Bromine is an Endogenous Component of a Vanadium Bromoperoxidase

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Brown algae such as *Ascophyllum nodosum* and *Laminaria digitata* have the ability to accumulate halogens like Br and I from seawater to an amazing extent. Haloperoxidases, enzymes that incorporate halide ions into organic substrates with H_2O_2 as a coreactant, are essential for the mediation of halogen uptake,[1] and are involved in the biosynthesis of various halogenated natural products.[2] They contain a V cofactor in the form of vanadate[3] which appears to interact with H_2O_2 only, not with Br^- or the organic substrate.[4] The absence of coordination of Br^- to V is well established by X-ray absorption spectroscopy (XAS) at the vanadium K edge.[5] In Br K edge extended X-ray absorption fine structure (EXAFS) studies[6] where *Ascophyllum nodosum* bromoperoxidase (*An*vBPO) was titrated with Br^- in the absence of H_2O_2, it was reported that Br is incorporated in the enzyme by formation of a covalent bond. This Br was proposed to be bound to an amino acid in the active site, viz. a Ser residue which is H-bonded to the vanadate, with implications for the catalytic cycle. We have recently interpreted the EXAFS of a number of relevant I and Br reference compounds,[7] and demonstrated that it is possible to distinguish the bond lengths between halogens and sp^2- and sp^3-hybridized carbons. This prompted us to investigate the proposed reactive Br intermediate in *An*vBPO in more detail.

Our experimental approach initially followed that described in the literature[5] in which XAS spectra at the Br K edge were taken of *An*vBPO in the presence of varying amounts of Br^-, up to 1 molar equivalent. We hardly detected EXAFS contributions characteristic[6,7] of non-covalently bound, hydrated Br^- in our titration.[8] The Fourier transform (FT) showed significant peaks up till approx. 6 Å, which is exceptional for biological EXAFS. The pattern of peaks reminded us of that characteristic of a phenyl ring which is meta-disubstituted with halogens,[7] and indeed a good match was found with the spectrum of 3,5-dibromotyrosine (Br_2Tyr). It is unlikely that the enzyme would be able to use the Br^- added in the titration to incorporate it into its aromatic groups in the absence of H_2O_2. We therefore hypothesized that the Br was already incorporated in the aromatic groups in the enzyme at the start of our titration, to such an extent that its EXAFS signal dominated that of the Br^- that had been added in near-equivalent amounts. This hypothesis was confirmed by the result of a blank experiment in which the Br EXAFS of *An*vBPO was measured in the absence of added Br^-; the agreement with the results for Br_2Tyr and the mixed halogen aromatic reference compound 3-bromo-5-iodobenzoic acid (BrIBA) is excellent (Fig. 1).

The enzyme has probably had opportunities to react with both Br^- and H_2O_2, either during its translation and folding or under physiological circumstances in the alga. It is also possible that the enzyme was halogenated during extraction and purification. If Br

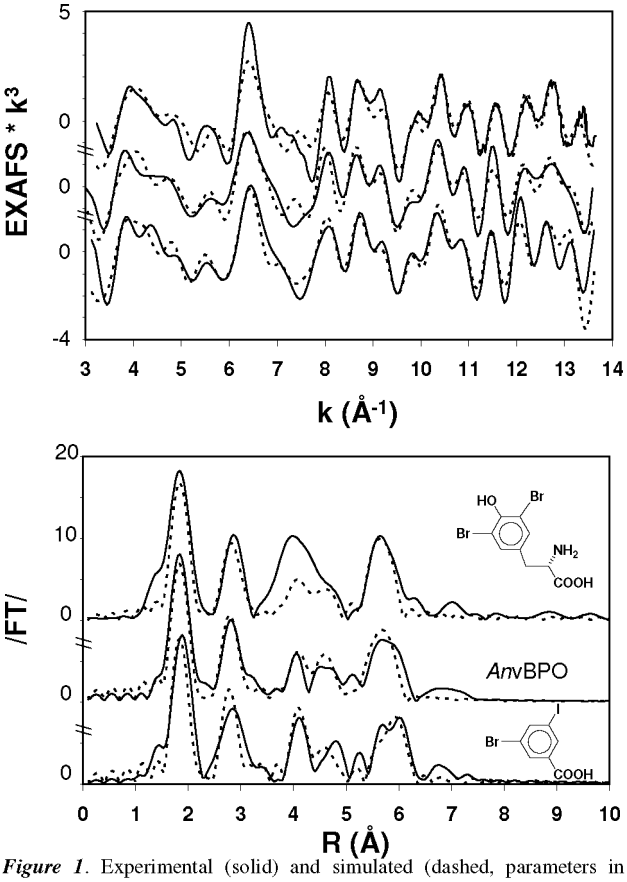

Figure 1. Experimental (solid) and simulated (dashed, parameters in Chart 1) Br K EXAFS (top panel) and phase-corrected FT (bottom panel) of (top to bottom) Br_2Tyr in BN (raw data), 1.3 mM *An*vBPO in 50 mM Tris-HCl, pH 9.0, and BrIBA in aqueous 1 M KOH (Fourier-filtered 0.8-7.0 Å).

appears to be incorporated on Tyr residues of the enzyme, there may have been opportunities for I to be incorporated as well. Preliminary XAS experiments at the iodine L3 edge revealed that the native enzyme indeed contains I.[8] Guided by the results for our reference compounds (Fig. 1) we undertook simulations of the *An*vBPO Br EXAFS to determine whether the other halogen atom in the brominated aromatic ring was Br or I. Following an approach described in detail elsewhere,[7] the EXAFS spectra were simulated with the programme EXCURVE,[9] using distances derived from the crystal structure[10] of the methoxy analogue of Br_2Tyr as restraints[11] for the ring atoms in the refinement. The results of the refined simulations (dashed lines in Fig. 1,

