Inactivation and site-specific oxidation of aquatic extracellular bacterial leucine aminopeptidase by singlet oxygen

Christine M. Egli,^{†,‡} Michael A. Stravs,[§] and Elisabeth M.L. Janssen^{*,‡}

[†]Institute of Biogeochemistry and Pollutant Dynamics, *ETH Zurich, Switzerland*

[‡]Eawag, Swiss Federal Institute of Aquatic Science and Technology, *Switzerland*

[§]Institute of Molecular Systems Biology, *ETH Zurich, Switzerland*

TOC Art

* E-mail: elisabeth.janssen@eawag.ch, Phone: +41 58 765 5428

Abstract

Extracellular enzymes are master recyclers of organic matter and to predict their functional lifetime, we need to understand their environmental transformation processes. In surface waters, direct and indirect photochemical transformation is a known driver of inactivation. We investigated molecular changes that occur along with inactivation in aminopeptidase, an abundant class of extracellular enzymes. We studied the inactivation kinetics and localized oxidation caused by singlet oxygen, $^1\text{O}_2$, a major photochemically-derived oxidant towards amino acids. Aminopeptidase showed second order inactivation rate constants with $^1\text{O}_2$ comparable to those of free amino acids. We then visualized site-specific oxidation kinetics within the three-dimensional protein and demonstrated that fastest oxidation occurred around the active site and at other reactive amino acids. However, second order oxidation rate constants did not correlate strictly with the $^1\text{O}_2$ -accessible surface areas of those amino acids. We inspected site-specific processes by a comprehensive suspect screening for 723'288 possible transformation products. We concluded that histidine involved in zinc coordination at the active site reacted slower than expected by its accessibility and we differentiated between two competing reaction pathways of $^1\text{O}_2$ with tryptophan residues. This systematic analysis can be directly applied to other proteins and transformation reactions.

Introduction

Extracellular enzymes are released by heterotrophic microorganisms and are drivers of biogeochemical cycles because they liberate bioavailable substrates from organic matter in the environment.^{1,2} Their extracellular state in surface waters makes them vulnerable to biotic and abiotic transformation processes that determine their active lifetime. A better understanding of the transformation processes of extracellular enzymes ultimately facilitates our ability to predict cycling of carbon and nutrients.³ Previous studies have shown that exposure to sunlight in surface waters is one major pathway that leads to enzyme inactivation both via direct and indirect photochemical processes.⁴⁻⁷ While direct photochemical processes are more important in the top centimeters of the water column, indirect photochemical processes dominate below the water surface in the upper meter.^{4,8-11} Indirect photochemistry involves light absorbance by chromophoric dissolved organic matter and formation of photochemically produced reactive intermediates. Singlet oxygen ($^1\text{O}_2$) and hydroxyl radicals are among the most abundant and reactive oxygen species produced by indirect photochemistry. Hydroxyl radical is a non-selective oxidant that can oxidize amino acids with up to diffusion-controlled reaction rate constants. However, the more selectively reacting $^1\text{O}_2$ has a significantly higher steady-state concentration in sunlit surface waters ranging from 10^{-12} to 10^{-14} M compared to hydroxyl radical (10^{-15} to 10^{-17} M).^{9,10,12-14}

In proteins, $^1\text{O}_2$ reacts predominantly with five amino acids: histidine (His, H), methionine (Met, M), cysteine (Cys, C), tyrosine (Tyr, Y) and tryptophan (Trp, W).^{8,15} While the reaction mechanisms and modifications for the reaction with $^1\text{O}_2$ have long been identified for freely dissolved amino acids, more recently, differences of oxidation rates have been observed for amino acid residues incorporated in proteins.¹⁶⁻²⁰ These studies demonstrate that the decay rate of amino acid residues is highly dependent on the accessibility to the oxidant in solution. Thus, highly buried residues in the protein are expected to experience a lower oxidant

concentration and consequently decay slower compared to exposed ones. For example, research showed that denaturation resulted in a 2-fold increase of $^1\text{O}_2$ oxidation rates compared to the folded cytochrome-c and site-specific oxidation was observed in capsid and assembly proteins during virus inactivation by $^1\text{O}_2$.¹⁸⁻²⁰ A linear relationship between the accessible surface area and reaction rate constant of histidine residues with $^1\text{O}_2$ has been demonstrated for two proteins (GAPDH and rhDNase).^{16, 17} Besides solvent accessibility, intramolecular interaction within proteins is another factor that can influence the decay rate of amino acid residues. For example, coordination of cofactors can reduce reactivity with $^1\text{O}_2$ for histidine residues,^{7, 21} and association with the photosensitizing organic matter of hydrophobic amino acids like phenylalanine can lead to faster decay of such peptides.²² Overall, the effects of intramolecular interactions on $^1\text{O}_2$ -reactivity in proteins and effects of solvent exposure for residues other than histidine have received much less attention thus far.

The state-of-the-art approach to study site-specific damage within proteins involves digestion with proteolytic enzymes and analysis of the peptides with liquid chromatography coupled to mass spectrometry.²³ Prerequisites for a comprehensive analysis are that proteins of interest are structurally resolved and that full sequence coverage is analytically achieved. Recent analytical advances also made it possible to study site-specific $^1\text{O}_2$ -induced transformation of amino acids including identification of transformation products (TPs).²⁴⁻³⁰ Most of these studies focused on individual amino acids or reaction pathways of interest with a rather limited range of TPs. To gain evidence for site-specific effects of $^1\text{O}_2$ -reactivity, a comprehensive analysis of known reaction kinetics of all residues and their local environments has to be considered. Work on posttranslational modifications based on *in silico* predictions of possible TPs demonstrated that the number of possible modifications and reactive sites quickly increased the search space, i.e., number of suspects to search for.^{24, 31} As demonstrated in our study here, hundreds of thousands of possible TPs of proteolytic peptides have to be considered

for one protein based on different reaction pathways of individual amino acids with $^1\text{O}_2$ and known transformation products. A comprehensive kinetic analysis of site-specific molecular transformation within proteins is still challenging to date and more sophisticated data analysis solutions are needed.

We present an approach to study site-specific $^1\text{O}_2$ -induced degradation and transformation in *Aeromonas proteolytica* aminopeptidase, an extracellular enzymatic function ubiquitous in surface waters. Therefore, we developed a comprehensive data analysis workflow by combining suspect screening from small molecules research with *in silico* predictions of possible TPs caused by the reaction with $^1\text{O}_2$ with the enzyme. We established the inactivation rate constant and the complete site-specific fingerprint of the oxidation kinetics. We then compared the observed reactivities of each site with expected values based on the degree of exposure to the oxidant in solution and inspected product formation kinetics.

Experimental Section

Materials. *Aeromonas proteolytica* aminopeptidase (BLAP, PDB 1RTQ, Sigma, 116.51 units mg^{-1}), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, BioUltra, 99.5-101.5 %), iodoacetamide (IAM, 99 %), DL-dithio-threitol (DTT, 99 %), perinaphthenone (PN, 97 %), 7-hydroxy-4-methylcoumarin, 7-leucine-7-amido-4-methylcoumarin hydrochloride, were purchased from Sigma. Furfuryl alcohol (FFA, Merck, 98%) was distilled prior to use. ProteaseMAXTM (Promega, lyophilized, V207A), sequencing grade chymotrypsin (Promega, lyophilized, V106A), iRT peptides (iRT Kit, Biognosys) were used for protein digestion and analytical measurements, respectively. Further chemicals purchased from commercial vendors are listed in Section S1.

