Vanadium Chloroperoxidase as a Catalyst for Hydrogen Peroxide Disproportionation to Singlet Oxygen in Mildly Acidic Aqueous Environment

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Abstract: Chemiluminescence studies show that the enzyme vanadium chloroperoxidase from the fungus *Curvularia inaequalis* is a highly efficient catalyst for the production of singlet oxygen from hydrogen peroxide in a mildly acidic aqueous environment, with a maximum turnover frequency of $100 \, s^{-1}$ with respect to H_2O_2 consumption. The enzyme studied here can be regarded as a supplementary catalyst to inorganic molybdate, which operates in basic aqueous media. The enzyme system is $10^3 - 10^4$ times faster than the molybdate system when compared at their optimal pH. The enzyme is very stable against singlet oxygen, in contrast to heme-containing peroxidases which, due to inactivation by singlet oxygen, only produce a short burst, presumably by oxidation of the labile cofactor. The vanadium chloroperoxidase was compared with vanadium bromoperoxidase from the seaweed *Ascophyllum nodosum* and was found to be even more stable; it is stable towards a continuous flow of singlet oxygen for at least 1 hour and also it is not inactivated by $500 \, mM \, H_2O_2$. An important difference with vanadium bromoperoxidase is that the vanadium chloroperoxidase is able to effectively use chloride as a cocatalyst ($20 \, s^{-1}$) instead of bromide. This offers an important advantage from an application point of view; using chloride, no side products are observed when the chemical trap anthracene-9,10-bis(ethanesulphonate) is fully converted by singlet oxygen into its corresponding endoperoxide. By contrast, when bromide is used, 20% side product is formed. During the conversion the enzyme remains fully stable for 25,000 turnovers.
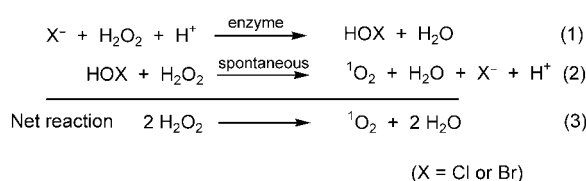
Keywords: biocatalysis; singlet oxygen production; vanadium chloroperoxidase (VCPO)

Introduction

A number of different processes are used to produce singlet oxygen ($^1O_2$, $^1\Delta_g$), a reactant that can oxidise various electron-rich organic compounds.[1,2] There are several photochemical processes in which dyes such as rose bengal, methylene blue or porphyrin are irradiated leading to the conversion of normal triplet oxygen to singlet oxygen.[3,4] In addition to these photochemical processes which require irradiation, several "dark" sources exist. For example, one can chemically trap $^1O_2$ in the form of naphthalenic endoperoxides,[5] after which these $^1O_2$ carriers are thermolysed to release $^1O_2$. Simply mixing hypochlorite (ClO^-) with H_2O_2 will also produce $^1O_2$,[6] however, hypohalites may give rise to unwanted side reactions with organic substrates. Therefore a catalyst is preferred such as molybdate,[7] which disproportionates H_2O_2 into $^1O_2$ generating a continuous flow of $^1O_2$. However, a drawback this system lies in the requirement of alkaline conditions to promote the formation of triperoxomolybdate, $MoO(O_2)_3^{2-}$, the precursor of $^1O_2$,[7] which lowers the synthetic scope of the molybdate-catalyzed "dark" singlet oxygenation. Very recently, we described the use of a heterogeneous lanthanum catalyst for "dark" singlet oxygenation[8] that also works under slightly acidic conditions and the synthetic advantage of this system over the molybdate system was demonstrated. This lanthanum system, however, yields only 45% $^1O_2$ in H_2O_2 disproportionation and, in addition, it is significantly slower than the molybdate system. For these reasons there is still an ongoing quest for more efficient catalyst systems that operate at acidic pH values.

The formation of $^1O_2$ under mildly acidic conditions (pH ~ 3 – 6) by haloperoxidases has been reported and is believed to occur *via* the mechanism shown in Scheme 1:[9–18]

$$X^- + H_2O_2 + H^+ \xrightarrow{\text{enzyme}} HOX + H_2O \quad (1)$$

$$HOX + H_2O_2 \xrightarrow{\text{spontaneous}} {}^1O_2 + H_2O + X^- + H^+ \quad (2)$$

$$\text{Net reaction} \quad 2\,H_2O_2 \longrightarrow {}^1O_2 + 2\,H_2O \quad (3)$$

(X = Cl or Br)

Scheme 1. Putative mechanism for singlet oxygen production by haloperoxidases.[9–18]

The haloperoxidases oxidise halides to hypohalous acids (Equation 1) and are named after the most electronegative element they are able to oxidise and hence a chloroperoxidase can oxidise chloride, bromide and iodide, whereas a bromoperoxidase only oxidises bromide and iodide at an appreciable rate. In the case of chloride and bromide, the formed hypohalous acid reacts non-enzymatically with H_2O_2 to give ${}^1O_2$ (Equation 2). In this process the halide is regenerated. Accordingly, the process is catalytic in halide provided that no halide is irreversibly lost by side-reactions of HOX. Such side reactions of HOX are minimised by a high H_2O_2 concentration in combination with a controlled continuous production of HOX, which is present in a low steady-state concentration. The gradual, controlled HOX release, together with the sub-stoichiometric halide requirement and the acidic processing conditions, make the enzymatic process advantageous to simple mixing of hypochlorite with H_2O_2. Production of ${}^1O_2$ has been demonstrated for some heme-containing haloperoxidases. They show, however, only a short burst of ${}^1O_2$ during which these enzymes are inactivated,[13–17] presumably by oxidation of the electron-rich heme

moiety by the generated ${}^1O_2$. Moreover, the heme containing haloperoxidases are not stable towards a high concentration of their substrate H_2O_2. In contrast, reports of ${}^1O_2$ production by a vanadium bromoperoxidase[18,19] show that this class of enzymes with the more robust vanadate cofactor is much more stable against ${}^1O_2$ and H_2O_2, making them more likely candidates for application in organic synthesis. This vanadate (HVO_4^{2-}) cofactor in these enzymes is covalently attached to a histidine residue, giving rise to a penta-coordinated vanadium atom (Figure 1).[20–23]

