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Role of Lysine during Protein Modification by HOCl and HOBr: Halogen-Transfer Agent or Sacrificial Antioxidant?

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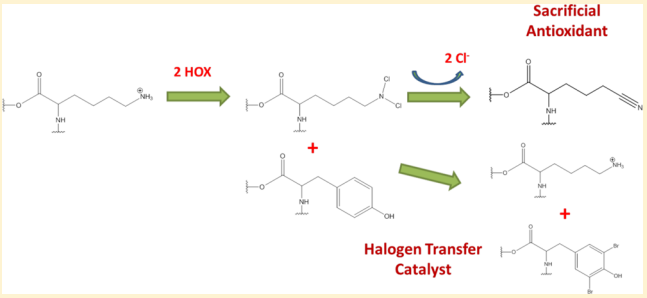
Supporting Information

ABSTRACT: Although protein degradation by neutrophil-derived hypochlorous acid (HOCl) and eosinophil-derived hypobromous acid (HOBr) can contribute to the inactivation of pathogens, collateral damage to host proteins can also occur and has been associated with inflammatory diseases ranging from arthritis to atherosclerosis. Though previous research suggested halotyrosines as biomarkers of protein damage and lysine as a mediator of the transfer of a halogen to tyrosine, these reactions within whole proteins are poorly understood. Herein, reactions of HOCl and HOBr with three well-characterized proteins [adenylate kinase (ADK), ribose binding protein, and bovine serum albumin] were characterized. Three assessments of oxidative modifications were evaluated for each of the proteins: (1) covalent modification of electron-rich amino acids (assessed via liquid chromatography and tandem mass spectrometry), (2) attenuation of secondary structure (via circular dichroism), and (3) fragmentation of protein backbones (via sodium dodecyl sulfate−polyacrylamide gel electrophoresis). In addition to forming halotyrosines, HOCl and HOBr converted lysine into lysine nitrile (2-amino-5-cyanopentanoic acid), a relatively stable and largely overlooked product, in yields of up to 80%. At uniform oxidant levels, fragmentation and loss of secondary structure correlated with protein size. To further examine the role of lysine, a lysine-free ADK variant was rationally designed. The absence of lysine increased yields of chlorinated tyrosines and decreased yields of brominated tyrosines following treatments with HOCl and HOBr, respectively, without influencing the susceptibility of ADK to HOX-mediated losses of secondary structure. These findings suggest that lysine serves predominantly as a sacrificial antioxidant (via formation of lysine nitrile) toward HOCl and as a halogen-transfer mediator [via reactions involving ε-*N*-(di)haloamines] with HOBr.



Hypochlorous acid (HOCl) and hypobromous acid (HOBr) are common ex vivo disinfectants applied to control pathogens in drinking waters, wastewaters, and recreational waters.[1] HOCl and HOBr exist in equilibrium with other free chlorine (e.g., OCl^-, Cl_2, and Cl_2O)[2] and free bromine (e.g., OBr^- and BrCl)[3] species; HOCl and HOBr are used herein to denote the sum of all free chlorine and free bromine species, respectively. Additionally, release of HOCl[4] and HOBr[5] from neutrophils and eosinophils, respectively, forms part of the host defense mechanism to pathogen invasions of human tissues.[6] In addition to inactivating pathogens, HOCl and HOBr (collectively, HOX) can damage host tissues via reactions with electron-rich biomolecules, including proteins,[7] lipids,[8] and nucleic acids,[9] despite competitive scavenging by endogenous antioxidants.[8] Enhanced peroxidase activity and HOX production have been associated with a variety of inflammatory diseases in the absence of pathogen infections (e.g., rheumatoid arthritis,[10] atherosclerosis,[11,12] asthma,[13] and Alzheimer's disease[14]), where HOX reactions with host molecules have been associated with both acute (e.g., promotion of myocardial infarction) and chronic (e.g., carcinogenesis) adverse health outcomes.[15−17]

Because of their abundance, high reactivity, and integral cellular function, proteins have been identified as particularly important targets of HOX.[18,19] Covalent modifications to amino acid residues by HOX are capable of altering bulk protein properties, including enzyme activity,[20] unfolding,[21−24] backbone fragmentation,[21,25] and aggregation.[22,23] These modifications can contribute to pathogen inactivation in vivo[20,21,25] and likely in disinfected waters. HOX−protein reactions also carry important implications for certain disease manifestations. For example, HOX modifications to β-amyloid promote plaque formation in Alzheimer's disease.[24,26,27] HOX-mediated alterations of low- and high-density lipoproteins can promote uptake by macrophages, which are transformed into

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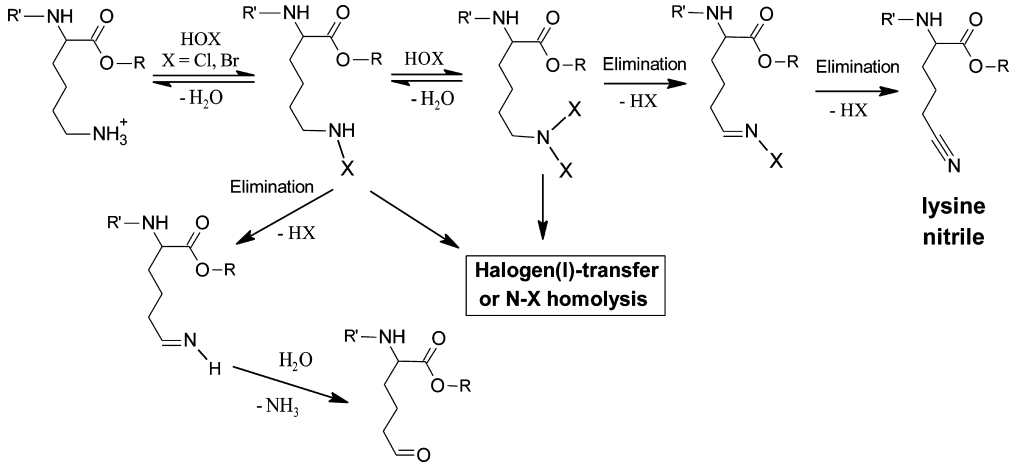

Figure 1. Putative reaction pathways of lysinyl side chains upon exposure to HOCl or HOBr.

foam cells prior to the development of atherosclerotic lesions.[12,28−30] Despite these documented associations between protein oxidation by HOX and pathogenesis, the relationship between protein structure and HOX-induced modifications remains poorly understood.

The susceptibility of the amino acid residues to oxidative modification by HOX depends on their inherent nucleophilicity, abundance, and accessibility within proteins. Amino acid moieties that can be oxidized by HOX include thiols (cysteine), sulfides (methionine), amines (lysine), and phenols (tyrosine).[18,19] Previous research has focused on quantifying rate constants for HOX, or subsequently generated reactive intermediates (e.g., organic chloramines), with free or α-N-protected amino acids, with rate constants generally in the following order: thiols ≈ sulfides > amines > phenols[18,19] (Table SI-1 of the Supporting Information). While kinetic models combining these individual rate constants largely matched experimental results for the loss of parent amino acids upon addition of HOX to mixtures of α-N-protected amino acids, they fail to match results for HOX treatment of full proteins.[31] For example, for treatment of lysozyme with a 25-fold molar excess of HOCl, an ∼60% loss of lysine and tyrosine was observed with the native protein.[31] However, an only ∼10% loss was observed with the same amino acid residues constituting lysozyme as an *N*-acetyl amino acid mixture; the kinetic model matched the results observed with the *N*-acetyl amino acid mixture rather than the native lysozyme.[31] These results suggest that the three-dimensional arrangement of amino acids within proteins plays a key role in determining the relative reactivity of the residues.

