Developing Tris(hydroxymethyl)aminomethane as an UV-active hydroxyl radical dosimeter

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Objectives

- **Hydroxyl radical protein footprinting (HRPF) is a technology for measuring protein topography based on measurement of the apparent rate of oxidation of amino acid side chains by hydroxyl radicals generated *in situ* in dilute aqueous solution with the protein.**
- **In order to measure the effective concentration of hydroxyl radical the protein analyte is exposed to (correcting for the amount of radical made, as well as the amount of radical scavengers in solution), a radical dosimeter with an easily measurable product is necessary.**
- **Tris(hydroxymethyl)aminomethane is a common biological buffer.**
- **Upon reaction with hydroxyl radicals, Tris forms a UV-absorbing product.**
- **Amount of UV-absorbing product generated correlates with peptide and protein oxidation up to the level of dosimeter saturation.**

Introduction

Fast Photochemical Oxidation of Proteins (FPOP) is a protein footprinting technique that uses hydroxyl radicals to oxidize solvent accessible protein sidechains faster than a protein folding event can occur, allowing researchers to measure changes in protein surfaces based on hydroxyl radical reactivity. In order to compare data between experiments, an adenine dosimeter was introduced that can be used to normalize the effective radical dose delivered to analyte proteins under different radical scavenging backgrounds. Adenine is a loss-of-signal dosimeter, lowering sensitivity. Additionally, many proteins have adenine binding sites, preventing the use of adenine as a dosimeter. Here, we show that the common organic buffer Tris(hydroxymethyl)aminomethane demonstrates dosimetry properties that make it suitable for FPOP experimentation. Specifically, Tris gains UV absorbance at 265 nm only after oxidation by hydroxyl radical. Tris absorbance increase has been correlated to oxidation of the model peptide [Glu[1]]-fibrinopeptide B, and we are in the process of correlating Tris dosimetry to myoglobin oxidation in the presence of competitive radical scavengers. Based on the typical mechanisms of hydroxyl radical organic chemistry and the UV absorbance spectrum of oxidized Tris, we believe that this absorbance gain results from one or more of the alcohol functional groups of Tris being converted to an aldehyde. This dosimeter also functions as the radical scavenger, equivalently replacing 20 mM glutamine to controls the lifetime of the radical.

Methods

Tris was diluted to a concentration of 8.5 mM, mixed with 100 mM hydrogen peroxide and oxidized by FPOP as previously reported, with Tris replacing the glutamine scavenger and adenine dosimeter. Negative controls samples either did not contain hydrogen peroxide or were not exposed to the laser. Full UV absorbance scans were performed on a Nanodrop UV/Vis spectrophotometer, with in-line dosimetry performed using a GenNext Pioneer dosimeter. Quadruplicate samples of Tris were oxidized with 5, 10, 15, 20, 40, 70, and 100 mM peroxide in the presence of 5 uM [Glu[1]]-fibrinopeptide B and collected in an identical quench solution as previously used. The absorbance at 265 nm was again taken for each sample. The catalase in each sample was digested with trypsin. Samples were analyzed on an Orbitrap Fusion Tribid Mass Spectrometer. QualBrowser was used to manually calculate the oxidation of the GluB.

Results

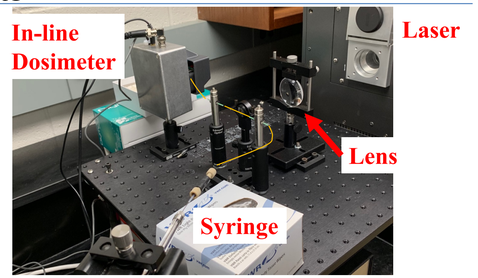

Figure 1: Bench Setup for all FPOP Experiments. Samples are pushed through a capillary system in line with a KrF excimer laser. Light is focused onto the capillary by a lens, and the size of the spot is measured before each experiment. Flow rate is coordinated such that each volume is only irradiated once. Capillary highlighted for clarity.

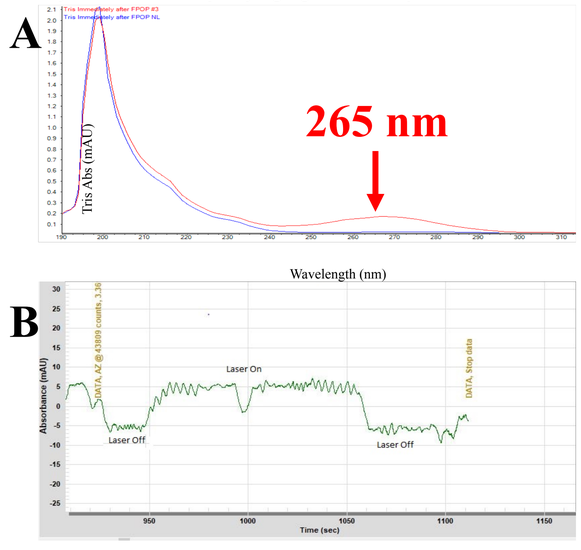

Figure 2: Tris absorbance increases at 265 nm after oxidation.
A: Tris buffer is inherently absorbent in the short wavelength region of the ultraviolet spectrum, and after oxidation by FPOP, this absorbance changes little. Contrastingly, unoxidized Tris buffer is non-absorbing in the longer wavelength UV region from 250-310 nm, but oxidation causes a substantial absorbance increase in this region with a maximum at 265 nm.
B: If the absorbance of Tris is measured at 265 nm as it flows through a capillary system, a rapid increase in absorbance can be seen when oxidation is begun. This absorbance can be averaged over both the Laser On and Laser Off regions and a difference can be taken to provide the net absorbance increase of the Tris buffer.

