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# Sulfoxidation mechanism of vanadium bromoperoxidase from *Ascophyllum nodosum*

## Evidence for direct oxygen transfer catalysis

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We have previously shown that vanadium bromoperoxidase from *Ascophyllum nodosum* mediates production of the (*R*)-enantiomer of methyl phenyl sulfoxide with 91% enantiomeric excess. Investigation of the intrinsic selectivity of vanadium bromoperoxidase reveals that the enzyme catalyzes the sulfoxidation of methyl phenyl sulfide in a purely enantioselective manner. The $K_m$ of the enzyme for methyl phenyl sulfide was determined to be $\approx$3.5 mM in the presence of 25% methanol or *tert*-butanol. The selectivity of the sulfoxidation of methyl phenyl sulfide is optimal in the temperature range 25–30 °C and can be further optimized by increasing the enzyme concentration, yielding selectivities with up to 96% enantiomeric excess. Furthermore, we established for the first time that vanadium bromoperoxidase is functional at temperatures up to 70 °C. A detailed investigation of the sulfoxidation activity of this enzyme using $^{18}$O-labeled hydrogen peroxide shows that vanadium bromoperoxidase mediates the direct transfer of the peroxide oxygen to the sulfide. A schematic model of the vanadium haloperoxidase sulfoxidation mechanism is presented.

*Keywords*: enantioselective sulfoxidation; selective oxygen-transfer; vanadium bromoperoxidase; vanadium chloroperoxidase.

In response to scientific and pharmaceutical interest in enantiomerically pure sulfoxides a number of catalytic methods for the production of these chiral synthons has been developed [1,2]. Several different methods for the preparation of chiral sulfoxides by enantioselective oxidation of the corresponding organic sulfides exist using chemical [3] and biological, whole-cell [4] and enzymatic [5,6] approaches. Inorganic vanadium (V) peroxo-complexes, some of which have been suggested to be functional models for the vanadium peroxidases [7], mediate oxygen-transfer reactions to a variety of organic compounds including sulfides [8]. Several chiral Schiff-base-ligated vanadium (V) peroxo-complexes catalyze the formation of optically active sulfoxides, with selectivities up to 78% enantiomeric excess [9,10]. It was recently demonstrated that the vanadium haloperoxidases are also capable of mediating selective sulfoxidation reactions in the presence of hydrogen peroxide [11–13]. Vanadium bromoperoxidase (VBPO) from the brown seaweed *Ascophyllum nodosum* promotes formation of the (*R*)-enantiomer of the methyl phenyl sulfoxide with 91% enantiomeric excess under optimal reaction conditions, whereas VBPO from the red seaweed *Corallina pilulifera* mediates formation of the (*S*)-enantiomer (55% enantiomeric excess). Recombinant vanadium chloroperoxidase (VCPO), however, produces a racemic mixture of the sulfoxides, which appeared to be an intrinsic characteristic of the enzyme. In addition, it has been reported [13,14] that VBPO from the red seaweed *C. officinalis* catalyzes the selective sulfoxidation of small aromatic sulfides and small sulfides possessing a *cis*-positioned carboxyl group to the (*S*)-enantiomer of the corresponding sulfoxides with selectivities exceeding 95% enantiomeric excess. However, this enzyme was observed to be incapable of converting methyl phenyl sulfide [13].

The vanadium haloperoxidases form a group of enzymes that possess a single bound vanadate ion as a prosthetic group. In the presence of hydrogen peroxide and halides these enzymes produce hypohalous acid (HOX) as a reactive product [15]. VBPO from the brown seaweed *A. nodosum* oxidizes bromide in the presence of hydrogen peroxide through a so-called 'bi-bi ping-pong mechanism' [15,16]. First, hydrogen peroxide binds to the vanadium metal forming an activated peroxo-intermediate, which facilitates the nucleophilic attack of the halide to yield hypobromous acid. This product may further react with either an organic compound or an additional equivalent of hydrogen peroxide to form the halogenated component or singlet molecular oxygen, respectively [15–17]. Because the tertiary structure of this enzyme has been determined [18] the nature of the active site in the enzyme is known in detail. An extraordinary feature of this enzyme from the brown seaweed, which is actually shared by all vanadium peroxidases, is its remarkable stability [19–22]. VBPO retains complete functionality upon storage in up to 60% methanol, ethanol and isopropanol, remains active when exposed to temperatures up to 70 °C and was seen to be unaffected by detergents. The enzyme also withstands oxidative inactivation in the presence of high concentrations of highly reactive oxidants such as hydrogen peroxide and HOX. These properties allow the

