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Leveraging the Mechanism of Oxidative Decay for Adenylate Kinase to Design Structural and Functional Resistances

Stanley C. Howell¹, David H. Richards², William A. Mitch³, and Corey J. Wilson^{1,2,4,*}

¹Department of Chemical and Environmental Engineering, Yale University, New Haven, CT

²Department of Molecular Biochemistry and Biophysics, Yale University, New Haven, CT

³Department of Civil and Environmental Engineering, Stanford University, Stanford, CA

⁴Department of Biomedical Engineering, Yale University, New Haven, CT

Abstract

Characterization of the mechanisms underlying hypohalous acid (*i.e.*, hypochlorous acid or hypobromous acid) degradation of proteins is important for understanding how the immune system deactivates pathogens during infections, and damages human tissues during inflammatory diseases. Proteins are particularly important hypohalous acid reaction targets in pathogens and in host tissues, as evidenced by the detection of chlorinated and brominated oxidizable residues. While a significant amount of work has been conducted for reactions of hypohalous acids with a range of individual amino acids and small peptides, the assessment of oxidative decay in full-length proteins has lagged in comparison. The most rigorous test of our understanding of oxidative decay of proteins is the rational redesign of proteins with conferred resistances to the decay of structure and function. Toward this end, in this study we experimentally determined a putative mechanism of oxidative decay using adenylate kinase as the model system. In turn, we leveraged this mechanism to rationally design new proteins and experimentally test each system for oxidative resistance to loss of structure and function. From our extensive assessment of secondary-structure, protein hydrodynamics and enzyme activity upon hypochlorous acid or hypobromous acid challenge, we have identified two key strategies for conferring structural and functional resistance. Namely, the design of proteins (adenylate kinase enzymes) that are resistant to oxidation requires complementary consideration of protein stability and the modification (elimination) of certain oxidizable residues proximal to catalytic sites.

*Corresponding author: corey.wilson@yale.edu.

**Additional Materials and Methods are given in Supporting Information.

Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

Notes

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Supporting Information. Gaussian reconstruction of oligomeric states, histogram reconstruction of the chromatograph/activity of the isolated fractions of bsAdK, tabulated (CD₅₀, MONO₅₀, and ACT₅₀) data, alignment of Design positions and supplemental materials and methods, assessment the role of relative exposure of oxidizable residues and dynamics on bsAdK resistance to oxidative decay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

Protein Oxidation Damage; Computational Protein Design; Oxidation Resistant Enzymes

INTRODUCTION

Both the immune system response to pathogen infections and chronic inflammatory diseases are associated with the production of reactive oxidants by a subset of white blood cells.^{1–3} In neutrophils, myeloperoxidase produces hypochlorous acid (HOCl) from hydrogen peroxide and chloride.^{1, 4, 5} Another class of white blood cells, eosinophils, generates hypobromous acid (HOBr) from hydrogen peroxide and bromide by eosinophil peroxidase.^{4, 6, 7} These hypohalous acids (HOX — *i.e.*, HOCl or HOBr) are considered important oxidants controlling the deactivation of pathogens and indeed are widely applied for disinfection of drinking water, wastewater and recreational waters.^{1–3, 5, 6, 8, 9} Due to their high reactivity with HOX and prevalence in biological systems, proteins are considered to be important targets for HOX oxidation^{10–14}, yet the mechanisms behind HOX deactivation of proteins remain poorly understood. Characterization of these mechanisms would improve our understanding of disinfectant deactivation of pathogens and how pathogens may evolve to resist oxidative deactivation *in vivo* or in engineered treatment systems.

Previous research has used rate constants measured for HOX reactions with individual amino acids in kinetic models to predict which residues will be the first targets of HOX attack.¹⁵ Products of HOX reactions have been characterized for certain amino acids (*e.g.*, halotyrosines).^{15–17} Although oxidation products, particularly halotyrosines, have been used as biomarkers for HOX oxidation in full proteins^{16, 18, 19}, the majority of studies have focused on simple model systems—either free amino acids or small peptides.^{20–22} While kinetic models combining these individual rate constants largely matched experimental results for loss of parent amino acids upon HOX application to mixtures of N-acetyl amino acids, they did not match results for HOX treatment of full proteins.¹⁵ For example, for treatment of lysozyme at a 25 molar excess with HOCl, ~60% loss of lysine and tyrosine were observed with the native protein, but only ~10% loss was predicted by the kinetic model or observed with the same amino acid residues constituting lysozyme as a N-acetyl amino acid mixture.¹⁵

The inability of current kinetic models employing rate constants for HOX reactions with free amino acids to predict degradation of amino acids in full proteins suggests that full proteins must be evaluated. A reasonable assumption is, the first sites of HOX attack are determined not just by the innate reactivity of residues with HOX, but are influenced by the three-dimensional arrangement of oxidizable residues, in addition to other physicochemical properties of the protein system. Additionally, the covalent modifications to amino acid side chains resulting from HOX oxidations can alter the amino acid interactions responsible for protein structure, stability, and function. In our initial foray into evaluating HOX oxidation of full proteins, model proteins treated with a wide range of molar equivalents of HOX were evaluated for formation of specific lysine and tyrosine oxidation products, and structural degradation.¹⁷ In the latter case, oxidative decay was accompanied by apparent aggregation,

with fragmentation observed at the highest HOX dosages. However, elucidating mechanisms of HOX decay was beyond the scope of the previous study.

To better understand the role of protein structure and amino-acid composition on oxidant-susceptibility, we focused our current study on evaluating the association between HOX-mediated structural damage and loss of enzymatic function using the model protein system adenylate kinase (AdK). This member of the class of ubiquitous nucleotide kinases was selected because nucleotide kinases have been extensively studied by numerous biophysical methods to probe the structure-function relationship in the absence of HOX exposure, which serves to provide a rich foundation to ultimately assess the role of oxidative modification and enzymatic inactivation. From our extensive assessment of secondary-structure, protein hydrodynamics and enzyme activity upon HOCl or HOBr challenge, we were able to construct a mechanism for HOX mediated decay of *Bacillus subtilis* adenylate kinase (bsAdK) to determine the sequence of events that lead to structural decay and deactivation. In addition, we experimentally characterized homologs of AdK to examine the role of variation in the composition and position of oxidizable residues, in a fixed topology. In turn, we leveraged our putative HOX decay mechanism (and knowledge of AdK homologue susceptibility) to rationally design novel bsAdK enzymes that are resistant to oxidative stress.

