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Characterization of a Cyanobacterial Haloperoxidase and Evaluation of its Biocatalytic Halogenation Potential

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Vanadium-dependent haloperoxidases (VHPOs) are a class of halogenating enzymes found in fungi, lichen, algae, and bacteria. We report the cloning, purification, and characterization of a functional VHPO from the cyanobacterium *Acaryochloris marina* (*Am*VHPO), including its structure determination by X-ray crystallography. Compared to other VHPOs, the *Am*VHPO features a unique set of disulfide bonds that stabilize the dodecameric assembly of the protein. Easy access by high-yield recombinant expression, as well as resistance towards organic solvents and temperature, together with a distinct halogenation reactivity, make this enzyme a promising starting point for the development of biocatalytic transformations.

A halogen substituent is often an essential structural feature of natural products, drugs, or signaling molecules and improves their biological properties.[1] Within the large number of over 5000 halogenated compounds described so far,[2] the majority contain bromine or chlorine, whereas iodine and fluorine are rare. Nature has evolved exquisite methods to introduce halogen atoms into organic compounds by the use of halogenating enzymes. Among these, the class of vanadium-dependent haloperoxidases (VHPOs) has attracted increasing attention because of the enzymes' biocatalytic properties.[3,4] These include an unusual stability and tolerance to heat and organic solvents.[5] An additional advantage is the more flexible substrate scope compared to other halogenating enzymes, such as flavin-dependent halogenases. Some members of the VHPO family, in particular the chloroperoxidases from *Actinomycete* species, even display high regio- and stereoselectivities in their halogenation reactions.[1c,d,g] VHPOs contain vanadate as a prosthetic group and catalyze the oxidation of halides (Cl^-, Br^-, or I^-)[6] in the presence of hydrogen peroxide as the oxidant. Over the course of this reaction, a peroxo intermediate is formed (Figure 1). Unlike the heme-dependent haloperoxidases,[1c,3d–f] the cofactor here is redox-neutral and thus does not suffer from oxidative inactivation during turnover. A classification according to the most electronegative halide they are able to oxidize differentiates between chloroperoxidases (VCPOs), bromoperoxidases (VBPOs), and iodoperoxidases (VIPOs).

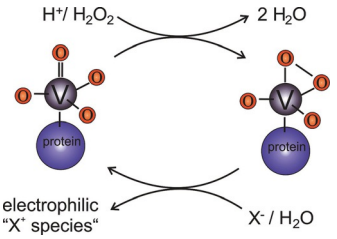

Figure 1. Simplified mechanism of oxidative halogenations catalyzed by VHPOs.

Structural and mechanistic studies of this class of enzymes have been carried out, but there remain unanswered questions regarding the defined substrate binding site or the origin of substrate specificity. The first functional VHPO from a cyanobacterium was recently identified in the marine cyanobacterium *Synechococcus* sp. CC9311.[7] As this VHPO appears to be part of a cluster of horizontally transferred genes, it was postulated that the epiphytic cyanobacterium might have acquired the enzyme from a red seaweed species or vice versa. In view of the significant catalytic potential observed for VBPOs, we decided to focus on a new bacterial member of this family (EC 1.11.X). Our aim was to discover and characterize a prokaryotic alternative to the existing catalysts. In addition to a natural variation in substrate scope and halogenation activities, this may provide a platform for diverse protein engineering efforts and thus an opportunity to establish a new chemoenzymatic halogenation tool in the future.

A BLAST search[8] using the characterized enzymes from *Corallina* species as a query identified a designated VBPO from the cyanobacterium *Acaryochloris marina* MBIC11017 (*Am*VHPO) with 47% sequence identity to both *C. pilulifera* and *C. officinalis* and 35% to *Synechococcus* sp. CC9311. After inserting the codon-optimized synthetic gene into a pET28 expression vector, we were able to produce up to 30 mg L^{-1} of soluble, recombinant protein in an *Escherichia coli* host system. Following affinity and size-exclusion chromatography, the purified VHPO remained stable and active for several weeks.

The enzyme's dodecameric structure was determined to a resolution of 3.1 Å (R_{work}/R_{free} = 20.7/24.9%) by X-ray crystallography, using the *Corallina pilulifera* VBPO coordinates (PDB ID: 1UP8) for Patterson search calculations[9] (Table 1). Structure analysis with the protein assembly tool PISA[10] calculated a buried surface area of 3340 $Å^2$ for each monomer in the 840 kD protein, which was also in line with retention times observed during size-exclusion chromatography (Figure 2A and Figure S14 in the Supporting Information).

