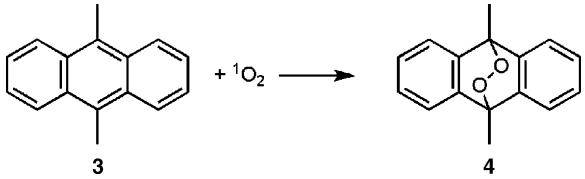


Fig. 6. Phase diagram for the system *n*-octane/water/$C_{10}E_4$ (●, solid line) [11] and *n*-octane/aqueous buffer (100 mM acetic acid pH 5.0, 20 mM NaCl)/$C_{10}E_4$ (▲, dashed line) as a function of temperature and surfactant concentration at a fixed ratio octane/water = 1:1.



Scheme 2. Reaction of 9,10-dimethylanthracene (**3**) with $^1O_2$ to give the corresponding endoperoxide (**4**).

In this reaction we used a high concentration of H_2O_2 (200 mM) to avoid any side-reaction between the HOCl intermediate and 9,10-dimethylanthracene. To test the added value of the microemulsion a similar experiment was performed using a biphasic system (with and without stirring) and the result is shown in Fig. 7.

The 9,10-dimethylanthracene is completely converted into the corresponding endoperoxide (DMA-O2) in about 50 h (80% in 20 h) and, obviously, stirring is not necessary. After an initial constant conversion the rate of conversion slowly drops, most likely due to a combined decrease in 9,10-dimethylanthracene and H_2O_2 concentration. Although the initial H_2O_2 concentration is clearly higher than the 9,10-dimethylanthracene, $^1O_2$ (and therefore also H_2O_2) is also lost via physical quenching before it can react with the 9,10-dimethylanthracene. This loss will be much

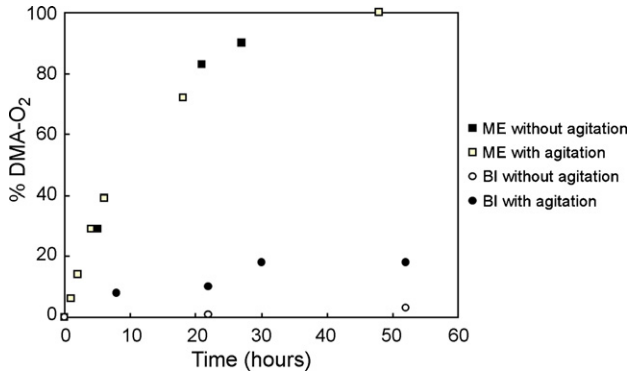


Fig. 7. Dark singlet oxygenation of 9,10-dimethylanthracene by vanadium chloroperoxidase in microemulsion (ME) (octane–aqueous buffer–$C_{10}E_4$) vs. a similar biphasic system (BI) and the effect of stirring. In both systems the aqueous buffer (100 mM acetic acid/NaOH pH 5.0) contained 0.36 μM VCPO, 200 mM H_2O_2 and 20 mM NaCl. The octane contained 10 mM 9,10-dimethylanthracene. $T = 23 \pm 0.1$ °C and aluminium foil was used to ensure dark conditions.

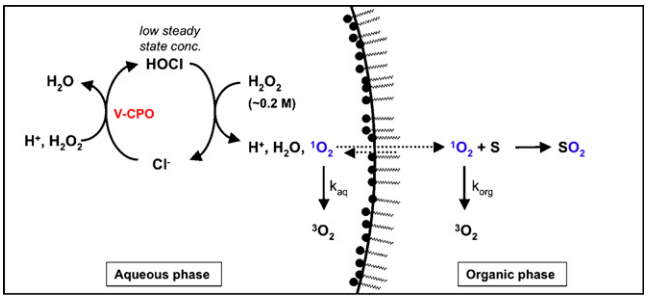


Fig. 8. Singlet oxygenation of apolar substrates (S) catalysed by VCPO in microemulsions. $^1O_2$ formation and oxygenation take place in separate microdomains, minimising direct contact between HOCl and substrate S. This contact is further minimised by using a high H_2O_2 concentration (at which VCPO remains stable). The lifetime of enzymatically produced $^1O_2$ is increased as compared to aqueous solution since overall physical quenching of $^1O_2$ is much lower ($k_{org} \ll k_{aq}$). Compared to a biphasic system the lifetime of $^1O_2$ and thus the product yield are maximized by the low mean travel distance of $^1O_2$ from the aqueous phase to the organic phase.

less than in the biphasic system, explaining the lower observed conversion rate in that system, even when stirred. The initial conversion rate in the microemulsion is clearly higher than the well-stirred biphasic system; in the well-stirred biphasic system the rate also drops in time and therefore even with the strong excess of H_2O_2 the 9,10-dimethylanthracene is not fully converted. The use of the microemulsion thus clearly increases efficiency of singlet oxygenation catalysis by VCPO. In a control experiment 9,10-dimethylanthracene was mixed under similar conditions with free HOCl; the resulting oxidation product was not observed using VCPO as catalyst. Similarly no oxidation product of the surfactant was found, showing its chemical neutrality in our system. The overall picture of using VCPO for singlet oxygenation in microemulsions is depicted in Fig. 8.

4. Conclusions

In summary we have shown that VCPO is active in chlorination and singlet oxygenation in non-ionic microemulsions. The chlorination of MCD shows that the conversion rate of the enzyme in microemulsion is of similar magnitude as in a simple aqueous solution. The oxygenation of 9,10-dimethylanthracene provides a proof of principle that in dark singlet oxygenation catalysis VCPO can be used as a green alternative for inorganic catalysts like molybdate, for which higher concentrations are needed due to 10^3 to 10^4 lower turnover numbers [4]. We have formulated two types of microemulsions in which VCPO is stable, using poly- and monodisperse ethoxylated fatty alcohols. The polydisperse situation offers the option for inexpensive bulk applications whereas the monodisperse situation offers a proof of principle for applications based on this currently expensive surfactant. The use of a biphasic system will give less complex purification of the end product, but takes much longer time and also requires more H_2O_2, since more $^1O_2$ is lost by physical quenching.

Acknowledgement

Dr. Sébastien Queste is kindly thanked for the gift of the pure $C_{10}E_4$ surfactant and for useful discussions.

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