Significant attention has been paid to specific oxidizable amino acid residues. Halogenated tyrosines, particularly 3-chlorotyrosine, have been widely employed as biomarkers of HOX-mediated protein damage.[12,17] Methionine has also been implicated as a facile target of oxidation by HOX to yield methionine sulfoxide.[12] Indeed, methionine may serve as an antioxidant by this reaction, potentially protecting other residues from HOX attack.[32]

Particular emphasis has been placed on the potential role of lysine as a key mediator of intramolecular halogen-transfer reactions,[28,31] protein aggregation,[33] and protein fragmentation.[25] The first step in reactions of HOX with protein-bound lysine likely involves transfer of Cl(+1) or Br(+1) to the ε-amino group to form chloramines or bromamines, respectively.[34] Lysine-ε-haloamines have been suggested to undergo further transformations (Figure 1). Several studies have suggested that lysine-ε-haloamines can promote the halogenation of tyrosine and other residues by halogen transfer, a process that would regenerate the parent lysine.[28,31,34−37] Cleavage of N−X bonds can generate N-centered radicals, which can possibly lead to protein fragmentation.[21,25] Lastly, abundant previous biomedical and environmental chemical research into the chlorination of the α-amino group of free amino acids has demonstrated that monochloramines form aldehydes by concerted decarboxylation, in which CO_2 and chloride both serve as leaving groups.[38] α-Dichloramines form nitriles by concerted decarboxylation coupled with elimination of HCl.[38] Similar products have been detected for reactions involving the α-amino terminus of peptides.[38−41] However, less work has focused on the products of lysine-ε-haloamines, where the haloamines are several bonds removed from electron-withdrawing carbonyls. Although lysine-ε-haloamine degradation to an aldehyde has been proposed to account for the carbonyls observed upon chlorination of proteins,[7,33] work with model peptides containing lysine indicated that the formation of aldehyde from lysine-ε-haloamines was of minor importance.[34,42] Lysine nitrile (Lys-nitrile) formation from lysine-ε-haloamines in proteins has not been demonstrated, although nitrile formation was observed from model primary alkylamines.[43] Additionally, Lys-nitrile formed via the chlorination of poly-L-lysine and was detected in a chlorinated tap water, presumably formed from intermediate lysine-ε-haloamine-containing peptides.[44]

The objective of this work is to evaluate the alterations in proteins resulting from treatments with HOCl and HOBr, with a particular emphasis on the roles played by lysine. To account for the three-dimensional arrangement of amino acids, proteins [adenylate kinase (ADK), ribose binding protein (RBP), and bovine serum albumin (BSA)], rather than model peptides, were employed. Though not directly relevant to pathogens or inflammatory diseases, the structures of these three model proteins are well-characterized and so are ideal systems for improving our understanding of protein oxidative damage. Protein modifications were evaluated using liquid chromatography with tandem mass spectrometry (LC−MS/MS) to quantify covalent modifications to lysine and tyrosine, circular dichroism (CD) to monitor secondary structure, and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−

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PAGE) gels to assess aggregation and fragmentation. To test the hypotheses of previous researchers regarding the role of lysine, we employed computational protein design (CPD) to engineer an ADK variant devoid of the 16 native lysines (ADK-Kfree) while preserving the structural and functional features of the wild-type protein (ADK-wt). Comparison of protein modifications observed between the two ADK variants isolates the role of lysine while accounting for the influence of the three-dimensional arrangement of amino acid residues. Our results demonstrate that Lys-nitrile can form at yields of up to 80% from proteins challenged with HOCl or HOBr. Concomitant with the formation of Lys-nitrile is the quenching of 2 equiv of halogen atoms, which are reduced to halides (Figure 1), indicating that lysine can function as a sacrificial antioxidant, protecting other residues from halogenation. Although lysine promoted tyrosine halogenation with HOBr, lysine served predominantly as a sacrificial antioxidant minimizing tyrosine halogenation by HOCl. While lysine did not have an appreciable effect on the loss of protein secondary structure or fragmentation, CD and SDS−PAGE data indicated that susceptibility to losses of secondary structure and fragmentation both correlated with protein molecular weight.

EXPERIMENTAL PROCEDURES

Chemical Reagents. Unless otherwise specified, all chemicals were of reagent-grade purity or greater and were used as received. Reference materials included Sigma-Aldrich 3-chlorotyrosine (Cl-Tyr), boc-3-chlorotyrosine, 3,5-dibromotyrosine (Br_2-Tyr), *N*-acetylphenylalanine, and Pronase E (Protease Type XIV from *Streptomyces griseus*), Chem-Impex boc-3,5-dibromotyrosine, Fisher laboratory grade sodium hypochlorite (~6%, w/v), Acros sodium bromide (99.5%), and Cambridge Isotopes Laboratories GFL peptide [an octapeptide (YGGFLRRI) containing a central glycyl-phenylalanyl-leucine tripeptide with uniform ^{13}C and ^{15}N labeling], L-phenylalanine (with uniform ^{13}C and ^{15}N labeling), and L-leucine (with uniform ^{13}C and ^{15}N labeling). All aqueous solutions were prepared with deionized water further treated with a Milli-Q purification system (Millipore, 18 MΩ cm resistivity; hereafter, Q-water). Syntheses of reference materials for 3,5-dichlorotyrosine (Cl_2-Tyr), 3-bromotyrosine (Br-Tyr), and Lys-nitrile are described in the Supporting Information.

Working solutions of free chlorine were prepared fresh daily by diluting sodium hypochlorite into Q-water and were standardized using UV−vis spectrophotometry in which absorbance measurements were simultaneously taken at 245 and 295 nm to quantify concentrations of HOCl and OCl^- (see Table SI-2 of the Supporting Information for extinction coefficients). Working solutions of free bromine were prepared fresh daily by reacting stoichiometric amounts of NaOCl with sodium bromide for 4 min in the dark at room temperature. In the presence of HOCl, bromide is rapidly oxidized to HOBr,[45] as shown in eq 1:

$$HOCl + Br^- \rightleftharpoons BrCl + OH^- \rightleftharpoons HOBr + Cl^- \quad (1)$$

Computational Design of an Adenylate Kinase Devoid of Lysine (ADK-Kfree). Computational redesign of an adenylate kinase in which the 16 native lysines are replaced was performed using the ORBIT protein design suite.[46−49] Designs were based on the crystal structure of adenylate kinase from *Bacillus subtilis* determined with a bound inhibitor [Protein Data Bank (PDB) entry 1P3J].[50] Each lysine position was allowed to float among the 16 low-reactivity amino acids [i.e., excluding amino acids featuring rate constants with HOCl or HOBr greater than or equal to those with tyrosine (Table SI-1 of the Supporting Information)], and ORBIT was used to optimize the structure by optimizing the side chain rotamer position and scoring the resultant structure. The design sequence with the most favorable score (Figure SI-4 of the Supporting Information) was then directed into gene fabrication.

Protein Expression and Purification. Genes encoding the adenylate kinases (AdK-WT and AdK-Kfree) and ribose binding protein (RBP) were cloned into a pET30b vector (Novagen) for expression with an N-terminal hexahistidine affinity tag. Modifications of the parental AdK-WT vector to generate AdK-Kfree were made using splice overlap extension (SOE) polymerase chain reaction.[51] Expression vectors were transformed into C43(DE3) cells (Lucigen) and cultivated at 37 °C in Luria-Bertani broth.[51] Protein expression was induced by the addition of 1 mM IPTG at an OD_{600} of 1. After a 6 h induction, cells were harvested by centrifugation [10000 relative centrifugal force (rcf) for 15 min at 5 °C]. Cell pellets were resuspended in ice-cold 50 mM potassium phosphate (pH 7.5) containing Complete Protease Inhibitor EDTA-free (Roche) and lysed by four passages through an Emulsi-flex C5 column. Unlysed cells and cellular debris were removed by centrifugation (16000 rcf for 30 min at 5 °C). Supernatants were loaded onto a Ni-charged 5 mL Hi-Trap IMAC column (GE Health Lifesciences) equilibrated in 50 mM potassium phosphate (pH 7.5). Protein was eluted using an imidazole gradient from 0 to 400 mM. Peak fractions from IMAC were further purified by gel filtration on a Hi-Prep 16/600 Superdex S200 column in 50 mM potassium phosphate (pH 7.5). Final samples were assessed for purity by SDS−PAGE and quantified by absorbance at 280 nm. The preservation of the enzymatic activity of the computationally designed adenylate kinase (ADK-Kfree) was demonstrated utilizing a coupled luciferase-based assay available from Lonza Biosciences (ToxiLight) (Figure SI-5 of the Supporting Information).