The resulting trigonal bipyramidal structure is stabilised by several neighbouring amino acid residues. The binding of the peroxide and the formation of the hypohalous acid have been shown to take place at this cofactor[21,25] and it has been shown that this class of enzymes is very stable towards high temperature and organic solvents.[26,27] These stable enzymes have already been shown to have high potential for application in organic synthesis: vanadium bromoperoxidases have been shown to perform enantioselective sulphoxidation[28–31] of a series of compounds of which both *R* and *S* enantiomeric excess can be formed, depending on which enzyme is used.[30]

A disadvantage of vanadium bromoperoxidase (VBPO) from the seaweed *Ascophyllum nodosum* is that it experiences slow inactivation during ${}^1O_2$ production.[18] This inactivation is likely to be worse at high H_2O_2 concentrations at which this enzyme (at low pH values) is inactivated by oxidation of a critical histidine.[32,33] These high H_2O_2 concentrations (100 – 1000 mM) in principle favour the application of haloperoxidases as a ${}^1O_2$ catalyst, since they minimise the
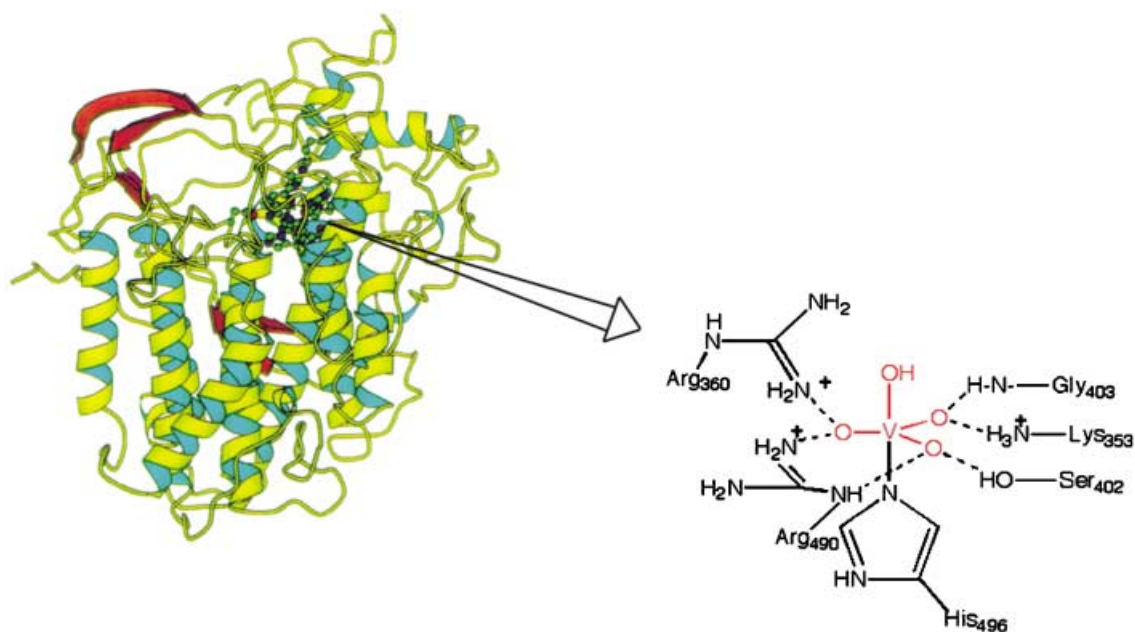
Figure 1. Vanadium chloroperoxidase from *Curvularia inaequalis* and its active site. The vanadate cofactor is shown in red. This figure was produced with Molscript.[24]

chance of side reactions of HOX with compounds other than H_2O_2. Thus a haloperoxidase with a high resistance towards H_2O_2 has an advantage in this application. Vanadium chloroperoxidase (VCPO) from the fungus *Curvularia inaequalis*[34] is not inhibited by H_2O_2, not even at high concentrations. Moreover, the VCPO is able to effectively use chloride instead of bromide which may affect the extent of side reactions of HOX. In addition, the monomeric VCPO is easily expressed as a recombinant protein,[35] which allows easy application in organic conversions.

For these reasons we have investigated $^1O_2$ release by VCPO from *C. inaequalis* since it may be a good catalyst for the production of $^1O_2$ under mildly acidic conditions and compared it to the $^1O_2$ release by VBPO from *A. nodosum*. For this study the near-infrared emission band at 1268 nm that is characteristic for $^1O_2$ ($^1\Delta_g$) was monitored. We also show the clean conversion of a water-soluble anthracene derivative into the corresponding endoperoxide by VCPO-generated $^1O_2$ and discuss the advantages of VCPO over VBPO in this respect.

