The Vanadium Iodoperoxidase from the Marine *Flavobacteriaceae* Species *Zobellia galactanivorans* Reveals Novel Molecular and Evolutionary Features of Halide Specificity in the Vanadium Haloperoxidase Enzyme Family

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Vanadium haloperoxidases (VHPO) are key enzymes that oxidize halides and are involved in the biosynthesis of organo-halogenes. Until now, only chloroperoxidases (VCPO) and bromoperoxidases (VBPO) have been characterized structurally, mainly from eukaryotic species. Three putative VHPO genes were predicted in the genome of the flavobacterium *Zobellia galactanivorans*, a marine bacterium associated with macroalgae. In a phylogenetic analysis, these putative bacterial VHPO were closely related to other VHPO from diverse bacterial phyla but clustered independently from eukaryotic algal VBPO and fungal VCPO. Two of these bacterial VHPO, heterogeneously produced in *Escherichia coli*, were found to be strictly specific for iodide oxidation. The crystal structure of one of these vanadium-dependent iodoperoxidases, Zg-VIPO1, was solved by multiwavelength anomalous diffraction at 1.8 Å, revealing a monomeric structure mainly folded into α -helices. This three-dimensional structure is relatively similar to those of VCPO of the fungus *Curvularia inaequalis* and of *Streptomyces* sp. and is superimposable onto the dimeric structure of algal VBPO. Surprisingly, the vanadate binding site of Zg-VIPO1 is strictly conserved with the fungal VCPO active site. Using site-directed mutagenesis, we showed that specific amino acids and the associated hydrogen bonding network around the vanadate center are essential for the catalytic properties and also the iodide specificity of Zg-VIPO1. Altogether, phylogeny and structure-function data support the finding that iodoperoxidase activities evolved independently in bacterial and algal lineages, and this sheds light on the evolution of the VHPO enzyme family.

Halogenated compounds have various biological functions in nature, ranging from chemical defense to signaling. Indeed, halogenation (i.e., iodination, bromination, or chlorination) is an efficient strategy used to increase the biological activity of secondary metabolites and involves many different halogenating enzymes (1–4). Among them, vanadium-dependent haloperoxidases (VHPO) contain the bare metal oxide vanadate as a prosthetic group. In the presence of hydrogen peroxide, VHPO enzymes catalyze the oxidation of halides according to the reaction $\text{H}_2\text{O}_2 + \text{X}^- + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{HOX}$, wherein X^- represents a halide ion and may be Cl^- , Br^- , or I^- (4). A variety of halocarbons can subsequently be generated if the appropriate nucleophilic acceptors are present. The nomenclature of vanadium-dependent haloperoxidases is based on the most electronegative halide they can oxidize: chloroperoxidases (VCPO) can catalyze the oxidation of three different halides, i.e., chloride, bromide and iodide; bromoperoxidases (VBPO) can oxidize only bromide and iodide; and iodoperoxidases (VIPO) are specific for iodide.

The first VBPO was discovered 30 years ago, in the brown alga *Ascophyllum nodosum* (5). Since then, structural and mechanistic studies have focused on two types of eukaryotic VHPO, namely, VCPO from the pathogenic fungus *Curvularia inaequalis* (6) and VBPO from *A. nodosum* (7) and the red algae *Corallina officinalis* (8) and *Corallina pilulifera* (9, 10). These eukaryotic VHPO are folded in alpha helices which combine into helical bundles. The high conservation of the tertiary structural motif and an identical arrangement of amino acid residues at the vanadium active site suggest that all VHPO derive from a common ancestor, sharing a

common evolutionary history with bacterial acid phosphatases (4). Surprisingly, quaternary VHPO structures differ dramatically between the different phyla. Fungal VCPO is monomeric in solution (6), whereas the VBPO from brown algae forms covalently bound dimers (7, 11, 12) and the dimers of the red algal VBPO self-rearrange into dodecamers (9, 10). Until now, only one vanadium-dependent iodoperoxidase (VIPO) protein has been characterized biochemically, from the brown alga *Laminaria digitata* (13), but no structural data are yet available for these iodide-specific enzymes.

In the active center of fungal VCPO and algal VBPO, the vanadate cofactor is covalently bound to the imidazole ring of a conserved histidine residue and finely coordinated by neighboring

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TABLE 1 Sequences of primers used for PCR-based cloning of *Z. galactanivorans* *VIPO1* and *VIPO2* genes and of site-directed mutants of Zg-*VIPO1*

Clone name	Primer direction	Primer sequence (5'–3')
Zg- <i>VIPO</i> genes ^a		
zg <i>VIPO1</i> (FP476056; <i>zobellia_1262</i>)	Forward	GGGGGGGGATCCAAAGCTCCACAAAAGAAGAACCTAT
	Reverse	CCCCCGGAATTCTAGTTTGGGCTACTTTCTTATCGGAT
zg <i>VIPO2</i> (FP476056; <i>zobellia_2088</i>)	Forward	ACCATCACGGATCCGCATGCGATACGTATTTGAAGGCGGTTTGTC
	Reverse	GCAGGTCGACCCGGGTACCTCAATGCTCCTTCTTAATCGCTCG
Zg- <i>VIPO1</i> mutants		
Y263A	Forward	CTTTTGGGATTGTAACCCCTGCTGTATCGGTTACCCGTGG
	Reverse	CCACGGGTAACCGATACAGCAGGGTTACAATCCCAAAG
Y263S	Forward	GGGATTGTAACCCCTTCTGTATCGGTTACCCG
	Reverse	CGGGTAACCGATACAGAAGGGTTACAATCCC
Y263F	Forward	CTTTTGGGATTGTAACCCCTTTGTATCGGTTACCCGTGGC
	Reverse	GCCACGGGTAACCGATACAAAAGGGTTACAATCCCAAAAG
W321R	Forward	GATGCCTTTATCAGTTGTGCGGACGAAAAGTACAGAAG
	Reverse	CTTCTGTACTTTTCGTCCCGACAAGTATAAAGGCATC
F353H	Forward	CTACAAACCCCTCCGCATCCAGAGTACACCAGC
	Reverse	GCTGGTGTACTCTGGATGCGGAGGGGTTTGTAG
S358A	Forward	CGTTTCCAGAGTACACCGCCGGACATAGTGTAGTC
	Reverse	GACTACACTATGTCCGGCGGTGTACTCTGGAAACG
H360A	Forward	GAGTACACCAGCGGAGCTAGTGTAGTCTCAGG
	Reverse	CCCTGAGACTACACTAGCTCCGCTGGTGTACTC
H360S	Forward	GAGTACACCAGCGGATCTAGTGTAGTCTCAG
	Reverse	CTGAGACTACACTAGATCCGCTGGTGTACTC
R410A	Forward	CGAAGCAGCGATCAGTGCCATGTACGGAGGCATAC
	Reverse	GTATGCCTCCGTACATGGCACTGATCGCTGCTTCG
C320S	Forward	GATGCCTTTATCAGTTCTTGGGACGAAAAGTAC
	Reverse	GTACTTTTTCGTCCCAAGAACTGATAAAGGCATC
D322K	Forward	GATGCCTTTATCAGTTGTTGGAAAGAAAAGTACAGAAGCAACCTC
	Reverse	GAGGTTGCTTCTGTACTTTTCTTTCCAACAACTGATAAAGGCATC
D322Y	Forward	GATGCCTTTATCAGTTGTTGGTATGAAAAGTACAGAAGCAACCTC
	Reverse	GAGGTTGCTTCTGTACTTTTCATACCAACAACTGATAAAGGCATC

^a Genome ID (GenBank accession no.) and locus tag are given parenthetically.

amino acid side chains through hydrogen bonds (1, 4, 14–16). The first step of the catalytic cycle is the coordination of hydrogen peroxide to vanadate, leading to the formation of a stable peroxo-vanadate intermediate as shown by X-ray diffraction and X-ray absorption spectroscopy (17–19). The second step, i.e., the oxidation of the halide, is the determinant for the enzymatic specificity of VHPO but has yet to be elucidated. Targeted active site mutants of *C. inaequalis* VCPO or *C. pilulifera* and *Gracilaria changii* VBPO have shown drastic reductions or increases of chlorinating activity, respectively (20–22). According to these structure-function studies, the catalytic properties and halide specificities of VCPO and VBPO are likely to be dependent on the electronic environment around the VO₄ moiety (4, 15, 23).