RESULTS AND DISCUSSION

Constructing a mechanism of oxidative structural decay for the model system adenylate kinase

Our previous work with *Bacillus subtilis* adenylate kinase (bsAdK) suggests a multi-stage process of HOX-mediated structural damage.¹⁷ At low HOX challenge (*i.e.*, 1–25 Molar Equivalents (ME), discrete steps under equilibrium), an initial abrupt loss of secondary structure was observed *via* far UV-circular dichroism (CD) (200 – 250 nm). As the oxidant challenge was increased (>25 ME), a second phase was observed in which the residual secondary structure continued to diminish, albeit with a substantially smaller amplitude. In addition, bsAdK progressively challenged with HOX was evaluated by way of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). During the initial abrupt loss of secondary structure (observed *via* far UV-CD), negligible changes were observed (*i.e.*, by way of SDS-PAGE) in the primary structure of the protein. When the residual secondary structure (far UV-CD) plateaued at ~30% residual structure, the appearance (*via* SDS-PAGE) of higher molecular weight species could be observed. Tentatively, the higher molecular weight species were attributed to either the formation of detergent resistant aggregates or oxidative crosslinking. At >12.5 ME oxidant, SDS-PAGE suggested the formation of smaller species by backbone fragmentation. Similar trends were observed for two additional proteins—*i.e.*, ribose binding protein and bovine serum albumin.¹⁷

In our previous study¹⁷, determination of the mechanism of oxidative decay for the model system bsAdK was limited by the low-resolution of the far UV-CD measurements and non-native conditions imposed by SDS-PAGE assessments. To develop a more accurate and detailed description of HOX-mediated decay of structure and function for bsAdK requires assessment of the system under native conditions in solution. Accordingly, in this study we

experimentally evaluated bsAdK hydrodynamics and enzyme activity upon transient (*i.e.*, 24 hour) exposure to increasing concentrations of HOX, to complement far UV-CD data, see **Materials and Methods**. In Figure 1A, we show the progressive loss of global secondary structure upon oxidative challenge with HOCl *via* far UV-CD, in the range of 0–25 ME. These data show a progressive loss of signal at 222 nm indicating a loss of secondary structure with increased HOX concentrations, consistent with our previous report.¹⁷ However, far UV-CD only reports on the global loss of secondary structure, which could result from protein unfolding, aggregation or degradation. To better resolve the cause of secondary structure loss we evaluated the decay of monomeric bsAdK using molecular-sieve chromatography. Shown in Figure 1B are the series of size-exclusion chromatographic separation changes exhibited by bsAdK across a discrete step gradient of HOCl oxidant dosages. Upon HOCl challenge, the dominant peak at 16mL retention volume (*i.e.*, monomeric bsAdK in solution) diminishes with a corresponding increase in the population of heterogeneous species with larger hydrodynamic radii—indicative of aggregated or unfolded states, a similar observation was made upon HOBr challenge, see Supporting Information, Figure S1. To estimate the number of putative non-native states, each of the chromatograms were decomposed using a Gaussian reconstruction (see Supporting Information, Figure S2) to quantify the changes in the observed aggregated and unfolded states. A maximum of 6 putative species were observed at 20 ME of HOCl (*i.e.*, 1 minor folded-monomeric state, 1 unfolded ensemble, and 4 aggregated states). The physical limitation of our molecular-sieve chromatography experiment prevented direct observation of degraded species. However, bsAdK fragmentation in solution was observed in the range tested *via* sedimentation velocity analytical ultra centrifugation, see Figure 1C. Analysis of these data suggests the global secondary structure measured *via* far UV-CD and fraction of monomer upon oxidative challenge show a slight decoupling of the decay curves—*i.e.*, the molar equivalents of HOCl where the population of monomeric bsAdK is ~50% (MONO₅₀) is lower than the signal in which 50% of the secondary structure (CD₅₀) is observed, see Figure 1D and Figure 2. Such that the ratio of the two metrics (*i.e.*, MONO₅₀/CD₅₀) represents the extent of oxidatively unfolded, aggregated, or fragmented species, after the removal of HOX (in the absence of substrate), see Figure 2. The decoupling of far UV-CD relative to the percent of monomer present upon exposure to HOBr has a similar mechanism but more salient, see Supporting Information, Figure S1C. Note: additional quantitative analysis for HOX exposure is given in Figure 2.

Constructing a mechanism of adenylate kinase oxidative-mediated functional decay

To quantify the susceptibility of enzyme function to HOX exposure, experimental assessment of bsAdK function (*i.e.*, the conversion of ATP + AMP to 2 ADP) was conducted (post-HOX challenge after 24 hour exposure) using an HOX step size coincidental to the CD and hydrodynamics experiments. Unlike the structural metrics, the profile of activity loss of total bsAdK enzyme upon oxidative challenge has an apparent sigmoidal deterioration with a mid-point (ACT₅₀) that occurs at a significantly higher concentration of HOX relative to the observed MONO₅₀, Figure 1E. Previous studies suggest that the presence of substrate can confer additional stability to the bsAdK enzyme, which can induce protein folding — resulting in catalytically active protein.^{23, 24} Accordingly, this apparent decoupling of HOX structural and functional resistance (*i.e.*, ACT₅₀>MONO₅₀) might be explained (at least in

part) by a similar paradigm. To test this assertion we assessed protein hydrodynamics after transient (24 hour) exposure to increasing concentrations of HOX, in the presence of a non-reactive substrate analog P_1, P_5 -Di(adenosine-5') pentaphosphate (AP_5A). Unlike the canonical substrates (*i.e.*, ATP and AMP), AP_5A is not transformed into products by the enzyme. As a result of the analogues strong interactions with oxidatively-challenged bsAdK, the effects of substrate binding can be captured *via* size exclusion chromatography. The result in Figure 1E, shows that at low-oxidant challenge (*i.e.*, < 10 ME) the substrate can induce a refolding reaction, increasing the $MONO_{50}$ population nearly 2-fold, respectively. In other words, the oxidative modifications associated with low HOX exposure can produce unfolded bsAdK that can refold in the presence of substrate and catalytically function. In Figure 1E bsAdK function was assessed on the total enzyme, to improve the resolution of our functional assessment discrete bsAdK fractions were collected over the course of the size exclusion chromatographic separation of the protein at several oxidant challenges and the individual fractions were assayed for function. Shown in Figure 1F is a representative comparison of the chromatographic trace and the histogram reconstruction of the chromatograph based on the observed activity of the isolated fractions of separated bsAdK, at 4 ME of HOCl. A more complete series of these comparisons is shown in Supporting Information, Figure S1. These species (*i.e.*, discrete monomer, unfolded, and aggregated states) targeted functional assessments of bsAdK reveals that the functional population occurs predominately within the monomer population, with some recovery in the unfolded state. Accordingly, this observation in conjunction with earlier data suggests that the oxidative deactivation of bsAdK occurs *via* irreversible unfolding, aggregation or site-specific deactivation of the active-site, and the extent of substrate induced refolding can be quantified *via* the $ACT_{50}/MONO_{50}$ ratio, see Figure 2.