Results and Discussion

Singlet Oxygen Production

In Figure 2 is shown chemiluminescence at 1268 nm produced by VCPO and VBPO, indicative for $^1O_2$ formation. With VCPO as catalyst (left panel), a clear emission at 1268 nm is observed and the activity even increases after the initial burst. After about 4 minutes

the signal drops because of complete conversion of the H_2O_2. For VBPO the signal appears almost constant when 2 mM H_2O_2 is used (right panel), but using 20 mM H_2O_2 a *decrease* of activity in time is observed (inset right panel). VBPO looses about 35% of its activity in 30 minutes, whereas VCPO shows no inactivation under these conditions, which gives VCPO a clear advantage over VBPO. It is expected that at lower pH the inactivation of VBPO will be even worse, especially when higher H_2O_2 concentrations are used, since a critical histidine residue is oxidised under those conditions.[32,33] VCPO and VBPO differ in their catalytic properties and therefore the bromide concentration in the VCPO experiment is chosen 200-fold lower than in the VBPO experiment. The VCPO has a much smaller K_M value for bromide than VBPO.[35] When 0.5 mM bromide is used in an assay, VBPO only exhibits a few percent of its maximal activity whereas VCPO is optimally active at this concentration. Conversely, the optimal concentration for VBPO of Br^- (100 mM) would be inhibitory for the VCPO.[35] The singlet oxygen production was measured at pH^{app} 5.4 for VCPO and pH^{app} 6.4 for VBPO which correspond to the pH optima with respect to the oxidation of halides[35,36] and with respect to singlet oxygen production as shown for VBPO by Butler and co-workers[18,19] and for VCPO in the left inset of Figure 2. The similarity of this optimum to the optimum observed for halide oxidation[35] indicates that the catalytic formation of the hypohalous acid, Equation (1), is rate limiting. Apparently the non-catalytic reaction between the hypohalous acid and hydrogen peroxide is much faster, even though it is known to slow down at low pH values.[6] The similarity of
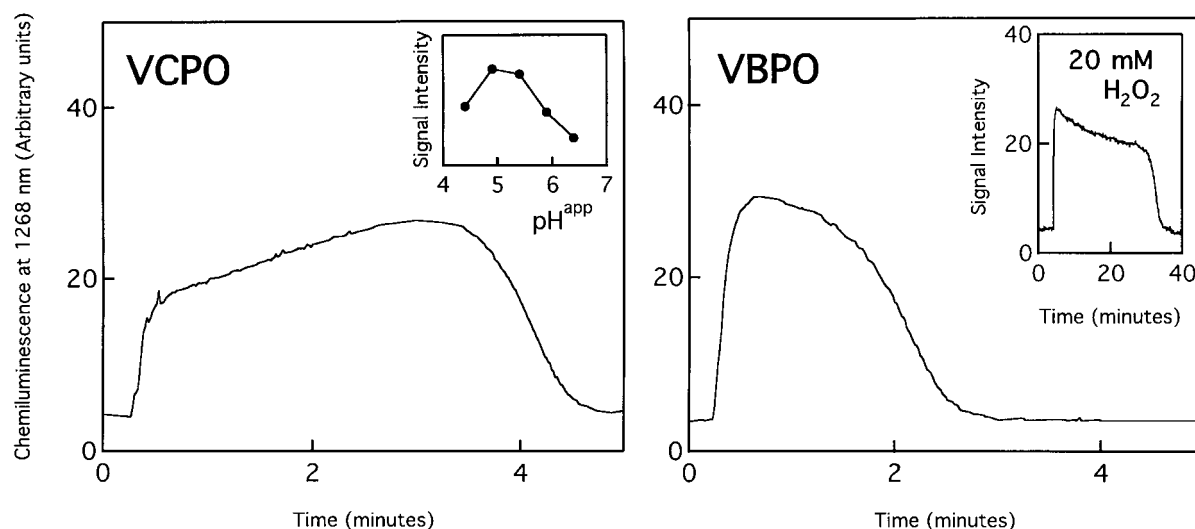
Figure 2. Chemiluminescence from singlet oxygen as produced by vanadium chloroperoxidase (*left*) and vanadium bromoperoxidase (*right*). *Left panel*: 100 nM VCPO, 20 mM citrate/citric acid buffer pH^{app} 5.4, 0.5 mM Br^-, ~98% D_2O. The reaction was started by addition of 2 mM H_2O_2. Inset: Apparent pH optimum for singlet oxygen production by vanadium chloroperoxidase. Conditions 100 nM VCPO, 5 mM H_2O_2, 0.5 mM Br^-, ~98% D_2O, 20 mM buffer (acetic acid/NaOH for pH^{app} 4.4, 4.9 and 5.4, MES/NaOH for pH^{app} 5.9 and 6.4). *Right panel:* 100 nM VBPO, 20 mM MES/NaOH buffer pH^{app} 6.4, 100 mM Br^-, ~98% D_2O. The reaction was started by addition of 2 mM H_2O_2. The inset shows VBPO at 20 mM H_2O_2.

Table 1. Effect of histidine on the 1268 nm emission from $^1O_2$ and on the bromination of monochlorodimedone.

	1268 nm emission[a]	MCD bromination[b]
VCPO Control	+	+
+4 mM histidine	−	+

[a] Conditions: 100 nM VCPO, 20 mM citrate buffer pH^{app} 5.4, 0.5 mM Br$^-$, ~98% D_2O. The reaction was started by addition of 50 mM H_2O_2. After 2 minutes 4 mM histidine was added.