The tertiary and quarternary structure of *Am*VHPO is dominated by so-called helix bundles (Figure S9). For the algal en-

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Table 1. Crystallographic data processing and refinement parameters for *Am*VHPO.

Crystal parameters	
space group	$F4_132$
cell constants [Å]	$a = 306.2$
subunits/asymmetric unit	1
Data collection	
X-ray source	ESRF, ID23-1
wavelength [Å]	0.972
resolution range [Å][a]	30.0–3.10 (3.20–3.10)
no. observations	84011
no. unique reflections	21760
completeness [%][c]	95.2 (96.9)
R_{merge} [%][a,c]	9.2 (57.2)
$CC_{1/2}$ [%]	99.7 (76.0)
I/σ (I)[a]	11.2 (2.2)
Refinement (REFMAC5)	
resolution range [Å]	15.0–3.10
no. reflections working set	20672
no. reflections test set	1088
no. non-hydrogen (protein)	4816
no. of ions	1
no. of solvent molecules	0
R_{work}/R_{free} [%][d]	20.7/24.9
rmsd bond lengths/angles [Å]/[°][e]	0.004/0.909
average B-factor [Å^2]	79.0
Ramachandran plot [%][f]	95.3/4.0/0.7
PDB ID	5LPC

[a] Values in parentheses for resolution range, completeness, R_{merge}, and I/σ (I) correspond to the last resolution shell. Datasets were collected from a single crystal. [b] Friedel pairs were treated as identical reflections. [c] $R_{merge}(I) = \Sigma hkl \Sigma j |I(hkl)j - \langle I(hkl)\rangle| / \Sigma hkl\ \Sigma j\ I(hkl)j$, where $I(hkl)j$ is the jth measurement of the intensity of reflection hkl, and $\langle I(hkl)\rangle$ is the average intensity; [d] $R = \Sigma_{hkl} ||F_{obs}| - |F_{calcd}|| / \Sigma_{hkl} |F_{obs}|$, where R_{free} was calculated without a sigma cutoff for a randomly chosen 5% of reflections, which were not used for structure refinement, and R_{work} was calculated for the remaining reflections.[e] Deviations from ideal bond lengths/angles. [f] Number of residues in favored region/allowed region/outlier region as determined in COOT.

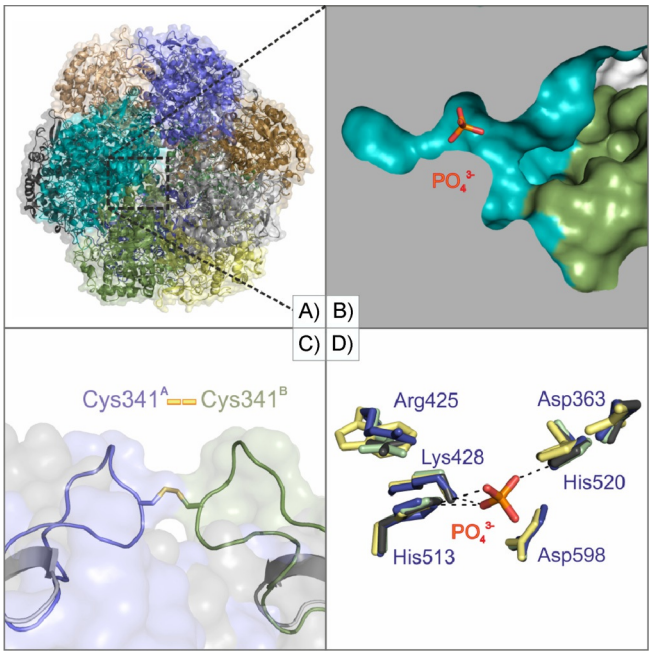


Figure 2. The *Am*VHPO crystal structure was refined to a resolution of 3.1 Å. A) The single monomer present in the asymmetric unit was predicted to assemble into a native dodecamer. B) The catalytic vanadate (replaced by the isosteric phosphate in the crystal structure) is situated in a small binding pocket to which the neighboring monomers form an access channel. C) An unusual feature of the *Am*VHPO structure is the presence of intermolecular disulfide bonds between the Cys341 side chains of two neighboring monomers. Superimposition with the *C. officinalis* VHPO (gray) shows the differences in secondary structure required to enable formation of this bond. D) Superimposition of the key catalytic residues in the VHPOs of *A. marina* (blue, labeled), *C. pilulifera* (gray), *C. officinalis* (green), and *A. nodosum* (yellow) reveals a clear structural conservation with the exception of a single tryptophan in the *A. nodosum* active site.