Quantification of Covalently Modified Amino Acid Residues. Proteins (4−8 μM) were treated in centrifuge tubes with a 0−600-fold molar excess of free chlorine or free bromine in 0.50 mL aqueous solutions buffered at pH 7.1 with 10 mM potassium phosphate for 24 h at room temperature (21 ± 1 °C). Residual oxidants were quenched with ascorbic acid (1.0 mM).

Proteins were digested following the method of Walse et al.,[44] with modifications as noted below. Briefly, the method involves liberation of modified amino acid monomers from oxidized proteins using a mixture of proteases. While the specific locus of the modified residue within the protein is lost upon monomer liberation, this liberation facilitates quantification of the overall conversion of particular amino acids to specific products. Although proteolytic digestion is less quantitative than alternative chemical treatments (e.g., strong acid digestion), it avoids extreme pH conditions that can destroy certain byproducts (e.g., lysine nitrile[44]). Because enzymatic digestion is not quantitative, digestion efficiency was simultaneously estimated for each sample by measuring the liberation of uniformly ^{13}C- and ^{15}N-labeled phenylalanine from a spike of GFL octapeptide (36 ± 25% standard deviation; n = 16; range of 11−72%). The concentrations of precursor amino acid residues were calculated from the known amino acid sequences of the three proteins. Yields of amino acid products, representing the percentage conversion of each amino acid to a

Table 1. Summary of Oxidizable Side Chains in Adenylate Kinase (ADK), Ribose Binding Protein (RBP), and Bovine Serum Albumin (BSA), Physical Properties of the Proteins Examined Herein, and Measured Circular Dichroism Midpoint Values (CD_{50})

oxidizable residue[a]	nucleophilic atom	no. of residues per protein			
		ADK-wt	ADK-Kfree	RBP	BSA[c]
Met	S	7	7	5	5
Cys		4	4	0	35
His	N	8	8	9	16
Lys		16	0	25	59
Trp		0	0	0	3
Tyr	C	9	9	3	21
total no. of oxidizable residues		44	28	42	139
total no. of residues (oxidizable and nonoxidizable)		224	224	282	607
molecular mass (kDa)		25.1	25.1	29.7	69.3
$CD_{50\ HOCl}$[b]		2.0 ± 0.9	2.2 ± 1.2	8.0 ± 2.7	259.2 ± 54.5
$CD_{50\ HOBr}$[b]		3.5 ± 2.2	4.1 ± 2.4	28.3 ± 2.5	227.8 ± 39.3

[a]Herein, oxidizable residues are defined as having a reactivity toward HOCl greater than or equal to that of the tyrosyl side chain, for which $k = 44\ M^{-1}\ s^{-1}$ at 22 °C.[10] [b]CD_{50} values correspond to the oxidant levels (as moles of oxidant per mole of protein) associated with a 50% loss of protein secondary structure determined via CD absorbance at 222 nm. All error estimates denote 95% confidence intervals. [c]Composition of bovine serum albumin as deposited for UniProtKB entry P02769.

specific product, were calculated by adjusting the measured concentration of each amino acid product for the digestion efficiency measured in each sample using the GFL octapeptide and comparing to the known initial concentrations of each amino acid within the proteins. This procedure assumes that the enzymatic digestion efficiency is similar for the GFL octapeptide and the proteins. Results presented below indicating ~95% conversion efficiency of tyrosine to bromotyrosines from treatment of RBP with 6 molar equiv of HOBr (Figure 3), a molar ratio below that at which significant degradation of secondary structure occurs [as measured by CD_{50} (see below)], suggest that this assumption is valid. If the more complex structure of a protein resulted in a lower enzymatic digestion efficiency compared to that measured using the octapeptide, the yield of bromotyrosines would have been >100% (i.e., physically meaningless).

Protein solutions were amended with 0.22 mg of $CaCl_2$ (10 μL of a 22 mg/mL working solution), 4 nmol of GFL octapeptide (4 μL of a 1 mM stock solution in DMSO), and 5 μg of Protease Type XIV (10 μL of a 0.5 mg/mL freshly prepared stock solution). Amended solutions were incubated at 37 °C for 24 h and centrifuged, and aliquots of the supernatant (400 μL) were transferred to 2 mL sample vials fit with 500 μL glass inserts. Internal standard [4 μL of an aqueous stock solution containing caffeine (108 mg/L) and boc-lysine (540 mg/L)] was added to each 400 μL sample prior to LC–MS/MS analysis. Five covalently modified amino acid residues (Lys-nitrile, 3-chlorotyrosine, 3,5-dichlorotyrosine, 3-bromotyrosine, and 3,5-dibromotyrosine), mass-labeled phenylalanine, and internal standards were quantified via LC–MS/MS (Thermo-Finnigan Surveyor LC system coupled with a LCQ Deca mass spectrometer) using electrospray ionization in positive ion mode. As the analyte suite contained small, polar molecules (e.g., Lys-nitrile) as well as larger, less hydrophilic molecules (e.g., halotyrosines), all samples were analyzed using separate full-loop (20 μL) injections on one of two columns. A Zorbax 300-SCX column (15 cm × 2.1 mm, 5 μm, Agilent) was used for polar analytes. An Ascentis RP-amide HPLC column (15 cm × 2.1 mm, 5 μm, Supelco) was employed for less hydrophilic molecules. Analytes were quantified using multiple-reaction monitoring. Column eluent conditions and MS parameters are provided in the Supporting Information.

Assessment of Changes in Protein Primary and Secondary Structure. To assess changes in the protein secondary structure, 5 μM protein was treated with HOCl or HOBr in 5 mM potassium phosphate (pH 7.0) for 24 h, as described above. Far-UV circular dichroism spectra of the samples were recorded on an Applied Photophysics Chirascan CD spectrophotometer, using a 1 mm path-length cell with the temperature regulated at 20 °C. Samples were measured in triplicate, scanning from 320 to 200 nm at 1 nm increments, averaging for 1 s at each wavelength. CD spectra were corrected using blank samples of oxidant and buffer alone. Figure 2 presents representative CD spectra. At low oxidant dosages, the CD signal, as quantified by the mean residue molar ellipticity at 222 nm, generally exhibited significant declines with an increasing oxidant dosage (Figure SI-2 of the Supporting Information). At the highest dosages, the decline in CD signal with oxidant dosage was lower and was associated with protein fragmentation as assessed by SDS–PAGE (see below). To compare the susceptibility of the three proteins to the two oxidants, the oxidant dosage associated with the 50% loss of CD signal (CD_{50}) was calculated within the low oxidant dosage range preceding fragmentation using eq 2:

$$I_C = I_0 e^{-rC} + I_{offset} \quad (2)$$

where I_C is the CD signal at the oxidant concentration C, I_0 is the amplitude of the CD signal change over oxidant dosages below those resulting in fragmentation, C is the concentration of oxidant in molar equivalents, r is a constant with units of per molar equivalent oxidant, and I_{offset} is the amplitude of the CD signal observed at oxidant dosages immediately prior to fragmentation. Fitting of the experimental data is performed by a global optimization of I_0, r, and I_{offset} to minimize the root-mean-square deviation (rmsd) between the experimental data and the fit data. CD_{50} is back-calculated from the optimized fit as the oxidant concentration at which $I_C = 0.5I_0$. Reported errors reflect the maximal deviation observed for calculated CD_{50} values when values for I_0, r, and I_{offset} were sampled within one rmsd of the values used to calculate the corresponding CD_{50} value.

Changes in the primary structure of the oxidatively challenged proteins were assessed by SDS−PAGE. Because of the low concentration of the samples assessed by circular dichroism, 5 μM, 250 μL of each sample was lyophilized and reconstituted in 25 μL of SDS−PAGE loading buffer (125 mM Tris, 140 mM SDS, 20% glycerol, 0.2% 2-mercaptoethanol, and 0.001% bromophenol blue) to achieve a loading concentration of 50 μM. Samples were heated to 80 °C and then centrifuged at 13000 rcf for 10 min to remove precipitated potassium dodecyl sulfate. Samples were run using NuPage 4 to 12% bis-tris gradient gels (Life Technologies) and MES running buffer [50 mM MES, 50 mM Tris, 3.5 mM SDS, and 1 mM EDTA (pH 7.3)]. Proteins were detected using Coomassie Brilliant Blue R-250 staining.