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*Abbreviations*: ee, enantiomeric excess; HOX, hypohalous acid; VBPO, vanadium bromoperoxidase; VCPO, vanadium chloroperoxidase.

*Enzymes*: vanadium bromoperoxidase (EC 1.11.1.7); vanadium chloroperoxidase.

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potential application of this vanadium peroxidase as a (industrial) biocatalyst in organic synthesis.

More recently, a vanadium chloroperoxidase was discovered which was produced by the terrestrial fungus *Curvularia inaequalis* and this enzyme has been studied in even greater detail [23–26]. Kinetic studies revealed that the chloride oxidation mechanism of this enzyme is similar to the bromide oxidation mechanism of the vanadium bromoperoxidase, although the values of the kinetic parameters differ [25]. The kinetic data indicate the presence of a vanadium peroxo-intermediate during catalysis. VCPO has been successfully expressed in the yeast *Saccharomyces cerevisiae*, therefore recombinant VCPO can easily be acquired in large quantities [27,28] and primary and X-ray structures of this enzyme are known [29,30]. In line with predictions based upon sequence comparisons [27,30], the architecture of the active site in VBPO and VCPO is very similar [18]. Direct evidence for the formation of an active enzyme–peroxo-intermediate in VCPO during catalysis has been obtained from X-ray crystallography [31]. The side-on coordination of the peroxide to the vanadium metal in the peroxo-intermediate of VCPO is similar to that found in vanadium (V) peroxo-complexes and probably also occurs in VBPO.

Another class of peroxidases, harboring a heme group in the active site, also catalyzes enantioselective oxygen-transfer reactions [32]. High enantioselectivity (> 99% enantiomeric excess) can be obtained using heme chloroperoxidase from the fungus *Caldariomyces fumago* in the oxidation of organic sulfides [6]. Other heme peroxidases, including *Coprinus cinereus* peroxidase and lactoperoxidase, mediate sulfoxidation reactions with a lower enantioselectivity (73 and 80% enantiomeric excess [33], respectively). In heme chloroperoxidase all the oxygen in the sulfoxide is derived from hydrogen peroxide [32], which was deduced from incorporation studies using $^{18}$O-labeled $H_2O_2$ [34]. Incorporation of peroxide oxygen into methyl phenyl sulfoxide was 85% for lactoperoxidase, while significant incorporation of oxygen derived from $^{18}O_2$ was also observed in sulfoxide produced by this enzyme [34]. Clearly, the heme peroxidases mediate enantioselective O-transfer by different mechanisms of oxidation [35]. The high enantioselectivity of the heme chloroperoxidase can be accounted for by a direct oxygen-transfer mechanism, whereas a oxygen-rebound mechanism is proposed for horseradish peroxidase and lactoperoxidase [33,35]. A major problem with the heme peroxidases is their rapid oxidative inactivation by hydrogen peroxide during catalysis. This inactivation did not occur when vanadium haloperoxidase was used as a biocatalyst [11]. In view of this, we investigated the sulfoxidation mechanism of vanadium bromoperoxidase from *A. nodosum* in greater detail. Here we present evidence that this enzyme directly promotes the direct transfer of oxygen from the peroxide to methyl phenyl sulfide in a purely enantioselective manner.

## EXPERIMENTAL PROCEDURES

Vanadium bromoperoxidase from *A. nodosum* was isolated as described previously [19,36], including the additional purification procedure using a Mono-Q column on a FPLC system yielding an uncolored pure enzyme preparation [11]. Recombinant vanadium chloroperoxidase was obtained from the developed *S. cerevisiae* expression system [27,28] and this enzyme has the same kinetic characteristics as the native enzyme from the fungus *Curvularia inaequalis* [27,28]. As this enzyme is purified in the apo-form vanadate was added as described previously [26–28]. Unless stated otherwise, all chemicals were obtained from Merck or Fluka.