Photochemical Experiments

Experiments were carried out in a photoreactor with 8 UVA lamps centered around 365 nm on a merry-go-round turn table by Rayonet Southern New England Ultraviolet Co (light spectrum in Figure S2.1A) combined with a polymer filter situated between the lamps and the samples to reduce heat transmission from the lamps (269 LEE Heat Shield, Lee Filters, Hampshire, UK). Stable light condition of UVA bulbs was verified over the experimental time by actinometry (8 μ M recrystallized p-nitroanisole and 0.5 mM pyridine, Figure S2.1B). Enzyme solutions were exposed in quartz tubes (ES quartz glass, transmission of 190-2500 nm, Starna GmbH Pfungstadt Germany) in presence of a photosensitizer PN (20 μ M) generating singlet oxygen with a quantum yield close to unity³², and FFA (40 μ M) as the probe molecule to obtain $^1\text{O}_2$ steady-state concentrations. Sampling took place at 6 time points up to 60 minutes with four replicates, and two replicates of the dark controls (covered in aluminum foil in the photoreactor). Samples were subjected to activity assays and protein digestion followed by peptide analysis.

Sample and Data Analysis

Enzyme activity. To assess the aminopeptidase activity, samples were incubated with 7-leucine-7-amido-4-methylcoumarin hydrochloride (114 μ M, pH 7.5) in 96-well plate (non-binding, clear, Greiner Bio One) in triplicates. These conditions ensured maximum enzyme rates, V_{max} as established earlier.⁷ The evolution of the fluorescence intensity of the hydrolysis products methylumbelliferone and 7-hydroxy-4-methylcoumarin was monitored at excitation/emission wavelengths of 320/450 nm, respectively (Tecan, Infinite M200, Männedorf, Switzerland). The activity was assessed as the slope of the linearly increasing fluorescence signal over time. Inactivation followed pseudo-first order kinetics and thus activity (a) values were log-normalized and the observed inactivation rate constants were determined as the slopes of $\ln(a_t/a_0)$ over $^1\text{O}_2$ -exposure time with a linear regression model. To

determine bimolecular inactivation rate constants with $^1\text{O}_2$ (in $\text{M}^{-1}\text{s}^{-1}$), the observed rate constants (k_{obs} in s^{-1}) were divided by $^1\text{O}_2$ steady-state concentrations (in M) as determined with FFA.

Singlet oxygen concentration. The pseudo-first order decay of the probe molecule FFA allowed to calculate steady-state $^1\text{O}_2$ concentrations. FFA was quantified by liquid chromatography (LC, Ultimate 3000, Dionex) equipped with a C18 column (XBridge, 3.5 μm , 2.1 x 50 mm, Waters) at a flow rate of 0.2 mL min^{-1} (5 μL injection volume). Separation was obtained by an isocratic flow of 40 % phosphate buffer (pH 7.5) and 60 % methanol, and FFA was detected by absorbance at 219 nm. The observed decay rate constant of FFA was determined as the slope of $\ln(C_t/C_0)$ over $^1\text{O}_2$ -exposure time with a linear regression model. A $^1\text{O}_2$ steady-state concentration of $(8.37 \pm 0.4) \times 10^{-11} \text{ M}$ was calculated by dividing the observed decay rate constant by the bimolecular reaction rate constant of FFA with $^1\text{O}_2$ ($1.06 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, 25°C)³³.

Protein digestion and peptide analysis. Samples were subjected to proteolytic digestion by chymotrypsin after metal chelation, reduction of disulfides, and alkylation of thiols. Details of the optimized digestion protocol are described in Section S1. Digested samples were injected (5 μL) to a LC (Ultimate 3000 RSLCnano, Dionex) with C18 trap column (Waters Nano Acquity UPLC, 2G-VMTrap, 5 μm , 180 μm x 20 mm) and C18 column (Nano Acquity UPLC, BEH130, 1.7 μm , 300 μm x 150 mm, Waters) at a flow rate of 5 $\mu\text{L min}^{-1}$. Peptide separation was obtained with a gradient set to 4:9:30:96:96:4:4 % mobile phase B at 0:3:53:62:71:71.1:75 min. The LC was coupled to a quadrupole-Orbitrap mass spectrometer (Q-Exactive-Plus, Thermo Scientific, Bremen) and operated with an electron spray ionization (ESI) source in positive mode at a spray voltage of 3 kV, capillary temperature of 275°C , S-lens RF level: 50, sheath gas of 15.00 and auxiliary gas of 5.00. Data was acquired in full scan mode (m/z range: 150-2000, resolution: 140000 (at 400 m/z), maximum injection time: 200 ms,

automatic gain control (AGC) target: 10^6 , profile mode. Additionally, MS² data was obtained with a top 6 experiment (m/z range: 150-2000, isolation window: 1 m/z , resolution: 17'500, maximum injection time: 80 ms, AGC target: 10^5 , profile mode, stepped (N)CE: 10, 20, 30, dynamic exclusion: 6 s, suspect list).

Peptide Transformation Kinetics. Parent peptide analysis and final TP identification was performed with Skyline (v3.7 and v4.1). The parent peptides were predicted by *in silico* digestion using the defined cleavage sites of chymotrypsin allowing up to 4 miscleaved sites (i.e., when the used protease did not cleave). We did not observe any statistically significant influence of the frequency or position of miscleaved sites relative to ¹O₂ exposure of individual amino acid residues. For the identification of the parent peptides we verified the correct identification based on the accuracy of the three most intense isotopes (M, M+1 and M+2, idotp > 0.95), three or more charge states (M+H, M+2H, ..., M+nH) and, where available, the MS² fragmentation pattern relative to *in silico* predictions. We did not quantify absolute peptide concentration and instead determined rates of peptide loss over time. Our analysis assumes directly proportional signals for absolute peptide abundance. For each peptide the sum of the peak area of the three most intense isotopes (A), from respective selected ion chromatograms, was log-normalized and the observed decay rate constants (k_{obs} in s⁻¹) were determined as the slopes of $\ln(A_t/A_0)$ over exposure time with a linear regression model. To exclude that some peptides may not have degraded by first order kinetics, we labeled rates with a linear correlation coefficient of $r^2 < 0.6$ and slopes with standard error larger than 40 % as not detected, “n.d.” (Table S3.1, coloured in grey for BLAP-18 in [Figure 1B](#)). To determine bimolecular reaction rate constants (in M⁻¹ s⁻¹), the observed rate constants (k_{obs} s⁻¹) were divided by ¹O₂ steady-state concentrations (M). Using UCSF Chimera software we visualized these rate constants in the three-dimensional structure of the protein based on the published crystal structure.³⁴⁻³⁶ The ¹O₂ accessible surface area (¹O₂-ASA) was calculated from the crystal structure using GETAREA

with a probe radius set to 1.22 Å for $^1\text{O}_2$ (details in Section S4).^{16, 37} To evaluate whether observed rate constants scale with $^1\text{O}_2$ -ASA, we calculated the predicted reaction rate constant ($k_{\text{rxn, predicted}}$) for all residues as the product of the bimolecular reaction rate constant of freely dissolved amino acids ($k_{\text{rxn, freeAA}}$) and the ratio of the site-chain ASA of a residue in the protein ($\text{ASA}_{\text{residue in protein}}$) relative to the ASA of the free amino acid side chain ($\text{ASA}_{\text{freeAA}}$). For each peptide of interest, these predicted rates were summed for all reactive amino acids (details in Section S4, all $^1\text{O}_2$ -ASA values in Tables S4.1):

$$k_{\text{rxn, predicted}} = \sum k_{\text{rxn, freeAA}} \cdot \frac{\text{ASA}_{\text{residue in protein}}}{\text{ASA}_{\text{freeAA}}} \quad (1)$$