RESULTS

Quantification of Oxidized Residues. For each protein assessed, the number of oxidizable residues is shown in Table 1. Oxidizable residues are defined as amino acids with side chains possessing a reactivity toward HOCl greater than or equal to that of tyrosine [i.e., Met, Cys, His, Lys, Trp, and Tyr (Table SI-1 of the Supporting Information)]. The structures of all three proteins are well-characterized, and the extent to which these oxidizable residues are surface-accessible (defined as having side chains with >10 Å^2 surface exposure[52]) is provided in Figure 2. Lysine is the most abundant oxidizable residue for

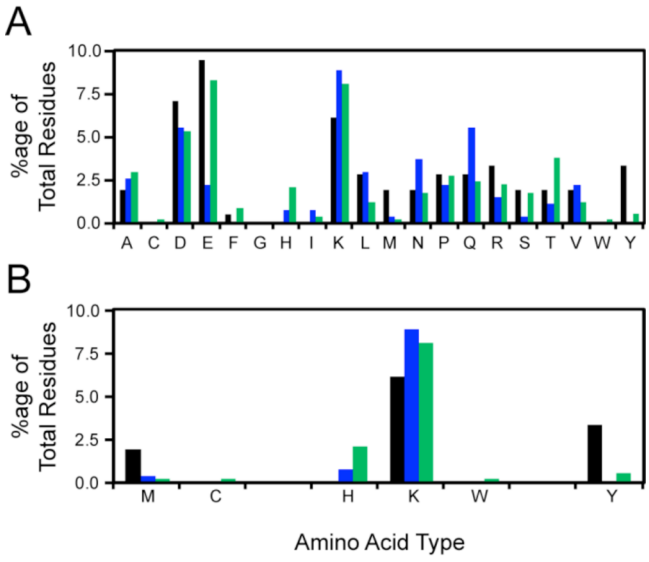

Figure 2. Surface accessibility of residues evaluated using WHATIF Accessible Molecular Surface and defined by residues having side chains with >10 Å^2 of area accessible. (A) Complete set of residues. (B) Oxidizable residues. The coloring of proteins in the plot is as follows: black for adenylate kinase (PDB entry 1P3J), blue for ribose-binding protein (PDB entry 2GX6), and green for bovine serum albumin (PDB entry 2VO3). Residues absent from the protein structure or part of affinity tags are considered to be accessible and included in the tally of accessible residues.

ADK (43%), RBP (69%), and BSA (42%), and the most surface-accessible, which suggests that lysine may influence the oxidative responses of these proteins. Methionine, histidine, and tyrosine are also fairly prevalent, but much less surface-accessible.

Proteins were treated with HOX for 24 h and enzymatically digested, and yields of oxidation products of Lys and Tyr [i.e., Lys-nitrile, 3-chlorotyrosine (Cl-Tyr), 3,5-dichlorotyrosine (Cl_2-Tyr), 3-bromotyrosine (Br-Tyr), and 3,5-dibromotyrosine (Br_2-Tyr)] were quantified via LC−MS/MS. None of the oxidized amino acids were detected in oxidant-free controls. Cl-Tyr and Cl_2-Tyr were commonly detected from proteins treated with HOCl (Figure 3). With the possible exception of BSA, chlorotyrosine yields reached a maximum at HOCl:protein concentration ratios of 12−60 and then decreased with larger HOCl doses. Lys-nitrile also was frequently detected, reaching maximal yields of 52 ± 6% at HOCl doses (HOCl:protein ratios of 60−600) larger than those corresponding to maximal yields of chlorotyrosines.

HOBr treatments generated Br-Tyr and Br_2-Tyr with yields peaking at a HOBr:protein concentration ratio of ≈12 and then decreasing at larger HOBr doses (Figure 3). Yields of Br_2-Tyr typically exceeded those of Br-Tyr, regardless of protein identity or HOBr dose. Yields of Lys-nitrile peaked at larger HOBr doses than for brominated tyrosines, reaching yields of 81 ± 9% at a HOBr:protein concentration ratio of 200. For HOX:protein concentration ratios of ≤60, yields of Lys-nitrile were greater for HOCl treatments than for HOBr treatments.

Assessment of Protein Structure as a Function of Oxidative Damage. In addition to covalent modifications of amino acids, exposure of proteins to HOX can result in the loss of protein structure because of unfolding,[20,22,53,54] fragmentation,[21,25] and aggregation.[22,23] Loss of CD absorbance is a comprehensive observable, in that protein fragmentation, unfolding, and aggregation can all result in the loss of CD signal. Accordingly, the CD midpoint (CD_{50}), the oxidant concentration at which 50% of the CD signal has been lost, was used as a principal metric of a given protein's susceptibility to secondary structural damage. The CD_{50} is interpreted as the point at which 50% of the protein ensemble or 50% of the protein secondary structure has been degraded. CD assessments of the three model proteins following 24 h treatments with HOCl or HOBr (HOX:protein concentration ratios of 0−600) are shown in Figure 4A. In general, all proteins are well-ordered in the absence of HOX and progressively lose secondary structure with an increasing oxidant dose. Table 1 summarizes CD_{50} values for proteins treated with HOCl and HOBr. The intrinsic resistance of the native proteins to oxidative structural modification, as measured by the CD_{50} (moles of HOX per mole of protein), correlated with protein size as measured by molecular mass (kilodaltons) (Figure SI-3A of the Supporting Information) for both HOCl (slope = 6.0 ± 0.5 standard error; p = 0.05) and HOBr (slope = 5.1 ± 0.03 standard error; p = 0.004). CD_{50} also correlated with the total number of oxidizable residues (Figure SI-3B of the Supporting Information) for both HOCl (slope = 2.6 ± 0.1 standard error; p = 0.03) and HOBr (slope = 2.2 ± 0.3 standard error; p = 0.08).

In addition, HOX-challenged ADK, RBP, and BSA were separated by SDS−PAGE (Figure 4B). In the case of ADK and RBP, the loss of the parental monomer band is observed across a range of HOX concentrations (HOX:protein concentration ratios of ∼10−50), where increasingly diffuse bands were observed with a coherent shift to smaller fragments at higher oxidative challenge, suggestive of fragmentation of the parents. In the case of BSA, loss of the parental species occurs only at the upper end of the range of HOX doses (HOX:protein concentration ratios of ∼100−300). Above each of the respective monomer bands in Figure 4B, higher-molecular mass bands are visible for each of the proteins tested, suggestive