In order to investigate the origin of the oxygen in the methyl phenyl sulfoxide produced by vanadium peroxidases, VBPO from *A. nodosum* (0.5 μM) was incubated in 100 mM sodium citrate/NaOH buffer, pH 5.0, with methyl phenyl sulfide (1.5 mM) and $^{18}$O-labeled hydrogen peroxide (1.5 mM final concentration) for 3 days at 25 °C. Labeled peroxide (> 90% enrichment, Icon) was added in five sequential steps, distributed over the 3 days, in order to prevent a significant contribution from the direct nonenzymatic reaction between sulfide and peroxide to the produced sulfoxide [11]. The same reaction conditions were used for the nonenzymatic reaction, however, buffer was added instead of enzyme. In addition, both enzyme-catalyzed and nonenzymatic reactions were carried out in the presence of either 25% *tert*-butanol or methanol. After 3 days the samples were quenched with sulfite, extracted with $CH_2Cl_2$ and the enantiomeric excess was analyzed by chiral HPLC analysis as described previously [11].

The products of both the catalyzed and uncatalyzed sulfoxidation reactions were analyzed by mass spectrometry (142 *m/z* for $^{18}$O-labeled sulfoxide and 140 *m/z* for $^{16}$O sulfoxide). Electron impact mass spectrometry was carried out using a JEOL JMS SX/SX102A four-sector mass spectrometer, coupled to a JEOL MS-MP7000 data system. Samples were introduced via the direct insertion probe into the ion source. Samples were measured at 15 eV. For recombinant VCPO [27,28] similar reaction conditions were applied, however, because the minimal turnover frequency was determined to be 18 $min^{-1}$ the reaction mixture was incubated for 20 h instead of 3 days, during which time the $^{18}$O-labeled and unlabeled peroxide was added in five consecutive steps. The recombinant VCPO-catalyzed sulfoxidation reaction was also performed in 50% $^{18}$O-labeled water (purchased from Icon). The samples were recovered and analyzed as described previously [11].

The minimal turnover frequency (mol of formed sulfoxide per mol of enzyme per min) of the recombinant VCPO in the sulfoxidation of methyl phenyl sulfide was determined by following the conversion of the sulfide at 290 nm ($\Delta\varepsilon = 0.44\ mM^{-1}\cdot cm^{-1}$) [11] in time with a Zeiss spectrophotometer for 2 h, after which time the reaction was completed. In this particular experiment recombinant VCPO (1 μM) was incubated in 100 mM sodium citrate/NaOH buffer, pH 5.0, with methyl phenyl sulfide (1.5 mM) and hydrogen peroxide (2 mM).

To determine the intrinsic selectivity of VBPO from *A. nodosum* in the sulfoxidation of methyl phenyl sulfide the experiments were conducted as follows. Incubations were performed in cuvettes (1.7 mL) with a capillary opening and no headspace containing 100 mM sodium citrate/NaOH buffer (pH 5.3), methyl phenyl sulfide (1.5 mM), $H_2O_2$ (0.5 mM) and 0.1, 0.25, 0.5 or 1 μM of enzyme. To continuously monitor formation of the sulfoxide the absorbance decrease at 290 nm was followed using a HP diode array spectrophotometer and an extinction coefficient of $0.44\ mM^{-1}\cdot cm^{-1}$ was used to determine the concentration of sulfoxide produced [11]. After 3 h the reaction was quenched, the mixture extracted, evaporated and dissolved as described previously and the enantioselectivity of the reaction was determined by chiral HPLC analysis [11]. The amount of (*R*)- and (*S*)-enantiomer methyl phenyl sulfoxide formed was calculated from these data.

