Studying disulfide damage at low X-ray doses with an engineered protein approach

Timothy R. Stachowski [1,2,4,#] Mary E. Snell [1] and Edward H. Snell [1,2,3]

1. Hauptman-Woodward Medical Research Institute, Buffalo, New York 14203, USA; 2. BioXFEL Center, Buffalo, New York, 14203, USA; 3. Department of Materials Design and Innovation, SUNY Buffalo, New York, 14260, USA; 4. Department of Cell Stress Biology, Roswell Park Comprehensive Cancer Center, Buffalo, 14203, USA.
Current Address: Department of Chemical Biology and Therapeutics, St. Jude Children's Research Hospital, Memphis, TN, 38103, USA.

BioXFEL — A National Science Foundation Science and Technology Center

HAUPTMAN-WOODWARD MEDICAL RESEARCH INSTITUTE

Abstract

A significant problem in biological X-ray crystallography is the radiation chemistry caused by the incident X-ray beam. This causes both global and site specific damage. Global damage manifests itself in the decay of the diffraction pattern and data processing parameters. Site specific damage targets glutamates, aspartates, methionines, and disulfide bonds. This can misdirect the biological interpretation of the structural models produced. Cryo-cooling crystals has been successful in mitigating but not eliminating damage; however, cooling has also been shown to limit functionally relevant protein conformations. Due to this, and the difficulty in cryoprotecting some systems, there has been interest in the return to near-physiological temperature crystallography. Radiation chemistry at these conditions is less well studied. The doses used for X-ray crystallography under both cryocooled and near physiological temperature are in the kGy to MGy range. X-rays are used therapeutically at much lower doses. Disulfide bonds are among the most significantly affected species in a protein in the crystalline state but limited information is known about their response to damage in vivo. In this work we engineered a protein that dimerizes through a vulnerable disulfide bond to understand if radiation damage processes seen in structural studies translate to conditions closer to physiology, specifically in solution. We monitored monomerization with small angle solution X-ray scattering (SAXS), simultaneously pumping the sample with X-rays and while probing for structural impact using doses that are therapeutically relevant and a fraction of that required for crystallographic studies. Our results show that X-ray radiation drives a dose dependent fragmentation of the engineered protein that can be explained by a dimer to monomer transition, indicating disulfide bond cleavage. This supports the crystalline mechanism and suggests that crystallographic damage results can be extrapolated to physiologic conditions. Fragmentation was observed to be pH dependent, suggesting radiation damage processes and future routes for investigation and mitigation. The engineered protein approach represents a promising tool for advancing radiation damage studies and studying physiologically relevant radiation-protein interactions [1]. Irradiating the engineered protein in the presence of radicals provides insights into the underlying chemical mechanism [2].

Stachowski, T.R., Snell, M.E., Snell, E.H. *PLoS One*. Accepted 2020.
Stachowski, T.R., Snell, M.E., Snell, E.H. *Manuscript in preparation.*

Radiation drives a change in the conformational ensemble of the dimer

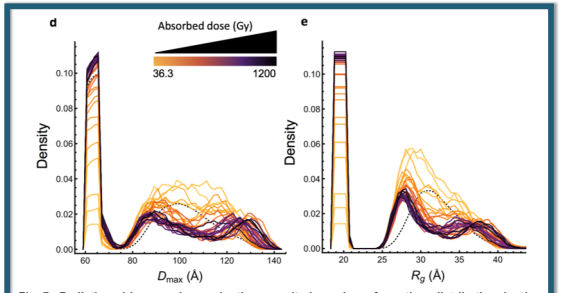


Fig 7. Radiation drives a change in the magnitude and conformation distribution in the engineered dimer. Conformational distributions of were calculated with the program EOM and are shown in (d) for the distribution of D_{max} and (e) R_g. Dotted line represents the random pool. The color gradient corresponds to the magnitude of the absorbed dose (36.3 Gy-1.2 kGy) delivered across 33 x 0.3 sec exposures where yellow is low dose and black is high dose.

Acknowledgments

University at Buffalo — The State University of New York

ROSWELL PARK COMPREHENSIVE CANCER CENTER

This work was funded by the National Science Foundation STC award number 1231306.

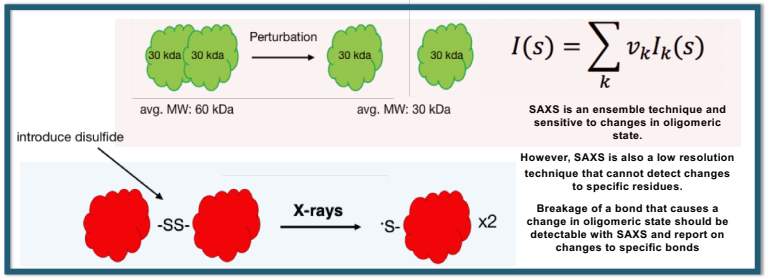
Connect with me!