Putative mechanism of HOX-mediated structural and functional decay of bsAdK

Observations in Figures 1 and 2 led us to postulate a general mechanism for HOX-mediated degradation of structure and function for wild-type bsAdK (Scheme 1). In the proposed mechanism, at low HOX concentrations oxidative damage occurs, which we suggest leads to protein unfolding and or aggregation (*i.e.*, $MONO_{50}/CD_{50} < 1$). While moderate oxidative damage promotes protein unfolding, activity can be maintained *via* substrate induced refolding (*i.e.*, $MONO_{50}/ACT_{50} > 1$), as long as this initial oxidative damage does not affect the active site (Scheme 1, steps 5 and 6). At higher oxidant exposure, irreversible unfolding and aggregation are associated with loss of enzymatic function (Scheme 1, steps 2 and 3). At the highest HOX doses, oxidation of the less reactive peptide bonds²² leads to degradation of the primary structure (Scheme 1, step 4). In general, the overall mechanisms of oxidative decay of bsAdK challenged with HOCl or HOBr are remarkably similar, given that the two oxidants can have quite profound differences in their reactivity with some residues.^{17, 25, 26} The most salient differences for the bsAdK case study, is that HOBr is a slightly stronger oxidant resulting in a higher susceptibility to structural (*i.e.*, CD_{50} and $MONO_{50}$) decay. However, a larger fraction of the HOBr unfolded protein can undergo substrate induced refolding, which leads to ACT_{50} that are nearly identical for both oxidants, see Figure 2.

Evaluating the role of protein stability in mitigating oxidative decay of adenylate kinase homologues structure and function

A reasonable hypothesis that can be drawn from our putative mechanism for oxidative decay is additional protein stability can mitigate oxidative decay of protein structure *via* reducing the probability of oxidative unfolding (Scheme 1, Step 5). To test this assertion we experimentally evaluated three additional homologues of adenylate kinase with similar structures but different thermal stabilities—*i.e.*, *Bacillus globisporus* (bgAdK), *Escherichia coli* (ecAdK), and *Geobacillus stearothermophilus* (gsAdK), Figure 3. The three-dimensional structures for bgAdK, ecAdK, and gsAdK are nearly identical to bsAdK, such that the all-atom structural alignment RMSD for bsAdK to ecAdK = 0.849 (Å); bsAdK to gsAdK = 0.498 (Å) and bsAdK to bgAdK = 0.505 (Å) (Figure S3) with nearly equivalent molecular weights (see Supporting Information, Table S1). The order of thermostability (*i.e.*, melting temperature (T_m)) for the AdK homologues measured *via* differential scanning calorimetry is bgAdK (T_m = 45 °C) < bsAdK (T_m = 51 °C) < ecAdK (T_m = 54 °C) < gsAdK (T_m = 72 °C). bgAdK, ecAdK and gsAdK homologues were evaluated for susceptibility to HOX-mediated decay *via* far-UV CD, hydrodynamics and activity assay. The susceptibility to structural decay is reported as molar equivalents (ME) of HOX where 50% loss of secondary structure (CD_{50}) and monomer population in solution ($MONO_{50}$) is observed. In addition, susceptibility of each AdK homologue to loss of activity upon HOX challenge was quantified, using the molar equivalents of HOX at which 50% activity is observed (ACT_{50}) as the principal metric, Figure 3A–B. Mechanistically, all of the homologues are similar to bsAdK reference experiments (see Figures 2 and 3). That is to say, each AdK homologue experiences oxidative decay that results in unfolded and/or aggregated species upon transient exposure to HOX (*i.e.*, $MONO_{50}/CD_{50} < 1$). Moreover, in each case a fraction of the HOX-mediated unfolded ensemble can undergo substrate induce refolding reaction and contribute to catalytic turnover (*i.e.*, $ACT_{50}/MONO_{50} > 1$), Figure 3A–B.

To quantify the role of protein stability in this collection of AdK homologs, we evaluated the CD_{50} , $MONO_{50}$, and ACT_{50} metrics relative to bsAdK—*i.e.*, resulting in CD_{50} ,

$MONO_{50}$, and ACT_{50} values, where Δ = (homologue value – bsAdK value) see Figure 2C–E. While each of the homologues has a HOX decay mechanistically similar to bsAdK, unlike bsAdK HOBr is not necessarily the stronger oxidant for each of the homologues—*i.e.*, CD_{50} , $MONO_{50}$, and ACT_{50} values for HOCl are typically lower than HOBr, see Figure 3C–D. In general, there is no apparent correlation for CD_{50} , $MONO_{50}$, and

ACT_{50} values with protein stability between the homologues. Interestingly, ecAdK transient exposure to HOCl and HOBr resulted in higher than expected increases in the

$MONO_{50}$ populations and ACT_{50} , given the protein stability. In other words, protein stability does not appear to be the sole determinant of structural or functional resistances, given proteins with nearly identical topology (three-dimensional structures). Accordingly, these data suggest that—while each of the AdK homologues have nearly identical topologies, and mechanism of HOX decay—the number and position of oxidizable residues (see Supporting Information, Table S2) can have a profound impact on resistance to oxidative stress, at least in the case study of AdK homologues.

