Overview

- Chemical crosslinking[1] combined with mass spectrometry offers great potential for studying protein structure[2] and interactions.[3]
- Enzymatic digestion of a crosslinked protein or protein complex yields pairs of crosslinked peptides that bear information on location of crosslinked residues in the 3D structure of a protein,[2] or on the identities and relative orientation of neighboring proteins in a complex.[3]
- Limitations currently include low abundance of crosslinked peptides in reaction mixtures, and limited MS/MS fragmentation.
- We are developing techniques for improved analysis of crosslinked peptides, including affinity separations for isolating crosslinked peptides, and MS^n for identifying the components of a crosslinked peptide pair.

Introduction

We describe experiments in progress on two model systems for applying chemical crosslinking and mass spectrometry to the study of proteins:

Ribonuclease A: model system for using intramolecular crosslinking for probing protein shape

Hemoglobin: model system for using intermolecular crosslinking for identifying subunits and adjacency relationships for protein complexes

Methods

- Chemical Crosslinking
  - BS^3, sulfo-SBED (Pierce)
- Mass Spectrometry
  - MALDI-TOF: PerSeptive Biosystems Voyager Elite-DE
  - ESI MS-MS: Finnigan Deca LCQ
- Separations
  - Monomeric avidin (Immunopure, Pierce)

Results

Ribonuclease A: intramolecular crosslinking

- RNAse A served as a model system for intramolecular crosslinking, with the aim of providing distance constraints for structure prediction.[2,4]
- Using BS^3 as the crosslinker, Fig. 1 shows that multiple crosslinks were introduced into the protein.
- A tryptic digest of crosslinked RNAse A shows one candidate crosslinked peptide pair at ca. 4650 Da (Fig. 2).