In contrast to algal and fungal VHPO, very few biochemical and structural studies are available for bacterial VHPO (4). Until now, a functional VBPO was reported for two strains of the marine cyanobacterium *Synechococcus* sp., and gene homologs have been detected in cyanobacterial environmental DNA samples (24). In marine *Streptomyces* sp. strains, some VCPO were demonstrated to be involved in the chlorination cyclization steps of antibiotic biosynthesis (25–27), and the corresponding bacterial VCPO three-dimensional (3D) structure was recently released in public databases (PDB accession number 3W36). In our laboratory, the marine flavobacterium *Zobellia galactanivorans* (28) is being studied by functional genomic approaches for the capacity

to degrade both red and brown algal polysaccharides (29–31). The present study reports the enzymatic and X-ray structural characterization of an iodide-specific VIPO whose gene was identified in the genome of this macroalga-associated marine bacterium. This novel recombinant VIPO allowed us to explore and identify amino acids essential for iodide specificity by using site-directed mutagenesis approaches. In light of phylogenetic analysis, these functional and structural data help us to understand the history and evolution of VHPO family members and to investigate the physiological roles of these enzymes among bacterial phyla.

MATERIALS AND METHODS

Cloning and site-directed mutagenesis of VHPO proteins. The *Z. galactanivorans* genomic DNA was isolated as described by Barbeyron et al. (28) and used as a template to amplify gene sequences by PCR, using *Pfu* DNA polymerase (Promega) (2 min at 96°C and then 35 cycles of 15 s at 96°C, 30 s at 60°C, and 6 min at 72°C, with a final hold of 4 min at 72°C). Specific oligonucleotides were designed to carry BamHI and EcoRI (*zobellia_1262*) or SphI and KpnI (*zobellia_2088*) sites (Table 1). The *zobellia_1262* (zg*VIPO1*) and *zobellia_2088* (zg*VIPO2*) gene PCR amplicons were cloned into the pFO4 [pFO4-zg*VIPO1*(wt)] (32) and pQE80L vectors (Qiagen, Netherlands), respectively. Zg-*VIPO1* mutants were created using the QuikChange site-directed mutagenesis method (Agilent), using the pFO4-zg*VIPO1*(wt) vector as the template in PCRs (primers used are listed in Table 1). After cloning and mutation validation by sequencing, all

vectors were introduced into the BL21(DE3) strain of *Escherichia coli* (EMD Millipore).

Overexpression and purification of VHPO proteins. The recombinant bacteria were grown in 200 ml autoinducible ZYP (consisting of a modified LB medium containing 0.5% glucose) in the presence of 100 µg/ml ampicillin for 3 days at 20°C. For crystallization setup and 3D structure resolution, the seleno-methionine (Se-Met) labeling procedure was performed by growing recombinant *E. coli* BL21(DE3)/pFO4-zgVIPO1(wt) in 200 ml of PASM 5052 medium (33) containing 200 µg/ml of ampicillin at 20°C for 10 days. The bacterial pellet was incubated for 1 h at 4°C in 100 mM NaCl, 50 mM Tris-HCl, pH 8.8, with lysozyme and bovine DNase I and harvested using a French press. After clarification, the supernatant was exchanged with buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM imidazole) and purified by a two-step chromatography protocol, using an Äkta purifier (GE Healthcare). The proteins were first fractionated on a Hisprep FF 16/10 Ni-Sepharose column (GE Healthcare), using an elution gradient of 50 mM to 500 mM imidazole in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl. For Zg-VIPO2 purification, 200 mM NaCl was used in exchange and elution buffers. The recombinant protein fractions, identified by Coomassie blue staining, were supplemented with 5 mM Na₃VO₄, concentrated by ultrafiltration on a CentriPrep 10-kDa centrifugal filter unit (EMD Millipore), and finally dialyzed against 50 mM Tris HCl, pH 7.5, 50 mM NaCl. The native vanadium bromoperoxidase I from *Ascophyllum nodosum* (An-VBPO1) was purified according to the method of Vilter (34).

DLS analyses. Prior to activity assay, the proper folding of purified recombinant proteins was analyzed by dynamic light scattering (DLS) in a quartz cuvette (120 µl with 1 mg/ml protein), using a Malvern Zetasizer Nano-S instrument (Malvern Instruments, United Kingdom). All recombinant and mutant proteins displayed the same monodisperse peak (data not shown). Thermostability of recombinant Zg-VIPO proteins from 10 to 64°C was also analyzed by DLS, using a heating rate of 1°C per 8 min, with 2-min intervals for temperature stabilization. The scattered light was collected at a fixed angle of 90°, and output data were processed with the DTS v4.10 program by use of multiple narrow modes to calculate hydrodynamic radius (Rh) distributions and melting temperatures (T_m). In parallel, recombinant Zg-VIPO1 was incubated at increasing temperatures for 10 min before testing of the iodoperoxidase activity by thymol blue (TB) assay.

Haloperoxidase activities. For in-gel activity assay, the VHPO activities were detected on nondenaturing electrophoresis gels, using *o*-dianisidine detection as previously described (35). The spectrophotometric assays used for bromo- and iodoperoxidase activity measurements are based on the bromination or iodination of TB and the production of a stable molecule, diiodothymolsulfonphthalein (TBI₂) or TBB₂, with spectral properties different from those of TB, as described by Verhaeghe et al. (36). At pH 7.8, TB (pK_a = 8.9) is responsible for yellow coloration (λ_{max} = 430 nm), while the TBI₂ (pK_a = 7.3) or TBB₂ (pK_a = 7.2) enzymatic product features a maximum absorption at 620 nm, corresponding to a deep blue coloration (36). All reactions were performed in quadruplicate at 20°C in clear flat-bottomed 96-well microplates (Greiner UV-Star 96-well plates). The assays were carried out as follows. Samples of 2.5 to 10 µg of purified proteins were added to the 250-µl reaction mixture, consisting of phosphate buffer (0.1 M, pH 7.2 and pH 7.8 for iodo- and bromoperoxidase assays, respectively), TB (100 µM), KI (80 µM to 2 mM) or KBr (1.5 to 10 mM), and H₂O₂ (0.42 mM and 0.1 mM for iodo- and bromoperoxidase assays, respectively). The absorbance at 620 nm was recorded on a Safire² spectrophotometer (Tecan Group Ltd., Switzerland) for 5 min. The A_{620} values were converted into millimoles of TBI₂ by using the following equation: $[TBI_2] = [A_{620}/(40.3 \text{ mM}^{-1} \text{ cm}^{-1} \times 0.71 \text{ cm})] \times 2$. The experimental initial (up to 112 s) velocities (v_i) were expressed in mM I[−] ([KI] range of 0.125 to 0.5 mM) converted per minute, and Lineweaver-Burk plots were used to calculate the kinetic parameters $K_m^{I^-}$ and V_{max} . $k_{\text{cat}}^{I^-}$ values were obtained by using the equation $k_{\text{cat}}^{I^-} =$

V_{max}/E_t , where E_t is the final concentration of Zg-VIPO1 (molecular weight [MW] = 49,404 g mol^{−1}) or Zg-VIPO2 (MW = 50,500 g mol^{−1}).