Evaluating the role of protein stability *via* the rational redesign of bsAdK

To isolate the role of stability (from the effects imposed *via* variation in the number, position and composition of oxidizable residues inherent to the AdK homologues) on HOX-mediated decay of structure and function, we explored three redesigned bsAdK homologues with greater thermostabilities relative to wild-type bsAdK. Unlike the AdK homologues (ecAdK, gsAdK, and bgAdK — in which topology is the only constraint) each stability design variant fixes the protein topology and total number of oxidizable residues (*i.e.* in terms of number, identity and positions), while varying bsAdK scaffold thermostability. Thus the bsAdK design variants are better suited to investigate the role of protein stability (in isolation) on resistance to HOX-mediated structural and functional decay. Additional bsAdK stability was achieved *via* computational protein design in which hydrophobic repacking of the enzyme core was the objective²⁷. Namely, conferred stability was achieved *via* increasing the number and quality of Van der Waals interactions in each of three variants, using a discreet multi-state design strategy outlined in²⁷. Here, we studied three redesigned bsAdK variants (ds1AdK, ds2AdK and ds3AdK) with progressively increased thermal stabilities and equivalent (identical) numbers of oxidizable residues (see Supporting Information Tables S3 and S4). Protein stability for each of the bsAdK variants was determined experimentally *via* differential scanning calorimetry (T_m)— *i.e.*, bsAdK ($T_m = 51\text{ }^{\circ}\text{C}$) < ds1AdK ($T_m = 56\text{ }^{\circ}\text{C}$) < ds2AdK ($T_m = 60\text{ }^{\circ}\text{C}$) < ds3AdK ($T_m = 68\text{ }^{\circ}\text{C}$). Each redesigned variant was non-trivial and required 7–10 modifications to the protein core to confer additional stability through a single non-bonded (hydrophobic) force, see Supporting Information, Tables S5.

Mechanistically, each of the variants responded to oxidative decay in a similar fashion to the parent bsAdK scaffold. Namely, exposure to HOX results in unfolding or aggregation (*i.e.*, $\text{MONO}_{50}/\text{CD}_{50} < 1$), such that a fraction of the unfolded species can undergo substrate induced refolding reaction (*i.e.*, $\text{ACT}_{50}/\text{MONO}_{50} > 1$), Figure 4A–B. Interestingly, with an increase in conferred stability the protein system has an apparent increase in the propensity to aggregate or unfold (*i.e.*, the $\text{MONO}_{50}/\text{CD}_{50}$ decrease with increased stability) upon exposure to HOX. In addition, there is a progressive decrease in the ability of the variants to undergo substrate induced refolding with increased stability (*i.e.*, the $\text{ACT}_{50}/\text{MONO}_{50}$ metric decreases with increased stability), Figure 4A–B. Evaluation of the design variants relative to wild-type bsAdK shows a clear correlation between resistance to HOX-mediated structural decay and protein stability, see Figure 4C–D. Namely, as protein stability increases both the CD_{50} and MONO_{50} values increase, where HOCl appears to be a slightly stronger oxidant. However, the structural resistance does not translate into functional resistance. Specifically, the ACT_{50} for HOCl-mediated decay has an upper limit of ~5 molar equivalents for all variants, despite having MONO_{50} that resist structural decay at > 5 ME. Similarly, ACT_{50} for HOBr has an upper limit of ~5ME for ds1AdK, and decreases with increased stability (ds2AdK and ds3AdK), despite having MONO_{50} values that exceed 10 ME (*i.e.*, ds3AdK). The observed functional susceptibility (relative to the observed structural resistance) is likely the result of site-specific damage to residues within or proximal to the active-site at higher HOX concentrations, with a higher sensitivity for HOBr-mediated decay of activity.

Assessing the role of relative exposure of oxidizable residues and dynamics on bsAdK resistance to oxidative decay

The observed HOX resistance to structure and activity (imposed by stability or changes in oxidizable residue composition) are not likely due to collateral physical properties—*i.e.*, slight variations in oxidant accessibility or moderate changes in ground state dynamics upon mutation. In a set of control experiments, we evaluated the role of the relative accessibility of oxidizable residues and protein dynamics in bsAdK, for 4 point-mutated bsAdK variants (*i.e.*, bsAdK: Q199R/G214R, Q199R/G213E, Q199R/A193V, and Q199R/T179I) where each variant has the same stability ($T_m = 54^\circ\text{C}$), identical oxidizable residues (*i.e.*, number and position) and greater than 99% sequence identity, identical topologies and high-resolution structural coordinates.^{23, 28, 29} However, due to mutations there are slight variations in the protein backbone and residue positions, relative to wild-type bsAdK²⁷. Analysis of detailed structural data *via* solvent-accessible surface area calculations^{30, 31} were used to estimate the exposure of oxidizable residues to HOX in solution; in addition, ground-state protein dynamics were measured *via* hydrogen-deuterium exchange collected in a previous study²³, Figure S4. Solvent-accessible surface area calculations revealed moderate changes to the relative position of several oxidizable residues in each of the point-mutated bsAdK variants, relative to wild-type bsAdK (see Figure S4A). However, the observed differences in rotamer (side-chain) position had no affect on structural (*i.e.*, CD_{50} and $MONO_{50}$) or functional (ACT_{50}) susceptibility. Likewise, moderate changes in ground-state dynamics observed in the Q199R/A193V bsAdK variant (measured *via* hydrogen-deuterium exchange) did not influence either property. Thus, conferred resistance to HOX in the AdK model system can be attributed directly to additional protein stability or modification to amino acid composition.

Conferring functional resistance to oxidative stress *via* rational design

In Figure 4 we were able to show that conferred protein stability can significantly decrease bsAdK susceptibility to oxidative structural decay—*i.e.*, stabilized bsAdK variants (with fixed number and composition of oxidizable residues) display both secondary structure (CD_{50}) and monomer populations ($MONO_{50}$) that persist at higher HOX concentrations, see Figure 5. However, additional stability conferred in the bsAdK enzyme system limits resistance to functional decay, which is likely the result of site-specific damage to residues within or proximal to the active-site at higher HOX concentrations. Accordingly, a reasonable supposition is that certain modifications to oxidizable residues proximal to the active-site could improve bsAdK resistance to HOX mediated functional decay. Case in point, clearly stability alone does not account for improved HOX resistance observed in the ecAdK system, see Figure 3. The most salient difference between ecAdK and the model system bsAdK is the absence of three cysteine residues in the (functionally required) lid domain in the ecAdK enzyme. Given the high reactivity of cysteines with HOX¹⁰, it is reasonable to assume the presents of these residues near the active-site in the bsAdK model system will increase the enzyme's susceptibility to HOX-mediated functional decay. To test this hypothesis, computational modeling was used to design and graft the cysteine-rich bsAdK lid domain onto the ecAdK core domain (bs/ecAdK), see Figure 5A. The bs/ecAdK exhibits a marked reduction (*i.e.*, 2 and 3-fold reduction for ACT_{50} HOCl and HOBr, respectively) in protein activity relative to wild-type ecAdK. In other words, the bs/ecAdK

variant has a HOX functional resistance that is on *par* with bsAdK, showing that the ecAdK-lid is required for the observed resistance to functional decay, relative to bsAdK, see Figure 6.