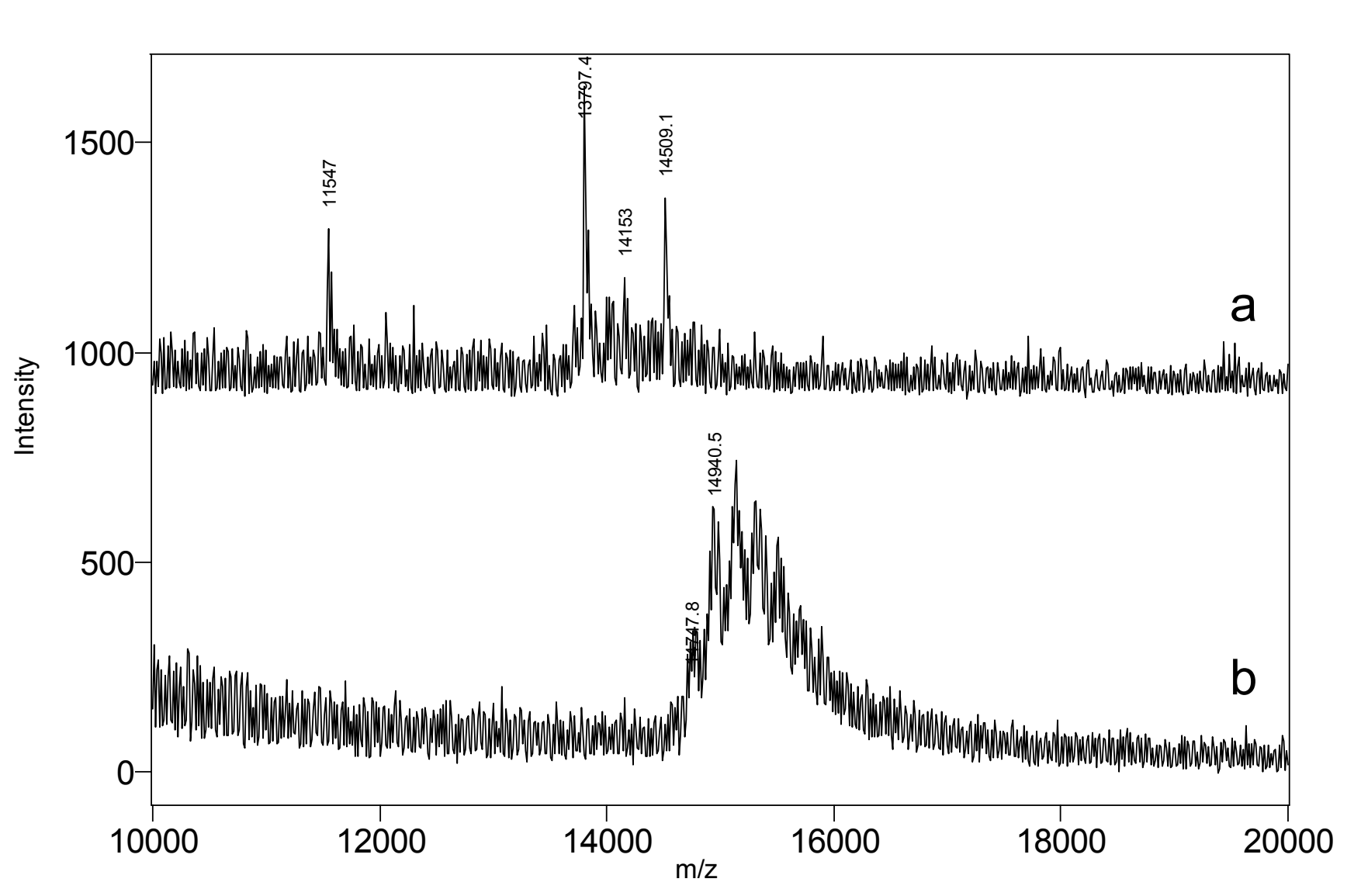


Figure 1. MALDI-TOF spectra of ribonuclease A (a) before and (b) after crosslinking with BS^3.

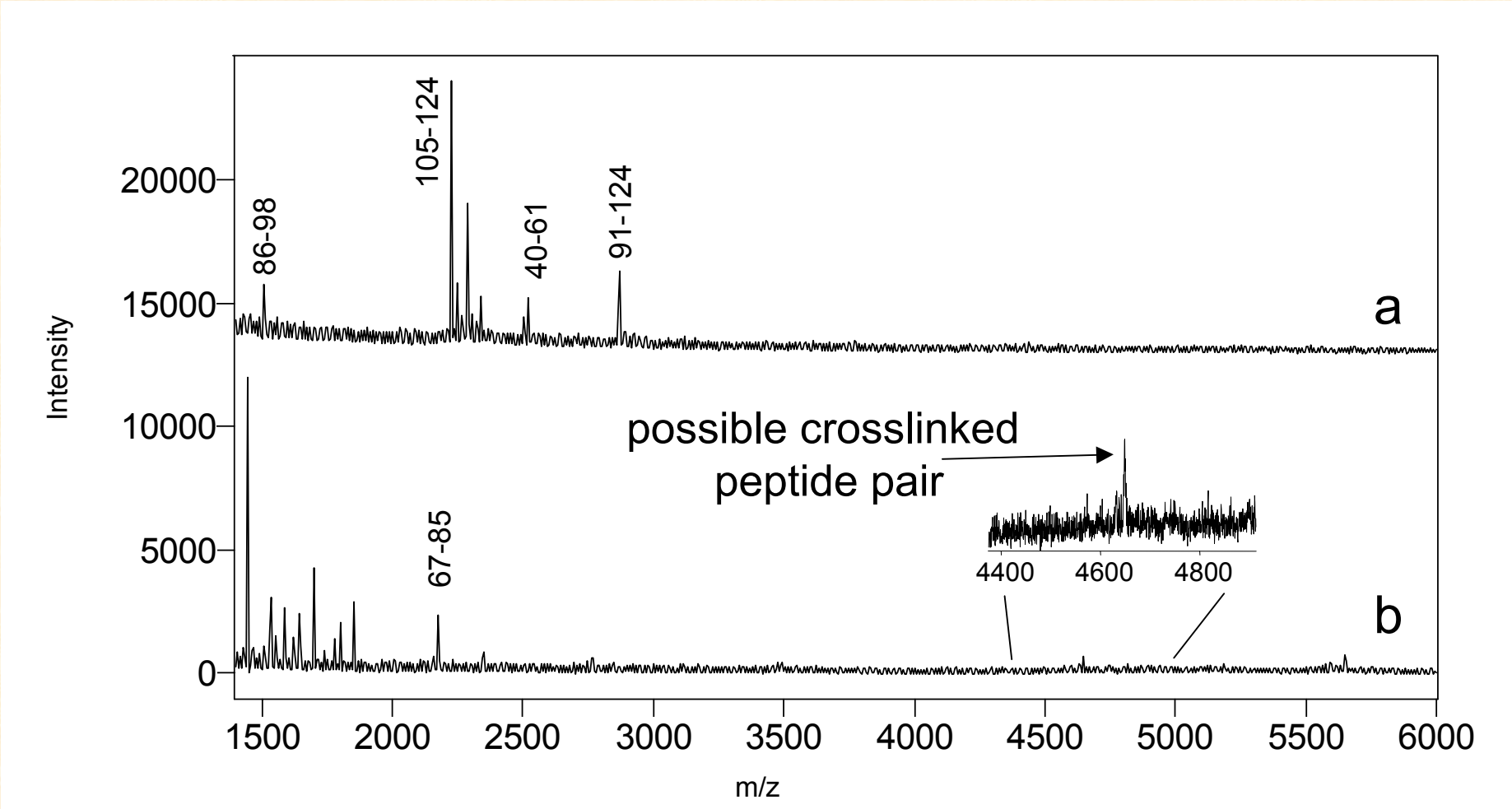


Figure 2. MALDI-TOF spectra of tryptic digests of ribonuclease A. (a) before, and (b) after crosslinking with BS^3