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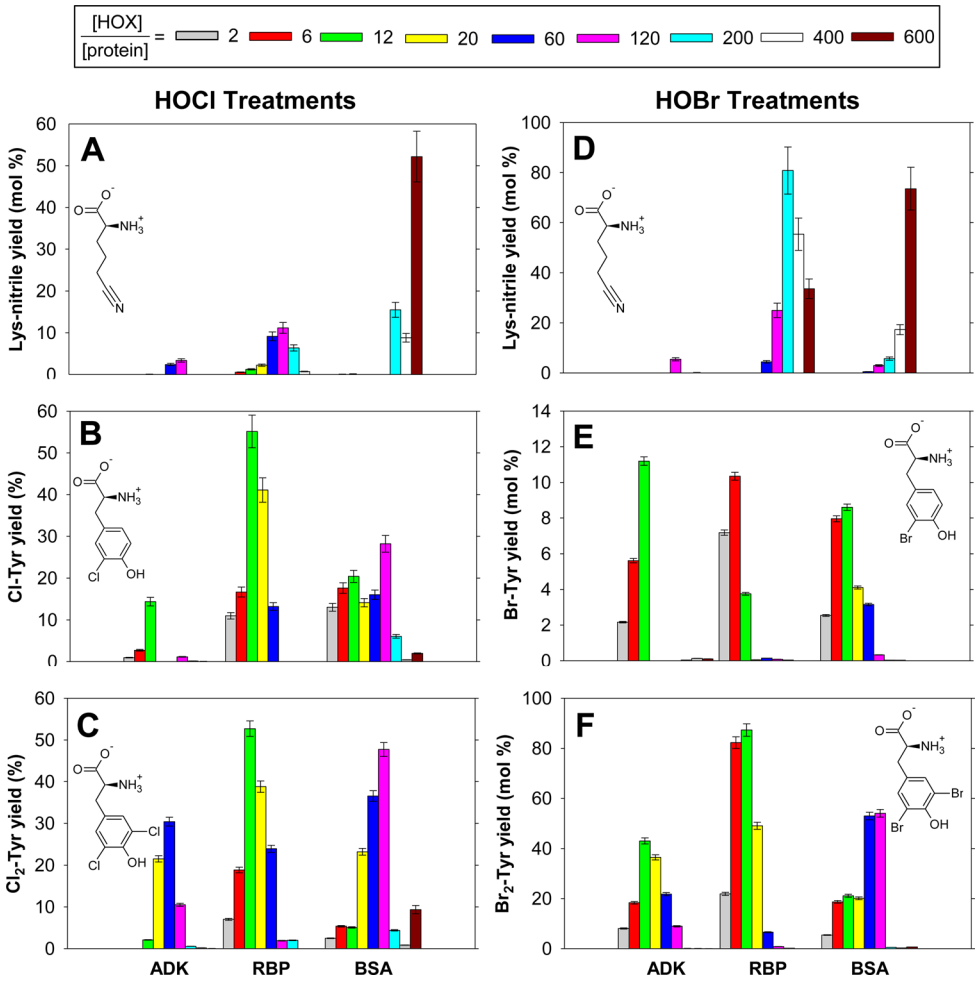

Figure 3. LC−MS/MS analysis of yields of amino acid oxidation products (as a percentage of precursor residues) following treatment of adenylate kinase (ADK), ribose binding protein (RBP), and bovine serum albumin (BSA) with HOCl (A−C) or HOBr (D−F) for 24 h prior to quenching with excess ascorbic acid. Oxidant:protein molar ratios ($[HOX]_T/[protein]$) ranged from 2 to 600 (see the legend). Monitored products: lysine nitrile (Lys-nitrile), 3-chlorotyrosine (Cl-Tyr), 3,5-dichlorotyrosine (Cl_2-Tyr), 3-bromotyrosine (Br-Tyr), and 3,5-dibromotyrosine (Br_2-Tyr). Additional reaction conditions: $[protein]_o$ = 8 μM (except $[BSA]_o$ = 4 μM), pH 7.0, [potassium phosphate] = 10 μM, T = 21 ± 1 °C. Error bars denote 95% confidence intervals. None of the monitored amino acid oxidation products were detected in control experiments performed in the absence of HOCl or HOBr.

of oligomers. This ladder persists through thermal denaturing of the proteins in SDS under reducing conditions (5 mM 2-mercaptoethanol). The SDS−PAGE technique cannot distinguish whether these apparent oligomers reflect the formation of detergent resistant aggregates or genuine covalent cross-links between the individual subunits. Nonetheless, at higher levels of oxidant challenge, the persistence of the oligomeric banding can clearly be observed to wane and exhibit the common downward trend in apparent molecular mass as noted for the parental monomer bands. In addition, there are some qualitative differences between HOCl- and HOBr-treated proteins (i.e., lane smearing). However, the putative mechanisms of oxidative damage via HOCl compared to HOBr will require additional analysis, beyond the scope of this study.

Binary Mixtures of Boc-Lysine and Boc-Tyrosine. HOX-modified Lys residues (ostensibly as Lys-haloamines) have been postulated to transfer active halogens to tyrosyl residues.[28,31,34−37] Our demonstration of Lys-nitrile formation suggests that lysine can also quench 2 equiv of active halogens. To further assess the possible dual role of lysine as an antioxidant (via Lys-nitrile formation) and/or halogen-transfer agent, mixtures of unbound Tyr and Lys, possessing boc-protected α-amino groups and unaltered carboxylic acid groups (bocTyr and bocLys, respectively), were treated with HOCl and HOBr. Products of both bocLys (bocLys-nitrile) and bocTyr [3-chloro-bocTyr (Cl-bocTyr), 3,5-dichloro-bocTyr (Cl_2-bocTyr), 3-bromo-bocTyr (Br-bocTyr), and 3,5-dibromo-bocTyr (Br_2-bocTyr)] were quantified via LC−MS (Figure 5). When bocTyr (100 μM) was treated with either 100 μM HOCl or 100 μM HOBr for 24 h, parental bocTyr concentrations decreased to ∼40 μM, and approximately equal mixtures of mono- and dihalogenated products were quantified. However, addition of 50−200 μM bocLys to the bocTyr solutions prior to HOX addition significantly attenuated losses of parental bocTyr and decreased yields of halogenated tyrosyl products. Indeed, at a bocLys:bocTyr concentration of 2, <10% of parental bocTyr was halogenated during the 24 h reaction time. BocLys-nitrile was observed in all bocLys-amended reactors and was maximal at 50 μM, where the HOX:bocLys molar ratio was 2.

Design of a Novel Adenylate Kinase To Probe the Role of Lysine. Computational protein design (CPD) was

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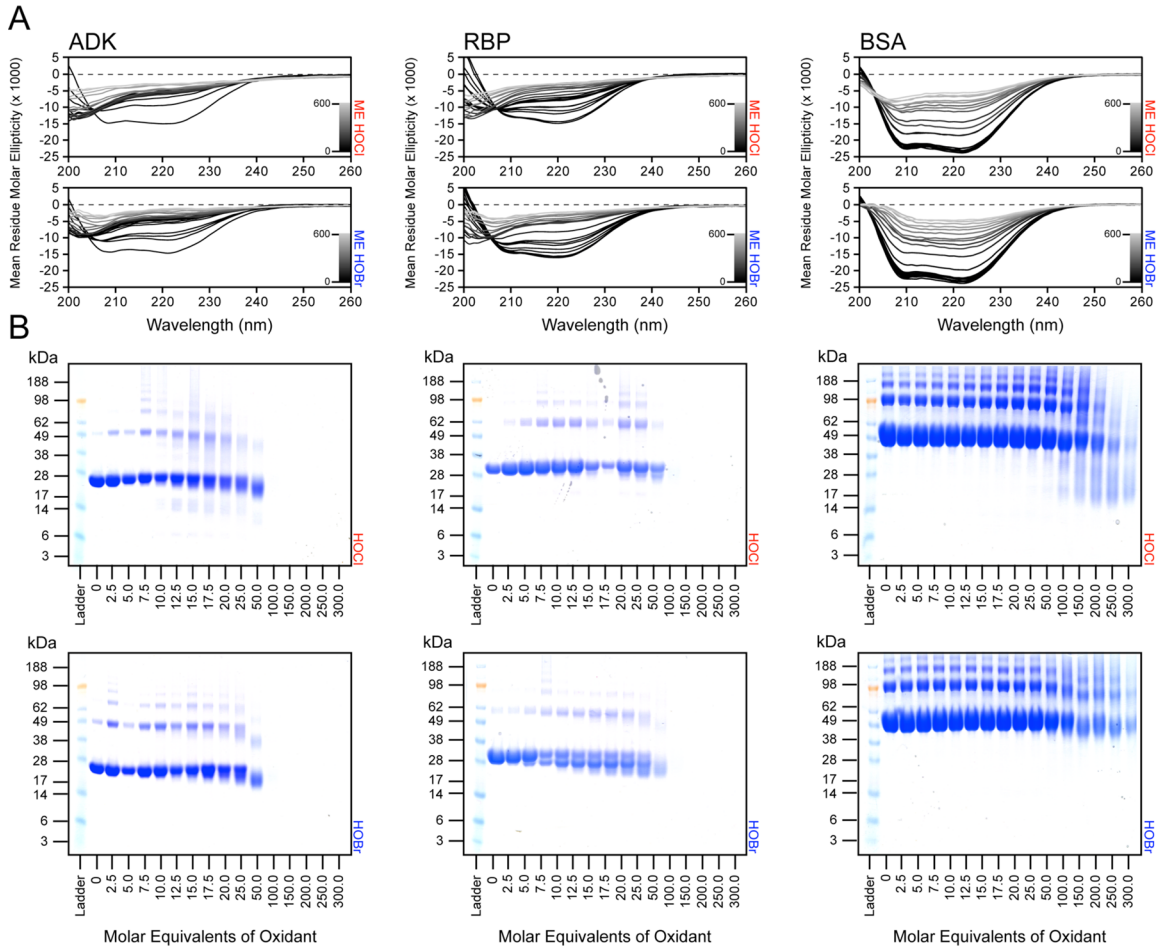