To determine the $K_m$ of VBPO for methyl phenyl sulfide the enzyme (0.5 μM) was incubated in 100 mM sodium citrate/NaOH buffer (pH 5.0) and 25% of either *tert*-butanol or

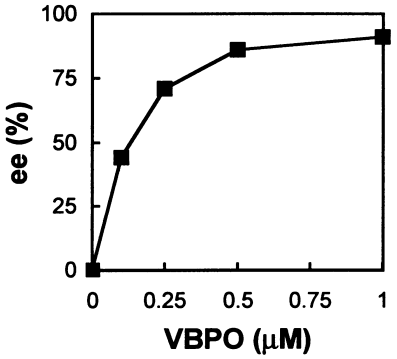


**Fig. 1. Influence of enzyme concentration on the selectivity of the sulfoxidation of methyl phenyl sulfide catalyzed by vanadium bromoperoxidase from *A. nodosum* at pH 5.3 for 3 h at 25 °C.**

methanol (in order to dissolve the sulfide at relatively high concentrations) with 1.5 mM $H_2O_2$ and 0.5, 1, 1.5, 2.5, 4, 5, 7.5, 10, 12.5, 15 or 20 mM of methyl phenyl sulfide at room temperature for 3 h. In addition, to study the uncatalyzed reaction in 100 mM sodium citrate/NaOH buffer (pH 5.0) and 25% *tert*-butanol or methanol, 5, 10 and 20 mM of methyl phenyl sulfide was incubated with 1.5 mM $H_2O_2$ at room temperature for 3 h. Formation of the sulfoxide was monitored at 290 nm on a Cary 50 spectrophotometer as mentioned above and after 3 h the yield and selectivity of the reactions were determined using a Diacel OD chiral HPLC column as described previously [11]. In order to determine the $K_m$ of VBPO for methyl phenyl sulfide the following procedure was used. The amount of (*R*)- and (*S*)-methyl phenyl sulfoxide was determined. Because the (*S*)-enantiomer is only formed due to the nonenzymatic sulfoxidation reaction, yielding both the (*R*)- and (*S*)-enantiomer, subtraction of the amount of (*R*)- and (*S*)-sulfoxide gives the yield of the (*R*)-enantiomer produced enzymatically. $K_m$ was calculated using ENZPACK for Windows version 1.4 (Biosoft).

The temperature dependence of the selective sulfoxidation of methyl phenyl sulfide by VBPO from *A. nodosum* was studied in 100 mM sodium citrate/NaOH buffer (pH 5.3) using similar reaction conditions as described previously [11], however, the concentration of methyl phenyl sulfide and hydrogen peroxide was changed to 1.5 mM. Buffer was added instead of enzyme in the control experiments. The reaction temperature dependence was studied using water baths to control the temperature in the range 0–70 °C during the incubation period of 20 h.

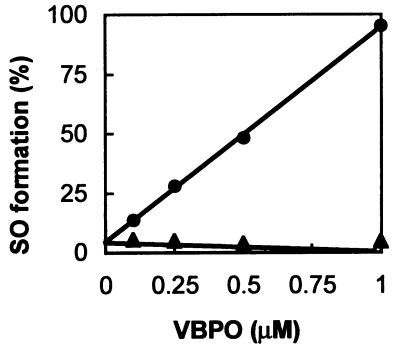


**Fig. 2. Effect of enzyme concentration on the formation of the (*R*)-enantiomer (●) and (*S*)-enantiomer (▲) of methyl phenyl sulfoxide in the sulfoxidation of methyl phenyl sulfide mediated by vanadium bromoperoxidase from *A. nodosum* at pH 5.3 for 3 h at 25 °C.**