Ribonuclease A: intramolecular crosslinking

- ESI-MS/MS of the 1550^{3+} ion corresponding to the candidate crosslinked peptide pair (Fig. 3) yielded only a limited number of fragment ions.
- MS^3 on 1870^{2+} ion from MS^2, Figs. 4 & 5, yields a more complete series of b and y sequence ions (Table 1).
- MS^3 sequence ions and intact molecular mass identify the m/z ~4650 ion: peptides Asn34-Arg39 and Tyr92-Val124, crosslinked through lysines K37 and K104.

Ribonuclease A: intramolecular crosslinking

- Protein Data Bank model for RNAse A (1FS3) indicates that the alpha carbons in K37 and K104 are 28.8Å apart.
- However, maximum Cα-Cα distance spanned by BS^3 is ~24Å.[2]
- ⇨Some distortion of the 3D structure of the RNAse may have resulted from the crosslinking. (Consistent with MALDI of intact protein showing insertion of multiple crosslinks--Fig. 1 b.)

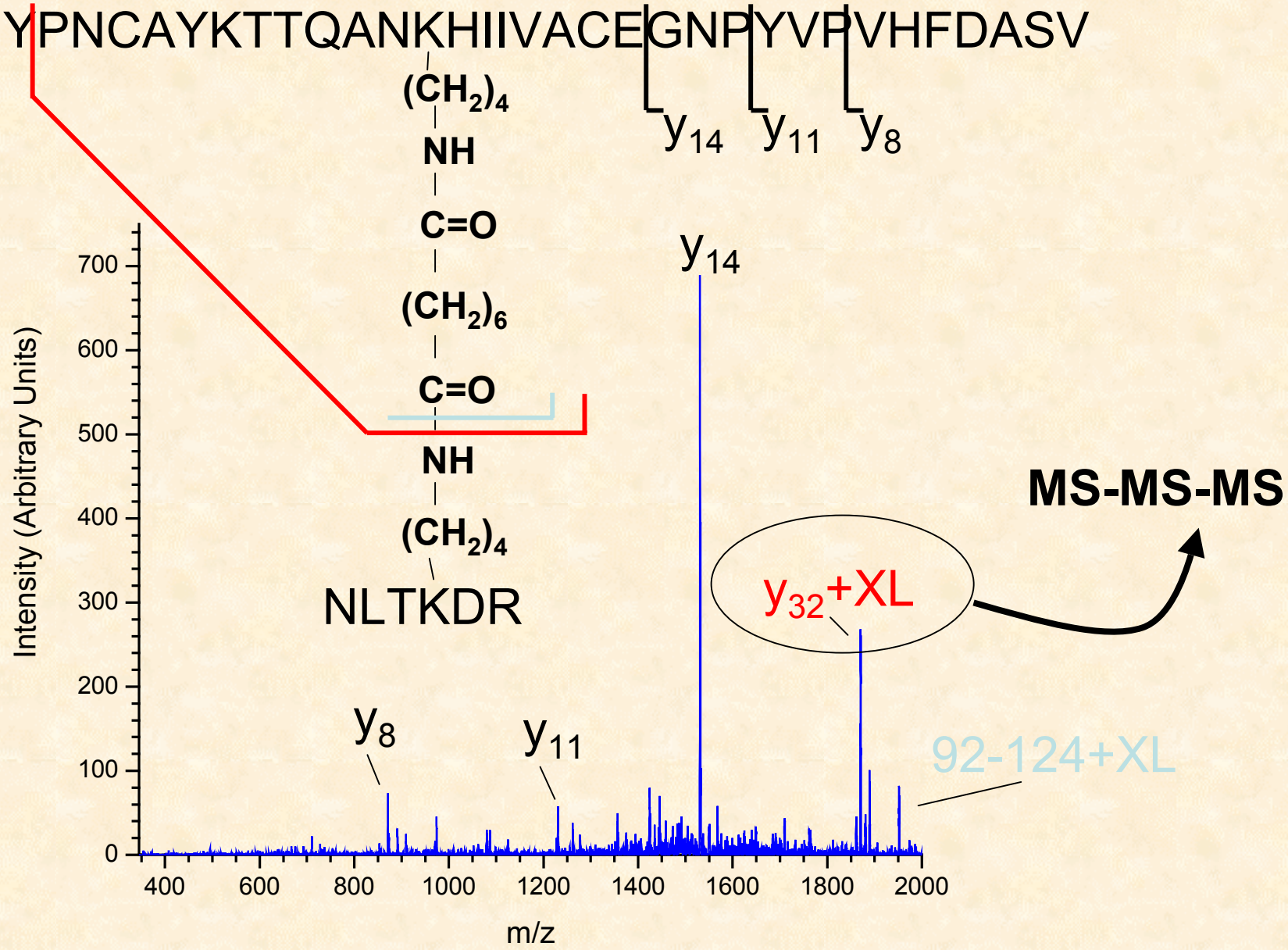


Figure 3. ESI-MS/MS (Deca LCQ, positive ion, direct infusion) of tryptic digest of BS^3-crosslinked RNAse A .