Phylogenetic analyses. The following protein sequences were used as queries for serial BLAST searches in the public databases: Zg-VIPO1, Zg-VHPO3, VIPO1 of *L. digitata* (13), VBPO of *Synechococcus* sp. CC9311 (24), VCPO of *C. inaequalis* (6), and NapH3 of *Streptomyces aculeolatus* (25). BLAST queries were conducted on all eukaryotic sequences in the nr protein database, but restricted to bacterial complete genomes by use of Concise BLAST (www.ncbi.nlm.nih.gov/genomes/prokhits.cgi), in order to gain a broader taxonomic representation of bacterial sequences. The protein sequences were aligned by use of MUSCLE (37) and manually filtered based on the presence of amino acid stretches putatively involved in the coordination of VO₄ (KxxxxxxxRP and RxxxGxH). Five sequences of bacterial nonspecific acid phosphatases were also included in the alignment as closely related proteins (4). Phylogenetic analyses were conducted using MEGA, version 6 (38), with a total of 129 protein sequences (detailed information is provided in Table S1 in the supplemental material). Gap positions or missing data were deleted when the site coverage was less than 95%. The best amino acid substitution model was selected according to the lowest Bayesian information criterion. Maximum likelihood analysis was carried out on 192 aligned amino acids, using the LG model with a discrete gamma distribution to model evolutionary rate differences among sites (5 categories; +G parameter = 4.4436), with some sites allowed to be evolutionarily invariable ([+I], 3.1250% sites). The tree with the highest log likelihood (−35,943.0984) is shown, and bootstrap analyses of 500 replicates were used to provide confidence estimates for the phylogenetic tree topology.

Crystallization, data collection, structure determination, and refinement. Single crystals of native and Se-Met Zg-VIPO1 were obtained using the hanging drop vapor diffusion method at 19°C. Crystallization drops were composed of 1 µl of enzyme and 0.5 µl of reservoir solution containing 22 to 25% polyethylene glycol 1150 (PEG 1150), 100 mM phosphate/citrate, pH 4.2, and 2% glycerol. Prior to data collection, crystals were quickly soaked in a cryo solution containing 23% PEG 1150, 100 mM phosphate/citrate, pH 4.2, and 4% glycerol and flash frozen at 100 K in liquid nitrogen. Native data were collected at the ESRF (Grenoble, France) on beamline BM30A, while multiple anomalous diffraction data were collected on beamline ID23-EH at three different wavelengths around the K absorption edge of selenium (Table 2). Both beamlines were equipped with an ADSC Q315R detector. For all data sets, the XDS package (39) was used for data reduction and scaling.

Searches of the selenium substructures and phasing were done using the SHELX suite (40) via the GUI HKL2MAP (41). Initial phases, with a figure of merit of 0.65, were improved by solvent flattening using DM (42) and provided an interpretable electron density map with a figure of merit of 0.77. An initial model of Zg-VIPO1 was automatically built using ARP/WARP (43). The model was further treated with cycles of positional refinement, using the program REFMAC5 (implemented within the CCP4 suite) (44), alternating with manual model building in COOT (45). The final model was refined against the native data, showing that both structures are identical, with a root mean square deviation (RMSD) of all atoms of 0.340 Å. General refinement statistics are presented in Table 3. The RMSDs (in Å) between VHPO structures from other organisms were obtained by the superposition of entire structures based on the alignment of secondary structures by use of SUPERPOSE software in the CCP4 suite (46). To compare the local 2F_o−F_c electron density maps at the active site, refinement was calculated with either a VO₄ or PO₄ moiety positioned in the active site. The hydrogen bonding network involving the nine residues around the VO₄ moiety was predicted using the HBAT 1.1 program and default settings, i.e., with bond angles between 90° and 180° and distances of 1.2 Å to 3.2 Å (47). From this generated extensive list of predicted close contacts, we selected the potential canonical hydrogen bonds on the basis of geometry and distance of N—H···O and O—H···O features (··· indicates a hydrogen bond). All structure figures were generated with the PyMOL molecular graphics system, version 1.3r1 (Schrödinger, LLC).

TABLE 2 Data collection statistics for native and MAD data sets for Zg-VIPO1 crystal structures^a

Parameter	Value or description for data set			
	BM30	ID23-1		
	Native	Peak	Inflection point	Remote
Wavelength (Å)	1.038	0.9793	0.9796	0.9685
Space group	P ₂ ₁ 2 ₁ 2 ₁	P ₂ ₁ 2 ₁ 2 ₁	P ₂ ₁ 2 ₁ 2 ₁	P ₂ ₁ 2 ₁ 2 ₁
Cell dimensions				
a, b, c (Å)	42.85, 85.88, 116.14	42.84, 84.36, 117.38	42.84, 84.36, 117.38	42.84, 84.36, 117.38
α, β, γ (°)	90	90	90	90
Resolution (Å)	69.04–2.00	42–1.80	42–1.80	42–1.80
High-resolution shell (Å)	2.052–2.00	1.85–1.80	1.85–1.80	1.95–1.90
No. of reflections (unique)	130,972 (34,478)	198,076 (72,492)	198,448 (74,005)	168,928 (66,137)
Completeness (%)	99.6 (99.8)	99.1 (99.6)	99.2 (99.7)	99.1 (99.5)
Redundancy	3.8 (3.9)	2.6 (2.6)	2.6 (2.6)	2.6 (2.6)
I/σI	12.4 (4.56)	13.74 (5.17)	10.91 (2.74)	10.60 (2.52)
R _{sym}	9.5 (30.8)	5.3 (20.7)	7.2 (44.2)	8.3 (45.4)
FOM of MAD phases		0.65		

^a Values in parentheses concern the high-resolution shell. FOM, figure of merit; MAD, multiple anomalous diffraction.

Protein structure accession numbers. The coordinates and structure factors of the Zg-VIPO1 model refined against native data and the data collected at the selenium edge have been deposited in the Protein Data Bank with PDB accession codes 4USZ and 4CIT, respectively.