[b] Conditions: 1 nM VCPO, 20 mM citrate buffer pH 5.0, 0.5 mM Br$^-$, 50 μM monochlorodimedone The reaction was started by addition of 50 mM H_2O_2. After 2 minutes 4 mM histidine was added.

the pH optimum for $^1O_2$ measurements and halide oxidation measurements indicates that the kinetic constants (K_M and V_{max}) for H_2O_2 and the halide substrates are identical. The magnitude of the activity for both the VCPO and the VBPO catalysts (using bromide as co-catalyst) are in the order of $1.0 - 2.0 \times 10^2$ s^{-1} (molecules of H_2O_2 consumed per molecule catalyst per second)[35], which is about $1.1 - 2.2 \times 10^4$ times higher than for the non-enzymatic molybdate catalyst at its optimal pH,[37] illustrating the efficiency of this biological catalyst.

To fully ensure that the chemiluminescence signal was due to $^1O_2$ additional control experiments were carried out. An effective quencher of chemiluminescence produced by singlet oxygen is histidine[18,38] and when 4 mM of histidine was added during measurement of the 1268 nm emission, the signal was immediately quenched (Table 1). Under the same conditions, histidine hardly inhibits the bromination of monochlorodimedone (Table 1), indicating that the quenching of the signal is not due to enzyme inhibition.

Moreover, in Figure 3 it is shown that the chemiluminescence signal was 20-fold lower in an H_2O-containing buffer as compared to the same experiment in a D_2O-containing buffer, in agreement with the 17 times longer lifetime of $^1O_2$ in D_2O than in H_2O.[39] No chemiluminescence signal was observed in the absence of either enzyme, halide or peroxide, indicating that all components are essential.

As expected from the bromide turnover numbers of these enzymes,[35] the signals of singlet oxygen production for VCPO and VBPO are similar in intensity, however as mentioned before, VCPO shows an increase of signal in time whereas the VBPO is slowly inactivated. For VCPO the type of buffer used influenced the shape of the trace somewhat but essentially the activation remained. The origin of this slow signal increase therefore remains unclear. From an application point of view it is important that the VCPO is stable against a (starting) concentration of 500 mM H_2O_2 and that it remains fully active for at least 1 hour of continuously producing singlet oxygen (Figure 4).
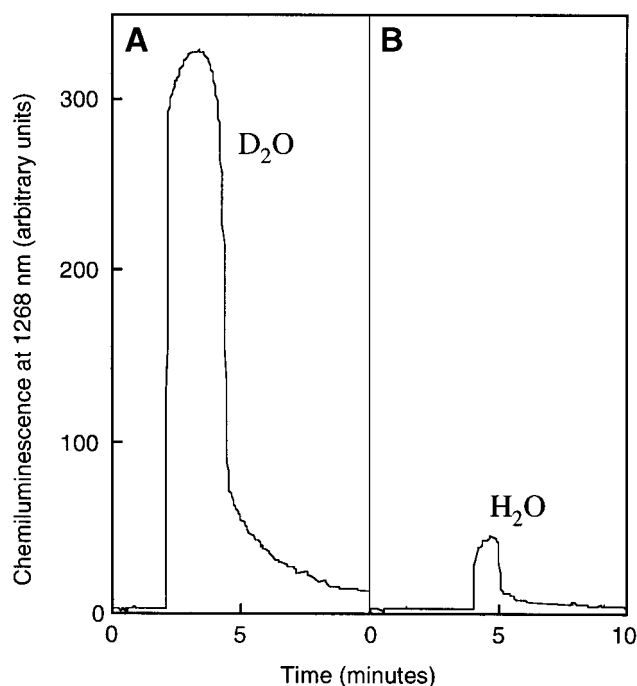

Figure 3. Comparison of chemiluminescence from singlet oxygen produced by vanadium chloroperoxidase in D_2O and H_2O. *Conditions:* 500 nM enzyme, 0.5 mM Br$^-$, reaction started by 20 mM H_2O_2. Panel **A**: Buffer in D_2O (20 mM acetic acid/NaOH pH^{app} 5.4, ~98% D_2O). Panel **B**: Buffer in H_2O (20 mM acetic acid/NaOH pH 5.0). The buffers were made by 50 times dilution of the same 1 M stock solution.

This stability towards both high concentrations of H_2O_2 and $^1O_2$ is in strong contrast to that of the heme-containing haloperoxidases.

The production of $^1O_2$ by VCPO *via* the cocatalyst chloride was also tested. Because the conversion rate of chloride is 5 times slower than that for bromide, a lower $^1O_2$ production rate is expected. $^1O_2$ is indeed produced at a 5 times lower rate, which is still 2.2×10^3 times higher than for peroxomolybdate (5 mM Cl$^-$ and 0.5 mM Br$^-$ were used, respectively, which are known to be well above the K_M values for the halogenating activity of VCPO[35] and thus saturating the $^1O_2$ production rate). This results again confirms that the catalytic halide oxidation, Equation (1), is the rate-determining step.
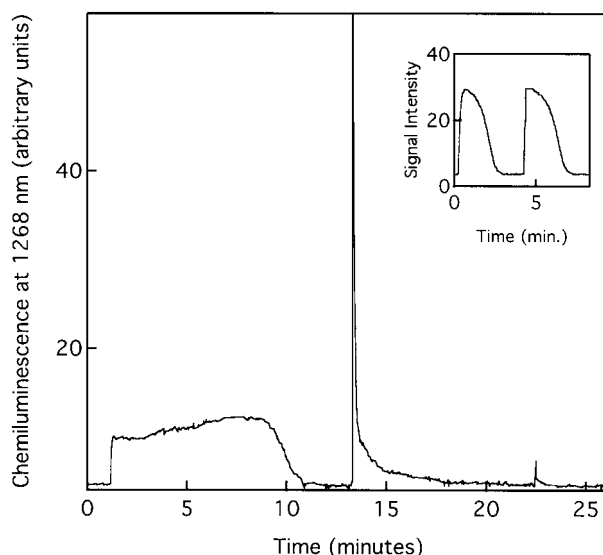
Inactivation of VCPO at Very Low H_2O_2