## RESULTS AND DISCUSSION

VBPO from *A. nodosum* was shown to mediate the sulfoxidation of methyl phenyl sulfide with selectivities up to 91% enantiomeric excess for the (*R*)-enantiomer of the sulfoxide [11]. Clearly this VBPO exhibits high enantioselectivity, however, enantioselectivity and versatility of the heme chloroperoxidase from *C. fumago* in the biotransformation of sulfides is better still [5,6]. We believe that this difference is due to the low turnover frequency of the vanadium peroxidase [11] in comparison with the heme chloroperoxidase [5,6], resulting in a relatively large contribution of the direct nonenzymatic reaction between hydrogen peroxide and the sulfide, and a subsequent decrease in measured selectivity. In addition, for all the heme peroxidase-catalyzed sulfoxidation reactions the concentration of enzyme used is ≈ 10-fold higher than the concentrations used in our studies, which may also contribute to higher selectivity of the heme chloroperoxidase catalyzed reaction. In view of this, we studied the dependence of the enantiomeric excess of the catalyzed sulfoxidation reaction on VBPO concentration. The results are presented in Fig. 1. When increasing amounts of enzyme are used to produce methyl phenyl sulfoxide, the selectivity of the reaction increases linearly at low concentrations of enzyme, but gradually levels off. An enantiomeric excess of ≈ 93% is reached when 5 μM of enzyme is used under these reaction conditions (results not shown).

When the amount of each enantiomer of methyl phenyl sulfoxide produced was determined as a function of the enzyme concentration a different trend is observed (Fig. 2). The amount of (*R*)-enantiomer of methyl phenyl sulfoxide formed increases linearly with the enzyme concentration in the incubation. In contrast, the amount of (*S*)-enantiomer of the sulfide decreases gradually. Formation of the (*S*)-enantiomer is therefore not an enzyme-related process and can be attributed purely to the direct reaction between hydrogen peroxide and methyl phenyl sulfide. At higher enzyme concentrations the concentration of $H_2O_2$ decreases more rapidly, leading to a decrease in the contribution of the nonenzymatic reaction. Consequently, we established that the vanadium bromoperoxidase from *A. nodosum* in is capable of catalyzing the asymmetric conversion of methyl phenyl sulfide with very high enantioselectivity [37]. It is likely that the selectivity of the catalyzed sulfoxidation reaction may improve further by decreasing and controlling the hydrogen peroxide concentration in the reaction, as demonstrated previously [11–13,33].

Earlier studies showed that the $K_m$ value of methyl phenyl sulfide was > 1.5 mM [11] and exceeds the maximal solubility of methyl phenyl sulfide in water (1.7 mM). Therefore, to obtain the $K_m$ value an organic solvent should be added to the medium. As shown previously the sulfoxidation activity of vanadium and heme peroxidases is affected only slightly by the presence of *tert*-butanol, especially compared with other cosolvents [11,13,38]. Moreover, it was established that the nonenzymatic sulfoxidation reaction between hydrogen peroxide and methyl phenyl sulfide did not proceed when sulfoxidation was carried out in 50% *tert*-butanol. A $K_m$ value of VBPO for methyl phenyl sulfide of ≈ 3.5 mM was found in the presence of either 25% *tert*-butanol or methanol at pH 5.0 (not shown). The rate of enzymatic sulfoxidation at 1.5 mM methyl phenyl sulfide is therefore suboptimal. However, in 25% methanol a clear effect was noted of the sulfide concentration on the selectivity of the sulfoxidation reaction. An optimal value of 48% enantiomeric excess for the (*R*)-sulfoxide was found at concentrations near the $K_m$ for

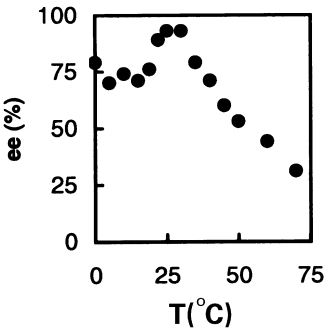


**Fig. 3. Temperature dependence of the enantiomeric excess of the sulfoxidation of methyl phenyl sulfide by vanadium bromoperoxidase from *A. nodosum* (1 μM) at pH 5.3.**

methyl phenyl sulfide, but at higher sulfide concentrations the selectivity decreased to 28% enantiomeric excess. The decrease is probably due to a larger contribution of the nonenzymatic formation of the sulfoxide at higher concentrations of sulfide (results not shown). A similar effect of the sulfide concentration on the enantiomeric excess, but less profound, was observed in the presence of *tert*-butanol.