To complement the bs/ecAdK variant, we created a second chimera in which we incorporated a cysteine-free ecAdK lid domain onto the bsAdK core domain (see Figure 5B), here the expectation is that the less reactive ecAdK lid will result in an increased resistance to bsAdK functional decay. While the lid-swapping introduced some thermo-destabilization (*i.e.*, $T_m = -12\text{ }^{\circ}\text{C}$ — see Supporting Information, Table S6), the chimeric ec/bsAdK variant shows a clear increase in resistance to HOX-mediated decay of protein function relative to bsAdK (*i.e.*, ~2 and 3.5-fold increase ACT_{50} for HOCl and HOBr, respectively) which is on *par* with ecAdK, see Figure 6. To assess how additional protein stability can influence the HOX functional resistance we created a third chimera in which we coupled the ds2AdK core with the ecAdK-lid (ec/ds2dK), see Figure 5C. Interestingly, the ec/ds2AdK variant showed a near 2-fold increase ACT_{50} (with HOCl) relative to the ec/bsAdK variant (*i.e.*, with a $T_m = 10\text{ }^{\circ}\text{C}$ relative to ec/bsAdK— see Supporting Information, Table S6). While the increase in protein stability resulted in a small increase in the propensity towards aggregation (Figure 6A), there is a modest increase in ec/ds2AdK substrate induced refolding, which can account for (at least in part) the apparent increase in protein activity observed for ec/ds2AdK exposed to HOCl compared to the ec/bsAdK variant.

CONCLUSIONS

In this study, we developed a mechanism for the oxidative decay of structure and function for the enzyme adenylate kinase, see Scheme 1. With few exceptions, AdK exposure to HOX results in unfolding, aggregation or degradation (*i.e.*, $MONO_{50}/CD_{50} < 1$); and a portion of the unfolded ensemble can undergo a substrate induced refolding reaction (*i.e.*, $ACT_{50}/MONO_{50} > 1$) that results in catalysis — where HOCl is typically the stronger oxidant. In turn, the putative mechanism (Scheme 1) was used to design novel bsAdK variants that are resistant to oxidative decay of structure and function. Here we were able to show that conferred stability (*i.e.*, with constraint to the number and position of oxidizable residues) can increase resistance to HOX mediated structural decay, see Figure 4. However, there appears to be an upper limit for activity (ACT_{50}) HOX resistance that can be achieved *via* conferred stability alone. To achieve HOX resistance to bsAdK structure and function required additional modification to residues proximal to the active site. Namely, three cysteine residues were removed from the bsAdK by means of designing a graft of the ecAdK lid onto the bsAdK core, resulting in a variant with a ACT_{50} resistance on *par* with wild-type ecAdK. Interestingly, when three cysteine residues were removed from the ds2AdK (replaced with the ecAdK cysteine-free lid), a 2-fold increase in HOCl ACT_{50} was achieved relative to the ec/bsAdK chimera. Moreover, the observed structural and functional resistances are not simply related to minor alteration in accessibility of oxidizable residues or protein dynamics, see control experiments in Supporting Information, Figure S4. Thus in this study we can conclude that: (i) structural resistance can be achieved *via* mitigating oxidative unfolding (*i.e.*, *via* conferring stability); however, this will not guarantee decreased functional susceptibility, see Figure 4; (ii) decreasing susceptibility of function to HOX

mediated decay can be achieved *via* strategic modification of certain oxidizable residues proximal to the active-site, and these modifications can be orthogonal to resistances achieved *via* conferred stability, see Figure 5 and 6; (iii) while both stability and modification of certain oxidizable residues can affect protein dynamics and accessibility of oxidizable residues, neither dynamics nor accessibility properties contributes significantly to structural or functional resistances. Moreover, it is our expectation that conferred stability and modification of certain oxidizable residues proximal to the active-site will result in similar resistances to structural and functional HOX decay, and these studies are currently underway.

MATERIALS AND METHODS

Design of stability enhanced adenylate kinases

Construction of the wild-type bsAdK and bsAdK temperature adapted variants was previously described²⁷. Generation of the gene for adenylate kinase of gsAdK was constructed by amplification driven mutagenesis starting from the coding sequence of the *bacillus subtilis* adenylate kinase. Generation of gene for bgAdK was synthetically constructed from a Gene String (Life Technologies). Generation of the gene for the coding sequence of adenylate kinase from ecAdK was constructed from colony PCR of the genomic DNA of the *e. coli* strain, BL21(λDE3) (Novagen). Amplified genomic ecAdK from this cell-line was observed to consistently contain two mutations (172Q and 177S) differing from previously reported ecAdKs and showing a diminished stability. Mutations were introduced by amplification driven mutagenesis into the amplified ecAdK to match previously reported ecAdK (172H and 177P), which will be considered to be the “wild-type” ecAdK for the remainder of this report. All AdK genes were inserted into a pET30b(+) vector containing an N-terminal hexa-histidine affinity tag by splice overlap extension.

Predictions for designing thermally enhanced ecAdK variants were made by generating a structural alignment of bsAdK (PDB:1P3J:A) and ecAdK (PDB:1AKE:A) using Multi-align in Chimera (UCSF). Critical residues identified from molecular evolution and computational design of bsAdK were mapped onto their counterparts in the ecAdK structure to comprise the sites targeted for redesign. All residues with 4 Angstroms of the design sites excluding surface and active site proximal were targeted for repacking to allow additional variation in the redesign sites. Construction of the lid-swap chimeras, were made from the previously prepared structural alignments of the bsAdK and ecAdK. Transitions between the two grafting sites were chosen at the sites of structural intersection at the hinge of the lid, where modest sequence conservation was observed between the two AdKs. Fragments of the ecAdK lid and the two pieces of the bsAdK core domain were generated by PCR amplification and assembled using splice overlap extension. A similar approach was used to generate all other chimera used in the study. Sequences for all variants used in this study were verified by DNA sequencing at the Keck DNA sequencing facility at Yale.