The $^1O_2$ release by VBPO is similar to that published before;[18] multiple aliquots of 2 mM H_2O_2 can be converted into $^1O_2$ (inset of Figure 5). In strong contrast to this, VCPO is not able to convert multiple aliquots of 2 mM H_2O_2 without being inactivated (Figure 5), but it converts amounts such as 20 mM H_2O_2 into $^1O_2$ while easily remaining fully active (Figure 3). Figure 5 shows that if a second aliquot of H_2O_2 is added to the reac-
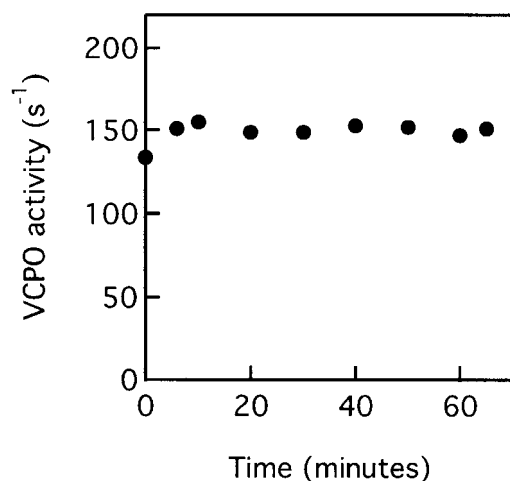
Figure 4. Stability of vanadium chloroperoxidase against 500 mM H_2O_2 and a continuous flow of $^1O_2$. In a 1-mL reaction mixture $^1O_2$ was continuously produced (200 nM VCPO, 500 mM H_2O_2, 5 mM Cl$^-$, 100 mM citrate pH 5.5). The solution was continuously stirred. From this mixture 25 μL aliquots were taken and enzyme activity was tested in 1 mL of monochlorodimedone assay mixture (100 mM acetic acid/NaOH pH 5.0, 50 μM monochlorodimedone, 0.5 mM Br$^-$, 100 μM vanadate, 13.5 mM H_2O_2).

Figure 5. Turnover of multiple aliquots of H_2O_2 by vanadium chloroperoxidase. *Conditions:* 50 nM VCPO, 20 mM citrate/ citric acid pH^{app} 5.4 (~98% D_2O), 0.5 mM Br$^-$. The reaction was started by addition of 2 mM H_2O_2. The second aliquot of H_2O_2 was added after 13 minutes and the third after 22.5 minutes. Inset: Similar experiment using vanadium bromo- peroxidase. 100 nM VBPO, 20 mM MES/NaOH buffer pH^{app} 6.4, 100 mM Br$^-$, ~98% D_2O, reaction started by addition of 2 mM H_2O_2. The second aliquot of H_2O_2 was added after 4.5 minutes.

tion mixture after consumption of the first aliquot, mainly a short intense burst of singlet oxygen is observed. After a third aliquot of H_2O_2 almost no $^1O_2$ formation is observed indicating that the enzyme is inactivated.

An explanation for this phenomenon may be found in the extreme affinity of VCPO for H_2O_2, as compared to VBPO.[35] Due to this VCPO is still producing HOBr at maximal rate at H_2O_2 concentrations as low as 10 – 100 μM. It is conceivable that at a certain point the H_2O_2 concentration becomes so low that the non- enzymatic reaction of H_2O_2 with HOBr hardly occurs. Consequently, there is a build-up of a relatively large concentration of HOBr, when nearly all the H_2O_2 is converted. This rationalizes both the intense burst of $^1O_2$ upon addition of the second aliquot of H_2O_2 as well as the inactivation of VCPO. The inactivation could, in principle, be caused by HOBr itself or by intermediates of the reaction between HOBr and $^1O_2$ during the burst. Inactivation of VCPO by the HOX product itself would be somewhat surprising, as it was previously publish- ed[27] that VCPO is stable towards HOCl under turnover conditions. However, unpublished results performed by us show that HOCl in that experiment (~4 mM) in fact reacted with citrate buffer and therefore a reaction product of citrate with HOCl rather than HOCl itself is present. In all experiments reported in the literature potential scavengers of HOX are present (such as H_2O_2, buffer, monochlorodimedone or dyes on the surface of the protein; these dyes are not found on the recombinant protein). We tested the resistance of VCPO towards

HOBr by mixing VCPO (9 nM) with chemically pro- duced HOBr (10 μM) under the same conditions as Figure 5 (for details, see Experimental Section). Normal activity was observed, excluding inactivation of native VCPO by HOBr. In principle, it is still possible that the inactivation as observed in Figure 5 is caused by interaction of an enzyme *intermediate* with HOBr during turnover (at low H_2O_2 concentration), however, we did not further investigate the origin of the inacti- vation since it is not relevant for the application of VCPO as a singlet oxygen catalyst as long as the H_2O_2 concentration is kept high.