RESULTS

Phylogenetic analyses of putative vanadium-dependent haloperoxidases from *Zobellia galactanivorans*. During manual annotation of the *Z. galactanivorans* genome, the *zobellia_1262*, *zobellia_2088*, and *zobellia_2250* gene loci, of 1,353, 1,326, and 1,455 bp, respectively, found to be part of two different gene clusters, were predicted to encode three putative VHPO, of 450, 441, and 484 amino acids, respectively. The *zobellia_1262* and *zobellia_2088* gene loci both encode ASPIC/UnbV-like proteins with conserved peptide repeats. The third gene, *zobellia_2250*, is clus-

tered with genes encoding a hypothetical protein (*zobellia_2251*) and a 4-hydroxybutyrate coenzyme A (CoA) transferase (*zobellia_2249*). The translated open reading frame of *zobellia_1262* (Zg-VIPO1) shares 46% amino acid identity with that of *zobellia_2088* (Zg-VIPO2) and 20% identity with that of *zobellia_2250* (Zg-VHPO3). Signal peptides (SP) and protein targeting were predicted using SIGNALP v.4.1 (48), LipoP v1.0, Cello v2.5, and PSORT. The protein products of *zobellia_1262* and *zobellia_2250* are expected to carry a type II and a type I SP, respectively, whereas the *zobellia_2088*-encoded protein is predicted to be cytoplasmic. BLASTP alignments of these proteins showed low but significant sequence identities with the VCPO of *C. inaequalis* (23 to 29% identity over an alignment covering 32 to 45% of the protein). The three putative VHPO feature conserved amino acid residues that were shown to be important in the coordination of the vanadate cofactor in 3D structures of fungal and bacterial VCPO and algal VBPO (7–10, 17). A phylogenetic analysis was carried out using protein maximum likelihood (PML) tree reconstruction based on alignment of 124 amino acid sequences, including those of all available eukaryotic homologous VHPO found in the NCBI protein database and of bacterial VHPO homologs from representative complete genomes (Fig. 1; see Fig. S1 and Table S1 in the supplemental material for full tree and sequence information). Five protein sequences of bacterial nonspecific acid phosphatases were included in the analysis, as an external outgroup (4). In the PML tree (Fig. 1), two heterogeneous, separated, and well-supported groups appeared, with a mix of both bacterial and eukaryotic sequences. The first and smallest one contains the VHPO from Stramenopiles and Rhodophyta algae which have been characterized at the biochemical and/or structural level and diverse bacterial proteins (e.g., from *Cyanobacteria*, *Firmicutes*, and *Actinobacteria*) for which no enzyme activity has been reported so far. Inside this cluster, all the brown algal VHPO are closely related and spread into two branches, with one including the VBPO1 of *A. nodosum* and the VIPO from *L. digitata* and the other bearing the second isoform of *A. nodosum* and the VBPO of *L. digitata*. The red algal VBPO form a strong monophyletic group (99% bootstrap support) with a few cyanobacterial VBPO proteins, one of

TABLE 3 Refinement statistics for native and MAD data sets for Zg-VIPO1 crystal structures

Parameter	Value	
	BM30	ID23-1
Resolution range	69.04–2.0	20.01–1.80
R _{work} (R _{free})	16.1 (20.7)	14.0 (18.0)
No. of atoms [B factor (Å ²)]		
Protein	3,280 (16.01)	3,335 (16.52)
Water	283 (22.82)	307 (26.60)
Cofactor	5 (20.87)	5 (14.21)
Ion	1 (18.6)	1 (14.38)
For Ramachandran plot analysis,		
no. (%) of residues in:		
Favored regions	395 (96.6)	397 (99.2)
Allowed regions	11 (2.7)	3 (0.8)
Outlier regions	3 (0.7)	0 (0)
RMSD		
Bond lengths (Å)	0.023	0.022
Bond angles (°)	1.95	1.93

FIG 1 Phylogenetic analysis of VHPO proteins. A PML tree was constructed using a multiple-sequence alignment of characterized and putative VHPO sequences from 124 eukaryotic and selected bacterial species and of five bacterial acid phosphatase sequences. A total of 192 informative residues were used for PML analyses, and bootstrap values (500 replicates) of >65% are provided. The full tree with species names and the corresponding NCBI protein accession numbers are provided in Fig. S1 and Table S1 in the supplemental material. The scale bar represents a difference of 0.1 substitution per site. Where characterized at the biochemical level, the VHPO specificity toward halides, i.e., iodoperoxidase (VIPO), bromoperoxidase (VBPO), or chloroperoxidase (VCPO) activity, is given as indicated by the legend. The available 3D structures of VHPO are indicated by stars.

which was recently characterized as a VBPO in a *Synechococcus* strain (24). On the other part of the tree (Fig. 1), many more bacterial sequences are present, with most of the bacterial phyla represented (mainly *Firmicutes*, *Cyanobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*). Two putative VHPO from *Z. galactanivorans*, encoded by the *zobellia_1262* and *zobellia_2088* gene loci, are grouped with other putative VHPO homologs from marine *Bacteroidetes* and are closely related to other proteins from freshwater and plant-associated *Bacteroidetes* and *Firmicutes* species. The third putative VHPO of *Z. galactanivorans* (encoded by *zobellia_2250*) merges into a separate group that is dominated by diverse bacterial origins. The fungal VCPO, including that of *C. inaequalis*, form a different strong monophyletic group and merge as a sister group of proteobacterial proteins from plant and terrestrial habitat species. Interestingly, the bacterial VCPO proteins characterized for *Streptomyces* species form another strong monophyletic cluster (95% bootstrap support), appearing at the base of algal putative VHPO-like proteins.

Characterization of recombinant vanadium-dependent iodoperoxidases from *Z. galactanivorans*. To characterize the biochemical function of the putative VHPO from *Z. galactanivorans*, the nucleotide sequences of protein-encoding regions, excluding

the sequences of the putative signal peptides, were cloned into expression vectors. With the exception of the third homologous gene (*zobellia_2250*), for which no overexpression was obtained, two recombinant proteins with the expected size of ~50 kDa were purified from the soluble fraction as monomeric proteins, as confirmed by DLS measurements (data not shown), and later were named Zg-VIPO1 (*zobellia_1262*) and Zg-VIPO2 (*zobellia_2088*). Using the TB colorimetric assay, blue or green colorations appeared after a few minutes when the purified native VBPO1 of *A. nodosum* and H_2O_2 were added to the yellow reaction mixture (Fig. 2A). As shown by Verhaeghe et al. (36), this color shift occurred in the presence of iodide or bromide at pH 7.8 and was due to the VHPO-catalyzed formation of TBI_2 or $TBBr_2$ in the reaction mixture, respectively (36) (Fig. 2A). Interestingly, this change of color was detected only in the presence of iodide for Zg-VIPO1 and Zg-VIPO2, not in the presence of bromide (Fig. 2A). Similarly, neither chloroperoxidase nor bromoperoxidase activity was detected using monochlorodimedone as a substrate (data not shown) or *o*-dianisidine in native gel activity assays (Fig. 2B), confirming the strict specificity for iodide oxidation of Zg-VIPO1 and Zg-VIPO2. For steady-state kinetic analyses, the TB iodoperoxidase assay is limited to a pH range of 7 to 8 but is much more

FIG 2 Haloperoxidase activities of purified recombinant VIPO1 (Zg-VIPO1) and VIPO2 (Zg-VIPO2) from *Z. galactanivorans*. (A) Thymol blue assay. The enzymatic reactions were performed at room temperature for 1 h in clear flat-bottomed microplate wells containing 250 μ l 0.1 M phosphate buffer, pH 7.8, 100 μ M thymol blue, 2.5 μ g of Zg-VIPO1, Zg-VIPO2, or purified native VBPO1 from *Ascomyllum nodosum* (An-VBPO1), and 1.5 mM KI and 0.42 mM H_2O_2 for iodoperoxidase assay (KI) or 10 mM KBr and 0.1 mM H_2O_2 for bromoperoxidase assay (KBr). Control assays consisted of no enzyme and/or no H_2O_2 additions in the reaction mixture. (B) In-gel *o*-dianisidine-based assays. Nondenaturing polyacrylamide gels were loaded with MilliQ water (lanes C) as a negative control or with 1 μ l (lanes 1) or 5 μ l (lanes 2) of the purified recombinant Zg-VIPO1 or Zg-VIPO2 enzyme and subsequently stained for chloroperoxidase activity (KCl lanes), bromoperoxidase activity (KBr lanes), and iodoperoxidase activity (KI lanes).