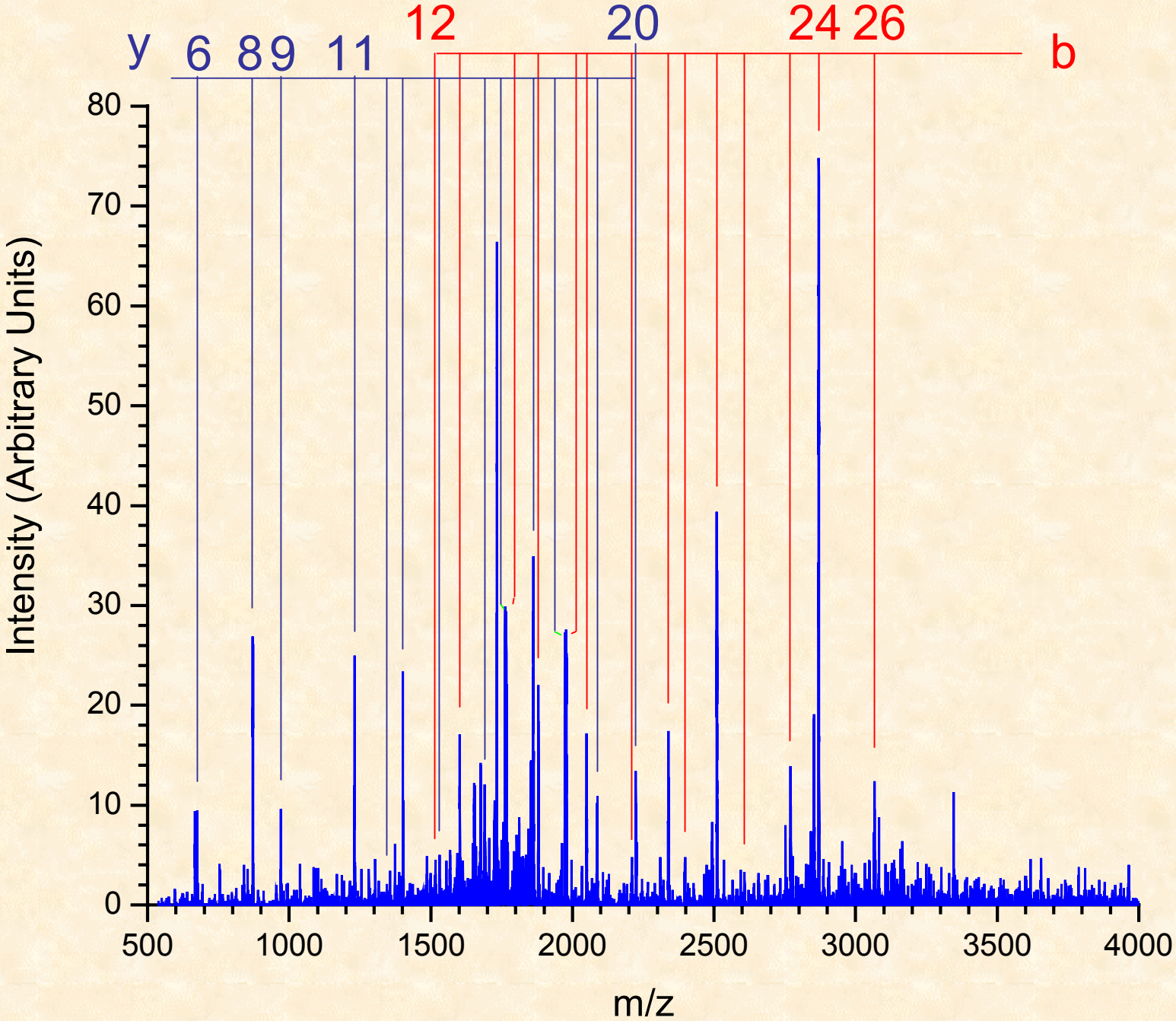


Figure 4: MS^3 (CAD of m/z 1870 from Figure 3) of crosslinked peptide from RNAse A