Suspect screening of transformation products. The potential TPs, also termed suspects, were predicted *in silico* including 24 known modifications (Table 1). Suspect screening was performed with the open-source workflow RMassScreening (<https://github.com/meowcat/RMassScreening>), which combines feature detection with the R package enviPick and builds cross-sample profiles using the R package enviMass.³⁸ The list of potential suspects was built in Skyline using 4 miscleaved sites allowing up to 4 variable modifications and one neutral loss based on reactions with $^1\text{O}_2$ (Table 1). Additional isobaric suspects were excluded. For each suspect, the masses for 3 isotopologues and 1 to 4 charge states were generated and screened on all profiles using exact mass match to identify potential hits. Hits were retained only if at least 3 ions (isotopes of any charge state) were found. The RMassScreening viewer was used to filter the screening hits according to multiple criteria. Features were retained if they (1) covered a fast degrading part of the sequence, (2) were absent in the dark controls, (3) absent or present in low intensity before irradiation, and (4) showed increasing intensities with increasing irradiation time. The ion chromatograms of putative suspects were extracted and categorized into three groups by confidence of identification according to corresponding levels defined by Schymanski *et al.*³⁹: Group 1: TPs showed

indicative MS² fragments for all modified sites detected (level 2b), group 2: TPs at least one indicative fragment covering transformed amino acids partially (level 3), and group 3: TPs MS¹ information with idotp > 0.95 (level 5). For information about TPs see Tables S5.1, and for chromatograms with all respective MS² information of parents and group 1 TPs see section S8. We acknowledge that biological and technical sources might have led to false negative results that we do not account for in the following, including missed peaks during automatic peak picking, an incomplete suspect list or inconclusive MS² fragmentation pattern (data analysis-based), varying ionization efficiency (MS-based), or unrecognized altered cleavage behavior during protein digestion (sample preparation-based).

Results and Discussion

Rapid Singlet Oxygen-induced Inactivation

Aminopeptidase was rapidly inactivated upon exposure to photochemically produced ¹O₂. The ¹O₂ concentration was (8.37±0.4) x 10⁻¹¹ M and activity loss showed pseudo-first order kinetics ([Figure 1A](#)). The system independent bimolecular inactivation rate constant for aminopeptidase with ¹O₂ was (3.69 ± 0.10) x 10⁷ M⁻¹ s⁻¹. This second order rate constant was comparable to ¹O₂-reactivity of freely dissolved amino acids ranging from (0.8 ± 0.24) x 10⁷ M⁻¹ s⁻¹ for tyrosine to (7.01 ± 0.10) x 10⁷ M⁻¹ s⁻¹ for histidine at pH 7 (Table S2.1).^{8, 40} No inactivation was observed in dark controls or during UVA light exposure without sensitizer (Figure S2.2). Aminopeptidases drive biogeochemical cycling by breaking peptide bonds of biological material. Particularly high concentrations of these enzymes are observed in near surface water where they become vulnerable to photoinactivation processes.^{3, 6, 41, 42} Our results demonstrate that indirect photooxidation by ¹O₂ likely becomes competitive with direct photoinactivation below the upper centimeters of the water column when UVB light intensity is minimal.⁴ The observed ¹O₂-induced inactivation further suggests that molecular changes

occurred either directly at the active site or upon conformational changes induced by oxidation elsewhere in the molecule, which was inspected in the following.

Three-dimensional Fingerprints of Oxidative Damage

The *in silico* predicted chymotryptic digestion of aminopeptidase resulted in 228 potential parent peptides. With the optimized digestion protocol, 75 peptides were analytically detectable after exposure to $^1\text{O}_2$ (Table S3.2). First, we selected 20 consecutive peptides that represent 100% of the aminopeptidase sequence and that we monitored for site-specific degradation (Table S3.1). The decay kinetics varied among peptides depending on the amino acid composition. Data in [Figure 1A](#) show the degradation kinetics of five areas of the enzyme, i.e., proteolytic peptides. All detected degradation rates were normalized by the $^1\text{O}_2$ -concentration to obtain bimolecular reaction rate constants that we mapped onto the three-dimensional protein structure in [Figure 1B](#) (tabulated data in Tables S3.1). All degrading peptides contain at least one amino acid known to react with $^1\text{O}_2$ (histidine (H), methionine (M), tryptophan (W), tyrosine (Y), cysteine (C)).^{8, 10, 15} The peptide that did not contain any of those amino acid residues, BLAP-2, showed only minimal degradation and all peptides remained stable throughout the experiment in dark controls (Figure S3.2). The faster degrading peptides in aminopeptidase were located around the active site (BLAP-4 and BLAP-8, $7.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $7.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, respectively) and their bimolecular reaction rate constants with $^1\text{O}_2$ exceeded the inactivation rate constant by 2-fold ($3.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$). The reaction kinetics varied among peptides, even where the same reactive residues were present, supporting that the local environment of the residues affects reactivity. Aminopeptidase contains multiple reactive residues (35 out of 299 residues, 12 %) distributed throughout the macromolecular structure. We detected transformation products induced by exposure to $^1\text{O}_2$ for half of these theoretically reactive sites (Table 1). In comparison, we demonstrated earlier that direct photoinactivation caused propagation of damage throughout larger areas of

aminopeptidase, initiated by only one chromophoric residue; tryptophan.⁴ In the following sections, we first evaluated the expected degradation based on the presence of amino acids that are reactive with $^1\text{O}_2$. We then inspected the chemical reaction kinetics and TP formation for those sites that showed fastest transformation by $^1\text{O}_2$ to demonstrate how not only solvent accessibility but also the local environment affected their reactivity.

Solvent Accessibility

Histidine containing peptides are expected to degrade the fastest because histidine is most reactive with $^1\text{O}_2$ among the amino acids with $(7.0 \pm 0.1) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at pH 7. Overall, aminopeptidase contains seven histidine residues distributed among six chymotryptic peptides (Table S3.1). Indeed, the fastest degrading part in aminopeptidase is the peptide BLAP-8 ($7.8 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$) that contains two histidines. Peptides with only one histidine showed slower and varying degradation kinetics ($1.7\text{-}2.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$) (Figure 1). Those $^1\text{O}_2$ -reactive amino acids most exposed to solution were expected to degrade the fastest, as $^1\text{O}_2$ was generated in the bulk solution from a photosensitizer (Figure 2A). Here, the fast degradation of BLAP-8 was in line with a high exposure of a histidine residue with $141.8 \text{ \AA}^2$, equaling 91.7 % relative to the accessible side chain surface area of free histidine. Peptides with the least accessible histidines (<3 % exposure) showed the lowest reactivities (BLAP-5, -15). Considering all reactive amino acid residues, we calculated the expected degradation rates based on (Equation 1). These values generally agreed with observed rates of three histidine-containing peptides (BLAP-8, -16, -17). However, $^1\text{O}_2$ -ASA was generally no adequate predictor for the reactivity and most peptides degraded 3 to 8-fold faster than expected by their $^1\text{O}_2$ -ASA (Figure 2B). Data in Figure 2B (inset) show that the actual observed reaction of slowly degrading peptides was underpredicted. The reactive moieties on these peptides have a very low $^1\text{O}_2$ -ASA (0.1-1.5 %, e.g., BLAP-6, -9, -19, -20) but degraded at faster rates than expected ($1.5\text{-}3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$).