Chemical Trapping of VCPO/Halide-Generated H_2O_2

Chemical trapping of VCPO/halide-generated singlet oxygen was studied by adding anthracene-9,10-bis- (ethanesulphonate), a water-soluble anthracene deriv- ative that is known to react with singlet oxygen[40,41] forming an endoperoxide (Scheme 2).

This conversion can be easily monitored by ^1H NMR; reactions were carried out directly within NMR tubes and followed in time. Figure 6 shows the ^1H NMR data of the conversion induced by VCPO, both with chloride and bromide as cocatalyst.
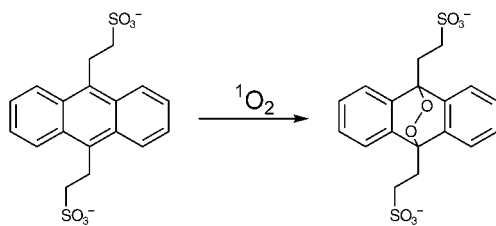
Scheme 2. Reaction of anthracene-9,10-bis(ethanesulphon-ate) with singlet oxygen.

Panel **A** shows the spectrum of the starting compound anthracene-9,10-bis(ethanesulphonate). With chloride as cocatalyst (panel **B**) anthracene-9,10-bis(ethane-

sulphonate) is completely converted into the corresponding endoperoxide. Side product formation from the HOCl intermediate with anthracene-9,10-bis(ethanesulphonate) is not observed. The small signal at 3.60 ppm in both the starting compound and product spectrum originates from Tris-buffer (in which the enzyme is routinely stored), as was confirmed by taking an NMR spectrum of 1 mM Tris under identical conditions (not shown). It should be noted that the chloride is substoichiometric with respect to anthracene-9,10-bis(ethanesulphonate), confirming the catalytic role of the halide. The spectrum obtained for the product is identical to panel **C**, which corresponds to the endoperoxide formed by molybdate released $^1O_2$.[42]
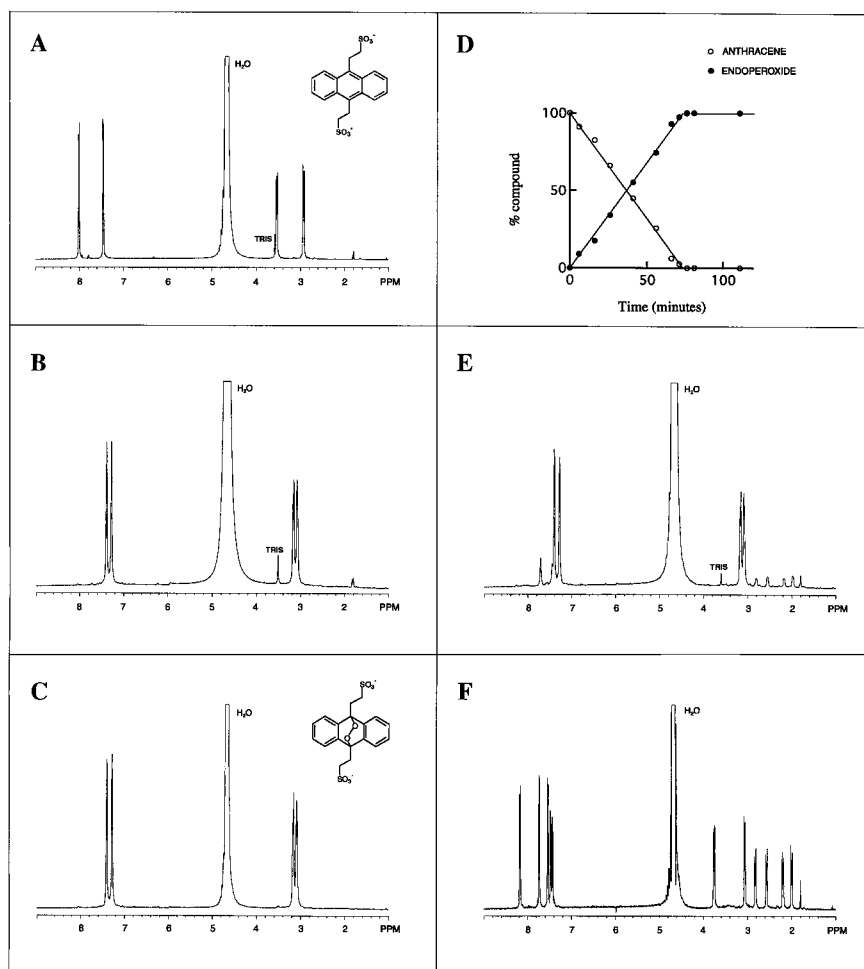
Figure 6. *In situ* conversion of anthracene-9,10-bis(ethanesulphonate) into the corresponding endoperoxide by vanadium chloroperoxidase, with either chloride or bromide as co-catalyst, as followed by ^{1}H NMR. **A:** 10 mM anthracene-9,10-bis(ethanesulphonate), 400 nM VCPO, 200 mM H_2O_2, 100 mM $CD_3COOD/NaOD$ buffer (pH^{app} 5.4), ~ 99% D_2O. **B:** Corresponding endoperoxide, formed 80 minutes after addition of the cocatalyst chloride (5 mM). **C:** Corresponding endoperoxide formed from 10 mM anthracene-9,10-bis(ethanesulphonate) by peroxomolybdate under alkaline conditions (pH^{app} 9.5). 1 mM MoO_4^{2-}, 100 mM H_2O_2, 99% D_2O. **D:** Conversion of anthracene-9,10-bis(ethanesulphonate) into its corresponding endoperoxide by VCPO as a function of time as calculated from integration of the NMR signals. ○ Anthracene-9,10-bis(ethanesulphonate), ● corresponding endoperoxide. **E:** Corresponding endoperoxide formed 120 minutes after addition of the cocatalyst bromide (0.5 mM). Starting conditions identical as in **A**, but 50 nM VCPO. **F:** 10 mM anthracene-9,10-bis(ethanesulphonate) was mixed with 5 mM HOBr (see Experimental Section) *in the absence of H_2O_2.* 100 mM $CD_3COOD/NaOD$ buffer (pH^{app} 5.4), ~ 99% D_2O.