Previously, it was shown for several lipases and a secondary alcohol dehydrogenase that the reaction temperature greatly affects the selectivity of certain reactions and that altering the reaction temperature can even change the stereochemistry of the reaction [39,40]. Therefore, the temperature-dependence of VBPO in the sulfoxidation of methyl phenyl sulfide was studied by conducting the reaction at different temperatures ranging from 0 to 70 °C for 20 h (see Experimental procedures). The influence of the different reaction temperatures on the selectivity of the enzymatic conversion of methyl phenyl sulfide is shown in Fig. 3. As this figure shows, the reaction temperature clearly affects the selectivity of the reaction and a distinct temperature optimum is found over a narrow range from ≈25 to 30 °C. Within this temperature range the enantiomeric excess of the product reaches a maximum of 96%, which is higher than the selectivities found for the conversion of methyl phenyl sulfide by vanadium peroxidases in a previous study [11].

A further increase in the reaction temperature results in a loss of selectivity, presumably due to the nonenzymatic reaction, which has an increased contribution at elevated temperatures (results not shown). In addition, higher reaction temperatures, such as 60 and 70 °C, which approach the melting temperature of 72 °C [20], may disturb the structure of the enzyme and reduce the selectivity of the conversion. Nevertheless, these results again highlight the remarkable stability of VBPO as it is active at these high temperatures. Decreasing the reaction temperature to below 20 °C does not induce great loss of selectivity, as the enantiomeric excess is found to remain at ≈73%. Low reaction temperatures decrease enzyme activity in general, however, and also constrain the enzyme structurally, which could explain the lack of an effect on the selectivity of the catalyzed sulfoxidation at temperatures below 20 °C. The rate of conversion of the enzyme-catalyzed sulfoxidation reaction was also strongly affected by the reaction temperature. At a reaction temperature of 40 °C or higher the conversion of methyl phenyl sulfide was complete within 20 h (results not shown).

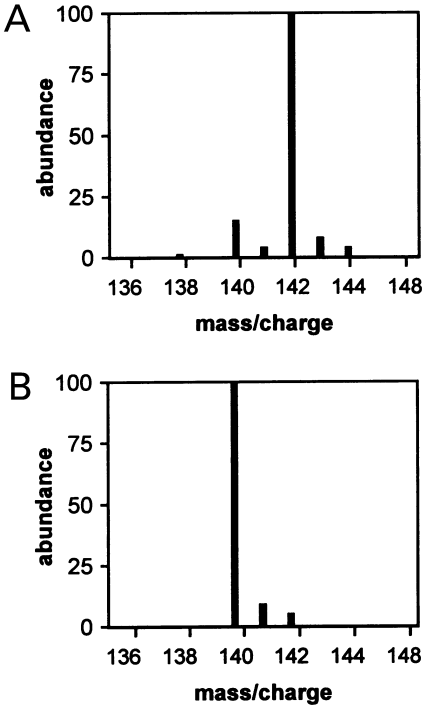


**Fig. 4. Mass chromatogram of (A) methyl phenyl sulfoxide formed by VBPO from *A. nodosum* in the presence of $H_2{}^{18}O_2$ and (B) methyl phenyl sulfoxide purchased from Sigma.** The peak at *m/z* 140 in (A) corresponds to enzymatically produced sulfoxide due to the presence of a small amount of unlabeled hydrogen peroxide in the labeled peroxide and to a minor amount of sulfoxide already present in the methyl phenyl sulfide used. The peaks with *m/z* values of 141, 143 and 144 in (A) and those of 141 and 142 in (B) originate from $^{33}S$ and $^{34}S$ isotopes in the sulfoxide.