Figure 4. Comparison of structural alterations to adenylate kinase (ADK, 25.0 kDa), ribose binding protein (RBP, 29.3 kDa), and bovine serum albumin (BSA, 69.3 kDa) upon increasing exposure to HOX. (A) Changes in the observed circular dichroism following treatments with HOCl or HOBr [0−600 molar equiv (ME)]. (B) Changes in the monomer bands observed by SDS−PAGE with Coomassie staining following treatments with HOCl and HOBr (0−300 molar equiv). All oxidation reactions were performed with 5 μM protein and 5 mM potassium phosphate (pH 7.0). Samples were incubated with oxidant for 24 h prior to assessment. Samples for SDS−PAGE were lyophilized to concentrate them 10-fold to 50 μM to allow for adequate detection.

employed to redesign ADK, extensively removing all native lysines while preserving the structural and functional properties of the native enzyme (Figure SI-4 of the Supporting Information). Biosynthetic expression and subsequent purification of ADK devoid of lysine (ADK-Kfree) demonstrated no divergence from the structural and functional properties observed for the wild-type adenylate kinase (ADK-wt) (Figure SI-5 of the Supporting Information). Both adenylate kinases were principally expressed to the soluble fraction as monomers in equivalent yields (~80 mg/L). Exposure to HOCl produced a modest difference in CD signal between ADK-wt and ADK-Kfree (Figure 6), where a minor segment of ADK-Kfree, accounting for approximately 11.5% of the total circular dichroism, appears to be more susceptible to HOCl than the wild type. No such differences were evident with HOBr. Nonetheless, convergence of the ADKs at higher levels of HOCl challenge is ultimately observed. No significant differences were observed for CD_{50} values for the two adenylate kinases (Table 1). Similarly, no notable differences in the protein fragmentation pattern are observed by SDS−PAGE between ADK-wt and ADK-Kfree following treatments with HOCl or HOBr.

To examine the influence of lysine on the generation of halogenated tyrosines, LC−MS/MS residue analyses were performed after ADK-wt and ADK-Kfree were treated with varying concentrations of HOCl and HOBr. Yields of Cl-Tyr and Cl_2-Tyr from ADK-Kfree exceeded those from ADK-wt at 20- or 60-fold molar excesses of HOCl but were similar at other HOCl doses (Figure 7). For treatments with HOBr, the opposite trend was observed: at intermediate HOBr doses (HOBr:protein concentration ratio of 12), yields of Br-Tyr and Br_2-Tyr from ADK-wt generally exceeded those obtained from ADK-Kfree but were similar at other HOBr doses.

DISCUSSION

Role of Lysine with Respect to Tyrosine Halogenation. Evaluating the effect of lysine on halotyrosine formation within proteins is an important focus of this study. Halotyrosines have been widely applied as biomarkers of neutrophil-derived HOCl and predominantly eosinophil-derived HOBr.[12,17,31] Indeed, previous examinations of proteins associated with chromatin,[36] neutrophils,[55] and bacteria[55] demonstrated that tyrosines were transformed following exposure to HOCl in appreciable yields to both Cl-Tyr and Cl_2-Tyr. Surface-accessible, oxidizable residues are likely to be

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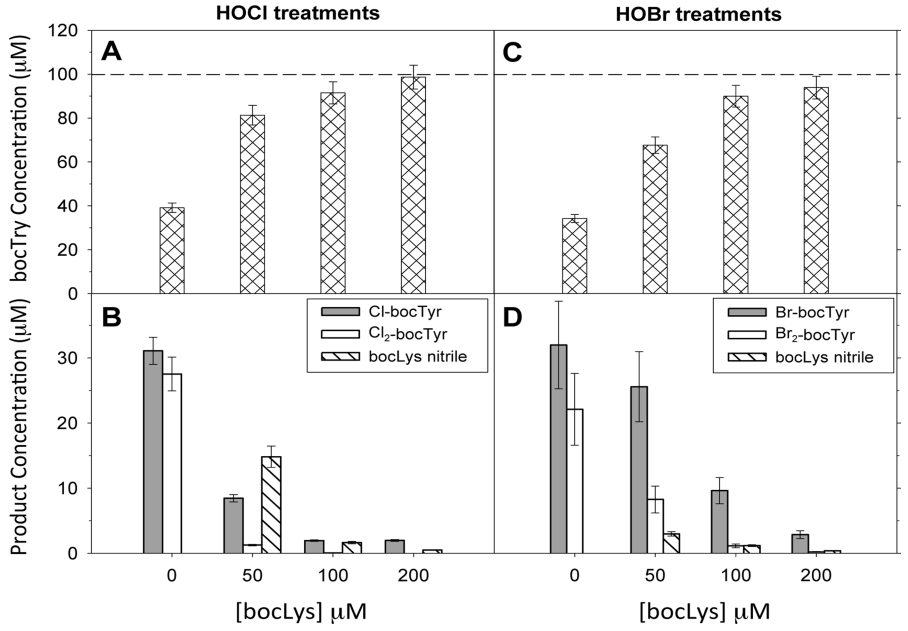

Figure 5. Influence of boc-lysine (bocLys, 0−200 μM) on the halogenation of boc-tyrosine (bocTyr, 100 μM, dashed line) following 24 h treatments with either 100 μM HOCl (A and B) or 100 μM HOBr (C and D). Measured concentrations of parental bocTyr are shown in the top row, and concentrations of halogenated tyrosyl products and boc-lysine nitrile are shown in the bottom row. Error bars denote 95% confidence intervals. Conditions: pH 7.1, [potassium phosphate] = 10 mM, T = 21 ± 1 °C.