Table 2. Catalysts for H_2O_2 disproportionation to $^1O_2$ in aqueous environment.

Catalyst system	Na_2MoO_4[7]	$La(NO_3)_3$/NaOH 1/2[8]	VCPO[a]	VBPO[b]	Heme CPO[c]
pH range	7.0 – 12.5	5.0 – 12.5	3.0 – 6.0	3.0 – 7.0	2.5 – 5.0
Cocatalyst	none	none	Cl^- (5 mM) or Br^- (0.5 mM)	Cl^- (1000 mM) or Br^- (100 mM)	Cl^- (20 mM)
Turnover H_2O_2	0.01 s^{-1}	0.005 s^{-1}	20 s^{-1} (Cl^-) 100 s^{-1} (Br^-)	0.6 s^{-1} (Cl^-) 200 s^{-1} (Br^-)	1200 s^{-1} (only initially)
$^1O_2$ yield from H_2O_2 in water	100%	45%	100%[d] (Cl^-)	80%[e] (Br^-)	100%[d] (Cl^-)
Oxidative stability	stable inorganic system	stable inorganic system	stable for at least 25,000 turnovers; max 1.0 M H_2O_2 tolerated	slow inactivation; max 0.1 M H_2O_2 tolerated	fast inactivation already at ~0.002 M H_2O_2
Organic synthetic scope	broad, but substrate must be inert toward peroxomolybdate, thus excluding i.a. allylic alcohols and alkenyl amines	very broad, including allylic alcohols and alkenyl amines	alkenes excluded that react efficiently with HOCl (in competition with H_2O_2)	alkenes excluded that react efficiently with HOBr (in competition with H_2O_2)	almost none due to inactivation

[a] Vanadium chloroperoxidase from the fungus *C. inaequalis*, recombinantly expressed in the yeast *S. cerevisiae*.[35]
[b] Vanadium bromoperoxidase from the seaweed *A. nodosum*.[36,43]
[c] Heme-containing chloroperoxidase from the mold *C. fumago*.[44,45]
[d] $^1O_2$ yield for the reaction of HOCl with H_2O_2.
[e] $^1O_2$ yield for the reaction of HOBr with H_2O_2.

Line broadening in the product spectra of panels **B** and **C** as compared to that of the starting compound spectrum in panel **A** is due to formation of oxygen within the NMR tubes. In panel **D** the time dependence of product formation catalysed by VCPO and substrate disappearance is depicted and a clear linearity is observed. From this linearity it can be concluded that the enzyme remains stable during the process. When instead of chloride, bromide is used as a cocatalyst in the enzymatic conversion about 20% of the anthracene-9,10-bis(ethanesulphonate) is converted into another product (panel **E**). This may be due a different reactivity of the HOCl formed *versus* HOBr towards H_2O_2 or towards anthracene-9,10-bis(ethanesulphonate) or a combination of both. Generally HOCl reacts more effectively with H_2O_2 than HOBr.[14] Therefore we assume that the enzymatically produced HOCl is fully scavenged by the high concentration of H_2O_2 whereas under the reaction conditions used part of the HOBr reacts with anthracene-9,10-bis(ethanesulphonate) instead of with H_2O_2. To test whether the observed side product is indeed due to reaction of HOBr with anthracene-9,10-bis(ethanesulphonate), 10 mM anthracene-9,10-bis(ethanesulphonate) was mixed with 5 mM HOBr *in the absence of H_2O_2* (panel **F**). About 3.5 mM product is formed with very similar chemical shifts as the

side product observed in panel **E**. The exact nature of the side product was not further investigated, but the presence of four different CH_2 resonances suggests a loss of symmetry due to aryl bromination.

In Table 2 we compare the properties of five different catalysts for $^1O_2$-production by H_2O_2 disproportionation in aqueous environment.

Table 2 demonstrates that VCPO is much more active (three to four orders of magnitude) than the inorganic molybdate and lanthanum systems. However, there are limitations with respect to its usefulness in preparative "dark" singlet oxygenations. Its scope is limited to substrates that are less reactive towards HOCl than H_2O_2 and this requirement excludes many alkenes.

Conclusion

In summary, we have shown that vanadium chloroperoxidase from *C. inaequalis* is a very effective singlet oxygen catalyst under mildly acidic conditions and is very stable towards H_2O_2 and singlet oxygen under turnover conditions. Its pH optimum is around 5.0. A potential drawback of this enzymatic system may be the formation of side products as a result of a reaction of the substrate or product with the hypohalous acid inter-