279 Recently, a linear relationship between the site-specific reaction rate constants of His-
280 containing peptides with $^1\text{O}_2$ and the respective $^1\text{O}_2$ -accessible surface area ($^1\text{O}_2$ -ASA) has been
281 reported for two model proteins.^{16, 17} Similar accessibility relationships have previously been
282 reported for protein oxidation by sulfate radicals and hydroxyl radicals.⁴³ In comparison to
283 published data for GAPDH, aminopeptidase also follows the general trend of increasing
284 reactivity with $^1\text{O}_2$ -ASA (dataset side-by-side in Figure S4.1). However, here, the predicted
285 reactivities based on $^1\text{O}_2$ -ASA deviated significantly from measured values. Some deviation is
286 likely related to the influence of the microenvironment of each residue. Uncertainties also
287 remain for accessible surface areas predicted from a rigid crystal structure compared to proteins
288 in solution with higher molecular flexibility.¹⁷ Another uncertainty can stem from the expected
289 concentration of $^1\text{O}_2$ that may be underpredicted if apolar regions of the molecule associate
290 with the sensitizer molecules, such as chromophoric dissolved organic matter or the model
291 sensitizer perinaphthenone used herein.²² However, the less systematic deviation from the
292 predicted values for most peptides suggests that additional factors may have influenced the
293 reactivity of amino acid residues with $^1\text{O}_2$.

294 In the following sections we provide further insights of site-specific oxidation including the
295 formation kinetics of oxidation products. A comprehensive data analysis workflow enabled us
296 to identify TPs by using a suspect screening approach established in small molecules
297 research.^{44, 45} The suspects are TPs that we predicted *in silico* based on $^1\text{O}_2$ -induced pathways
298 of reactive amino acids that we assembled from the current literature (Figures S7.1, [Figure 4](#))
299 including 24 potential modifications listed in

Table 1 1. The chymotryptic digestion of aminopeptidase resulted in 228 potential parent peptides and combined with 24 $^1\text{O}_2$ -induced modifications and 2-4 charge states for each peptide the suspect list contained 723'288 possible TPs.

Influence of Metal Coordination on Histidine Reactivity

Oxidation of histidine contributed to the overall degradation in aminopeptidase, but the detected TPs were not dominating. The formation kinetics of the TPs indicate that histidine oxidation generally occurred immediately after exposure started, underlining the generally fast histidine reactivity with $^1\text{O}_2$. Here, we based the product analysis on the $^1\text{O}_2$ -induced degradation pathways for histidine that we compiled from the current literature (Figure S7.1). We observed one oxygen addition (+16 Da, e.g., on H234 of BLAP-16, **Figure 3B** inset) as well as TPs from further oxidation of histidine all the way to asparagine (e.g., +48, +32 and -23 Da on H97 of BLAP-8, Figure S7.2B).

Histidines are often found in active sites of enzymes where the imidazole moiety can play an important roles in catalytic mechanisms and can participate in coordinating metal cofactors.⁴⁶ Here, we identified a combined effect of solvent exposure and Zn-coordination for BLAP-8 by inspecting its TPs. BLAP-8 is the overall fastest degrading peptide in aminopeptidase and reacted with $(7.83 \pm 1.72) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, at a comparable rate to free histidine $(7.01 \pm 0.10 \times 10^7 \text{ M}^{-1}\text{s}^{-1})$,¹¹ even though it contains two histidine residues. Under the assumption that reactivity is additive when multiple targets are located on the same peptide, one would expect a faster rate constant for BLAP-8. The H97 is involved in Zn-coordination and mostly buried (0.2% exposure) which may explain the reduced reactivity of this peptide. Metal coordination gives histidine a quasi-protonated character and its reactivity is expected to be further reduced as HisH^+ reacts 20-fold slower with $^1\text{O}_2$.¹⁶ Indeed, we observe delayed

formation of hydroxy-imidazolones (+32 Da) and triple oxidized histidine (+48 Da) of the buried H97 (see Figure S7.2B). The second histidine on this peptide, H105, is highly exposed (91.7 %) but we were not able to identify TPs, which may be due to too rapid oxidation and subsequent decay kinetics of TPs. These observations suggest that this peptide shows similar reactivity as freely dissolved histidine because H105 is almost completely exposed while H97 is almost completely buried. Metal coordination may further suppress the reactivity.

Readily Oxidizable Methionines Contribute to Initial Degradation

Methionine oxidation is a common reaction in proteins that plays important regulative and protective roles and can affect protein conformation.^{47, 48} Methionine sulfoxide is formed via a formal oxygen transfer by a two-electron oxidation in the presence of appropriate oxidants. Around 4-5 % spontaneous oxidation to methionine sulfoxide has been reported under aerobic conditions and can be reversed, e.g., by methionine sulfoxide reductases inside cells.⁴⁹ Here, methionine sulfoxide formation (+16 Da) was observed for all seven methionine residues with varying reaction rate constants (Figures S6.1). Data in [Figure 3](#) show examples of two peptides where oxidation to methionine sulfoxide was observed. Most Met-containing peptides also contained additional reactive amino acids that influenced the observed decay. Data in [Figure 3B](#) show an example where histidine and methionine were both present on the peptide BLAP-16. The oxidation of BLAP-16 led to formation of methionine sulfoxide (+16 Da on M242 with 2.9 % exposure) and also further oxidation to Met-sulfone (+32 Da) but also oxidation of histidine, as discussed above (+16 Da on H234 with 40.2 % exposure). Overall, the kinetic analysis of methionine oxidation suggests that methionine sulfoxide formation is a dominating process for the initial degradation processes in aminopeptidase, even at relatively low solvent exposure. Methionine is also referred to as an endogenous antioxidant that can protect other moieties in biomolecules and its oxidation can lead to critical accumulation of damage that causes inactivation of enzymes.⁴⁹⁻⁵¹ The interconversion of solvent exposed methionine to

methionine sulfoxide alters the local hydrophobicity in biomolecules and can affect their site-specific conformation, interaction with the environment, and their catalytic activity.⁵²⁻⁵⁴