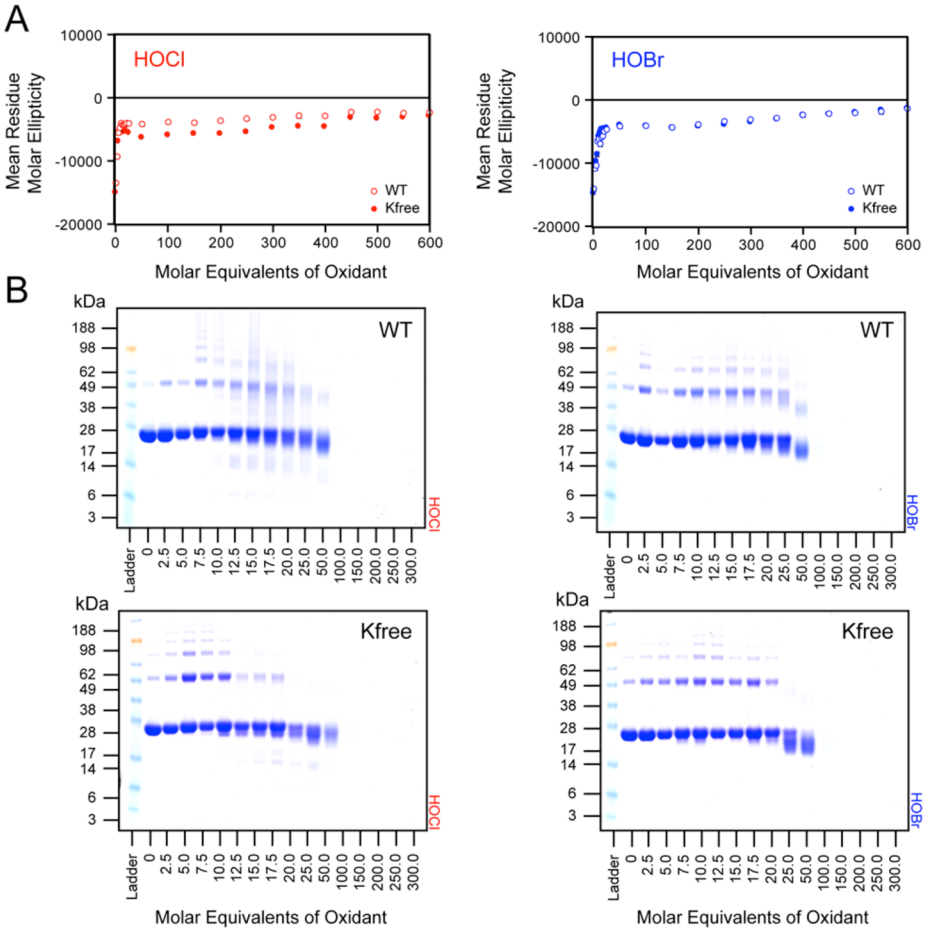

Figure 6. Comparison of the structural changes to the parental adenylate kinase (WT) and the design devoid of all lysines (Kfree) upon HOX exposure. (A) Changes in circular dichroism monitored at 222 nm: WT (filled circles) and Kfree (empty circles). (B) Changes in the bands observed by SDS−PAGE with Coomassie staining.

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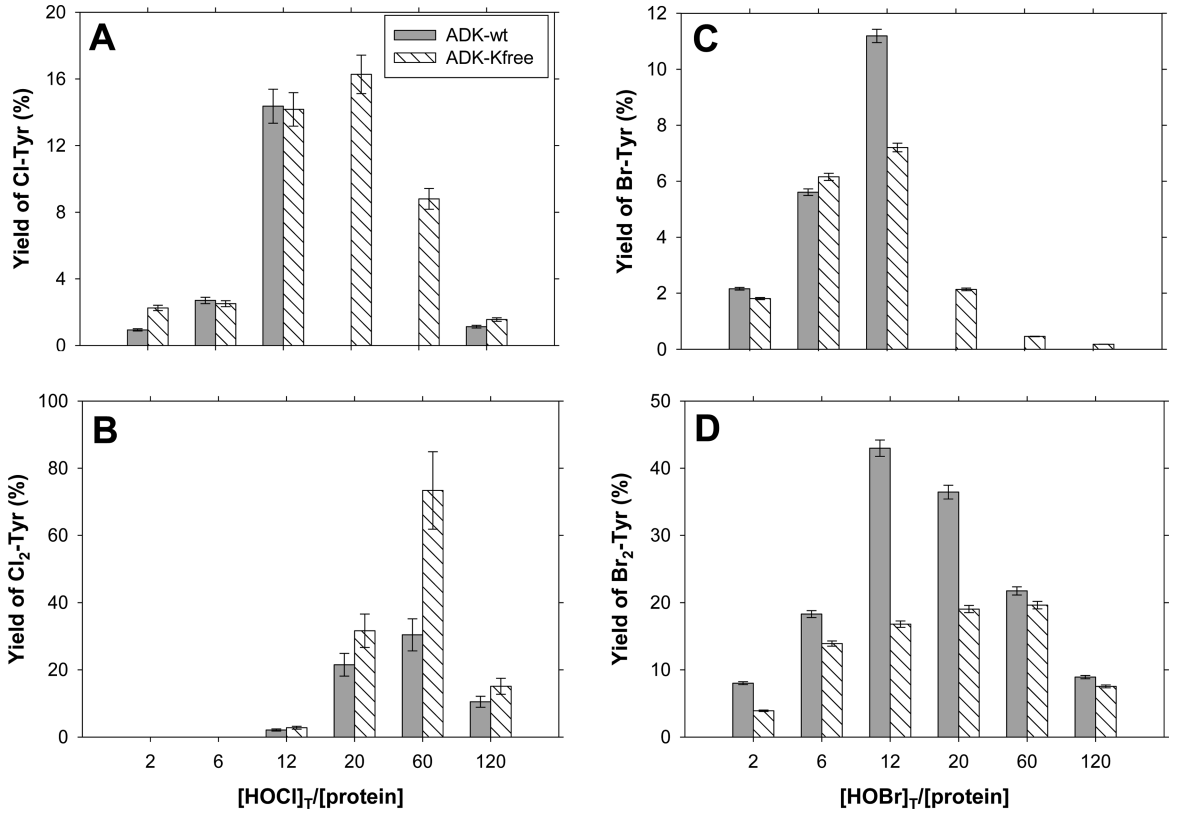

Figure 7. Yields of amino acid oxidation products (as a percentage of precursor residues) from treatments of wild-type adenylate kinase (ADK-wt; filled bars) and lysine-free adenylate kinase (ADK-Kfree; hatched bars) as a function of oxidant dose: (A and B) HOCl and (C and D) HOBr. Reactions proceeded for 24 h prior to being quenched with ascorbic acid (980 μM). Monitored products: 3-chlorotyrosine (Cl-Tyr), 3,5-dichlorotyrosine (Cl_2-Tyr), 3-bromotyrosine (Br-Tyr), and 3,5-dibromotyrosine (Br_2-Tyr). Additional reaction conditions: $[protein]_o$ = 8 μM, pH 7.0, [potassium phosphate] = 10 μM, T = 21 ± 1 °C. Error bars denote 95% confidence intervals (smaller than symbols when not shown). None of the tyrosine halogenation products were detected in control experiments performed in the absence of HOCl or HOBr.

the first targets of attack for HOX, although oxidizable residues within the protein interior will become more accessible upon degradation of protein secondary structure.[56] For all three proteins, lysine is the most abundant surface-accessible, oxidizable residue (Figure 2), suggesting that its reactions with HOX may affect the halogenation of other residues, including tyrosine. Indeed, the fact that lysine promotes the halogenation of tyrosine via transfer of a halogen from lysine-derived haloamine intermediates has been a key contention of previous research.[28,31,34−37]

Previous researchers have hypothesized that halotyrosines are stable in the presence of excess HOX.[6] For all proteins examined herein (except perhaps BSA), yields of halotyrosines reached their maxima and then decreased with increasing concentrations of HOX (Figure 3). Our results employing parental proteins corroborate recent findings of Curtis et al.,[37] who observed that Cl-Tyr and Cl_2-Tyr (derived from small peptides) are unstable in the presence of excess HOCl. In the study of Curtis et al.,[37] chlorination of unbound Cl-Tyr (to give Cl_2-Tyr) was approximately 3 times faster than chlorination of unbound Tyr by HOCl at 37 °C and pH 7.4, and Cl_2-Tyr reacted further with HOCl to give uncharacterized products. Previous investigations[57] of reactions between HOCl and phenol, the reactive functional group of the tyrosyl side chain, indicate that ring fragmentation can occur and can produce trihalomethanes.

Previous research has suggested that lysine haloamines can transfer halogen to other residues (e.g., tyrosine), regenerating the parent lysine.[28,31,34−37] Lysine haloamines also can conceivably react to give aldehydes via elimination of HX[6,28,31,33−37] (Figure 1). However, other research has suggested that significant aldehyde formation is only observed upon chlorination of α-amino groups, not from ε-chloramines of the lysinyl side chain.[34,42] Similarly, previous research has demonstrated nitrile formation via elimination of 2 equiv of HX from α-terminal dichloramines.[38−41] Although not measured, Lys-nitrile was suggested as a likely product of in vitro HOCl treatments of a Lys-containing hexapeptide.[37] Lys-nitrile was detected in chlorinated drinking water and was demonstrated to form via chlorination of poly-L-lysine.[44] Our results with model proteins indicate that Lys-nitrile can be an important product, forming on similar time scales and at yields comparable to those of any of the examined halotyrosines (Figure 3). At many oxidant concentrations, HOBr treatments gave higher yields of Lys-nitrile than HOCl treatments (Figure 3). This trend may result from the greater nucleofugality (leaving group ability) of bromine relative to that of chlorine.