zymes, two bundles of neighboring subunits associate closely and jointly contribute to the wider active site spheres. This arrangement has been implicated in multimerization and is found in dimers of the brown seaweed *Ascophyllum nodosum*, as well as in the dodecamers of *Corallina* species.[11]

Although helical bundles are present in other eubacterial haloperoxidases, the *Am*VHPO is, to the best of our knowledge, the first bacterial enzyme showing this particular type of arrangement. A second unexpected feature that stabilizes the monomer, dimer, and, ultimately, the hexamer of dimers, is the presence of intra- and intermolecular disulfide bonds. These occur between Cys206/308 and Cys341 of two neighboring monomers, respectively (Figure 2c, Figure S10), and are likely to contribute to the enzyme's significant tolerance to heat and solvents as described below. On a monomeric level, each subunit consists of a single, compact $\alpha+\beta$ type domain with a short N-terminal helix protruding out of the core peptide. Again, this architecture does not resemble any of the bacterial VHPO structures determined to date but is highly similar to the homologous enzymes from red and brown seaweed species. Structural alignment of *Am*VHPO with the *C. pilulifera*, *C. officinalis*, and *A. nodosum* VHPOs yields accordingly low root mean square deviations (RMSDs) of 0.7, 0.6, and 1.1 Å, respectively.

This strong resemblance in folding and multimeric assembly raises interesting questions about their evolutionary relationship. All species share a similar marine environment, and cyanobacteria are frequently found in symbiotic relationships with algal species.[12] As a result, horizontal gene transfer may have contributed to the distribution of VHPOs, even between different kingdoms. A closer inspection of the phylogenetic tree (Figure S7) shows separate branches for marine and terrestrial VHPO-producing organisms, which further supports this hypothesis. A second possibility is that the cyanobacterial haloperoxidase genes were acquired by algae through an early process of endocytosis.[13] The latter has been suggested for the evolutionary origin of various plastid types and would account for the observed similarities more convincingly than simple convergent evolution.

Although the protein was crystallized in the presence of excess vanadate, an isosteric phosphate obtained from the reservoir solution replaced the cofactor in the crystal structure. It is bound in a small, defined pocket and protected from solvent by the close assembly of the monomer with its neighboring two subunits. This substructure also forms an access tunnel to

Table 2. Comparison of Michaelis–Menten constants, turnover numbers, and specific activity.

		K_M [mM]				k_{cat} [s^{-1}]	A_{spec} [U mg^{-1}]	$k_{cat}K_M^{-1}$ [$M^{-1}s^{-1}$]
		Br^{-}[c]	H_2O_2[c]	I^{-}[a]	Cl^{-}[c]	Br^{-}		
cyano-bacteria	*Am*VBPO	0.40	0.06	6.40	–	8.4	23.3 (Br)[d]	2×10^4
	*Syn*VHPO[7]	1.50	–	0.02	–	–	0.56 (Br)	
red seaweed	*Co*VBPO[24]	1.00	0.06	–	–	–	470 (Br)	
	r*Co*VBPO[25]	1.20	0.02	1.80	–	–	–	
	*Cp*VBPO[11c]	11.0	0.09	–	–	–	26.3 (Br)	
brown algae	*An*IVBPO[18,26]	3.70	0.06	0.20[b]	344	85	127 (Br) 0.49 (Cl)	2×10^4
fungi	*Ed*VCPO[27]	0.005	0.06	–	1.20	60	–	
	*Ci*VCPO[28]	0.007	0.003	–	0.01	41	7.50 (Cl)	5×10^7 [28c]

[a] Triiodide assay. [b] Thymolblue assay. [c] Monochlorodimedone (MCD) assay. [d] Highest detected activity. *Syn*VHPO = *Synechococcus* sp. PCC 7335; *Co*VBPO = *C. officinalis*; r*Co*VBPO = recombinant *C. officinalis*; *Cp*VBPO = *C. pilulifera*; *An*IVBPO = *A. nodosum;* isoenzyme I; *Ed*VCPO = *Embellisia didymospora*; *Ci*VCPO = *Curvularia inaequalis.*