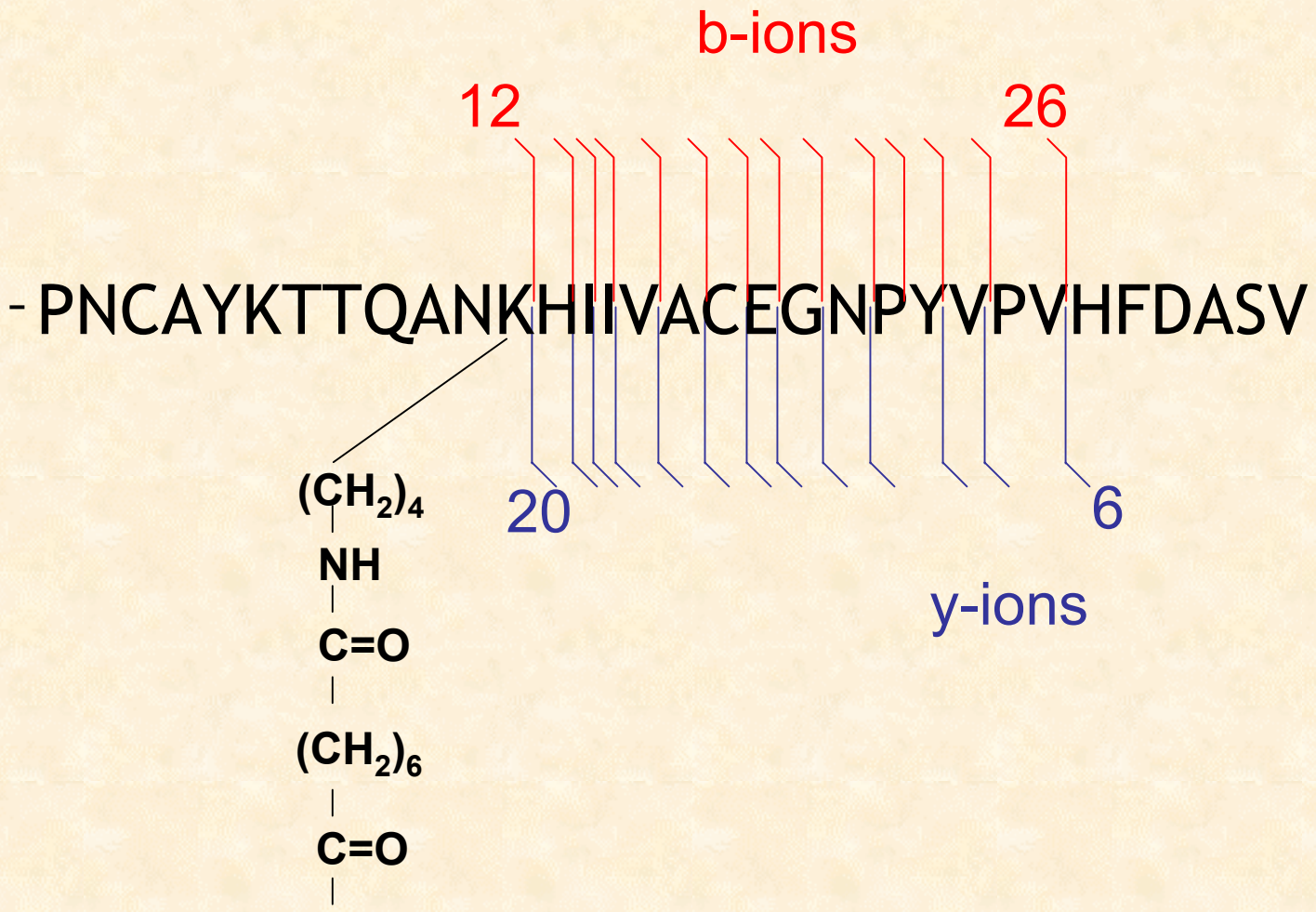


Figure 5. Sequence information from MS^3 of BS^3-crosslinked RNAse A.

