

Experimental and Informatic Aspects of Electrochemical Oxidation as a Surface Mapping Probe of Higher Order Protein Structure

Carlee McClintock,¹ Vilmos Kertesz,² Susie Y. Dai,² Robert L. Hettich^{1,2}

¹UT-ORNL Genome Science & Technology Program, 1060 Commerce Park, Oak Ridge, TN, ²Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN



OVERVIEW

- Protein structure is important for understanding protein functions, and typically involves evaluation of multiple dynamic conformations of a resolved static structure
- A variety of computational modeling approaches can be employed to estimate unknown or static protein structures:¹
 - Homology modeling (>30% seq. ID)
 - Protein threading (domain parsing)
 - Ab initio structure prediction
 - Molecular dynamics (from PDB entry)
- Protein structures built *in silico* can provide insight, but experimental data provides critical guidance for model evaluation
- Sidechain solvent accessibility can be probed with mass tags identified by mass spectrometry (MS), providing useful data for evaluating computational models²
- Highly reactive hydroxyl radicals (*OH) can be generated *in situ* by a variety of methods for oxidative protein surface mapping applications³
- Boron-doped diamond (BDD) electrodes can generate *OH without reagents or radiation, in addition to being accessible and affordable
- While experimental trials remain in optimizing this methodology, exhaustive data mining for oxidative modifications poses a tough challenge

EXPERIMENTAL APPROACH

Electrochemical Oxidation Protocol

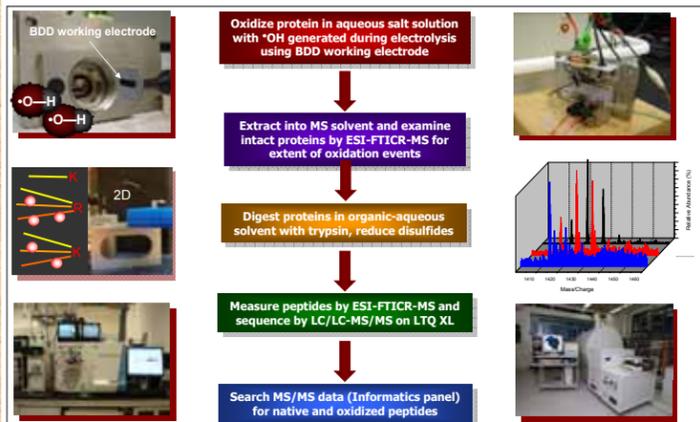


Figure 1. Hydroxyl radicals generated during electrolysis of aqueous solutions using a boron-doped diamond (BDD) electrode (ESA, Inc.) can react with thiol, aromatic, and aliphatic groups of amino acids with sufficient solvent-accessible surface area (SASA).³ Proteins dissolved in 0.25 M K₂SO₄ (pH 8) were oxidized by application of positive voltage to BDD working electrode (anode) during passage through the electrochemical flow-by cell at 1 μL/min flow rate. Samples were collected upon exit from cell and proteins were extracted by C4 ZipTip (Millipore) and infused directly into the Z-spray source for electrospray ionization (ESI) into the FTICR-MS (Varian, IonSpec) housed inside an actively-shielded 9.4-Tesla superconducting magnet for high-resolution measurement of oxidation events. Oxidized proteins eluted from C4 by 50-100% acetonitrile were digested in 60-80% ACN (aqueous phase: 50 mM Tris-HCl, 10 mM CaCl₂) with trypsin (Promega), and proteolytic peptides were separated with reversed-phase (RP) (1D) or multidimensional (MudPIT, 2D) RP–strong cation exchange (RP-SCX) high-pressure liquid chromatography (HPLC) on-line with an LTQ XL (ThermoScientific) equipped with ETD.

Parameters for Electrochemical Oxidation Experiments

- General considerations for oxidizing proteins with electrochemistry via the BDD working electrode:
 - Use solution with ionic strength ≥ 150 mM and buffering capacity against electrogenerated H⁺ (250 mM K₂SO₄)
 - Apply voltage to BDD that achieves the target current for moderate oxidation (53 events) → ~100-150 μA
 - Test range of protein concentrations and flow rates to ensure proper ratio analyte to *OH → ~50 ng/μL @ 1 μL/min
 - Collect discrete fractions and test absorbance at 215 nm and/or 280 nm to gauge potential adsorption
 - Acquire optimal sample after testing conditions; consider adding catalase and/or *OH quencher to protocol