Competing Reaction Pathways of Tryptophan

Analogous to histidine and methionine, we assembled the ¹O₂-induced degradation pathways for tryptophan from the current literature (Figure 4A).⁵⁵⁻⁵⁷ We successfully identified tryptophan oxidation products in aminopeptidase for three out of five tryptophan residues. Based on the kinetics of product formation, we differentiated two routes of tryptophan oxidation by ¹O₂. After an initial 2,3-cycloaddition of ¹O₂ to the indole, either a hydroxy tryptophan (+16 Da, product 1A) or a *N*-formylkynurenine via ring opening (+32 Da, product 2A) can be formed. The kinetic analysis of all oxidized tryptophan residues in aminopeptidase suggests that the reaction proceeded predominantly via the *N*-formylkynurenine pathway. The products formed from W91 on BLAP-7 correspond to hydroxylated *N*-formylkynurenine (+48 Da, product 3B), hydroxylated kynurenine (+20 Da product 4B), and dihydroxylated *N*-formylkynurenine (+64 Da, product 5B), that appeared with some delay as secondary products (Figure S7.2B). Data in Figure 4B and C demonstrate how the product analysis was utilized to differentiate between the two pathways. BLAP-4 was one of the fastest degrading peptides in aminopeptidase with $(7.40 \pm 0.92) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, almost twice as fast as free tryptophan $(3.43 \pm 0.02 \times 10^7 \text{ M}^{-1}\text{s}^{-1})$.¹⁸ BLAP-4 contains two tryptophan residues that would explain the high rate constant, but based on the solvent exposure a slower rate would be predicted with only W48 being highly solvent exposed (29.4 % exposure) and W53 being mostly buried (0.3 % exposure). For comparison, BLAP-7, which only contains only one exposed tryptophan (29.2 % exposure) degrades significantly slower $(2.44 \pm 0.12 \times 10^7 \text{ M}^{-1}\text{s}^{-1})$, Figure 4B inset). In contrast to the prediction based on solvent exposure, our product analysis supports that both tryptophan residues of BLAP-4 react significantly with ¹O₂, which is in agreement with the

high reaction rate that we measured. Initially, the dominant products were +32 Da for W48 and W53 (Figure 4B yellow diamonds and triangles). The +32 Da product can correspond to either of the isobaric products of the competing pathways; *N*-formylkynurenine or dihydroxylated tryptophan (products 1B and 2A in Figure 4A). Hydroxylated tryptophan (+16 Da) is the precursor of dihydroxylated tryptophan and was observed simultaneously but with much lower intensities (Figure 4B yellow circles). We could follow W53 on a different peptide (BLAP-4, fragment 2) and data in Figure 4C show that kynurenine was subsequently formed for W53 (green squares, +4 Da). This observation supports that the initially formed +32 Da was dominated by *N*-formylkynurenine that subsequently underwent deformylation. Data in Figure 4C also show the formation of another +32 Da product for the W53, which also appeared later, suggesting that this is rather the dihydroxylated tryptophan as a secondary product of monohydroxylated tryptophan (+16 Da, note lower overall intensities).

Tryptophan is a rather hydrophobic residue with a large surface area, which plays a key role in structural stability of proteins and can participate in a wide range of intermolecular interactions.^{58,59} Chemical alteration by ¹O₂, opening of the indole ring, or hydroxylation might therefore affect the conformation and activity of the enzyme. Kynurenine has been suggested as a molecular marker for the presence of ¹O₂.⁵⁷ While we observed kynurenine formation as a ¹O₂-mediated reaction, it may stay undetected when the other ¹O₂-reaction pathway is dominating. Also, the tryptophan TPs further support that the reactivity with ¹O₂ cannot always be explained by the solvent accessibility alone. The accessible surface area of dissolved proteins may also not be accurately presented by the rigid crystal structure. While the product analysis strongly suggest that the major decay proceeded predominantly through reaction with ¹O₂, we cannot exclude that variation in observed reactivity relative to the expected ¹O₂-ASA resulted from an interaction of more apolar residues with the ¹O₂-sensitizer perinaphthenone. In such cases, higher ¹O₂ concentrations around those sites can lead to faster observed

degradation.²²

Reactivities of Cysteine and Tyrosine

The protein contains two cysteine residues; C223 and C227, that form a disulfide bridge between a relatively small part of the protein (5 amino acids). We followed these cysteine residues on BLAP-14 and BLAP-15 and both peptides showed significant degradation. From the digestion procedure, cysteines were capped by IAM and we observed these peptides with a mass of +57 Da accordingly. Even though both peptides degraded quickly in the presence of ¹O₂, this reactivity can only be ascribed in part to the cysteine themselves, which are mostly buried within the protein structure (2.4 % and 8.8 % side chain exposure, respectively). For BLAP-14, most reactivity came from the highly exposed tyrosine residue (64.7 % exposure) and for BLAP-15 the high reactivity of histidine and tryptophan contributed to its decay. While oxidation of disulfide bridges linking larger parts of the peptide chain can have a great effect on protein confirmation, this was not observed to play a major role for the aminopeptidase presented here.

Thirteen tyrosine residues are located on 11 chymotryptic peptides from aminopeptidase. The reactivities of these peptides range from $1.29 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for BLAP-3 with the only reactive amino acid being partially exposed Y38 (19.2 % exposed) to $2.71 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for BLAP-14 with Y225 being mostly exposed (64.7 %). We observed a general trend of faster decay with higher exposure. However, we did not identify a systematic observation for Tyr-containing peptides that explained the overestimation of reactivity with ¹O₂ based on solvent-accessible surface area as discussed above ([Figure 2B](#)). We identified transformation products of +16 Da (DOPA), +32 Da (TOPA) and +14 (Dopa-quinone) and observed their formation kinetics following parent decay (Figure S.8.1).⁶⁰⁻⁶² The reactivity of tyrosine increases

significantly in its phenolate form.⁶³ One can suspect that local pH within the macromolecule as well as local pK_a of the residues may also contribute to differences in observed rates. In relation to this hypothesis, it has been observed that integration of tyrosine into peptides affected its reactivity with 1O_2 . Yet, the local effects of tyrosine reactivity within proteins has not been investigated sufficiently to date.⁶³

Implications

The functional lifetime of extracellular enzymes defines their influence on biogeochemical cycling in the aquatic environment. Understanding the environmental inactivation and transformation processes enables us to more accurately predict these lifetimes. We demonstrate that the extracellular aminopeptidase was highly susceptible to inactivation by aqueous 1O_2 at bimolecular inactivation rates comparable to degradation rates of free amino acids. The data strongly suggests that photooxidation by 1O_2 plays a critical role for the inactivation of extracellular enzymatic functions below the upper centimeters of the water column and becomes compatible with direct photoinactivation. Our approach to study site-specific 1O_2 -induced degradation and transformation in the molecular structure of proteins made it possible to also assess the influence of the local environment on the reactivity of amino acid residues. The oxidative damage was focused on the active site of aminopeptidase and caused a fast inactivation. Our results demonstrate how not only solvent accessibility but also other intramolecular interactions play an important role in predicting molecular damage by 1O_2 . Our comprehensive data analysis workflow with suspect screening of *in silico* predicted transformation products allowed us to draw further conclusions about the site-specific reaction pathways. This approach can be applied to other amino acid-based molecules and oxidation reactions of interest.

446 **Acknowledgement**

447 We gratefully acknowledge support of the Swiss National Science Foundation (Grant
448 number 200020-159809). We thank Simon Wullschleger for assistance with data analysis and
449 Jennifer Schollée for support with MS² data representation.

450 **Supporting Information**

451 Additional information and data on measured and predicted reaction rate constants,
452 transformation products and pathways.

453

Figure 1. A) Log normalized degradation of peptides and activity with exposure time and corresponding dark controls. Error bars correspond to one standard deviation of three to four replicates. B) Color map with bimolecular singlet oxygen reaction rate constants for individual regions of *Aeromonas proteolytica* leucyl aminopeptidase (BLAP, EC3.4.11.10, PDB 1RTQ). Selected peptides are indicated with the amino acid sequence as well as sequence number with reactive residues highlighted in red: methionine (M), histidine (H), tryptophan (W), tyrosine (Y), and cysteine (C). For peptide BLAP-18 (grey) the degradation did not follow first order kinetics and no rate was assessed. For tabulated data see Table S3.1 and for colormap with complete annotation see Figure S3.1.