appropriate than triiodide (I_3^-) detection, which presents a number of limits due to the high chemical instability of I_3^- in solution (36). Indeed, the aqueous chemistry of iodine species is very complex and strongly depends on pH conditions. For instance, the chemical dismutation of H_2O_2 in the presence of iodine species should occur at pHs above 7. It is thus not possible to miniaturize the control of the H_2O_2 concentration and to calculate an accurate K_m value for H_2O_2 by using the iodoperoxidase assays. In contrast, and following the protocol proposed by Verhaeghe et al. (36), the kinetic parameters of iodide for both enzymes were determined at pH 7.2 and resulted in $K_m^{\text{I}^-}$ and $k_{\text{cat}}^{\text{I}^-}$ values of 0.22 ± 0.01 mM and 1.98 ± 0.05 s $^{-1}$, respectively, for Zg-VIPO1 and of 0.22 ± 0.02 mM and 2.04 ± 0.08 s $^{-1}$, respectively, for Zg-VIPO2. The structural stability of the recombinant enzymes was also investigated by DLS analyses, which established the melting temperatures of Zg-VIPO1 and Zg-VIPO2 at 42°C and 36°C, respectively. Upon heating for 10 min, purified Zg-VIPO1 remained fully active at temperatures up to 40°C.

Crystal structure determination and overall structure of Zg-VIPO1. The crystal structure of Zg-VIPO1 was solved by multi-wavelength anomalous diffraction (MAD) at 1.8 Å, using a single crystal of seleno-methionine-labeled protein. The native and Se-Met-labeled Zg-VIPO1 proteins crystallized in space group P2₁2₁2₁, with the unit cell parameters reported in Table 2. The final structural models were obtained at 1.8 (Se-Met-labeled protein)- and 2.0 (native crystals)-Å resolutions and showed perfectly superimposable features. Since the data collected at the Se edge diffracted to a higher resolution than that of the native crystals, further structural analysis was performed against these data, unless otherwise mentioned. The asymmetric unit corresponds to a monomer, giving a solvent content of 43% and a crystal volume per protein mass of 2.17 Å³ Da⁻¹ (49). The overall phasing statistics showed that most of the 12 Se-Met sites were not completely substituted (data not shown), with half of them having very low occupancies, down to 0.2. Keeping the methionine residues as such in the structure refinement did not lead to extensive positive

electron density peaks at the methionine positions. In both refinements, the final model of Zg-VIPO1 contained 400 residues of the expected 445 present in the protein construct, together with the vanadate cofactor, one sodium cation, and 307 or 283 water molecules. No halide ion was observed in the vicinity of VO_4 or in the global structure of Zg-VIPO1. More than 99% of the residues were in the most favored regions of the Ramachandran plot, with no outliers. In the crystal collected at the Se edge, Cys260 appeared to be an oxy-cysteine with two alternative conformations. Loops formed by residues Phe173 to Asp178 and Val266 to Thr275 were not visible in the 2F_o-F_c electron density map at 1.8 Å and are presumed to be highly flexible. In the native crystal at 2.0 Å, residues 173 to 176 of the first loop could be modeled; however, the electron density defining this region still remains rather poor.

Zg-VIPO1 had a cylindrical shape with an approximate length of 85 Å and a diameter of 50 Å. Its global monomeric structure was folded into 14 α -helices and two 3_{10} -helices (helices η_1 and η_2), which represented 49.7% of the model (Fig. 3A). In Zg-VIPO1, two β -strands, constituting residues Phe381 to Asp383 and Arg394 to Phe396, formed an antiparallel β -sheet at the surface of the protein. The core of the overall structure was composed of two bundles of five α -helices (Fig. 3A): helices 1 to 5 for bundle 1 and helices 9, 10, and 12 to 14 for bundle 2 (Zg-VIPO1 numbering). The sodium binding site was located at the end of helix 2 and interacted with the protein through three carbonyl groups of the main chain (residues Ala54, Asn57, and Tyr60). The position of this ion formed the end of helix 2 at this position by inducing a change in the direction of the main chain, suggesting a structural role for this sodium ion. The two bundles were related by a 2-fold axis of symmetry parallel to the helices, located between the two bundles (Fig. 3A). Based on amino acid alignment, the Zg-VIPO2 protein shared 13 of the 14 α -helices present in Zg-VIPO1 (see Fig. S2 in the supplemental material).

Comparison to other known VHPO structures showed that the structural topology of Zg-VIPO1 was overall relatively similar to those of both known VCPO structures (Fig. 3A to C), whereas the RMSD between the structure of Zg-VIPO1 and that of *C. inaequalis* VCPO (Ci-VCPO) (1.79 Å) was elevated (Table 4). In agreement with secondary structure topology comparisons (Fig. 3A and D), these deviations were even more pronounced compared to the dimeric algal VBPO of *A. nodosum*, *C. officinalis*, and *C. pilulifera* (Table 4). Helix bundle 1 of Zg-VIPO1 did not have insertions in loops between helices, unlike the helix inserted between helices 2 and 3 in the VCPO of *C. inaequalis* and of *Streptomyces* sp. (Fig. 3B and C). Moreover, the VCPO of *Streptomyces* sp. lacks helix 1 of bundle 1 (Fig. 3B). There were two sites that appeared to be favorable regarding insertions in helix bundle 2. The first was between helices 10 and 12 and contained helix 11, which was also conserved in all other VHPO. The second insertion was found between helices 12 and 13 and was formed by two β -strands separated by a 3_{10} -helix. Insertions in this loop were also observed in other structures of VHPO, but with different motifs (Fig. 3B to D). Furthermore, in Zg-VIPO1, an additional highly flexible loop was present between helices 8 and 9 and seemed to block the entrance of the active site (Fig. 4A). At the N-terminal part of this loop, the side chain of Tyr263 appeared to be the main barrier to the active site (Fig. 4A). Absent in Ci-VCPO and other VHPO structures (Fig. 3B to D), this amino acid insertion was found at the same position in only the 10 closest protein homologs

FIG 3 Comparison of secondary structure topologies of VHPO. (A) VIPO1 from *Z. galactanivorans* (Zg-VIPO1) (PDB accession number 4CIT). (B) VCPO from *Streptomyces* sp. CNQ525 (Ssp-VCPO) (PDB accession number 3W36). (C) VCPO from *Curvularia inaequalis* (Ci-VCPO) (PDB accession number 1IDQ). (D) VBPO1 from *A. nodosum* (An-VBPO1) (PDB accession number 1Q19). The first-bundle helices (numbered 1 to 5) are colored green for Zg-VIPO1, Ci-VCPO, and Ssp-VCPO, and the conserved helices between the four VHPO are represented in dark blue. The second monomer of An-VBPO1 is drawn in light blue. The monomers of Zg-VIPO1, Ci-VCPO, and Ssp-VCPO and the homodimer of An-VBPO1 are drawn in both 2D and 3D representations.

TABLE 4 RMSD between the 3D structures of different VHPO, based on the entire 3D atomic backbones^a

VHPO	RMSD relative to:			
	An-VBPO1 (1QI9)	Co-VBPO (1QHB)	Cp-VBPO (1UP8)	Ci-VCPO (1IDQ)
Zg-VIPO1	2.608	2.259	2.548	1.791
An-VBPO1		1.418	1.633	2.948
Co-VBPO			0.482	3.164
Cp-VBPO				2.584

^a Data in parentheses are PDB accession numbers. Zg, *Zobellia galactanivorans*; An, *Ascophyllum nodosum*; Co, *Corallina officinalis*; Cp, *Corallina pilulifera*; Ci, *Curvularia inaequalis*.

of Zg-VIPO1, including Zg-VIPO2 (see Fig. S2 in the supplemental material).