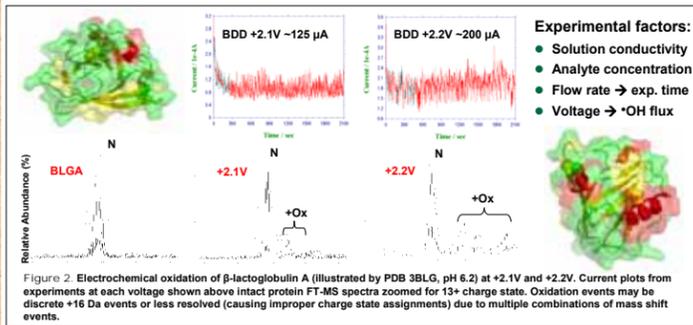


Figure 2. Electrochemical oxidation of β-lactoglobulin A (illustrated by PDB 3BLG, pH 6.2) at +2.1V and +2.2V. Current plots from experiments at each voltage shown above intact protein FT-MS spectra zoomed for 13+ charge state. Oxidation events may be discrete +16 Da events or less resolved (causing improper charge state assignments) due to multiple combinations of mass shift events.

DATA ACQUISITION

Characterization by High Performance Mass Spectrometry

- Intact proteins are extracted from buffer and monitored by FT-MS for identity before and after oxidation
- Oxidation is observed as an increase in MS heterogeneity with mass shifts relative to the unoxidized MS
- Circular dichroism allows evaluation of protein secondary structure before and after oxidation, although electrode adsorption occurring in some cases affects spectral intensity

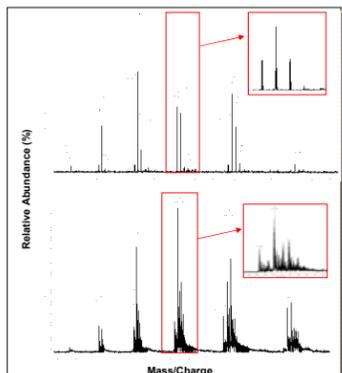


Figure 3. Intact lysozyme FT-MS spectra showing oxidation at +2.1V. Satellite peaks present in both spectra may be due to sulfate adducts.

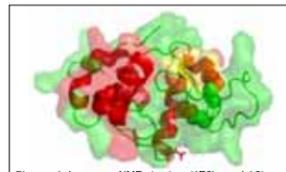


Figure 4. Lysozyme NMR structure (1E8L, model 6) showing surface with underlying secondary structure, disulfides, and substrate binding site.

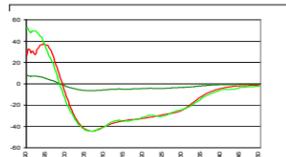


Figure 5. Lysozyme CD spectra before and after oxidation at +2.1V.

Expanding Analytical Capabilities with MudPIT⁶ and CID/ETD

- Multidimensional chromatography (Fig.6) increases sampling of peptides within rich mixture due to distribution of oxidation tags
 - Increased dynamic range promotes detection of low-abundance oxidized peptides represented by single spectral counts
 - Repeat sampling of all peptides lends greater confidence in IDs, both new and redundant spectra improve statistical significance of data
- Different collision activation modes may be incorporated to obtain complementary information for evaluating validity of modified peptide matches as used in *de novo* methods (Fig.7)

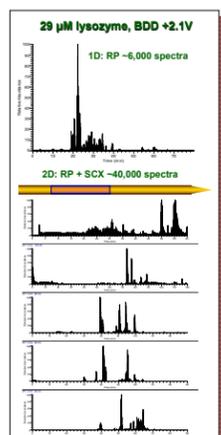


Figure 6. Base peak chromatograms from 1D (top) and 2D (bottom) LC-MS/MS runs of same lysozyme +2.1V sample (results in Table 1).

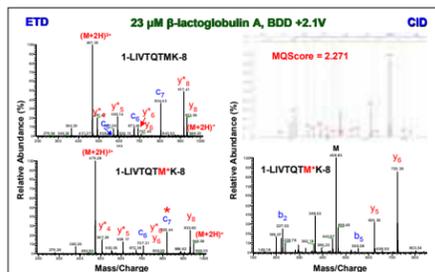


Figure 7. ETD MS/MS spectra showing peptides from both native (unoxidized) and oxidized β-lactoglobulin A, after aqueous-organic tryptic digest of oxidized proteins shown in Figure 2. CID spectra from 2D LC-MS/MS run, with InsPect⁷ match (p-value 0.00058) on top and raw data below.