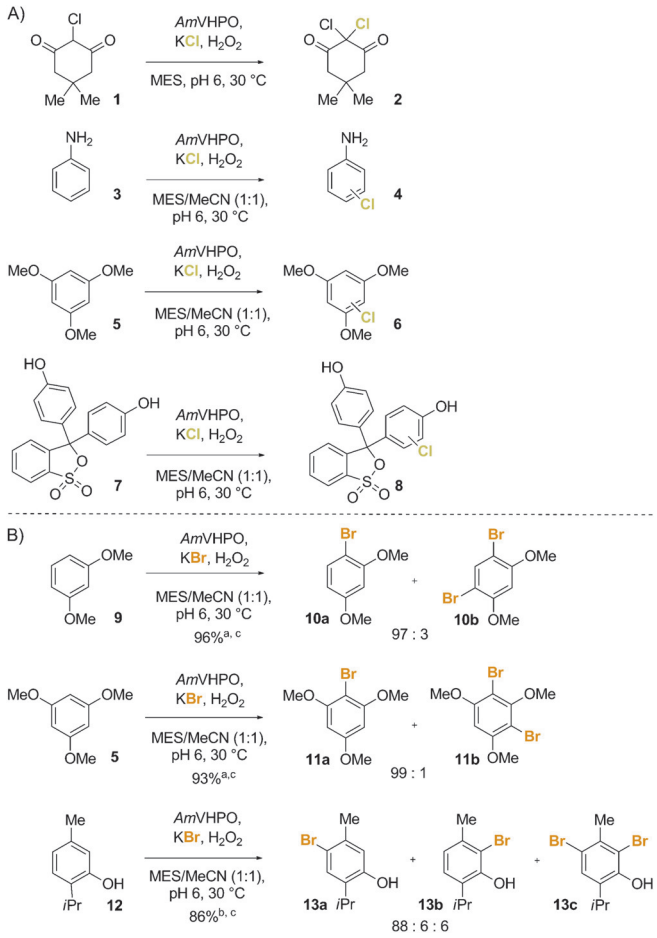
the cofactor and active site cavity (Figure 2B). Most of the protein's key catalytic residues involved in cofactor binding and coordination of the peroxo intermediate are conserved in their position and orientation within this class of enzymes (Figure 2D).[9,11,14] Interestingly, if the alignment of the *Am*VHPO active site is extended to homologous structures of the *A. nodosum* VBPO and various known VCPOs, small differences become apparent. These include the presence of an additional histidine residue in VBPOs (phenylalanine in VCPOs) and the substitution of a vanadate-coordinating arginine with a tryptophan, which has been suggested to be involved in halogen specificity (Figures 2D, S8, and S12).[15] Such comparisons, as well as the substantially higher bromide affinity observed for several VCPOs compared to designated VBPOs (Table 2), hint at a more complex mode of action than suggested by the simple classification into bromo- and chloroperoxidases.

The enzyme was subsequently analyzed with respect to its halogenation potential. As recombinant expression yielded the *Am*VHPO *apo* form, pre-incubation with K_3VO_4 was necessary to restore its activity prior to enzymatic assays. Colorimetric assays served as a preliminary qualitative proof for halogenating activity.[3a,16] Optimal turnover was observed at a pH of 6. The kinetics of VHPOs follow a complex bi–bi ping-pong mechanism due to sequential reaction with several substrates, such as H_2O_2 and X^-.[17] When using saturating levels of one of the employed substrates, however, enzyme activities could be simplified to pseudo-first order kinetics. As shown in Table 2, the determined kinetic parameters were comparable with those of other VBPOs. In addition to iodination and bromination activities, generally assigned to VBPOs, even the unexpected oxidative chlorination was achieved by *Am*VHPO. It was not surprising that, in the latter case, the detected activity was notably lower than that for the corresponding bromination. Similar findings were described for VBPOs from *A. nodosum*[18] and *C. pilulifera*.[19] Because of the high chloride concentration needed to achieve sufficient turnover, the kinetic parameters for chlorination were not determined. In order to confirm that the observed activity indeed stemmed from chlorination and not from bromination, caused, for example, by impurities of the applied KCl or the buffer salts, we conducted ESI MS investigations of the reaction mixture. The chlorinated products **4**, **6**, and **8** were unequivocally identified (Scheme 1, see the Supporting Information for details). Although the specificity constant ($k_{cat}K_M^{-1}$) of fungal VCPOs is much higher for bromination than that of our bacterial halogenating enzyme, the intrinsic ability to catalyze chlorination, combined with all of the other versatile properties exhibited by *Am*VHPO (see below), constitutes a valuable starting point for further protein engineering and investigation of the structure–function relationship.

