

Probing Native Structures Of Homologous Large Proteins With Differential Covalent Labeling and Mass Spectrometry Characterization

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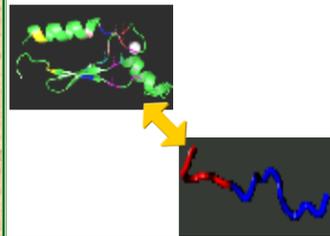
OVERVIEW

- Electrochemical induced oxidative and chemical labeling are complementary methods to covalently label solvent accessible residues in proteins.
- This integrated approach is applied to human serum albumin (HSA) and bovine serum albumin (BSA).
- Identification of modified sites was achieved by protease digestion and LC-MS/MS analysis
- The differential labeling can target residues of different hydrophobicities and map the protein native structure.
- Assisted with computational modeling, the technique can be used to elucidate protein structure.

INTRODUCTION

- Modification to the protein native folds can provide structural information.
- Side chain labeling experiments rely on protein structure dynamics, the amino acid reactivity, solvent accessible surface area (SASA) and the micro-environment of the residue.
- A single labeling experiment can be both selective (like chemical labeling) and non-selective (like oxidative labeling).
- Combination of different labeling techniques are more likely to provide SASA information of amino acid residues (Figure 1).

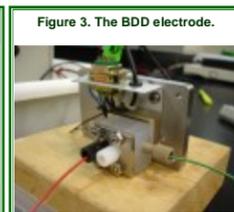
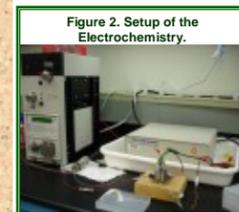
Figure 1. Native Versus Denatured Protein Structures, Revealing Dramatic Differences in Solvent Accessibility.



EXPERIMENTAL

Electrochemical Labeling Experiments

The electrochemical labeling was carried out with a BDD electrode.¹ Briefly, 6 μ M of HSA in 250 μ M K_2SO_4 solution was flowed through the electrode at 1 μ l/min flow rate. The sample solution was collected at 2.1 volts for half an hour (Figures 2 and 3).

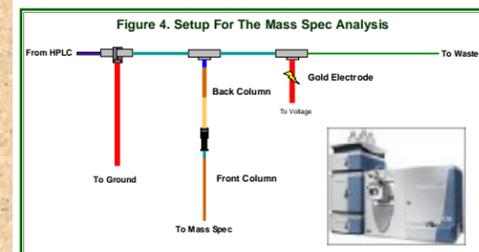


Chemical Labeling Experiments

The chemical labeling was achieved by using Traut's reagent (2-Iminothiolane-HCl, abbreviated as 2-IT), Sulfo-NHS acetate (S-NHS) or tetranitromethane (TNM). Labeling with either Traut's reagent or S-NHS acetate was carried out at the protein: chemical molar ratio at 1:8 for HSA in the buffer solution which contains 56 mM NaH_2PO_4 , 144 mM Na_2HPO_4 at room temperature for 1 hr. Labeling with TNM for tyrosine was carried at the protein: chemical molar ratio at 1:1.1 in the 50 mM tris-HCl and 10 mM $CaCl_2$ buffer. The reaction solution was then purified with C2 Sep Pack (Waters, Milford, MA) and the protein elution was lyophilized and kept at -80 $^{\circ}C$ for protease digestion.

Protein Digestion and Mass Spectrometry Analysis

The protein solution was mixed with trypsin at 20:1 weight ratio with an organic digestion protocol². Peptides were separated with a five step run by Multidimensional Protein Identification Technology (MudPIT)³ and subjected to a LTQ mass spectrometer for analysis (Figure 4).



Data Analysis

The modification sites were identified with Inspect⁴ software search with MS/MS spectra. The modified peptide spectra were verified manually.