INFORMATICS

Data Mining Strategy to Enhance Detection of Modifications

- Challenges abound in mining MS/MS data for many possible oxidative modifications
 - Expanding search space significantly with each additional mod:
 - Combinatorial possibilities inflate scores which shifts score distribution
 - Allowing multiple modifications per peptide compounds problem
 - Must compromise by weighting probability of observation based on empirical data (reactivity rates, oxid product stability and abundance, etc.)
 - Establishing filter levels that maintain balance between true positive retention and false positive elimination:
 - Complex mixtures and/or high-throughput applications will benefit from reliable search and filtering strategy without exhaustive manual validation
 - Extracting spectral count ratios allows qualitative analysis prior to relative quantitation by integration of chromatographic peak area:
 - Optimizing search capabilities will enhance information extraction from full-scan LC-MS mode
 - Positional isomers differing only in site of oxidation may be identified by differential elution during 2D LC/LC-MS/MS, lending awareness of isobaric oxidized peptide matches potentially co-fragmented during 1D run
- Solution resides in robust sampling followed by targeted searches with InsPect⁷ software tailored for hunting modifications

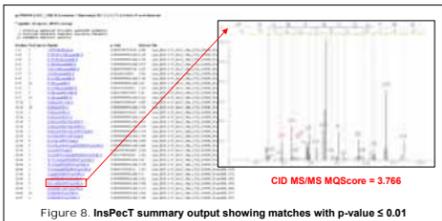
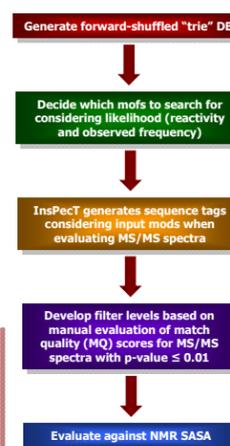


Figure 8. InsPect summary output showing matches with p-value ≤ 0.01

MS/MS Search Results After Database Filtering and Scoring

- P-value assignments are dependent upon database and entire set of data being evaluated
- Data reported in Table 1 passed p-value ≤ 0.01 criterion, though additional filtering based on MQScore should be considered on basis of data-specific evaluation
- Search is very limited in terms of mod list and only single mod allowed per peptide
- Methionine oxidation is significant in results from control lysozyme digest, could result from sample processing
- Cysteine oxidation is minimal though not indicative of SASA when considering control oxidations
- Oxidation of F34 (also shown in Fig.8) corroborated by 3 MS/MS spectra (2D) when compared with neighboring F38
- Background oxidation is non-zero, should be considered in surface mapping experiments, especially when experimenting with larger proteins difficult to resolve by intact MS

Table 1. InsPect search results (p-value ≤ 0.01) for control and oxidized lysozyme sampled by 1D and 2D LC-MS/MS on LTQ XL (CID only).

Peptide	Sequence	SASA	1D Lys Control	2D Lys Control	1D Lys +2.1V	2D Lys +2.1V
1	P	223	5.6	16	0	0
2	F	223	5.6	16	0	0
3	C	118	6.6	22	1	0
4	L	81	6.2	20	0	0
5	M	81	6.2	20	0	0
6	L	81	6.2	20	0	0
7	L	81	6.2	20	0	0
8	L	81	6.2	20	0	0
9	L	81	6.2	20	0	0
10	L	81	6.2	20	0	0
11	L	81	6.2	20	0	0
12	L	81	6.2	20	0	0
13	L	81	6.2	20	0	0
14	L	81	6.2	20	0	0
15	L	81	6.2	20	0	0
16	L	81	6.2	20	0	0
17	L	81	6.2	20	0	0
18	L	81	6.2	20	0	0
19	L	81	6.2	20	0	0
20	L	81	6.2	20	0	0
21	L	81	6.2	20	0	0
22	L	81	6.2	20	0	0
23	L	81	6.2	20	0	0
24	L	81	6.2	20	0	0
25	L	81	6.2	20	0	0
26	L	81	6.2	20	0	0
27	L	81	6.2	20	0	0
28	L	81	6.2	20	0	0
29	L	81	6.2	20	0	0
30	L	81	6.2	20	0	0
31	L	81	6.2	20	0	0
32	L	81	6.2	20	0	0
33	L	81	6.2	20	0	0
34	L	81	6.2	20	0	0
35	L	81	6.2	20	0	0
36	L	81	6.2	20	0	0
37	L	81	6.2	20	0	0
38	L	81	6.2	20	0	0
39	L	81	6.2	20	0	0
40	L	81	6.2	20	0	0
41	L	81	6.2	20	0	0
42	L	81	6.2	20	0	0
43	L	81	6.2	20	0	0
44	L	81	6.2	20	0	0
45	L	81	6.2	20	0	0
46	L	81	6.2	20	0	0
47	L	81	6.2	20	0	0
48	L	81	6.2	20	0	0
49	L	81	6.2	20	0	0
50	L	81	6.2	20	0	0
51	L	81	6.2	20	0	0
52	L	81	6.2	20	0	0
53	L	81	6.2	20	0	0
54	L	81	6.2	20	0	0
55	L	81	6.2	20	0	0
56	L	81	6.2	20	0	0
57	L	81	6.2	20	0	0
58	L	81	6.2	20	0	0
59	L	81	6.2	20	0	0
60	L	81	6.2	20	0	0
61	L	81	6.2	20	0	0
62	L	81	6.2	20	0	0
63	L	81	6.2	20	0	0
64	L	81	6.2	20	0	0
65	L	81	6.2	20	0	0
66	L	81	6.2	20	0	0
67	L	81	6.2	20	0	0
68	L	81	6.2	20	0	0
69	L	81	6.2	20	0	0
70	L	81	6.2	20	0	0
71	L	81	6.2	20	0	0
72	L	81	6.2	20	0	0
73	L	81	6.2	20	0	0
74	L	81	6.2	20	0	0
75	L	81	6.2	20	0	0
76	L	81	6.2	20	0	0
77	L	81	6.2	20	0	0
78	L	81	6.2	20	0	0
79	L	81	6.2	20	0	0
80	L	81	6.2	20	0	0
81	L	81	6.2	20	0	0
82	L	81	6.2	20	0	0
83	L	81	6.2	20	0	0
84	L	81	6.2	20	0	0
85	L	81	6.2	20	0	0
86	L	81	6.2	20	0	0
87	L	81	6.2	20	0	0
88	L	81	6.2	20	0	0
89	L	81	6.2	20	0	0
90	L	81	6.2	20	0	0
91	L	81	6.2	20	0	0
92	L	81	6.2	20	0	0
93	L	81	6.2	20	0	0
94	L	81	6.2	20	0	0
95	L	81	6.2	20	0	0
96	L	81	6.2	20	0	0
97	L	81	6.2	20	0	0
98	L	81	6.2	20	0	0
99	L	81	6.2	20	0	0
100	L	81	6.2	20	0	0