Remarkably, *Am*VHPO is stable for several days in a number of commonly used organic solvents, such as alcohols, acetonitrile, and acetone, even at concentrations of 75 vol% (Figure S4). In addition, significant thermostability was detected: the activity of the enzyme decreased by 60% after heating to 65 °C for 30 min, but was fully recovered after cooling to 4 °C. In the dimeric *A. nodosum* VHPO, disulfide bonds were suggested to contribute to the enzyme's stability.[11e] While the same is likely to apply to *Am*VHPO, the effect may be enhanced even further by its dodecameric state. The hexamer of stable dimers increases the percentage of protein interfaces protected from solvent, simply by interactions between neighboring monomers (Figure S11).

VHPOs are commonly inactivated by high H_2O_2 concentrations, but this limitation is generally circumvented by sequential addition of the oxidant.[20] Initial experiments showed that exposure of *Am*VHPO to high H_2O_2 concentrations (100 mM) for 7 h led to a significant decrease in activity (90%; Figure S5). However, the inactivation appeared to be reversible upon H_2O_2 consumption. This striking feature became evident, as no significant difference in the halogenation activity was observed, regardless of whether the oxidant was added slowly or in one addition.

As no halogenated natural products have been isolated from *A. marina* to date,[21] we started our investigation with bromocyclizations of terpenes, as this is the preferred reaction of VHPOs originating from *Corallina* species.[11f] To our surprise, we observed no conversion for these substrates, either in the presence or absence of organic co-solvents. Halogenation of dicarbonyl compounds such as **1** and activated arenes **3**–**12**, however, proceeded smoothly. Further studies of aromatic bromination showed that monobrominated products **10a** and **11a** were generated with excellent conversions and regioselec-



Scheme 1. *Am*VHPO-catalyzed aromatic halogenations. [a] Determined by HPLC. [b] Determined by gas chromatography (GC). [c] Conversion calculated from the remaining starting material.

tivities (Scheme 1 B). Optimum results were achieved with equimolar amounts of KBr and H_2O_2 at pH 6.[22] The regioisomeric ratios obtained from the bromination of methoxy arenes **5** and **9** closely resemble those achieved by oxidative brominations simply applying NH_4VO_3 (see the Supporting Information). Nevertheless, the biocatalytic method is still preferable to the abiotic one, as significantly milder conditions are applicable and thus, in principle, a broader substrate range can be addressed. For example, the enzymatic synthesis of **10a** and **11a** proceeds smoothly at pH 6, whereas NH_4VO_3-mediated halogenation requires strongly acidic conditions and is only operative at pH 1. In addition, higher levels of conversion were observed using the enzyme. Interestingly, *Am*VHPO also worked in a highly chemoselective manner, even allowing differentiation between electron-rich aryl compounds, such as anisole (no conversion) and dimethoxy benzene **9**, a task that is hard to accomplish with standard synthetic halogenation procedures.

The distinct reactivity displayed by *Am*VHPO is particularly beneficial when employed in late-stage halogenation, for example, of a specific, activated aromatic subunit in a structurally complex target compound. Application of our chemoenzymatic bromination was likewise possible using the monoterpene thymol (**12**). Halogenated thymol derivatives possess antibacterial and antifungal properties, making their synthesis valuable for a number of applications in the pharmaceutical industry.[23] With *Am*VHPO as a catalyst, *p*-bromothymol (**13a**) was predominantly formed (88% yield), with the *o*-isomer (**13b**) being only a minor product. This result is in clear contrast to the conversion obtained by applying the VBPO from *A. nodosum*,[4b] and, in this case, rather resembles the unusual product selectivity of fungal VCPOs.[4a]

In summary, the structural and functional characterization of *Am*VHPO has revealed an unusually robust biocatalyst that is available in high yield using a recombinant *E. coli* expression system. The structural similarities found between this cyanobacterial haloperoxidase and its eukaryotic homologues show a fascinating evolutionary relationship between VHPOs from two different kingdoms of life. The enzyme's successful bromination of aromatic substrates, as well as its ability to chlorinate, point to a promiscuous substrate tolerance combined with selective halogenation activities. These properties enable protein engineering and evolution strategies so as to improve this catalyst's activity, widen its substrate scope, and contribute to more-selective, sustainable, and environmentally benign halogenations in the future.

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[21] Although, to our knowledge, no halogenated secondary metabolites have previously been isolated from *A. marina*, cyanobacteria are responsible for the production of significant amounts of bromoform and similar brominated compounds in the ocean. As an example, see ref. [1b].

[22] No reaction was observed if *Am*VHPO, KBr, or H_2O_2 was omitted.

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