Objectives

Question: Can we characterize X-ray induced disulfide bond cleavage with SAXS using *low* X-ray doses?

Steps:
1. Develop new data collection strategies to obtain reliable data from low (Gy vs. KGy and MGy) X-ray doses (Stachowski *et al., JSR*, 2019)
2. Develop a method to monitor a residue specific change with a low-resolution technique
3. Develop methods of quantifying and analyzing these data
4. Use the system to interrogate the underlying chemical mechanism

Monitoring a dimer to monomer transition with SAXS



SAXS is an ensemble technique and sensitive to changes in oligomeric state.

However, SAXS is also a low resolution technique that cannot detect changes to specific residues.

Breakage of a bond that causes a change in oligomeric state should be detectable with SAXS and report on changes to specific bonds

Engineering a dimer linked by a susceptible disulfide

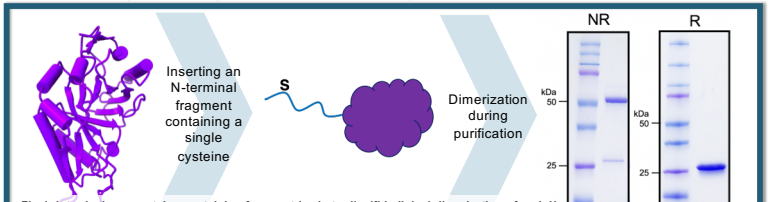


Fig 1. Introducing a cysteine containing fragment leads to disulfide linked dimerization of endoH. EndoH is a suitable protein to engineer in a radiation sensitive disulfide since it is thermally stable, natively monomeric, and contains no native cysteines. (left) The original crystal structure of native endoH solved by Rao et al., 1995. (middle) A schematic showing the placement of the engineered fragment. (right) SDS-PAGE analysis shows that the apparent MW of endoH$_{CYS}$ decreased in the presence of a reducing agent and indicates the presence of a disulfide bond.

Fragmentation is dependent on pH and protein concentration

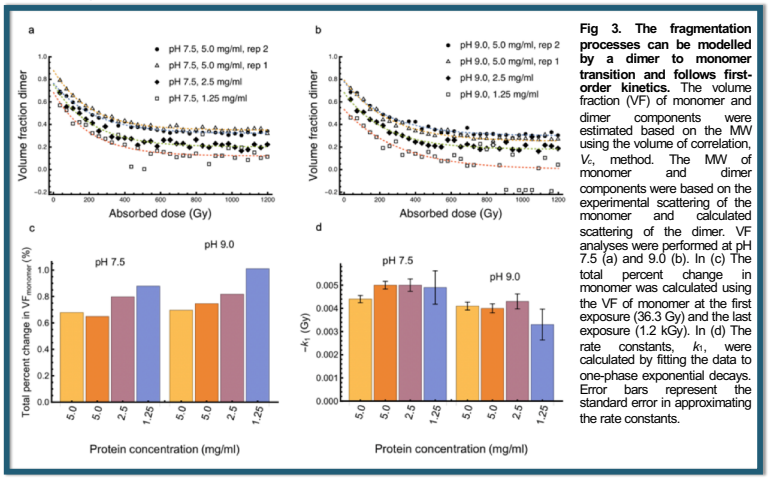


Fig 3. The fragmentation processes can be modelled by a dimer to monomer transition and follows first-order kinetics. The volume fraction (VF) of monomer and dimer components were estimated based on the MW using the volume of correlation, V_c, method. The MW of monomer and dimer components were based on the experimental scattering of the monomer and calculated scattering of the dimer. VF analyses were performed at pH 7.5 (a) and 9.0 (b). In (c) The total percent change in monomer was calculated using the VF of monomer at the first exposure (36.3 Gy) and the last exposure (1.2 kGy). In (d) The rate constants, k_1, were calculated by fitting the data to one-phase exponential decays. Error bars represent the standard error in approximating the rate constants.