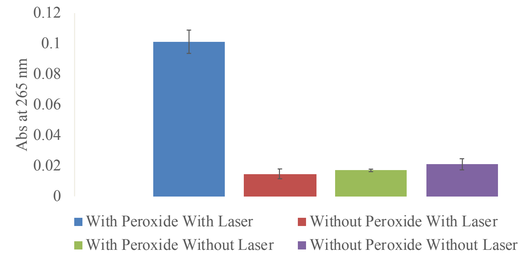

Figure 3: Tris absorbance as a function of different reaction conditions. Hydrogen peroxide and laser alone does not alter Tris absorbance at 265 nm. The UV absorbance of Tris buffer becomes much greater after the buffer reacts with hydroxyl radicals.

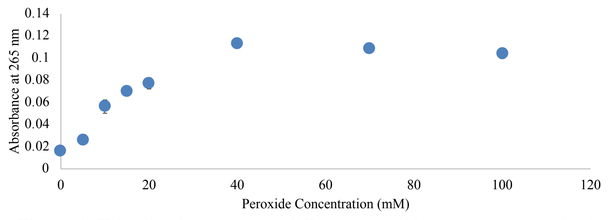

Figure 4: Tris absorbance versus different peroxide concentrations. As the concentration of peroxide present in the sample is increased, the absorbance of Tris increases until the buffer becomes fully saturated. Error bars represent one standard deviation in the absorbance of quadruplicate samples.

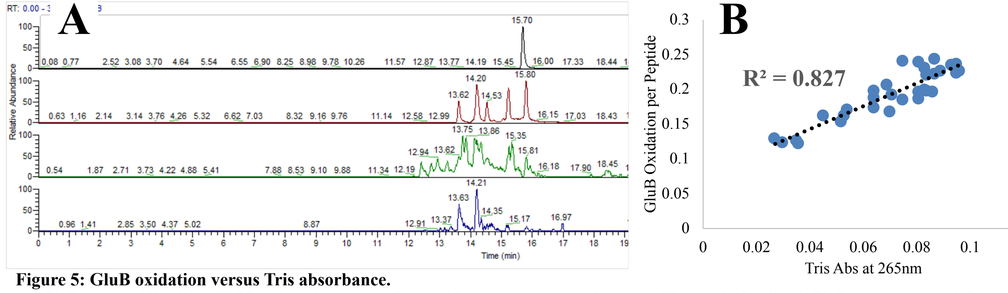

Figure 5: GluB oxidation versus Tris absorbance.
A: GluB was oxidized by FPOP using the range of peroxide concentrations shown in **Figure 4**. Oxidized GluB was separated from catalase peptides by HPLC before being analyzed on an Orbitrap Fusion Tribid Mass Spec. The spectra shown represent the unoxidized, singly, doubly, and triply oxidized GluB peptides, respectively from top to bottom, for a sample oxidized in 15 mM peroxide. Oxidation per peptide was determined by taking a weighted average of the peak areas.
B: GluB oxidation can be correlated to Tris absorbance gain. Each point represents one individual sample at a peroxide concentration between 5 mM and 40 mM.

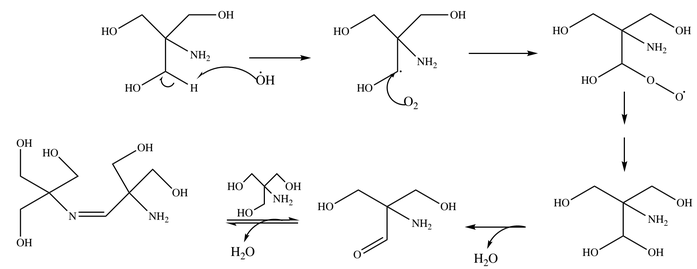

Figure 6: Proposed scheme for the reaction of Tris with hydroxyl radical. We hypothesize that a hydroxyl radical abstracts a hydrogen from the C-H bond of Tris. Reaction with molecular oxygen ultimately forms a *gem*-diol, which spontaneously eliminates water to form an aldehyde. This aldehyde may then condense with the amine from a second Tris molecule to form an imine. The absorbance spectrum of Tris is consistent with formation of an aldehyde and/or imine.

Conclusion

- **Tris(hydroxymethyl)aminomethane becomes UV active at 265 nm after reacting with hydroxyl radical.**
- **Tris demonstrates dosimetry capability within a moderate range of effective hydroxyl radical doses typical for FPOP.**
- **UV Spectrum suggests production of an aldehyde and/or imine via standard hydrogen abstraction/O_2 oxidation chemistry.**

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