Assessment of changes in secondary structure by far-UV circular dichroism

Oxidant challenged samples were diluted to a final protein concentration of 10 μM with 50 mM potassium phosphate, pH 7.5. Far-UV circular dichroism spectra were acquired using a

Chirascan CD Spectrophotometer, using a 1 mm pathlength quartz cuvette. Scans were conducted in triplicate at 20 °C, scanning from 260 to 200 nm, with a one second sampling at each wavelength. Replicate scans were averaged and buffer subtracted. Changes in the dichroism are reported at 222 nm owing to the large extinction coefficient and significant α -helical content of AdK. Fits for loss of structure are made using an offset exponential decay, described previously.¹⁷ The concentration of oxidant at the midpoint of the decay is reported for each variant as the CD₅₀. All data was collected in at least triplicate, and statistical analysis was conducted on these data following the approach we used in ¹⁷.

Assessment of morphological changes by gel-filtration chromatography

Oxidatively challenged protein samples were assessed using gel filtration chromatography under naturing conditions. Proteins were transiently exposed to HOX for 24 hours, pH = 7.5 was strictly controlled at different levels of HOX challenges. Samples were injected at 100 μ M protein concentration onto a Superdex 200 10/300 GL column with a 50 mM potassium phosphate mobile phase (GE Healthcare Life Sciences). Chromatographic traces were collected by simultaneous detection at 230, 254, and 280 nm. Collected chromatographs decomposed using a Gaussian deconvolution, where a maximum of six Gaussian populations were allowed. The first four Gaussian populations were centered on retention volumes corresponding to the mono-, di-, tri-, and tetra-meric populations. The fifth and sixth distributions were utilized to fit higher order-states and near void-volume peaks respectively. Due to the ability of the oxidants to strongly influence or interfere with the commonly utilized near-UV chromaphoric sidechains, assessment of the gel-filtration chromatographs were made using the absorbance monitored at 230 nm. Integration of the peak fit to the Gaussian corresponding to the monomeric population are correlated to the oxidant challenge and fit to a simple exponential decay function. The concentration of oxidant at the midpoint of the decay is reported for each variant as the MONO₅₀. All data was collected in at least triplicate, and statistical analysis was conducted on these data following the approach we used in ¹⁷.

Oxidant challenge of AdK variants

Preparation of oxidants for challenging AdK variants were prepared immediately prior to use. Hypochlorite solutions were prepared in deionized water from a reagent grade sodium hypochlorite solution (10–15% available chlorine, Sigma). Dilution of neat hypochlorite solution was quantified spectrophotometrically by absorbance at 245 and 295nm. The sodium hypobromite solution was prepared by adding hypochlorite to a stoichiometric amount of sodium bromide to a final concentration of 10 mM. The hypobromite solution was allowed to develop sealed in the dark for 4 minutes at room temperature before use. From the quantified stocks of sodium hypochlorite, stocks of 10mM sodium hypochlorite and 10mM sodium hypobromite were prepared in 50mM potassium phosphate and used to challenge protein samples, where a final protein concentration of 100 μ M and 50mM potassium phosphate were maintained. Oxidant challenged protein samples were allowed to react for 24 hours in the dark at room temperature before characterization. The typical range of molar excesses of oxidants was 0–25ME; however, HOX exceeds 25ME in given experiment neither dynamics nor accessibility properties contributes significantly if a post-transition is not observed.

Analytical Ultracentrifugation of bsAdK

Sedimentation velocity experiments were performed to determine the Svedberg value (s) for bsAdK under oxidative stress 0–25ME —identifying monomeric, aggregated and fragmented species. Analytical ultracentrifugation data were collected on a Beckman XL-A analytical ultracentrifuge using the vendor's software. The s-values were determined as outlined in ^{32, 33}. Experiments were conducted with protein concentrations in the range of 10–100 μ M in 50mM potassium phosphate, pH 7.5. Experiments were conducted in triplicate.

Stability assessment of AdK variants

Stability of the AdK variants were made using a differential scanning calorimeter (DSC) as previously described²⁷. Briefly, samples were prepared at 100 μ M concentrations in 50mM potassium phosphate in 1mM β -mercaptoethanol, pH = 7.5 was strictly controlled at different levels of HOX challenges. After passage through a 0.22 μ m filter and 20 minutes of degassing at room temperature, samples were run using a NanoDSC (TA Instruments) at 3 atm, cycling from 20 to 90 °C at a rate of 1 °C/minute. Samples were collected in triplicate and corrected with an initial subtraction of an independent buffer run. Data fits were performed using NanoAnalyze v2.3.6 using the built-in General Fitting Function (TA Instruments). All data was collected in at least triplicate, and statistical analysis was conducted on these data following the approach we used in²⁷.