Table 1. MS^3 of Crosslinked Peptide Pair from RNAse A

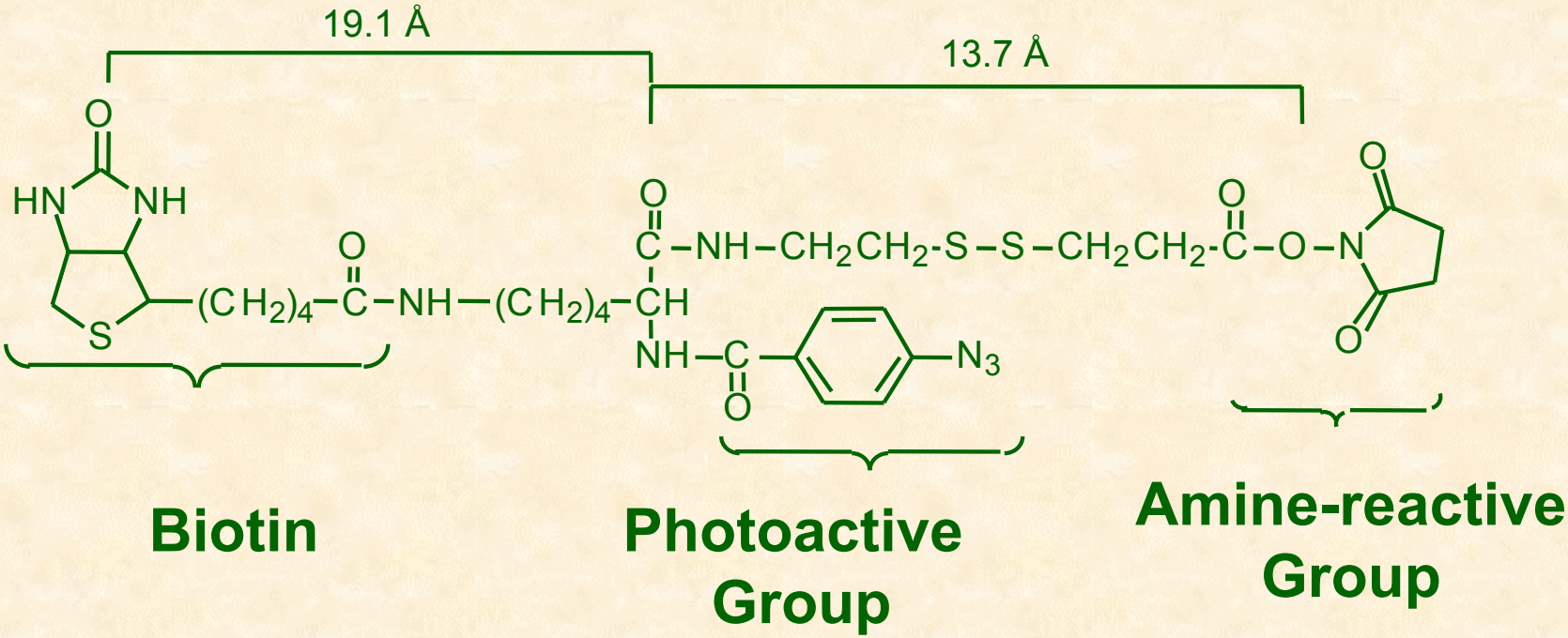
Observed m/z	assignment	assigned charge state	error
667.1	-		
674.9	y"6	+1	0.40
754	-		
853.1	-		
871.2	y"8	+1	0.22
954.6	-		
970.2	y"9	+1	0.29
1230.6	y"11	+1	0.01
1345	y"12	+1	0.35
1373.1	-		
1401.5	y"13	+1	0.17
1486.7	-		
1516	b12	+1	0.74
1530.7	y"14	+1	0.01
1567.1	y"27	+2	0.69
1602.4	b27	+2	0.61
1653.2	b13	+1	0.60
1675.7	b28	+2	0.38
	y*29	+2	0.64
1690.5	y"15	+1	0.25
1724.9	b*29	+2	0.56
1733.4	b29	+2	0.56
1761.7	y"16	+1	0.08
1766.5	b14	+1	0.38
1767.2	b14	+1	0.32
1803.5	b*31	+2	0.13
1811.7	b31	+2	0.17
1844.5	y*17	+1	0.65
1853	b*32	+2	0.09
1862	b*15	+1	0.96
	b32	+2	0.59
	y*32	+2	0.09
1879.6	b15	+1	0.36

Observed m/z	assignment	assigned charge state	error
1897.7	-		
1950.1	-		
1960.2	-		
1973.9	y"18	+1	0.03
1978.5	b16	+1	0.53
1996.4	-		
2033.5	b*17	+1	0.43
2049.7	b17	+1	0.37
2087.1	y"19	+1	0.08
2209.2	b18	+1	0.90
2224.1	y"20	+1	0.02
2309.7	-		
2321.6	b*19	+1	0.54
2339.5	b19	+1	0.36
2396.6	b20	+1	0.44
2493.5	b*21	+1	0.29
2509.7	b21	+1	0.51
2606.9	b22	+1	0.36
2752.7	b*23	+1	0.62
2769.7	b23	+1	0.62
2825.4	-		
2841.7	-		
2851.7	b*24	+1	0.69
2870	b24	+1	0.61
3066.6	-		
3082.4	-		
3346.4	-		

Hemoglobin: intermolecular crosslinking

- Bovine hemoglobin--model protein complex
  - Tetramer: two α chains (15,053 Da); two β chains (15,954 Da)

- Crosslinked with sulfo-SBED, which has 3 important functional groups (see Figure 6):
  - NHS ester -- covalently attaches to lysine
  - Aryl azide -- photoactivated; inserts into N-H and C-H bonds
  - Biotin -- allows selective isolation of SBED-crosslinked species via affinity chromatography (avidin).