Vanadium-binding active site of Zg-VIPO1. The nature of the cofactor bound in the active site pocket was further investigated because of the presence of phosphate (PO_4) during the crystallization process. Electron density maps were obtained after refinement with either vanadate or inorganic phosphate in the active site of Zg-VIPO1 (Fig. 4B and C). The $F_o - F_c$ Fourier difference maps

revealed a lack of electrons close to the P atom for the PO_4 -refined structure (Fig. 4C), suggesting the presence of a heavier atom. Moreover, the electron density clearly showed a coordination sphere of five ligands around the central atom, which is incompatible with binding of a PO_4 group. These observations were in favor of a VO_4 moiety in the active site of the recombinant Zg-VIPO1 protein, covalently bound to the $\text{N}^{\epsilon 2}$ of His416 at a distance of 2.16 Å (Fig. 4B). This histidine residue was located between helices 13 and 14 at the GIH motif (residues 414 to 416) (see Fig. S2 in the supplemental material) and was strictly conserved in all VHPO structures. In Zg-VIPO1 (Fig. 4D), residues of the first coordination sphere of vanadate were Trp321 and Lys324, in the C-terminal part of helix 10; Arg331, in the C-terminal part of helix 11; Phe353, between helices 11 and 12; Ser358, Gly359, and His360, in the N-terminal part of helix 12; and Arg410, in the C-terminal part of helix 13. Zg-VIPO2 features identical amino acids at these positions (see Fig. S2). Interestingly, all nine of these residues were also strictly conserved with the fungal *C. inaequalis* VCPO (Fig. 4E), and both active site structures were very similar, with an RMSD between these residues of 0.258 Å. Compared to the Zg-VIPO1 active site (Fig. 4D), the *Streptomyces* sp. VCPO and all red and brown algal VBPO had a histidine residue in place of Phe353.

FIG 4 Vanadate active site in Zg-VIPO1 structure. (A) External loop at the entrance of the Zg-VIPO1 active site, between helices 8 and 9. The ribbon diagram is colored according to the α -carbon thermal motion based on B-factor calculation, showing stable (cold, blue) to highly mobile (warm, orange) residues. (B and C) Electron density maps calculated in the vicinity of the His416 residue after refinement with VO_4 (B) or PO_4 (C) atoms. Blue maps correspond to the Fourier maps ($2F_o - F_c$) and green maps to the positive Fourier difference maps ($F_o - F_c$), contoured at the 1σ and 3σ levels, respectively. (D and E) Vanadate binding site and surrounding amino acid residues in Zg-VIPO1 (D) compared to those of Ci-VCPO (PDB accession number 1IDQ) (E). For clarity, only the hydrogen bonds involving the VO_4 moiety and surrounding residues are represented with yellow dashed lines. In all figures, the selected residues and cofactors are represented as sticks and numbered according to the Zg-VIPO1 and Ci-VCPO protein sequences.

Moreover, the bacterial VCPO had a serine residue (Ser427) instead of His360 in Zg-VIPO1, and the two red algal VBPO, from *C. pilulifera* and *C. officinalis* (10), displayed an arginine in place of Trp321 of Zg-VIPO1.

To further analyze the coordination of VO_4 in the active site of Zg-VIPO1, the local hydrogen bonding network was compared with that of Ci-VCPO (see Table S2 in the supplemental material). Six of nine residues surrounding VO_4 were directly involved in hydrogen bonds with the oxygen atoms of vanadate in Zg-VIPO1, in the same way as in Ci-VCPO (Fig. 4D and E). For both enzymes, these nine residues were involved in a total of 16 H bonds between them. Analyzing the second coordination sphere of the VO_4 center, additional hydrogen bonds were identified as linking other amino acid residues to the nine residues of the first sphere, through 30 and 33 hydrogen bonds in Zg-VIPO1 and Ci-VCPO, respectively (see Table S2). Some significant differences in this second sphere of VO_4 coordination appeared between Zg-VIPO1 and Ci-VCPO. The Ser358 residue of Zg-VIPO1 was H bonded to six residues, including three present in the active site (His360, Arg410, and His416), while the corresponding residue of Ci-VCPO (Ser402) was linked to four residues (see Table S2). Comparatively, the VO_4 -coordinating His416 residue in Zg-VIPO1 was coordinated by six H bonds, while eight H bonds stabilized the corresponding residue (His496) in Ci-VCPO (see Table S2). Two other differences were notable between the two active site H-bonding networks: the first concerned Gly359 (Gly403 in Ci-VCPO), which was H bonded to Cys320 in Zg-VIPO1, whereas the corresponding glycine in Ci-VCPO was bound to His404; the second concerned Arg331 (in Zg-VIPO1), which featured three H bonds instead of five for the equivalent residue (Arg360) in Ci-VCPO (see Table S2).

Site-directed mutagenesis and steady-state kinetics of mutants. A site-directed mutagenesis approach was undertaken, based on structural differences observed between the different VHPO active sites, in order to identify amino acids essential for the catalytic mechanism and for iodide specificity in Zg-VIPO1. Five residues in the close vicinity of the VO_4 moiety of Zg-VIPO1 (Fig. 4D) were targeted, to produce His360Ala, Arg410Ala, Phe353His, Trp321Ala, and Ser358Ala single mutations. Two residues belonging to the second coordination sphere, Cys320 and Asp322, were mutated to serine and to lysine and tyrosine, respectively. Finally, to explore the structural role of the extra loop between helices 8 and 9, Tyr263 (Fig. 4A) was replaced by phenylalanine, serine, or alanine.

The enzymatic activity and specificity for halide of the 12 mutants of Zg-VIPO1 were analyzed using TB colorimetric assays in the presence of iodide or bromide at pH 7.8 (Fig. 5). For the Arg410Ala, Asp322Tyr, His360Ala, and His360Ser mutants, the reaction mixtures remained yellow, as in the control without enzyme, suggesting a complete loss of haloperoxidase activity. Similarly to the wild-type (WT) Zg-VIPO1 enzyme reaction, blue or green coloration was observed only with iodide for the Tyr263Phe, Tyr263Ser, Cys320Ser, Trp321Arg, Asp322Lys, and Phe353His mutants. This coloration also appeared in the presence of bromide for two mutants, the Ser358Ala and Tyr263Ala mutants, showing the formation of TBBBr_2 in the reaction mixture. In order to compare the enzymatic properties of the mutants and the wild-type recombinant Zg-VIPO1 enzyme, the kinetic parameters were determined using the TB assay for iodoperoxidase activity at pH 7.2 (Table 5). Bromoperoxidase activity, observed for the Ser358Ala

FIG 5 Thymol blue colorimetric assays of purified recombinant wild-type and site-directed mutant Zg-VIPO1 from *Z. galactanivorans*. Both thymol blue iodoperoxidase (in the presence of KI) and bromoperoxidase (in the presence of KBr) assays were performed at room temperature and pH 7.8. Control assays consisted of no enzyme as a negative control (Control) and purified native VBPO1 from *Ascophyllum nodosum* (An-VBPO1) as a positive control.

and Tyr263Ala mutants, was too low for determination of corresponding kinetic parameters. In agreement with the colorimetric assays (Fig. 5), four mutants (Arg410Ala, Asp322Tyr, His360Ala, and His360Ser) showed a drastic decrease in catalytic efficiency ($k_{\text{cat}}^{I^-}/K_m^{I^-}$). Among those showing TB iodination activity, the Ser358Ala mutant had a $K_m^{I^-}$ similar to that of WT Zg-VIPO1, whereas the $K_m^{I^-}$ values of the other mutants increased by factors of ~ 2 to ~ 12 . The catalytic turnover constants ($k_{\text{cat}}^{I^-}$) of the Trp321Arg and Ser358Ala mutants were not significantly changed compared to that of the wild-type enzyme. In contrast, the Tyr263Ala/Phe/Ser, Cys320Ser, Asp322Lys, and Phe353His mutations increased the $k_{\text{cat}}^{I^-}$, up to 26-fold (Table 5).