476 **Table 1.** Tabulated modifications included in the transformation product analysis with
477 respective amino acid (AA), elemental changes of the amino acid (elemental Δ), respective
478 mass shift in Dalton (Δ Da). Identified modifications at a specific site in aminopeptidase
479 (BLAP) are indicated with the respective sequence number of the residue in the enzyme.
480 Listed are all modifications that were identified on a transformation product with confidence
481 level 2b, level 3 with the identifying MS² fragment or level 3 with no other combination
482 possibilities. Other modification that were considered in the analysis but have not been
483 detected (n.d.) are listed last.

AA	modification	references	elemental Δ	mass shift	#AA in BLAP
W	kynurenine	26, 55, 60, 62, 64	+O-C	+4	53
W	<i>N</i> -formylkynurenine	26, 60, 62, 64	+2O	+32	48
W	OH- <i>N</i> -formylkynurenine	26, 60	+3O	+48	48, 53, 91
W	diOH- <i>N</i> -formylkynurenine	26, 60	+4O	+64	53, 91
W	OH-tryptophan	26, 28, 60	+O	+16	48, 53
W	diOH-tryptophan	26, 60	+2O	+32	48, 53
Y	DOPA	60-62	+O	+16	37, 196, 204
Y	TOPA	60-62	+2O	+32	282
Y	DOPA-quinone	60-62	+O-2H	+14	73
H	2-/4-/5-hydroxy-histidine	27	+O	+16	234
H	triply oxidized histidine	29	+3O	+48	97
H	4-/5-hydroxy-imidazolone	27, 29, 30	+2O	+32	97
M	methionine sulfoxide	26, 28, 62	+O	+16	1, 80, 146, 180, 209, 242, 286
M	methionine sulfone	26, 28, 62	+2O	+32	1, 146, 242, 286
W	tryptophan-dione	26, 60	+2O-2H	+30	n.d.
W	2,4-bis-tryptophandione	26, 60	+2O-4H	+28	n.d.
W	OH-bis-tryptophandione	26, 60	+3O-4H	+44	n.d.
W	OH-kynurenine	26, 60, 62, 64	+2O-C	+20	n.d.
Y	TOPA-quinone	60-62	2O-2H	+30	n.d.
H	asparagine	26, 62, 65, 66	-2C-N-H+O	-23	n.d.
H	aspartic acid	26, 28, 62, 65, 66	-2C-2N-2H+2O	-22	n.d.
H	2-oxo-histidine	27, 28, 30	+O-2H	+14	n.d.
C	cysteic acid	26, 62, 67	+3O	+48	n.d.
C	sulfinic acid	67	+2O	+32	n.d.

486

487

488 **Figure 3.** Degradation of Met-containing parent peptides A) BLAP-16 and B) BLAP-20, as
 489 well as formation of various TPs over the time course of the $^1\text{O}_2$ exposure experiment.
 490 Methionine sulfoxide (+16 Da) was formed for all methionine residues with different formation
 491 kinetics, whereas methionine sulfone (+32 Da) was a minor TP (panel B). Sulfoxide TPs in
 492 combined peptides showed initial formation and subsequent decay kinetics due to formation of
 493 combined products from histidine and methionine oxidation (panel B and inset). Sequence
 494 number of the respective peptides are indicated and $^1\text{O}_2$ -reactive amino acid residues are
 495 marked in bold and red in the sequence. All depicted TPs were identified with confidence level
 496 2b and kinetics of all sulfoxide products can be found in Figure S6.1), error bars correspond to
 497 one standard deviation of three to four replicates.

498

Figure 4. A) Tryptophan oxidation pathways with $^1\text{O}_2$ initiated by endoperoxide formation and two subsequent pathways: pathway A proceeding to hydroxy tryptophan (1A), dihydroxy tryptophan (2A) followed by various diones (3A-5A) and pathway B proceeding to formylkynurenine (1B) by ring opening followed either by kynurenine (2B) or hydroxylated N-formylkynurenine (3B) that can both react further to hydroxylated kynurenine (4B) or dihydroxylated N-formylkynurenine (5B) from 3B. B) Kinetic data showing degradation of BLAP-4 (fragment 1) containing W49 and W53 (black circles) and formation and decay of three transformation products (yellow symbols), the inset shows log-normalized decay for peptide BLAP-4 and BLAP-7. C) Kinetic data showing degradation of a shorter proteolytic peptides BLAP-4 (fragment 2) containing only W53 (black circles) with formation of two products (green symbols). Solid lines correspond to a product identified with confidence level 2b, long-dashed lines to level 3, dotted lines to level 5 (compare Table S5.1), error bars correspond to one standard deviation of three to four replicates.

References

1. Arnosti, C., Microbial Extracellular Enzymes and the Marine Carbon Cycle. *Annu Rev Mar Sci* **2011**, 3, 401-425.
2. Sinsabaugh, R. L.; Shah, J. J. F.; Hill, B. H.; Elonen, C. M., Ecoenzymatic stoichiometry of stream sediments with comparison to terrestrial soils. *Biogeochemistry* **2012**, 111, (1-3), 455-467.
3. Arnosti, C.; Bell, C.; Moorhead, D. L.; Sinsabaugh, R. L.; Steen, A. D.; Stromberger, M.; Wallenstein, M.; Weintraub, M. N., Extracellular enzymes in terrestrial, freshwater, and marine environments: perspectives on system variability and common research needs. *Biogeochemistry* **2014**, 117, (1), 5-21.
4. Egli, C. M.; Janssen, E. M. L., Proteomics Approach To Trace Site-Specific Damage in Aquatic Extracellular Enzymes During Photoinactivation. *Environ Sci Technol* **2018**, 52, (14), 7671-7679.
5. Janssen, E. M. L.; McNeill, K., Environmental Photoinactivation of Extracellular Phosphatases and the Effects of Dissolved Organic Matter. *Environ Sci Technol* **2015**, 49, (2), 889-896.
6. Steen, A. D.; Arnosti, C., Long lifetimes of beta-glucosidase, leucine aminopeptidase, and phosphatase in Arctic seawater. *Mar Chem* **2011**, 123, (1-4), 127-132.
7. Suto, D.; Iuchi, Y.; Ikeda, Y.; Sato, K.; Ohba, Y.; Fujii, J., Inactivation of cysteine and serine proteases by singlet oxygen. *Arch Biochem Biophys* **2007**, 461, (2), 151-158.
8. Lundeen, R. A.; Janssen, E. M. L.; Chu, C. H.; McNeill, K., Environmental Photochemistry of Amino Acids, Peptides and Proteins. *Chimia* **2014**, 68, (11), 812-817.
9. Page, S. E.; Arnold, W. A.; McNeill, K., Assessing the Contribution of Free Hydroxyl Radical in Organic Matter-Sensitized Photohydroxylation Reactions. *Environ Sci Technol* **2011**, 45, (7), 2818-2825.