$C_{32}H_{42}S_4N_9O_{11}Na$

Figure 6. Sulfo-SBED (Pierce)

Hemoglobin: intermolecular crosslinking

- MALDI-TOF of crosslinked and non-crosslinked hemoglobin (Fig. 7) shows addition of sulfo-SBED and accompanying loss of NHS and N_2 (net addition of 667 Da; $C_{28}H_{39}S_4N_6O_5$).

- Monomeric avidin affinity chromatography allowed isolation of biotinylated (and therefore crosslinked) hemoglobin (See Fig. 8).

- The presence of both biotinylated and non-biotinylated α and β chains in the biotinylated fraction (Fig. 7b), combined with the persistence of heme group absorbance (Fig. 8), suggests that intact tetramers survive the crosslinking and separation.

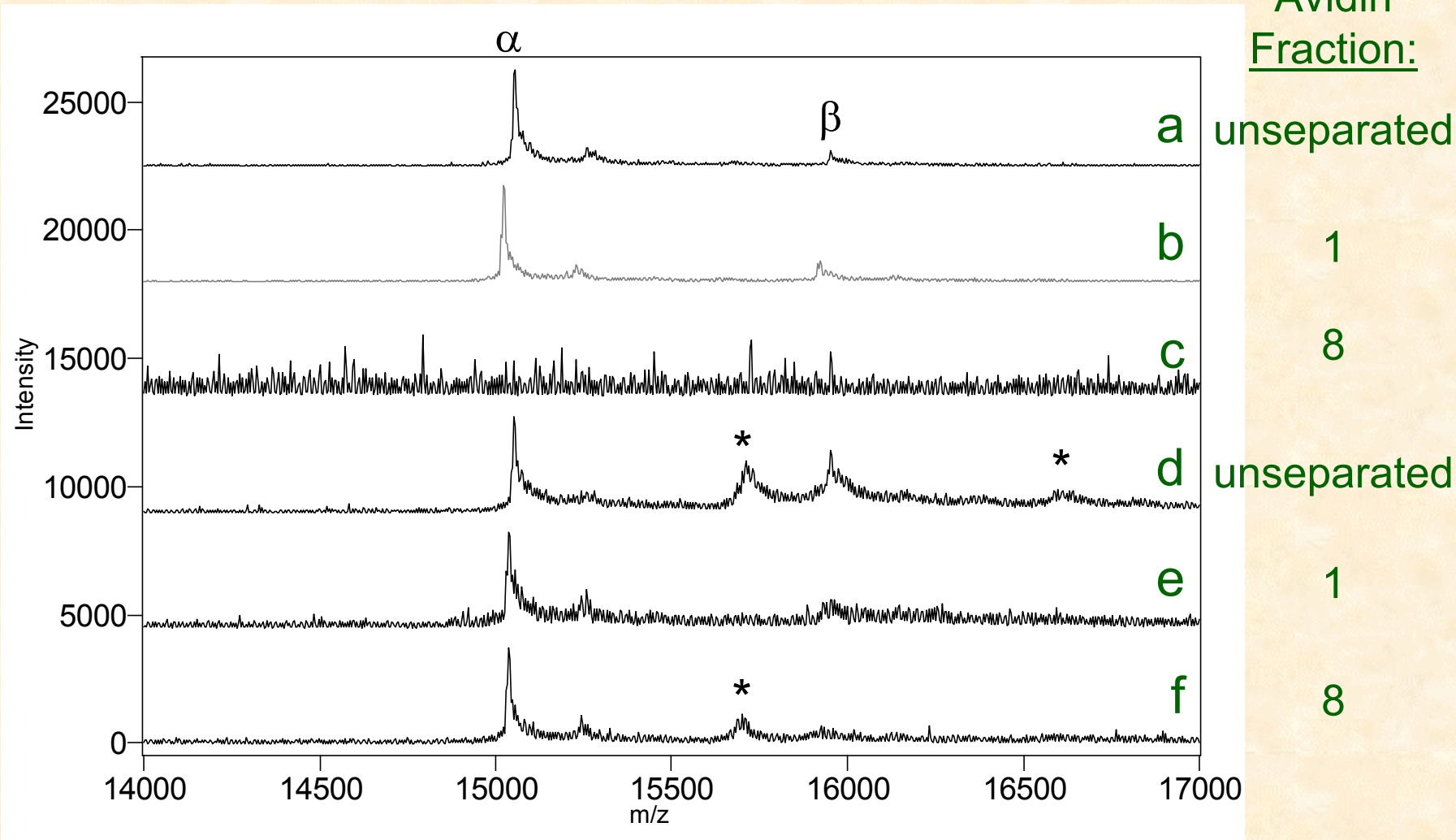


Figure 7. MALDI-TOF spectra of hemoglobin. a,b,c: not crosslinked. d,e,f: sulfo-SBED crosslinked. *=addition of sulfo-SBED