Assessment of changes in functionality by coupled luciferase assay

AdK function was assessed through the use of a coupled enzymatic assay. In this assay, the reaction is started by an excess of adenosine diphosphate (ADP, 2 mM) to a dilute adenylate kinase, 10 nM, and luciferin/luciferase cocktail (Toxilight Non-destructive Cytotoxicity Bioassay, Lonza) in 50 mM potassium phosphate. Adenosine triphosphate generated by turnover of the ADP by AdK is utilized by the luciferase for conversion of luciferin to an excited form of oxyluciferin, which results in the ejection of a photon upon relaxation of the oxyluciferin to the ground state. Reactions are set up in 96-well white, solid bottomed microplates and monitored at 20 °C using a Synergy HT microplate reader operating in luminescence mode. Proteins were transiently exposed to HOX for 24 hours, pH = 7.5 strictly controlled at different levels of HOX challenges. Luminescence is monitored until a constant rate of luminescence is observed. The maximum rate of luminescence observed is corrected for luminescence observed for the plate prior to addition of the ADP and normalized to the luminescence measured for the unchallenged protein sample. Normalized luminescence is then correlated to the oxidant challenged and fit to a sigmoidal decay. The concentration of oxidant at the midpoint of the decay is reported for each variant as the ACT₅₀. All data was collected in at least triplicate, and statistical analysis was conducted on these data following the approach we used in ¹⁷.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: Oxidants, myeloperoxidase, and bacterial killing. *Blood*. 1998; 92:3007–3017. [PubMed: 9787133]
2. Heinecke JW. Mechanisms of oxidative damage by myeloperoxidase in atherosclerosis and other inflammatory disorders. *The Journal of laboratory and clinical medicine*. 1999; 133:321–325. [PubMed: 10218761]
3. Nicholls SJ, Hazen SL. Myeloperoxidase and cardiovascular disease. *Arteriosclerosis, thrombosis, and vascular biology*. 2005; 25:1102–1111.
4. Weiss SJ. Tissue Destruction by Neutrophils. *New Engl J Med*. 1989; 320:365–376. [PubMed: 2536474]
5. Davies MJ, Hawkins CL, Pattison DI, Rees MD. Mammalian heme peroxidases: from molecular mechanisms to health implications. *Antioxidants & redox signaling*. 2008; 10:1199–1234. [PubMed: 18331199]
6. Meeusen ENT, Balic A. Do eosinophils have a role in the killing of helminth parasites? *Parasitol Today*. 2000; 16:95–101. [PubMed: 10689327]
7. Wu W, Chen Y, d'Avignon A, Hazen SL. 3-Bromotyrosine and 3,5-dibromotyrosine are major products of protein oxidation by eosinophil peroxidase: potential markers for eosinophil-dependent tissue injury in vivo. *Biochemistry*. 1999; 38:3538–3548. [PubMed: 10090740]
8. Regelman WE, Schneider LA, Fahrenkrug SC, Gray BH, Johnson S, Herron JM, Clawson CC, Clawson DJ, Wangenstein OD. Proteinase-free myeloperoxidase increases airway epithelial permeability in a whole trachea model. *Pediatric pulmonology*. 1997; 24:29–34. [PubMed: 9261850]
9. Schiller J, Arnhold J, Sonntag K, Arnold K. NMR studies on human, pathologically changed synovial fluids: role of hypochlorous acid. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 1996; 35:848–853.
10. Chapman AL, Hampton MB, Senthilmohan R, Winterbourn CC, Kettle AJ. Chlorination of bacterial and neutrophil proteins during phagocytosis and killing of *Staphylococcus aureus*. *The Journal of biological chemistry*. 2002; 277:9757–9762. [PubMed: 11733505]
11. Page MA, Shisler JL, Marinas BJ. Mechanistic aspects of adenovirus serotype 2 inactivation with free chlorine. *Applied and environmental microbiology*. 2010; 76:2946–2954. [PubMed: 20305026]
12. Rosen H, Crowley JR, Heinecke JW. Human neutrophils use the myeloperoxidase-hydrogen peroxide-chloride system to chlorinate but not nitrate bacterial proteins during phagocytosis. *The Journal of biological chemistry*. 2002; 277:30463–30468. [PubMed: 12060654]
13. Wigginton KR, Kohn T. Virus disinfection mechanisms: the role of virus composition, structure, and function. *Current opinion in virology*. 2012; 2:84–89. [PubMed: 22440970]
14. Wigginton KR, Pecson BM, Sigstam T, Bosshard F, Kohn T. Virus inactivation mechanisms: impact of disinfectants on virus function and structural integrity. *Environmental science & technology*. 2012; 46:12069–12078. [PubMed: 23098102]
15. Pattison DI, Hawkins CL, Davies MJ. Hypochlorous acid-mediated protein oxidation: how important are chloramine transfer reactions and protein tertiary structure? *Biochemistry*. 2007; 46:9853–9864. [PubMed: 17676767]
16. Kang JI, Neidigh JW. Hypochlorous acid damages histone proteins forming 3-chlorotyrosine and 3,5-dichlorotyrosine. *Chem Res Toxicol*. 2008; 21:1028–1038. [PubMed: 18452314]

17. Sivey JD, Howell SC, Bean DJ, McCurry DL, Mitch WA, Wilson CJ. Role of lysine during protein modification by HOCl and HOBr: halogen-transfer agent or sacrificial antioxidant? *Biochemistry*. 2013; 52:1260–1271. [PubMed: 23327477]
18. Salavej P, Spalteholz H, Arnhold J. Modification of amino acid residues in human serum albumin by myeloperoxidase. *Free Radical Bio Med*. 2006; 40:516–525. [PubMed: 16443167]
19. Brock JWC, Ames JM, Thorpe SR, Baynes JW. Formation of methionine sulfoxide during glycoxidation and lipoxidation of ribonuclease A. *Arch Biochem Biophys*. 2007; 457:170–176. [PubMed: 17141728]
20. Nightingale ZD, Lancha AH Jr, Handelman SK, Dolnikowski GG, Busse SC, Dratz EA, Blumberg JB, Handelman GJ. Relative reactivity of lysine and other peptide-bound amino acids to oxidation by hypochlorite. *Free Radical Bio Med*. 2000; 29:425–433. [PubMed: 11020664]
21. Hawkins LC, Davies JM. Reaction of HOCl with amino acids and peptides: EPR evidence for rapid rearrangement and fragmentation reactions of nitrogen-centred radicals. *Journal of the Chemical Society, Perkin Transactions*. 1998; 2:1937–1946.
22. Pattison DI, Davies MJ. Kinetic Analysis of the Reactions of Hypobromous Acid with Protein Components:3 Implications for Cellular Damage and Use of 3-Bromotyrosine as a Marker of Oxidative Stress†. *Biochemistry*. 2004; 43:4799–4809. [PubMed: 15096049]
23. Miller C, Davlieva M, Wilson C, White KI, Counago R, Wu G, Myers JC, Wittung-Stafshede P, Shamoo Y. Experimental evolution of adenylate kinase reveals contrasting strategies toward protein thermostability. *Biophysical journal*. 2010; 99:887–896. [PubMed: 20682267]
24. Pena MI, Davlieva M, Bennett MR, Olson JS, Shamoo Y. Evolutionary fates within a microbial population highlight an essential role for protein folding during natural selection. *Mol Syst Biol*. 2010; 6
25. Pattison DI, Davies MJ. Absolute rate constants for the reaction of hypochlorous acid with protein side chains and peptide bonds. *Chem Res Toxicol*. 2001; 14:1453–1464. [PubMed: 1159938]
26. Pattison DI, Davies MJ. Kinetic analysis of the reactions of hypobromous acid with protein components: implications for cellular damage and use of 3-bromotyrosine as a marker of oxidative stress. *Biochemistry*. 2004; 43:4799–4809. [PubMed: 15096049]
27. Howell SC, Inampudi KK, Bean DP, Wilson CJ. Understanding thermal adaptation of enzymes through the multistate rational design and stability prediction of 100 adenylate kinases. *Structure*. 2014; 22:218–229. [PubMed: 24361272]
28. Counago R, Chen S, Shamoo Y. In vivo molecular evolution reveals biophysical origins of organismal fitness. *Mol Cell*. 2006; 22:441–449. [PubMed: 16713575]
29. Bae E, Phillips GN. Structures and analysis of highly homologous psychrophilic, mesophilic, and thermophilic adenylate kinases. *J Biol Chem*. 2004; 279:28202–28208. [PubMed: 15100224]
30. Connolly ML. Solvent-accessible surfaces of proteins and nucleic acids. *Science*. 1983; 221:709–713. [PubMed: 6879170]
31. Lee B, Richards FM. The interpretation of protein structures: estimation of static accessibility. *Journal of molecular biology*. 1971; 55:379–400. [PubMed: 5551392]
32. Nichols JC, Matthews KS. Combinatorial mutations of lac repressor. Stability of monomer-monomer interface is increased by apolar substitution at position 84. *The Journal of biological chemistry*. 1997; 272:18550–18557. [PubMed: 9228020]
33. Wilson CJ, Das P, Clementi C, Matthews KS, Wittung-Stafshede P. The experimental folding landscape of monomeric lactose repressor, a large two-domain protein, involves two kinetic intermediates. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:14563–14568. [PubMed: 16203983]