10. Boreen, A. L.; Edhlund, B. L.; Cotner, J. B.; McNeill, K., Indirect photodegradation of dissolved free amino acids: The contribution of singlet oxygen and the differential reactivity of DOM from various sources. *Environ Sci Technol* **2008**, *42*, (15), 5492-5498.
11. Davis, C. A.; McNeill, K.; Janssen, E. M. L., Non-Singlet Oxygen Kinetic Solvent Isotope Effects in Aquatic Photochemistry. *Environ Sci Technol* **2018**, *52*, (17), 9908-9916.
12. Haag, W. R.; Hoigne, J., Photo-Sensitized Oxidation in Natural-Water Via .Oh Radicals. *Chemosphere* **1985**, *14*, (11-12), 1659-1671.
13. Wolff, C. J. M.; Halmans, M. T. H.; Vanderheijde, H. B., The Formation of Singlet Oxygen in Surface Waters. *Chemosphere* **1981**, *10*, (1), 59-62.
14. Zepp, R. G.; Wolfe, N. L.; Baughman, G. L.; Hollis, R. C., Singlet Oxygen in Natural-Waters. *Nature* **1977**, *267*, (5610), 421-423.
15. Pattison, D. I.; Rahmanto, A. S.; Davies, M. J., Photo-oxidation of proteins. *Photoch Photobio Sci* **2012**, *11*, (1), 38-53.
16. Lundeen, R. A.; McNeill, K., Reactivity Differences of Combined and Free Amino Acids: Quantifying the Relationship between Three-Dimensional Protein Structure and Singlet Oxygen Reaction Rates. *Environ Sci Technol* **2013**, *47*, (24), 14215-14223.
17. Lei, M.; Carcelen, T.; Walters, B. T.; Zamiri, C.; Quan, C.; Hu, Y. Z.; Nishihara, J.; Yip, H.; Woon, N.; Zhang, T.; Kao, Y. H.; Schoneich, C., Structure-Based Correlation of Light-Induced Histidine Reactivity in A Model Protein. *Anal Chem* **2017**, *89*, (13), 7225-7231.
18. Jensen, R. L.; Arnbjerg, J.; Birkedal, H.; Ogilby, P. R., Singlet Oxygen's Response to Protein Dynamics. *J Am Chem Soc* **2011**, *133*, (18), 7166-7173.
19. Wigginton, K. R.; Menin, L.; Montoya, J. P.; Kohn, T., Oxidation of Virus Proteins during UV254 and Singlet Oxygen Mediated Inactivation. *Environ Sci Technol* **2010**, *44*, (14), 5437-5443.
20. Wigginton, K. R.; Pecson, B. M.; Sigstam, T.; Bosshard, F.; Kohn, T., Virus Inactivation Mechanisms: Impact of Disinfectants on Virus Function and Structural Integrity. *Environ Sci Technol* **2012**, *46*, (21), 12069-12078.

- 566 21. Lundeen, R. A.; Chu, C. H.; Sander, M.; McNeill, K., Photooxidation of the Antimicrobial,
567 Nonribosomal Peptide Bacitracin A by Singlet Oxygen under Environmentally Relevant
568 Conditions. *Environ Sci Technol* **2016**, 50, (16), 8586-8595.
- 569 22. Chu, C. H.; Lundeen, R. A.; Sander, M.; McNeill, K., Assessing the Indirect Photochemical
570 Transformation of Dissolved Combined Amino Acids through the Use of Systematically
571 Designed Histidine-Containing Oligopeptides. *Environ Sci Technol* **2015**, 49, (21), 12798-
572 12807.
- 573 23. Schoneich, C.; Sharov, V. S., Mass spectrometry of protein modifications by reactive
574 oxygen and nitrogen species. *Free Radical Bio Med* **2006**, 41, (10), 1507-1520.
- 575 24. Rykaer, M.; Svensson, B.; Davies, M. J.; Hagglund, P., Unrestricted Mass Spectrometric
576 Data Analysis for Identification, Localization, and Quantification of Oxidative Protein
577 Modifications. *J Proteome Res* **2017**, 16, (11), 3978-3988.
- 578 25. Leinisch, F.; Mariotti, M.; Hagglund, P.; Davies, M. J., Structural and functional changes
579 in RNase A originating from tyrosine and histidine cross-linking and oxidation induced by
580 singlet oxygen and peroxy radicals. *Free Radical Bio Med* **2018**, 126, 73-86.
- 581 26. Kerkaert, B.; Mestdagh, F.; Obando, M.; Cucu, T.; De Meulenaer, B., Identification of
582 Modified Lysozyme Peptides upon Photo-oxidation by LC-TOF-MS. *J Agr Food Chem*
583 **2013**, 61, (51), 12727-12736.
- 584 27. Marques, E. F.; Medeiros, M. H. G.; Di Mascio, P., Lysozyme oxidation by singlet
585 molecular oxygen: Peptide characterization using [O-18]-labeling oxygen and nLC-
586 MS/MS. *J Mass Spectrom* **2017**, 52, (11), 739-751.
- 587 28. Kim, J.; Rodriguez, M. E.; Guo, M.; Kenney, M. E.; Oleinick, N. L.; Anderson, V. E.,
588 Oxidative modification of cytochrome c by singlet oxygen. *Free Radical Bio Med* **2008**,
589 44, (9), 1700-1711.
- 590 29. Bane, J.; Mozziconacci, O.; Yi, L.; Wang, Y. J.; Sreedhara, A.; Schoneich, C., Photo-
591 oxidation of IgG1 and Model Peptides: Detection and Analysis of Triply Oxidized His and
592 Trp Side Chain Cleavage Products. *Pharm Res-Dordr* **2017**, 34, (1), 229-242.

30. Amano, M.; Kobayashi, N.; Yabuta, M.; Uchiyama, S.; Fukui, K., Detection of Histidine Oxidation in a Monoclonal Immunoglobulin Gamma (IgG) 1 Antibody. *Anal Chem* **2014**, 86, (15), 7536-7543.
31. Savitski, M. M.; Nielsen, M. L.; Zubarev, R. A., ModifiComb, a new proteomic tool for mapping substoichiometric post-translational modifications, finding novel types of modifications, and fingerprinting complex protein mixtures. *Mol Cell Proteomics* **2006**, 5, (5), 935-948.
32. Oliveros, E.; Bossmann, S. H.; Nonell, S.; Marti, C.; Heit, G.; Troscher, G.; Neuner, A.; Martinez, C.; Braun, A. M., Photochemistry of the singlet oxygen [O-2((1)Delta(g))] sensitizer perinaphthenone (phenalenone) in N,N '-dimethylacetamide and 1,4-dioxane. *New J Chem* **1999**, 23, (1), 85-93.
33. Appiani, E.; Ossola, R.; Latch, D. E.; Erickson, P. R.; McNeill, K., Aqueous singlet oxygen reaction kinetics of furfuryl alcohol: effect of temperature, pH, and salt content. *Environ Sci-Proc Imp* **2017**, 19, (4), 507-516.
34. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E., UCSF chimera - A visualization system for exploratory research and analysis. *J Comput Chem* **2004**, 25, (13), 1605-1612.
35. Stec, B.; Holtz, K. M.; Kantrowitz, E. R., A revised mechanism for the alkaline phosphatase reaction involving three metal ions. *J Mol Biol* **2000**, 299, (5), 1303-1311.
36. Desmarais, W.; Bienvenue, D. L.; Bzymek, K. P.; Petsko, G. A.; Ringe, D.; Holz, R. C., The high-resolution structures of the neutral and the low pH crystals of aminopeptidase from *Aeromonas proteolytica*. *J Biol Inorg Chem* **2006**, 11, (4), 398-408.
37. Fraczekiewicz, R.; Braun, W., Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. *J Comput Chem* **1998**, 19, (3), 319-333.
38. Loos, M., enviMass, version 3.5, LC-HRMS trend detection workflow -- R package. *Zenobo* **2018**.