Radiation exposure drives protein fragmentation

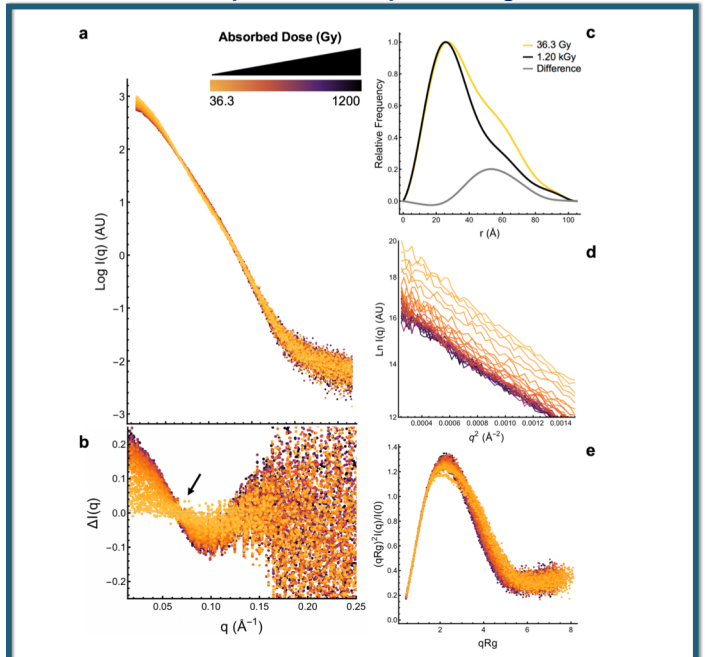


Fig 2. X-ray solution scattering analysis indicating that fragmentation is radiation dose dependent. The color gradient corresponds to the magnitude of the absorbed dose (36.3 Gy-1.2 kGy) delivered across 33 x 0.3 sec exposures where yellow is low dose and black is high dose. EndoH$_{CYS}$ was irradiated at pH 7.5 and the results shown are averages of two replicates at a concentration of 5.0 mg/ml. In (a) buffer subtracted scattering curves exhibit a dose-dependent decrease in intensity. In (b) residuals calculated between the first exposure and each sequential exposure show that intensity decreases at low-q and increases at high-q with increasing absorbed dose. The arrow identifies an isoscattering point at q ~ 0.07 Å^{-1}, characteristic of a transition between two states. In (c) a pairwise distance distribution plot, $P(r)$, normalized to MW, of the first, last exposure, and difference between first and last exposure shows that the fraction of longer distances in the protein decreased. The slope of the Guinier region remains linear (d) but decreases, indicating that the size of the protein is decreasing. A Dimensionless Kratky plot (e) also indicates that the protein is changing shape during irradiation.

Radical scavengers change the magnitude of fragmentation

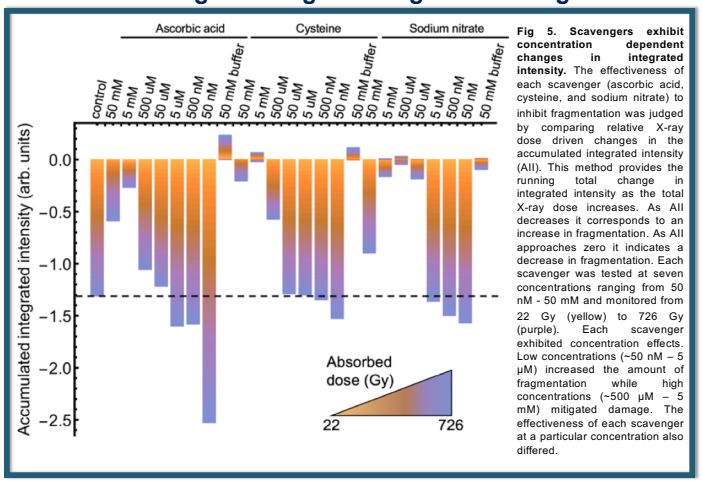


Fig 5. Scavengers exhibit concentration dependent changes in integrated intensity. The effectiveness of each scavenger (ascorbic acid, cysteine, and sodium nitrate) to inhibit fragmentation was judged by comparing relative X-ray dose driven changes in the accumulated integrated intensity (AII). This method provides the running total change in integrated intensity as the total X-ray dose increases. As AII decreases it corresponds to an increase in fragmentation. As AII approaches zero it indicates a decrease in fragmentation. Each scavenger was tested at seven concentrations ranging from 50 nM - 50 mM and monitored from 22 Gy (yellow) to 726 Gy (purple). Each scavenger exhibited concentration effects. Low concentrations (~50 nM – 5 µM) increased the amount of fragmentation while high concentrations (~500 µM – 5 mM) mitigated damage. The effectiveness of each scavenger at a particular concentration also differed.