Maximal Lys-nitrile yields generally were observed at higher HOX:protein molar ratios than for halotyrosines, suggesting that Lys-nitrile is more stable in the presence of excess HOX. Lys-nitrile was detected at HOCl:protein molar ratios ranging from 6 to 600 (Figure 3), corresponding to HOCl:Lys molar ratios from 0.24 to 24. In adult plasma, an average lysine concentration of 191 μM has been reported.[58] At sites of inflammation, concentrations of HOCl of up to 5 mM have been postulated,[4] although scavenging by antioxidants (e.g.,

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glutathione) could rapidly attenuate these concentrations. These concentrations suggest that [HOCl]/[Lys] ≤ 26 in plasma. Accordingly, the [HOCl]/[Lys] range wherein Lys-nitrile was observed herein likely encompasses the in vivo range at sites of inflammation.

The potential formation of Lys-nitrile in vivo suggests its possible use as a biomarker of HOX-mediated oxidative stress. Despite the lack of halogen in Lys-nitrile, its formation appears to be specific to HOX reactions. The putative mechanism of Lys-nitrile formation from lysine-dihaloamines involves two sequential dehydrohalogenation steps in which HCl or HBr is eliminated[43,44] (Figure 1). Accordingly, of the possible oxidants generated in vivo, only HOCl and HOBr are anticipated to form Lys-nitrile. For example, treatment of bocLys (50 μM) at pH 6 with H_2O_2 (294 μM for 24 h) or with hydroxyl radicals (formed by photolysis of 294 μM H_2O_2 with 1000 mJ/cm^2 of 254 nm light provided by a low-pressure mercury lamp) formed no bocLys-nitrile. Similarly, treatment of bocLys (40 μM) at pH 7 with 450 μM ozone produced no bocLys-nitrile. Additionally, lysinyl residues are more abundant than tyrosyl residues (Table 1). With yields comparable to those of halotyrosines, the greater frequency of lysinyl residues would promote higher absolute concentrations, facilitating detection. Together with their higher stability, these factors suggest that Lys-nitrile could be a useful biomarker for HOX-mediated protein damage. However, further work is needed to confirm that Lys-nitrile formation is specific to HOX and that it occurs in vivo at sites of inflammation.

Several previous studies[28,31,34−37] have suggested Lys-derived chloramines and bromamines [ε-*N*-chlorolysine, ε-*N*-bromolysine, and their dihalo analogues (Figure 1)] transfer halogen to tyrosine, thereby promoting tyrosine halogenation while regenerating the parent lysine. However, conversion of Lys-dihaloamines to Lys-nitrile reduces two halogen atoms to halide ions, thereby eliminating two oxidizing equivalents (Figure 1). Our results demonstrating Lys-nitrile formation suggest that Lys could serve as a "sacrificial antioxidant" with respect to tyrosine, scavenging HOX to protect tyrosines from halogenation by HOX. The ability of Lys to function as a sacrificial antioxidant was demonstrated via experiments with mixtures of unbound bocTyr and bocLys (Figure 5), in which even substoichiometric amounts of bocLys (relative to HOX) could significantly attenuate halogenation of Tyr by HOCl and HOBr. These results are likely due to the quenching of active halogens via Lys-nitrile formation and the inherently slower halogenation rates of Lys-haloamines relative to those of HOX.[18,19] Using mixtures of N-acetylated amino acids and small peptides, Nightingale et al.[34] also observed that Lys scavenged HOX to reduce the level of halogenation of Tyr, although Lys-nitrile formation was not measured.

Through rational, computational design of the adenylate kinase (ADK), the highly surface-represented, oxidizable residue, Lys, was systematically replaced with nonoxidizable residues without significantly perturbing the fundamental physical properties of the enzyme, which is evident in the ability of the enzyme to catalyze the formation of ATP from ADP at rates comparable to that of the wild-type enzyme (Figure SI-5 of the Supporting Information). Experiments comparing halotyrosine yields from ADK-wt to those of ADK-Kfree (Figure 7) indicated that, in the absence of Lys (ADK-Kfree), tyrosine residues are more likely to be chlorinated and less likely to be brominated at intermediate doses of HOCl and HOBr, respectively. These results suggest that Lys may be more likely to serve as a sacrificial antioxidant with respect to tyrosine when proteins are challenged with HOCl and to promote the transfer of a halogen to tyrosine when challenged with HOBr. These results contradict previous suggestions that Lys serves predominantly as a chlorine-transfer agent in the presence of HOCl, promoting chlorotyrosine formation.[31] However, they are consistent with the work of Wu et al.,[35] who found that Lys-bromamines can efficiently shuttle active bromine to tyrosyl residues. These results also concur with previous research in water disinfection indicating that chloramines are less reactive halogenating agents than the analogous bromamines.[1] While highlighting lysine's role as a sacrificial antioxidant, the use of free amino acids failed to detect important differences between HOCl and HOBr reactions in full proteins (Figure 5). These results demonstrate the importance of employing full-length proteins, in particular selectively engineered protein variants, capable of manifesting effects arising from the three-dimensional arrangement of amino acid residues. The ability of Lys to scavenge HOX and form Lys-nitrile has been largely overlooked in the literature. While the focus here was the effect of these competing pathways of lysine on tyrosine halogenation, we are currently evaluating the effect of lysine on other oxidizable residues. Additionally, because of its prevalence as a surface-accessible residue, it is anticipated that the conversion of lysine, with a positively charged ε-amine at pH 7.4, to lysine nitrile, with no charge on the ε-nitrogen, would itself impact protein behavior.

Factors Affecting Secondary Structural Modifications. Ultimately, loss of secondary structure must derive from a combination of the alterations in the physical properties (e.g., hydrophobicity or hydrophilicity) of the individual amino acid residues resulting from HOX-mediated covalent modifications, and the interactions of these modified residues within the three-dimensional conformation of the protein. Accordingly, understanding the importance of specific amino acid residue modifications is important. For example, previous research had suggested that formation of aminyl radicals from lysine chloramines could play an important role in the loss of protein secondary structure by promoting fragmentation of peptide bonds.[21,25]

Our results with native proteins indicate that loss of secondary structure (quantified as CD_{50}) correlated with protein size for both HOCl and HOBr (Table 1 and Figure SI-3 of the Supporting Information). Such correlations are reasonable given that the greater number of residues in larger proteins should necessitate larger oxidant doses to achieve a similar percentage reduction in protein secondary structure. Drawing from the availability of high-resolution structures of the proteins assessed in this study, we evaluated the abundance of oxidizable residues and their surface accessibility. Lysine stands out among the known oxidizable residues presented at the protein surface (Figure 2). Broad correlations were also observed between CD_{50} and total oxidizable residues (TOR) for native proteins (Figure SI-3 of the Supporting Information).

However, in the engineered ADK experiments, depletion of Lys residues (7.14% of all residues, 12.4% of surface-accessible residues, and 34.0% of the oxidizable residues) produced no significant change in the progressive dysfunction observed upon oxidative stress when assessed by CD and SDS−PAGE (Table 1 and Figure 6). These results suggest that lysine does not play a critical role in determining the susceptibility of proteins to HOX-mediated loss of secondary structure. Furthermore, these results indicate that factors other than molecular mass and the

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number of oxidizable residues must be considered. For example, the degree of structural stability conferred by the three-dimensional interactions of amino acid residues likely affects the resistance of proteins to HOX-mediated decay, a possibility we are currently evaluating using model protein variants with different stabilities. Although oxidative transformations of protein secondary structure likely ultimately derive from covalent modifications to amino acid residues altering their physical properties, understanding how these alterations translate into secondary structural alterations will be a complex process. The engineering of computationally designed protein variants that encompass effects deriving from the three-dimensional conformation of the protein should prove to be useful in unraveling such effects.

■ ASSOCIATED CONTENT

Supporting Information

Details of synthesis, analytical methods, enzymatic activity assays, rate constants, additional CD spectra, and correlations between structural decay as assessed by CD and protein molecular mass or the prevalence of oxidizable residues. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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