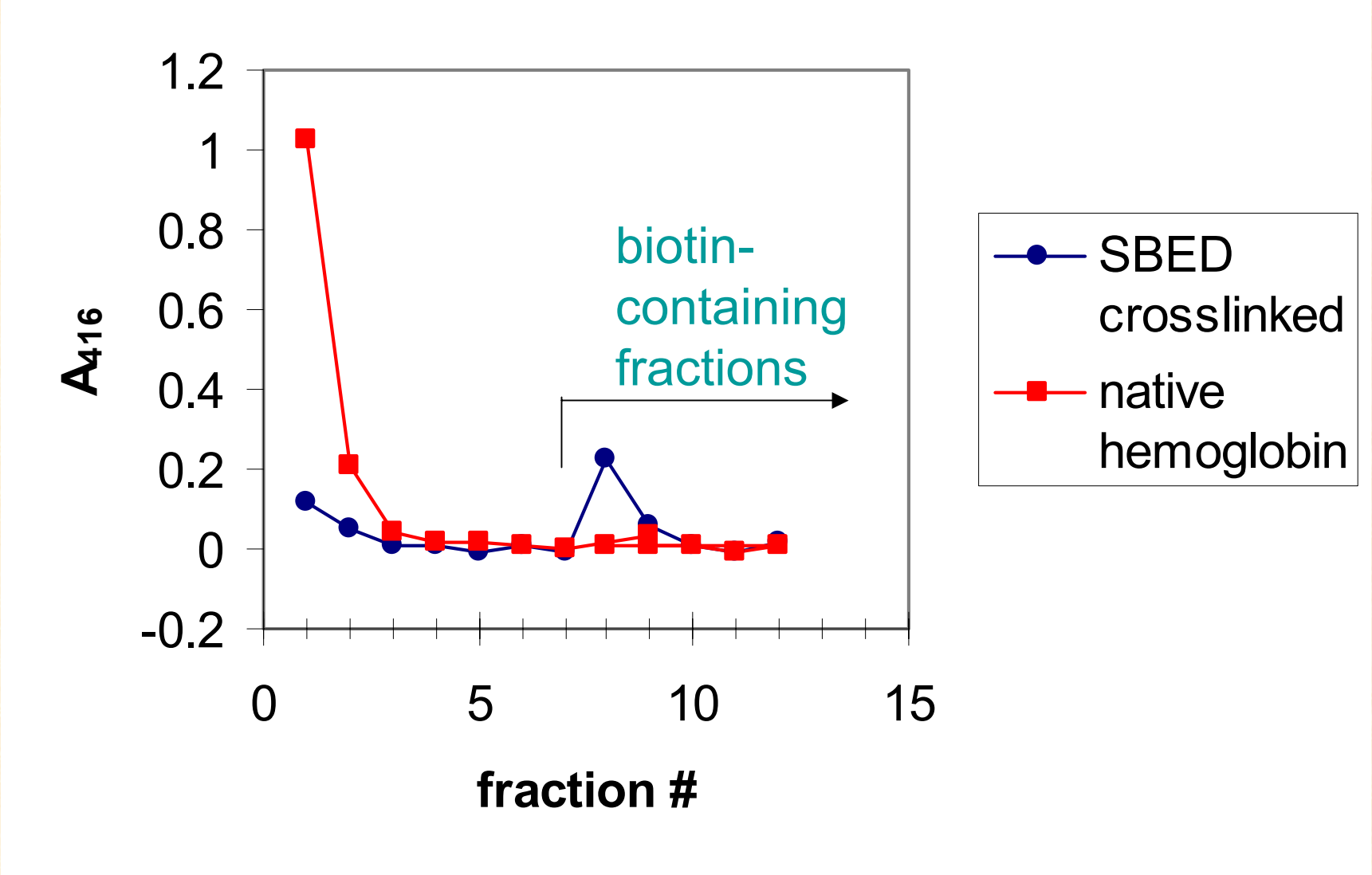


Figure 8. Avidin fractionation of sulfo-SBED crosslinked hemoglobin

Conclusions

- Crosslinked peptide pairs are present in low abundance, and require extra steps for detection and identification
  - MS^3
  - Enrichment via affinity chromatography

- Work in Progress:
  - Avidin chromatography on digests of sulfo-SBED crosslinked protein complexes
  - Accurate mass identification of crosslinked peptide pairs by FTICR (R.L. Hettich)
  - Improved data analysis software

    (Y Xu, J. Razumovskaya)

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