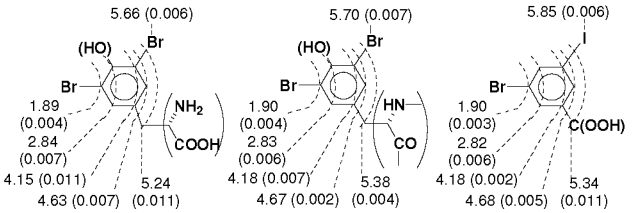

Chart 1. Distances (Å) to the Br absorber atom found in the refined simulations for the spectra in Fig.1. Left, Br_2Tyr; middle, native *An*vBPO; right, BrIBA. Atoms in parentheses were not included in the simulation. Debye-Waller-type factors as $2\sigma^2$ (Å^2) in parentheses. Errors for 1st-shell distances 0.02 Å, other distances 0.05 Å.

geometries in Chart 1) for the Br_2Tyr EXAFS are consistent with those of the crystal structure,[10] including the Br-Br distance (5.664 Å vs. 5.672 Å), while the Br-I distance in BrIBA was somewhat longer (5.845 Å), in line with the larger atomic radius of I. For *An*vBPO the best fit (Fig. 1) was with Br in the meta-position and an optimum occupancy of 90 % of that of the ring atoms. Mixtures of Br and I in ratios between 1:1 and 2:1 also gave reasonable fits[8] (fit index 0.4034) but not as good as Br alone (0.3266).

ESI-Q-TOF mass spectrometry (MS) analyses were performed on trypsin-digested *An*vBPO, in order to determine which and how many amino acids were halogenated. Many observed peptides correspond with a good accuracy to the predicted ones and cover 63% of the protein sequence. However, only one of them appears to contain 2 Br as indicated by the addition of 157.8 Da (Table 1) to the second Tyr, Y447, in the corresponding MS/MS spectra;[8] both the non-halogenated and the doubly brominated peptides are observed, but no mono-brominated or iodinated peptides.

Interestingly, the crystal structure of *An*vBPO[12] reveals the presence of two iodinated Tyr residues at the surface of each of both monomers, Y447 and Y398. The choice of I to fit the residual electron density in the crystal structure determination appears to be rather arbitrary, and it was proposed that the crystal contains different conformers of a mono-iodinated Tyr. However, the modeling of I atoms is problematic, since it results in strong negative peaks in the F_o-F_c electron density map, even with a partial occupancy of 0.5. Therefore, the compatibility of bromination of these tyrosines in the crystal structure was tested by reconducting structural refinement with REFMAC[13] (version 5). To this purpose, the coordinates and structure factors of *An*vBPO[12] (id codes 1QI9 and r1qi9sf) were submitted to refinement replacing the iodinated Tyr by Br_2Tyr (using distances obtained by EXAFS, see below). Clearly, the structural model assuming Br_2Tyr as residue 447 in both monomers and a 3-monobrominated Tyr as residue 398 of monomer A is in better agreement with the experimental crystallographic data.[8] This is demonstrated by the lower R_{free} value obtained (18.5%) after one refinement cycle. However, the occupancy of the Br positions is not 100%. The best R (15.3%) and R_{free} (18.5%) values were obtained with 70% 3-bromo- and 55% 5-bromotyrosines at position 447, and the results were identical for the two monomers. In comparison, the R values for the deposited structure were 16.5% (R) and 21.9% (R_{free}), respectively.[12] At these occupancy values, negative peaks almost disappeared from the F_o-F_c electron density map.[8] The occupancy of the Br position in Y398 remains very low (~20%), in agreement with MS analyses, where no halogenation of this Tyr was detected.

In conclusion, using a combination of experiments (EXAFS, MS/MS of tryptic digests) and modeling (structure refinement)

Table 1. MS/MS analyses of the 438-454 residues tryptic peptides from *An*vBPO

MH^+(obs.) Da	MH^+(calc.) Da	ΔM Da	*Peptide sequence*
1881.86	1881.77	0.09	GGDCYPDPVYPDDDGLK
2039.67	2039.57	0.10	GGDCYPDPVXPDDDGLK

X = Br_2Tyr

we find that native *An*vBPO contains Br in the surface Tyr residues 447 (frequently di-brominated) and 398 (rarely mono-brominated). These brominated Tyr residues are not likely to be reactive intermediates in the catalytic cycle of *An*vBPO due to their location at the surface and the nature of the Br-Tyr bond. I XAS reveals I incorporation to a lower extent, which is not detectable by MS/MS. Our results are an important correction to the iodinated Tyr and reactive brominated Ser residues that were proposed in earlier crystallographic[12] and spectroscopic[6] studies, respectively. The reactive role of this Ser residue in haloperoxidases was already controversial because of lack of both solvent accessibility[14] and an effect of mutation to Ala.[15] In future work, we will address the questions of the origin of this posttranslational modification in *An*VBPO, of possible differences in enzymatic efficiency between halogenated and non-halogenated *An*vBPO, and of the halogenation of algal vanadium haloperoxidases in general.

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Supporting Information Available: protein experimental, XAS and MS details and figures, calculated electron density map.

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Abstract:
Based on EXAFS and MS/MS experimental results and a reinterpretation of the electron density map obtained by X-ray crystallography, we describe a new post-translational modification, viz. a 3,5-dibromotyrosine residue that is incorporated in the polypeptide chain of a vanadium haloperoxidase.

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