We investigated the source of oxygen in methyl phenyl sulfoxide produced by VBPO from *A. nodosum* from methyl phenyl sulfide using $^{18}O$-labeled hydrogen peroxide. As the reaction rate was found to be 1 $min^{-1}$ under these conditions (see Experimental procedures) [11], 3 days are needed for VBPO to complete the conversion. Methyl phenyl sulfide was also incubated with $^{18}O$-labeled peroxide in the absence of enzyme using the same reaction conditions. The incorporation of $^{18}O$ into methyl phenyl sulfide was determined by mass spectrometry. Figure 4 represents the mass chromatograms of the sulfoxide produced in the presence of VBPO and $^{18}O$-labeled peroxide and that of unlabeled commercially available methyl phenyl sulfoxide. A chromatogram of the enzymatically produced sulfoxide shows that only $^{18}O$-labeled sulfoxide is formed by VBPO. In addition, the uncatalyzed sulfoxidation reaction produces only $^{18}O$-labeled methyl phenyl sulfoxide in the presence of $H_2^{18}O_2$ (results not shown). Therefore, spontaneous exchange of oxygen atoms between peroxide and water or molecular oxygen does not occur during the reaction. These experiments demonstrate that VBPO from *A. nodosum* mediates the conversion of the sulfide with essentially quantitative incorporation of oxygen atoms derived from peroxide. Although both the enzyme-catalyzed and direct sulfoxidation reaction produce $^{18}O$-labeled methyl phenyl sulfoxide in the presence of $H_2^{18}O_2$, VBPO catalyzes the formation of the (*R*)-sulfoxide with 63% enantiomeric excess, whereas a racemic mixture is produced in the absence of the biocatalyst. The selectivity of the enzyme-catalyzed reaction in this particular experiment is lower than that expected from prior results [11], because a lower enzyme concentration was used.

The addition of several solvents decreases the selectivity of the VBPO-catalyzed sulfoxidation reaction [11,13]. In order to study the origin of the suppressed selectivity, labeling studies were also carried out in the presence of methanol or

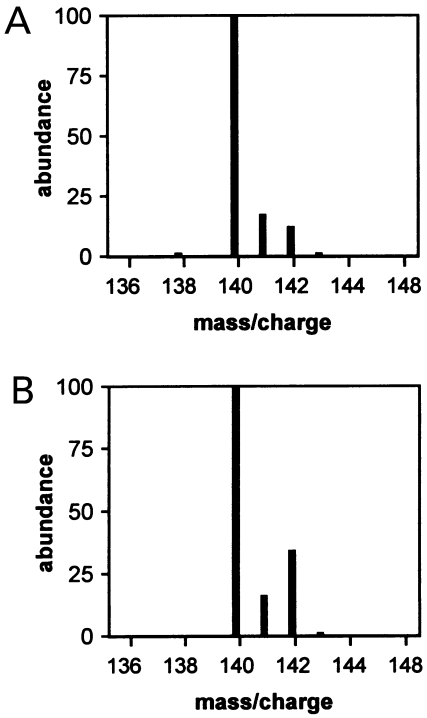


**Fig. 5. Mass chromatogram of methyl phenyl sulfoxide formed by recombinant VCPO in the presence of (A) $H_2{}^{18}O_2$ and (B) unlabeled $H_2O_2$ in 50% $H_2{}^{18}O$.**

*tert*-butanol. VBPO was incubated with methyl phenyl sulfide and $^{18}$O-labeled hydrogen peroxide in either 25% *tert*-butanol or methanol for 3 days as described. In addition, these experiments were conducted in the absence of enzyme. The chromatograms of the methyl phenyl sulfoxides either produced in the presence or absence of the biocatalyst show that only $^{18}$O-labeled sulfoxide is produced (results obtained resemble Fig. 4A and are therefore not shown). Consequently, it is clear that also in the presence of 25% *tert*-butanol or methanol, VBPO still catalyzes the direct transfer of oxygen from hydrogen peroxide to methyl phenyl sulfide to form the corresponding sulfoxide. However, the selectivity of the conversion of methyl phenyl sulfide catalyzed by the vanadium enzyme decreases due to the presence of these alcohols. A slight decrease from 63% to 58% enantiomeric excess is found for the (*R*)-enantiomer of the sulfoxide produced by VBPO in the presence 25% *tert*-butanol and the enantiomeric excess decreases to 34% in the presence of 25% methanol. Similar effects were observed in an earlier study [11]. The origin of the decrease is probably due to a distortion of the three-dimensional structure of the enzyme and penetration of the solvent in the active site of the enzyme affecting the intrinsic selectivity of the enzyme. These experiments show that *tert*-butanol influences only slightly the selectivity of VBPO and that *tert*-butanol is the solvent most likely to be used in further studies.