mediate. However, we have shown that it is possible to cleanly convert a typical singlet oxygen trap using chloride as the co-catalyst. During the conversion of this trap the enzymatic system remains fully active for more than one hour. The clean conversion upon the use of the cocatalyst chloride and the high stability versus singlet oxygen make VCPO superior to VBPO from *A. nodosum*. VBPO can in principle also catalyse the oxidation of Cl^-, however at a 36-fold lower rate[43] and at a very high Cl^- concentration (~1.0 M). Although VCPO has been shown to be superior to VBPO, each substrate should be tested for side reactions since each individual substrate may have a different reactivity towards the HOCl intermediate. Since effectively a competition exists between H_2O_2 and the substrate for HOCl, the H_2O_2 concentration should be kept high during the application. If this concentration is kept high, the observed inactivation of the enzyme at very low H_2O_2 concentrations will not occur. 10 mM anthracene-9,10-bis(ethanesulphonate) was converted by 400 nM VCPO catalyst (25,000 turnovers) within 75 minutes, corresponding to a turnover frequency of $5.6\ s^{-1}$. The production of the HOCl intermediate is maximally $20\ s^{-1}$,[35] which means that under these conditions part of the singlet oxygen is physically quenched before it can react with the anthracene molecule. However, this is a satisfying efficiency which may be increased using a higher anthracene concentration. Also changing the target molecule will affect this efficiency. In H_2O this efficiency will be lower due to physical quenching of $^1O_2$, but even then from an application point of view a very good conversion rate will be obtained as compared with the inorganic molybdate and lanthanum systems. In addition, these systems are not effective at such low pH values. These features make the enzyme system an interesting additional tool in the box of singlet oxygen catalysts.

Experimental Section

Chemiluminescence Detector

The luminescence originating from $^1O_2$ at 1268 nm was detected by an N_2-cooled Ge-diode detector from North Coast Scientific Corp., Model EO-817L, 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. The geometry of the setup[37] was kept constant for all experiments. Experiments were performed in a 3-mL sample cell. For experimental details, see figure legends.

^{1}H NMR Spectroscopy

Spectra were recorded on a 500 MHz Varian Inova NMR spectrometer. Reactions were carried out within the NMR tubes. A little hole was made in the plastic cap of the NMR tube to release excess oxygen.

Activity Measurements

The oxidation of bromide by VCPO in the absence and presence of histidine was measured in an assay containing monochlorodimedone (MCD). Upon bromination or chlorination by the hypohalous acid formed by the VCPO a loss of absorption is observed ($\Delta\varepsilon_{290\ nm} = 20\ mM^{-1}\ cm^{-1}$).[46]

Chemicals

Sodium hypochlorite was purchased from Acros organics as a 5% solution (in chlorine), corresponding to 0.70 M ClO^-. The concentration of ClO^- was checked by titration of a monochlorodimedone solution ($\varepsilon_{290\ nm} = 20.2\ mM^{-1}\ cm^{-1}$), which is converted into dichlorodimedone ($\varepsilon_{290\ nm} = 0.2\ mM^{-1}\ cm^{-1}$). A 400 mM BrO^- stock solution was made by mixing ClO^- with Br^-. At pH 7–9, this ClO^- is known to react very fast with Br^- to give BrO^- and Cl^-.[47] This stock was made freshly and used within one minute. A slight excess of 10 mM Br^- was used to ensure full conversion of the ClO^-. A drawback of this excess Br^- could be reaction with BrO^- to form Br_2 and Br_3^-, however, upon further dilution of the "BrO^-" stock, BrO^- is the main component and the contributions of Br_2 and Br_3^- become minimal.[47]

The synthesis of the sodium salt of anthracene-9,10-(ethanesulphonate) was described previously.[40,41,42]

Buffers

Buffers used for chemiluminescence measurements were prepared by dilution of 1.0 M stock solutions (H_2O based) in D_2O giving an H_2O contribution of about 2% to the reaction mixtures. In the final experiments the H_2O content is slightly higher than 2% due to contributions from H_2O_2 and enzyme stocks. In accordance with the literature the resulting buffers give an apparent pH (pH^{app}), which is about 0.4 pH units higher than the stock due to the D_2O.[48,49] In the ^{1}H NMR experiments the presence of protons was minimised by using a deuterated buffer (> 99 atom % D). A 1.0 M stock solution of CD_3COOD in D_2O was adjusted to pH^{app} 5.4 with an NaOD solution in D_2O and further diluted with D_2O for the experiments.

Enzymes

Vanadium chloroperoxidase from the fungus *Curvularia inaequalis* was recombinantly expressed in the yeast *Saccharomyces cerevisiae* and purified as described before.[35] The turnover frequency (molecules of substrate converted per second per molecule of catalyst) of this enzyme is about $100\ s^{-1}$ with respect to bromide (0.5 mM Br^-, pH 5.0, 2 mM H_2O_2, at 25 °C, monochlorodimedone assay) and $20\ s^{-1}$ with respect to chloride (5 mM Cl^-, pH 5.0, 2 mM H_2O_2, at 25 °C, monochlorodimedone assay). Vanadium bromoperoxidase from the seaweed *Ascophyllum nodosum* was purified as described before.[50] The turnover frequency of this catalyst is about $120\ s^{-1}$ at pH 6.0, 100 mM Br^-, 2 mM H_2O_2, at 25 °C. Both enzymes are

standardly stored in 100 mM TrisSO$_4$ buffer pH 8.3 containing 100 µM vanadate.

VCPO Inactivation Test

Freshly prepared 4.0 mM HOBr stock (diluted from the 400 mM stock which was described above) was added to 2.5 mL reaction mixture containing 20 mM citrate pH 5.0 and 18 nM VCPO to a final concentration of 10 µM. After 4 minutes of incubation the activity of VCPO was tested by addition of 100 µM monochlorodimedone, 0.5 mM Br$^-$ and 1 mM H$_2$O$_2$.

Acknowledgements

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