39. Schymanski, E. L.; Jeon, J.; Gulde, R.; Fenner, K.; Ruff, M.; Singer, H. P.; Hollender, J., Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. *Environ Sci Technol* **2014**, *48*, (4), 2097-2098.
40. Matheson, I. B. C.; Lee, J., Chemical-Reaction Rates of Amino-Acids with Singlet Oxygen. *Photochem Photobiol* **1979**, *29*, (5), 879-881.
41. Keith, S. C.; Arnosti, C., Extracellular enzyme activity in a river-bay-shelf transect: variations in polysaccharide hydrolysis rates with substrate and size class. *Aquat Microb Ecol* **2001**, *24*, (3), 243-253.
42. Baltar, F.; Aristegui, J.; Sintes, E.; van Aken, H. M.; Gasol, J. M.; Herndl, G. J., Prokaryotic extracellular enzymatic activity in relation to biomass production and respiration in the meso- and bathypelagic waters of the (sub)tropical Atlantic. *Environ Microbiol* **2009**, *11*, (8), 1998-2014.
43. Gau, B. C.; Chen, H.; Zhang, Y.; Gross, M. L., Sulfate Radical Anion as a New Reagent for Fast Photochemical Oxidation of Proteins. *Anal Chem* **2010**, *82*, (18), 7821-7827.
44. Stravs, M. A.; Pomati, F.; Hollender, J., Exploring micropollutant biotransformation in three freshwater phytoplankton species. *Environ Sci-Proc Imp* **2017**, *19*, (6), 822-832.
45. MacLean, B.; Tomazela, D. M.; Shulman, N.; Chambers, M.; Finney, G. L.; Frewen, B.; Kern, R.; Tabb, D. L.; Liebler, D. C.; MacCoss, M. J., Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* **2010**, *26*, (7), 966-968.
46. Schneider, F., Histidine in Enzyme Active-Centers. *Angew Chem Int Edit* **1978**, *17*, (8), 583-592.
47. Lavine, T. F., The Formation, Resolution, and Optical Properties of the Diastereoisomeric Sulfoxides Derived from L-Methionine. *J Biol Chem* **1947**, *169*, (3), 477-491.
48. Vogt, W., Oxidation of Methionyl Residues in Proteins - Tools, Targets, and Reversal. *Free Radical Bio Med* **1995**, *18*, (1), 93-105.

- 646 49. Levine, R. L.; Berlett, B. S.; Moskovitz, J.; Mosoni, L.; Stadtman, E. R., Methionine
647 residues may protect proteins from critical oxidative damage. *Mech Ageing Dev* **1999**,
648 *107*, (3), 323-332.
- 649 50. Shechter, Y.; Burstein, Y.; Patchornik, A., Selective Oxidation of Methionine Residues in
650 Proteins. *Biochemistry-Ussr* **1975**, *14*, (20), 4497-4503.
- 651 51. Luo, S.; Levine, R. L., Methionine in proteins defends against oxidative stress. *Faseb J*
652 **2009**, *23*, (2), 464-472.
- 653 52. Garner, B.; Witting, P. K.; Waldeck, A. R.; Christison, J. K.; Raftery, M.; Stocker, P.,
654 Oxidation of high density lipoproteins - I. Formation of methionine sulfoxide in
655 apolipoproteins AI and AII is an early event that accompanies lipid peroxidation and can
656 be enhanced by alpha-tocopherol. *J Biol Chem* **1998**, *273*, (11), 6080-6087.
- 657 53. Caldwell, P.; Luk, D. C.; Weissbach, H.; Brot, N., Oxidation of Methionine Residues of
658 Escherichia-Coli Ribosomal-Protein L12 Decreases Proteins Biological-Activity. *P Natl*
659 *Acad Sci USA* **1978**, *75*, (11), 5349-5352.
- 660 54. Johnson, D.; Travis, J., Oxidative Inactivation of Human Alpha-1-Proteinase Inhibitor -
661 Further Evidence for Methionine at the Reactive Center. *J Biol Chem* **1979**, *254*, (10),
662 4022-4026.
- 663 55. Nakagawa, M.; Watanabe, H.; Kodato, S.; Okajima, H.; Hino, T.; Flippen, J. L.; Witkop,
664 B., Valid Model for Mechanism of Oxidation of Tryptophan to Formylkynurenine - 25 Years
665 Later. *P Natl Acad Sci USA* **1977**, *74*, (11), 4730-4733.
- 666 56. Davies, M. J.; Truscott, R. J. W., Photo-oxidation of proteins and its role in
667 cataractogenesis. *J Photoch Photobio B* **2001**, *63*, (1-3), 114-125.
- 668 57. Plowman, J. E.; Deb-Choudhury, S.; Grosvenor, A. J.; Dyer, J. M., Protein oxidation:
669 identification and utilisation of molecular markers to differentiate singlet oxygen and
670 hydroxyl radical-mediated oxidative pathways. *Photoch Photobio Sci* **2013**, *12*, (11),
671 1960-1967.
- 672 58. Dougherty, D. A., Cation-pi interactions in chemistry and biology: A new view of benzene,
673 Phe, Tyr, and Trp. *Science* **1996**, *271*, (5246), 163-168.

59. Pochapsky, T. C.; Gopen, Q., A Chromatographic Approach to the Determination of Relative Free-Energies of Interaction between Hydrophobic and Amphiphilic Amino-Acid Side-Chains. *Protein Sci* **1992**, 1, (6), 786-795.
60. Grosvenor, A. J.; Morton, J. D.; Dyer, J. M., Profiling of residue-level photo-oxidative damage in peptides. *Amino Acids* **2010**, 39, (1), 285-296.
61. Dyer, J. M.; Bringans, S. D.; Bryson, W. G., Determination of photo-oxidation products within photoyellowed bleached wool proteins. *Photochem Photobiol* **2006**, 82, (2), 551-557.
62. Davies, M. J., Singlet oxygen-mediated damage to proteins and its consequences. *Biochem Bioph Res Co* **2003**, 305, (3), 761-770.
63. Bertolotti, S. G.; Garcia, N. A.; Arguello, G. A., Effect of the Peptide-Bond on the Singlet-Molecular-Oxygen-Mediated Sensitized Photooxidation of Tyrosine and Tryptophan Dipeptides - a Kinetic-Study. *J Photoch Photobio B* **1991**, 10, (1-2), 57-70.
64. Gracanin, M.; Hawkins, C. L.; Pattison, D. I.; Davies, M. J., Singlet-oxygen-mediated amino acid and protein oxidation: Formation of tryptophan peroxides and decomposition products. *Free Radical Bio Med* **2009**, 47, (1), 92-102.
65. Agon, V. V.; Bubbs, W. A.; Wright, A.; Hawkins, C. L.; Davies, M. J., Sensitizer-mediated photooxidation of histidine residues: Evidence for the formation of reactive side-chain peroxides. *Free Radical Bio Med* **2006**, 40, (4), 698-710.
66. Tomita, M.; Irie, M.; Ukita, T., Sensitized Photooxidation of Histidine and Its Derivatives . Products and Mechanism of Reaction. *Biochemistry-Us* **1969**, 8, (12), 5149-&.
67. Chu, C. H.; Erickson, P. R.; Lundeen, R. A.; Stamatelatos, D.; Alaimo, P. J.; Latch, D. E.; McNeill, K., Photochemical and Nonphotochemical Transformations of Cysteine with Dissolved Organic Matter. *Environ Sci Technol* **2016**, 50, (12), 6363-6373.