The origin of the oxygen in methyl phenyl sulfoxide produced by recombinant VCPO was also studied, because, as demonstrated previously, this enzyme produces a racemic mixture [11]. Methyl phenyl sulfide was incubated for 20 h with $^{18}$O-labeled hydrogen peroxide in the presence of recombinant VCPO. The mass chromatogram of the product (Fig. 5A) clearly shows that $< 5\%$ of $^{18}$O is present in the sulfoxide. Similar results are obtained when unlabeled $H_2O_2$ is used (results not shown). Analysis of the products using chiral HPLC yielded racemic methyl phenyl sulfoxide as expected. In order to reveal the origin of the oxygen in the sulfoxide formed by VCPO, the same experiment was performed in $^{18}$O-labeled water (50%) using unlabeled hydrogen peroxide. Now $\approx 25\%$ of the product contains the labeled oxygen atom (Fig. 5B) corresponding to 50% of the sulfoxide oxygen originating from water.

Our data show that VBPO from *A. nodosum* promotes the direct transfer of oxygen from the vanadium bound peroxide to the sulfide in a selective manner, strongly suggesting that the aromatic sulfide binds near/in the active site with a relatively low affinity (Fig. 6). The presence of a binding site in VBPO from *A. nodosum* for organic substrates is in agreement with an earlier study performed by Tschirret-Guth & Butler [41]. A direct oxygen-transfer pathway has been suggested for the enantioselective sulfoxidation catalyzed by heme chloroperoxidase from *C. fumago* [32,34,35]. Because of the accessibility of the active site, substrates are presumed to bind near the activated oxygen of heme compound I facilitating sulfoxidation via a direct oxygen-transfer mechanism.

VCPO, in contrast to VBPO, is not capable of mediating the direct and selective transfer of peroxide oxygen to methyl phenyl sulfide and a different oxidation pathway should be present in VCPO. We suggest that the aromatic sulfide is oxidized by a one-electron transfer step by the peroxo-intermediate of the enzyme [31], forming a positively charged sulfur radical, which migrates from the enzyme and is subsequently converted to the product via chemical steps (Fig. 6) [32,34,35]. The additional electron still present in the enzyme can either reside on the vanadium peroxo-intermediate [31] or could be transferred to an aromatic amino acid in the vicinity of the prosthetic group, until a second substrate molecule arrives to be oxidized.

In conclusion, we have established that VBPO from *A. nodosum* produces only the (*R*)-enantiomer of methyl phenyl sulfoxide in the enzymatic sulfoxidation of methyl phenyl sulfide. Formation of the (*S*)-enantiomer of the sulfoxide should be attributed to the nonenzymatic reaction between the sulfide and hydrogen peroxide. Clearly, the main reason that it is difficult to obtain sulfoxides with very high enantiomeric purity using vanadium peroxidases is the significant contribution of this reaction.

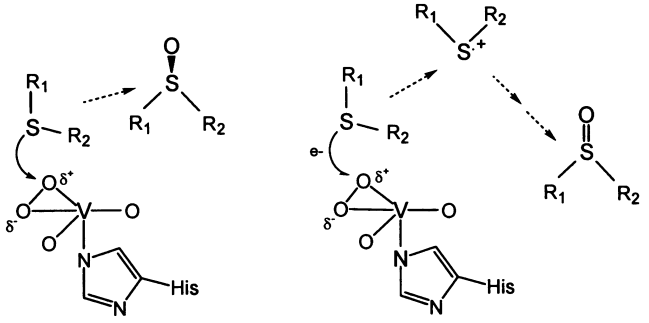


**Fig. 6. Schematic models for the sulfoxidation mechanism of the VBPO from *A. nodosum* (left) and recombinant VCPO (right).** The schematic representations of the active sites of the enzymes, in the presence of peroxide, are based on the tertiary structure of the peroxo-intermediate of the VCPO [25]. The sulfide is shown as $R_1SR_2$. For methyl phenyl sulfide $R_1$ is the methyl and $R_2$ the phenyl group.

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