DISCUSSION

Although exponentially increasing sequence data on VHPO-like genes are currently available for numerous bacterial lineages, the biochemistry and structure of bacterial VHPO have barely been explored (4), and the molecular bases of their specificity for oxidation of halides have remained elusive until now. In the current study, we characterized a novel bacterial vanadium-dependent iodoperoxidase from the marine bacterium *Zobellia galactanivorans*, described its 3D crystallographic structure, and investigated the structural basis of its enzymatic activity and iodide specificity by using site-directed mutagenesis.

Two bacterial recombinant VIPO, named Zg-VIPO1 and Zg-VIPO2, were shown to specifically oxidize iodide (Fig. 2) with similar catalytic turnover rates ($k_{\text{cat}}^{I^-}/K_m^{I^-}$ of 9.14 and 9.32 $\text{s}^{-1} \text{mM}^{-1}$ for Zg-VIPO1 and Zg-VIPO2, respectively). They present a $K_m^{I^-}$ similar to that of VBPO1 from *A. nodosum* (An-VBPO1), based on the thymol blue assay (36), but display a catalytic turnover rate reduced by a factor of 40 compared to that of An-VBPO1 ($k_{\text{cat}}^{I^-}$ of 75 s^{-1}). For *L. digitata*, the iodide-specific VIPO was shown to be more closely related to this algal VBPO (13), and there is no similarity between the bacterial and algal vanadate active centers of VIPO. This is also illustrated by their distant

TABLE 5 Kinetic parameters of WT Zg-UIPO1 and mutants, determined by thymol blue colorimetric assay

Enzyme	K_m^{-1} (mM) ^a	% of WT value	k_{cat}^{-1} (s ⁻¹) ^a	% of WT value	k_{cat}^{-1}/K_m^{-1} (s ⁻¹ mM ⁻¹)	% of WT value
Zg-UIPO1 (WT)	0.22 ± 0.01	100	1.98 ± 0.05	100	9.00	100
Tyr263Ala mutant	0.44 ± 0.17	200	3.53 ± 0.25	178	8.02	89
Tyr263Phe mutant	0.79 ± 0.07	359	2.67 ± 0.08	135	3.38	38
Tyr263Ser mutant	1.58 ± 0.30	718	13.97 ± 2.43	706	8.84	98
Cys320Ser mutant	0.91 ± 0.28	414	2.76 ± 0.87	139	3.03	34
Trp321Arg mutant	0.39 ± 0.11	177	1.78 ± 0.22	90	4.56	51
Asp322Lys mutant	1.38 ± 0.43	627	3.86 ± 1.1	195	2.80	31
Asp322Tyr mutant	0.03 ± 0.03	14	0.0007 ± 0.0006	0.04	0.02	0.3
Phe353His mutant	2.63 ± 0.53	1,195	53.27 ± 10.77	2,690	20.25	225
Ser358Ala mutant	0.19 ± 0.06	86	2.19 ± 0.07	111	11.53	128
His360Ala mutant	0.02 ± 0.01	9	0.01 ± 0.0003	0.5	0.50	6
His360Ser mutant	0.05 ± 0.08	23	0.002 ± 0.001	0.1	0.04	0.4
Arg410Ala mutant	3.45 ± 1.07	1,568	0.03 ± 0.01	1.5	0.01	0.1

^a Values are means ± SD.

positions in the PML tree (Fig. 1). Altogether, these results strongly support the fact that iodide specificity appeared at least twice, and independently, during the evolution of VHPO enzymes. While the iodine metabolism in brown algal kelps (50) is related to defense mechanisms, the presence of two UIPO genes in the genome of *Z. galactanivorans* calls into question their biological role in marine flavobacteria. *Z. galactanivorans* efficiently degrades brown algal cell walls due to specialized enzymes, such as glycoside hydrolases or alginate lyases (30, 31). The enzymatic degradation of the cell wall of kelps is likely to lead to a rapid liberation of reactive oxygen species (ROS) and a remobilization of the algal iodide pool (51, 52). During iodine-rich cell wall degradation, *Z. galactanivorans* may specifically mobilize UIPO, putatively secreted, to cope not only with ROS but also with large amounts of I⁻, by converting I⁻ and H₂O₂ into I₂, H₂O, and O₂. Our data mining shows that homologs of VHPO with unknown functions are also widespread in many bacterial lineages. Their natural substrates and physiological roles should be explored further by functional approaches. In marine bacteria, VHPO might play important roles in the biosynthesis of bioactive secondary metabolites, such as antibiotics in actinobacteria (26, 27), or in the emission of volatile bromocarbons during biotic interactions (53). The large distribution of these enzymes also suggests a significant bacterial contribution of VHPO-mediated processes to the iodine cycling in marine environments (54).

The structural characterization of Zg-UIPO1 from *Z. galactanivorans* showed that the enzyme is mainly folded into α-helices (Fig. 3), similar to the case in the five 3D structures of VHPO already available (6–8, 10; PDB accession number 3W36). The two helix bundle domains of the Zg-UIPO1 monomer can be superimposed with those of *C. inaequalis* (Ci-VCPO) and *Streptomyces* sp. (Ssp-VCPO) VCPO and with the homodimeric algal VBPO (Fig. 3A to D), in agreement with a common origin of VHPO (4). The putative ancestral core domain is defined by only eight helices, i.e., the five helices of helix bundle 2 and helices 7, 8, and 11 (shown in dark blue in Fig. 3), which are conserved in the bacterial UIPO, fungal and bacterial VCPO, and algal VBPO structures.

Our phylogenetic analysis suggests that the monomeric type of VHPO, those close to Zg-UIPO1, Ssp-VCPO, and Ci-VCPO, is overrepresented in bacterial lineages (Fig. 1), especially marine ones. Based on screening of eukaryotic protein databases, it appears that relatively few eukaryotic lineages feature VHPO ho-

mologs. This “patchy” emergence of eukaryote VHPO among bacterial lineages is likely a consequence of independent lateral gene transfers from bacterial counterparts, as illustrated by the external position of fungal VCPO in the phylogenetic tree (Fig. 1). The three algal VBPO for which 3D structures are available merge independently from the “monomeric-type” VHPO in the phylogenetic tree. These dimeric enzymes do not possess helix bundle 1 (shown in green in Fig. 3A to C), which is replaced by the helix bundle of a second, identical monomer (shown in light blue in Fig. 3D). The loss of helix bundle 1, through incomplete gene transfer, for instance, most likely exposed a hydrophobic patch at the surface; the replacement of this helix bundle thus may have facilitated dimerization, later on further stabilized by multimerization (55) in red algal VBPO dodecamers (8) or by covalent disulfide bonds between the two monomers, such as in brown algal VHPO homodimers (7). Other putative algal VHPO genes closely related to VCPO genes from *Streptomyces* sp. (Fig. 1) have been identified in whole sequenced genomes of the red alga *Chondrus crispus* (56), the brown alga *Ectocarpus siliculosus* (57), and the diatom *Thalassiosira oceanica* (58), suggesting that marine algae evolved both types of VHPO. Consequently, and based on phylogeny (Fig. 1) and topology comparisons (Fig. 3), we propose that VHPO most likely derived from a bacterial ancestral enzyme with a monomeric conformation, probably from marine prokaryotes.