Figure 1. Biophysical assessment of oxidative damage in bsAdK

A. Collection of CD spectra of bsAdK challenged by HOCl, 0–25 ME. Dashed line shows reference wavelength (222 nm) for tracking secondary structure changes. **B.** A series of size-exclusion chromatographic separations for wild-type bsAdK incrementally challenged with increasing HOCl oxidant, 0–25 ME. Dashed line indicates the monomeric population, the distribution of the unfolded ensemble, and aggregated states are given in Supporting Information, Figure S2. **C.** Sedimentation velocity analytical ultra centrifugation for wild-type bsAdK incrementally challenged with HOCl, 0–25 ME. **D.** Overlay of progressive changes in secondary structure (far-UV CD at 22nm, closed circles) and the monomer population (MONO) for bsAdK challenged with HOCl. **E.** Correlation shown loss of activity under increasing challenge of HOCl, fits using an exponential decay as shown for MONO size-exclusion chromatography measurements (blue, open circles [– Ap5A]; open squares [+ Ap5A]) and decay for loss of activity (red) are shown. **F.** A representative comparison of the chromatographic trace and the histogram reconstruction of the chromatograph based on the observed activity of the isolated fractions of separated bsAdK, at 4 ME of HOCl. Note: Complementary HOBt experiments are given in Supporting Information, Figure S1. Molar Equivalent (ME) = the stoichiometric amount of HOX added to the sample (*e.g.*, 2ME = 2moles of HOX to 1 mole of protein).

Figure 2. Quantitative analysis of HOX structural and functional decay for wild-type bsAdK
 CD₅₀, MONO₅₀, and ACT₅₀ are reported for wild-type bsAdK challenged with HOCl or HOBr. In addition, MONO₅₀/CD₅₀ and ACT₅₀/MONO₅₀ are reported to indicate the mechanisms of HOX decay of structure and function. Note: A threshold of one (red-dashed line) was selected because given CD₅₀ = MONO₅₀ = ACT₅₀ (*i.e.*, MONO₅₀/CD₅₀ = 1 and ACT₅₀/MONO₅₀ = 1) would imply no loss of protein (AdK) due to oxidative unfolding or aggregation — under the conditions tested.

Figure 3. Structural and functional assessment of AdK homologues upon HOX challenge

Assessment of CD₅₀, MONO₅₀, and ACT₅₀ upon oxidative challenge with HOCl (A) and HOBr (B). In addition, ratios for MONO₅₀/CD₅₀ and ACT₅₀/MONO₅₀ upon oxidative challenge with HOCl (C) and HOBr (D)—metrics defined in Figure 2. Note: propagated errors for the ratios falls within the circle above each bar and are reported in Supporting Information, Table S1. Comparison of homologues relative to bsAdK *via* CD₅₀ (E),

MONO₅₀ (F) and ACT₅₀ (G). Statistical error based on 5–6 independent experiments for each homologue, gray box represents and dashed lines the statistical error for wild-type bsAdK, for HOCl and HOBr—respectively. Tabulated data given in Supporting Information,

Table S1. Molar Equivalent (ME) = the stoichiometric amount of HOX added to the sample (*e.g.*, 2ME = 2moles of HOX to 1 mole of protein). Note: statistically significant differences between and two values was determined via T-Test for 2 Independent Means, see Supporting Information.

Figure 4. Structural and functional assessment of redesigned bsAdK with conferred stability, upon HOX challenge

Assessment of CD₅₀, MONO₅₀, and ACT₅₀ upon oxidative challenge with HOCl (A) and HOBr (B), with ratios for MONO₅₀/ CD₅₀ and ACT₅₀/ MONO₅₀, for HOCl (C) and HOBr (D). Comparison of designs relative to bsAdK *via* CD₅₀ (E), MONO₅₀, (F) and ACT₅₀ (G). Statistical error based on 5–6 independent experiments for each of the design variants, gray box represents with solid line (HOCl) and dashed lines (HOBr) the statistical error for wild-type bsAdK. Tabulated data given in Supporting Information, Table S3. Molar Equivalent (ME) = the stoichiometric amount of HOX added to the sample (*e.g.*, 2ME =

2moles of HOX to 1 mole of protein). Note: statistically significant differences between and two values was determined via T-Test for 2 Independent Means, see Supporting Information.

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Figure 5. Design of an oxidant resistant AdK

Sequence alignment of ecAdK and bsAdK with lid swap illustrated with black arrows. **A.** bsAdK lid + ecAdK core; **B.** ecAdK lid + bsAdK core; **C.** ecAdK lid + ds2AK core. Below: Cartoon of chimeras (white = bsAdK core, green = ecAdK core, blue = bsAdK lid, red = ecAdK lid). Also see Figure 6 for quantitative analysis.

Scheme 1. Putative mechanism of oxidative protein decay

Cartoon illustration depicting the progressive morphological changes brought on by oxidative (HOX) damage, with molar equivalents (ME) at which species are observed.