RESULTS

HSA Lysine Differential Labeling Results

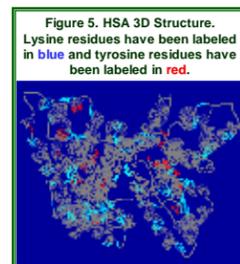
Residue	SASA(Å)	2-IT	S-NHS	Oxidation	Three methods
12	92.2	*	*	*	2/3
20	57.74	*	*	*	2/3
51	90.55	ND	*	*	2/3
64	90.7	*	*	*	2/3
61	61.53	*	*	*	2/3
73	43.71	*	*	*	2/3
93	80.82	*	*	*	2/3
106	3.84	*	*	*	2/3
136	52.05	ND	ND	*	2/3
137	88.82	*	*	*	2/3
159	73.34	*	*	*	2/3
162	76.12	*	*	*	2/3
174	63.95	*	*	*	2/3
181	63.95	*	*	*	2/3
190	67.29	*	*	*	2/3
195	91.71	*	*	*	2/3
199	26.11	*	*	*	2/3
205	100.64	*	*	*	2/3
212	69.21	*	*	*	2/3
225	78.56	*	*	*	2/3
233	75.3	*	*	*	2/3
240	106.8	*	*	*	2/3
262	114.1	*	*	*	2/3
274	44.96	*	*	*	2/3
276	114.96	*	*	*	2/3
281	85.45	*	*	*	2/3
286	36.14	*	*	*	2/3
313	139.78	*	*	*	2/3
317	88.77	*	ND	*	2/3
323	102.72	*	*	*	2/3
359	131.68	*	*	*	2/3
372	117.73	*	*	*	2/3
378	117.73	*	*	*	2/3
389	115.83	*	*	*	2/3
402	106.05	*	*	*	2/3
413	42.39	ND	*	*	2/3
414	12.53	*	*	*	2/3
432	65.26	*	*	*	2/3
436	79.12	*	*	*	2/3
439	157.6	*	*	*	2/3
444	101.71	ND	*	*	2/3
465	64.35	ND	*	*	2/3
475	161.75	*	*	*	2/3
500	151.12	*	*	*	2/3
519	121.08	*	*	*	2/3
524	98.73	ND	ND	*	2/3
525	9.69	*	*	*	2/3
534	20.87	*	*	*	2/3
536	49.87	ND	ND	*	2/3
538	158.95	ND	ND	*	2/3
541	149.59	*	*	*	2/3
545	166.9	*	*	*	2/3
557	112.43	*	*	*	2/3
560	140.86	*	*	*	2/3
564	159.57	*	*	*	2/3
573	132.65	*	*	*	2/3
574	141.84	*	*	*	2/3

ND: Not detected

HSA Tyrosine Differential Labeling Results

Residue	SASA(Å)	TNM	S-NHS	Oxidation	Three methods
30	9.06	*	ND	*	2/3
84	4.91	*	*	*	2/3
138	23.29	*	*	*	2/3
140	6.34	*	*	*	2/3
148	19.15	*	*	*	2/3
150	13.73	*	*	*	2/3
161	18.96	*	*	*	2/3
263	52.2	*	*	*	2/3
319	11.38	*	*	*	2/3
332	12.66	*	*	*	2/3
334	12.41	*	*	*	2/3
341	23.56	*	*	*	2/3
353	0.05	*	*	*	2/3
370	1.83	*	*	*	2/3
401	58.28	*	*	*	2/3
411	11.34	*	ND	*	2/3
452	28.43	*	*	*	2/3
497	23.27	*	*	*	2/3

ND: Not detected



- Residues labeled by all three methods and detected in the MS/MS analysis have large SASA.
- Sampling problem in the MS/MS analysis may contribute to the observation that solvent exposed residues are not detected or not labeled.
- Multiple labeling probes are more likely to confidently assign residues that are solvent exposed (Figure 5).
- Although modified sites can be identified by Inspect search, manual validation is necessary due to the complication of possible isomers (Figures 6 and 7).