The enzymatic characterization of Zg-UIPO1 single amino acid mutants showed that both His360 and Arg410 have important roles in the catalytic cycle, since changing them into alanine or serine totally switched off iodoperoxidase activity (Fig. 5 and Table 5). The same importance was already shown for the corresponding residues, His404 and Arg490, in Ci-VCPO (59, 60). This underlines that the conserved histidine residue (e.g., His360 in Zg-UIPO1, His404 in Ci-VCPO, and His418 in An-VBPO1), often presented as the catalytic residue (60), is certainly not the only essential residue for VHPO activity. Taken together, these results suggest that the coordination of three oxygen atoms of the vanadate cofactor through hydrogen bonds with both arginine (Arg410 in Zg-UIPO1) and histidine (His360 in Zg-UIPO1) residues plays a key role in the catalytic cycle. In the active site of VCPO from *Streptomyces* sp. (PDB accession number 3W36), a serine residue replaces the histidine residue. Interestingly, we found that the mutation of residues outside the active site can also suppress the enzymatic activity of Zg-UIPO1, as observed for the

Asp322Tyr mutant (Fig. 5 and Table 5). In this case, we hypothesize that the presence of a tyrosine residue at the molecular surface may deeply alter the conformation of this part of helix 11, which bears two residues of the first coordination sphere of vanadate, i.e., Trp321 and Lys324 (Fig. 4D). In Ci-VCPO, a homologous lysine residue (Lys353) was proposed to be involved in the interaction with the peroxo-vanadate intermediate during the catalytic cycle (59). In the same way, in Zg-VIPO1, mutations of residues in the active site, i.e., Trp321Arg and Phe353His, had important effects on kinetic parameters of the enzymes (Table 5). This was also observed for Cys320Ser and Asp322Lys mutations, located at two residues of the second coordination sphere which interact with Trp321, Lys324, Arg331, and Gly359 (see Table S2 in the supplemental material). For instance, the Phe353His mutant, which mimics the vanadate binding site of VBPO1 from *A. nodosum* (7), showed the strongest increases of the $K_m^{I^-}$ and $k_{cat}^{I^-}$ values, of 12- and 26-fold, respectively, compared to those of the wild-type enzyme, with a highly improved catalytic efficiency (Table 5). In contrast, the Phe397His mutant of Ci-VCPO presented decreases in chloro- and bromoperoxidase activities (21). Altogether, our results suggest that the catalytic properties of Zg-VIPO1 are dependent on the structural conservation of amino acids flanking the VO_4 moiety, including at least Lys324, His360, and Arg410, but also Trp321 and Phe353, which are themselves stabilized by a complex network of interacting residues.

Concerning the specificity of VIPO, the similar active sites of Zg-VIPO1 and Ci-VCPO reveal that the exclusive iodide oxidation by Zg-VIPO1 must be controlled by something beyond the nine residues directly surrounding the VO_4 moiety, as Ci-VCPO is able to react with chloride, bromide, and iodide. If a transitory binding pocket for halide exists in the vicinity of the vanadate cofactor (23), this cannot by itself explain the different reactivities of these two enzymes toward halides. In agreement with this hypothesis, the transformation of the Zg-VIPO1 active site into a VBPO-like (Phe353His mutant) or bacterial VCPO-like (His360Ser mutant) active site does not confer bromoperoxidase or chloroperoxidase activity. Interestingly, conversion of two amino acid residues to alanine, one inside the active site (Ser358), as found in the active site of VIPO from *L. digitata* (13), and the other (Tyr263) located on the external loop of Zg-VIPO1 (Fig. 4A), led to a slight bromoperoxidase activity of Zg-VIPO1 (Fig. 5). The $K_m^{I^-}$ of the Ser358Ala Zg-VIPO1 mutant was not significantly modified (Table 5), suggesting that the change of halide specificity in Zg-VIPO1, i.e., the switch from iodoperoxidase to bromoperoxidase activity, is linked not only to the alteration of iodide oxidation but rather to the increase of redox potential of the peroxo-vanadium intermediate, favoring its reactivity with bromide. In the Ser358Ala Zg-VIPO1 mutant, the lack of Ser358-mediated hydrogen bonds with the equatorial oxygen atom of vanadate and with His360, Arg410, and His416 residues is likely to strongly modify the redox potential of the Zg-VIPO1 active site. In Ci-VCPO, a similar mutation (Ser402Ala) had a different effect and reduced the rate of enzyme activity toward chloride and bromide oxidation (21). However, significant differences in the H-bonding network have been identified surrounding the active site residues of these two VHPO (see Table S2 in the supplemental material). As already proposed (4, 15, 36), our results on Zg-VIPO1 support the current hypothesis that explains the change of halide specificity by modifications of H-bond coordination and redox potential of the VO_4 moiety rather than by selective halide

binding. We further demonstrate that this redox potential is fine-tuned not only locally but also by remote amino acid residues, as illustrated by the change of specificity of the Tyr263Ala mutant. This tyrosine residue is present at the N-terminal part of the additional flexible loop (Fig. 4A) in Zg-VIPO1. The Ala/Phe/Ser mutations of this Tyr263 also affected the kinetic constants of the enzyme (Table 5). This suggests a structural role in catalysis for this loop, which seems only to be present in the enzymes of the same clade (see Fig. S2).

In conclusion, both the structural comparison and phylogeny suggest that VHPO derived from a common marine bacterial ancestor that was closely related to bacterial acid phosphatases and that the specificity for iodide oxidation resulted in a convergent evolution of the divergent phyla *Bacteroidetes* and *Stramenopiles*. In terms of structural characteristics, the Zg-VIPO1 active site shares the same residues around the vanadate as the fungal chloroperoxidase Ci-VCPO. Given their different enzymatic specificities, the nine residues surrounding the vanadate are thus important for the fixation of the VO_4 cofactor and the first reaction step (coordination of hydrogen peroxide to vanadate) but are not the unique factors for the catalytic properties and halide specificity of VHPO. Indeed, our data clearly support the fact that a complex hydrogen bonding network, involving a large number of residues that directly or indirectly coordinate the vanadate center, is essential for the catalytic properties and also for halide specificity. The structural characterization of this bacterial VIPO, combined with site-directed mutagenesis, has filled some gaps in our knowledge about bacterial and eukaryotic VHPO. To further understand the fine-tuning of the VO_4 moiety and its importance for the catalytic cycle and halide reactivity, additional biochemical and local structural studies, such as X-ray absorption spectrum studies of the vanadium cofactor, will be necessary to infer the global evolution of VHPO enzymatic mechanisms, especially within bacterial species. These studies are likely to lead to novel biotechnological developments to exploit the potential of halogenation reactions catalyzed by these enzymes. Finally, these studies lay the basis to gain more knowledge about the physiological roles of VHPO in marine bacteria.

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