DISCUSSION

- Electrochemically-generated hydroxyl radicals can be used to controllably oxidize proteins by applying voltage that achieves target current
 - Extent of intact protein oxidation is relatively consistent among proteins when equivalent masses are compared
 - Variable level of adsorption occurs with proteins containing cysteine residues (currently under investigation)
- Oxidation events distributed among exposed, *OH-reactive residues results in a rich variety of oxidized peptides
 - Two-dimensional separations strategy effectively increases dynamic range to allow detection of low-abundance peptides
 - Repeat sampling of modified peptides increases confidence in IDs without manual evaluation to foster high-throughput applications
 - Database search methodology and scoring filters under evaluation to balance exclusion of false positives with retention of true positives
 - Accuracy is compromised for any scoring algorithm when search space is expanded
 - With additional mods, more peaks can be annotated and score distribution altered
 - Combinatorial problem when multiple modifications are allowed per peptide
- With further development, the BDD electrode could become an easily accessible research tool for probing solvent accessible surfaces of unknown protein structures

REFERENCES

- Floudas, C. A. *Biotechnol. Bioeng.* 2007, 97, 207-213.
- Chance, M. R.; Fiser, A.; Sali, A.; Pieper, U.; Eswar, N.; Xu, G.; Fajardo, J. E.; Radhakannan, T.; Marinkovic, N. *Genome Res.* 2004, 14, 2145-2154.
- Xu, G.; Chance, M. R. *Chem. Rev.* 2007, 107, 3514-3543.
- Zhu, X. P.; Shi, S. Y.; Wei, J. J.; Lv, F. X.; Zhao, H. Z.; Kong, J. T.; He, Q.; Ni, J. R. *Environ. Sci. Technol.* 2007, 41, 6541-6546.
- McClintock, C.; Kertesz, V.; Hettich, R. L. *Anal. Chem.* 2008, 80(9), 3304-17.
- Washburn, M. P.; Wolters, D.; Yates J. R. 3rd. *Nat. Biotech.* 2001, 19(3), 242-7.
- Tanner, S.; Shu, H.; Frank, A.; Wang, L.; Zandi, E.; Mummy, M.; Pevzner, P. A.; Bafna, V. *Anal. Chem.* 2005, 77(14), 4626-39.

ACKNOWLEDGMENTS

This research was sponsored by the National Institutes of Health under 1R01-GM070754. The authors thank Dr. Gary Van Berkel for use of lab equipment, and ESA, Inc. for supplying electrodes. C.M. acknowledges support from the UT-ORNL Genome Science and Technology Program. Oak Ridge National Laboratory, managed by UT-Battelle, LLC, under contract DE-AC05-00OR22725 with the U.S. Department of Energy.