Identification Of Modified Sites: Isomers

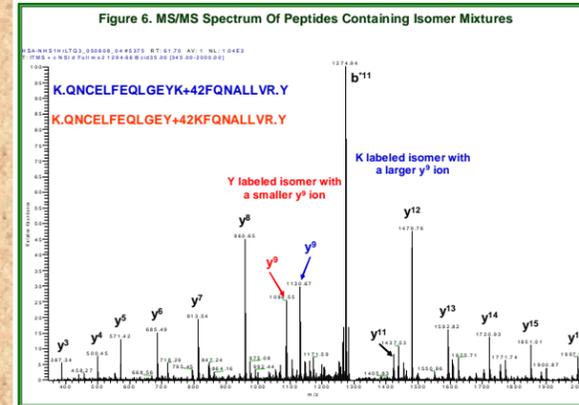
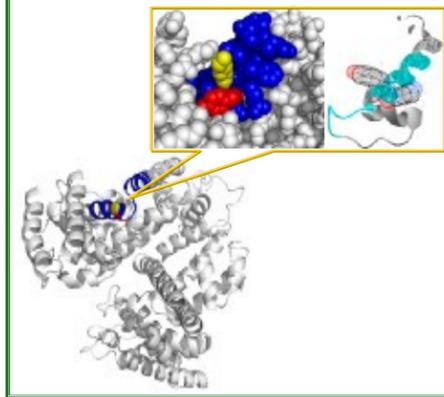
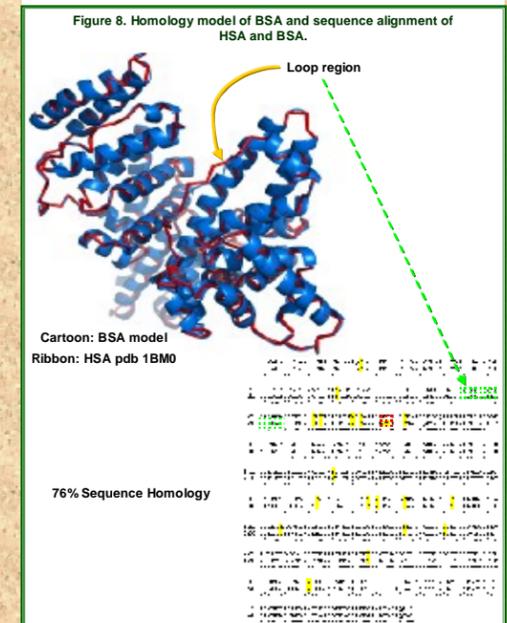


Figure 7. 3D view of the modified peptide with neighboring Y401 and K402 residues. Solvent accessible surface area is shown in the up right. Both residues are highly exposed to solvent with SASA at 58 and 100 Å each.



DISCUSSION

- Selective labeling and non-selective labeling can be effectively combined to target residues of interest.
- Multiple probes in the labeling experiments will help to identify surface exposed residues more confidently.
- This approach is tested on BSA (no X-ray data available), based on HSA crystal structure (Figure 8).



- The biggest deviation between the BSA model and HSA X-ray data is the loop region between residue 111-125.
- The labeling pattern is quite similar between the two proteins, which is supportive of the homology model.
- The two tyrosines (Y161 and Y162) in BSA (highlighted in pink) have been predicted to have 22 and 7 Å SASA, respectively.
- Y162 was found to be oxidized in the labeling experiments. However, neither of the two residues were confirmed by all three labeling methods. This implies that Y161 and Y162 in BSA may not have significant solvent accessibility.
- The homology model of BSA is consistent with the experimental data and this methodology should be applicable to other protein families.

PERSPECTIVES

- Dose-dependent chemical labeling experiments can be used to further confirm the SASA of reactive residues.
- Residues besides lysine and tyrosine can be selectively labeled with other chemical reagents.
- Computational approaches, such as *ab initio* prediction strategies, can be developed and the experimental data can be used to evaluate the computational models.
- The integration of differential labeling and oxidative labeling techniques should be applicable to protein complexes and protein mixtures.
- Implementation of the MudPIT and Inspect data-mining strategy helps to identify many modified sites. The methodology developed here will accelerate extracting more information out of the mass- spectrometry-based labeling experimental data.

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