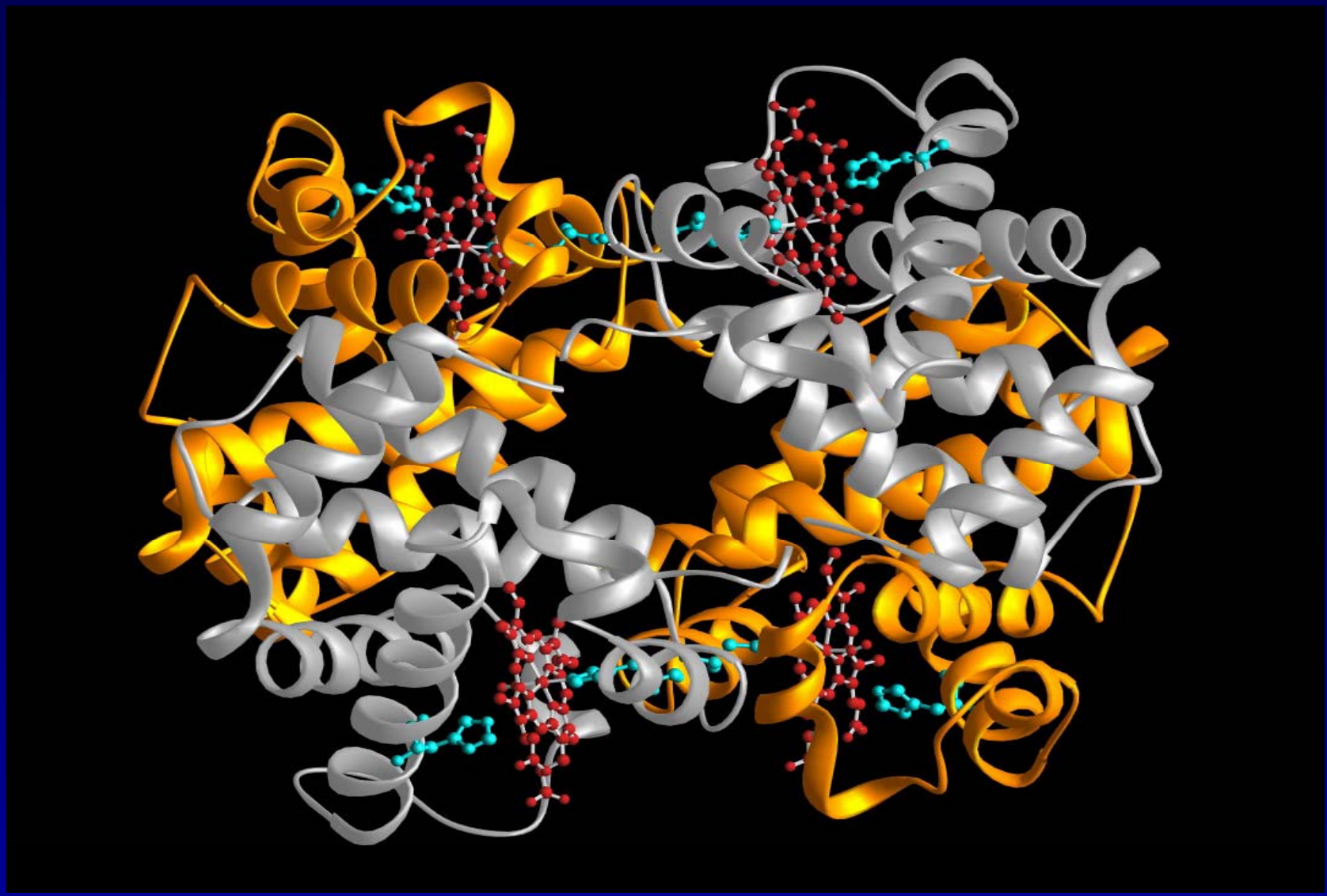
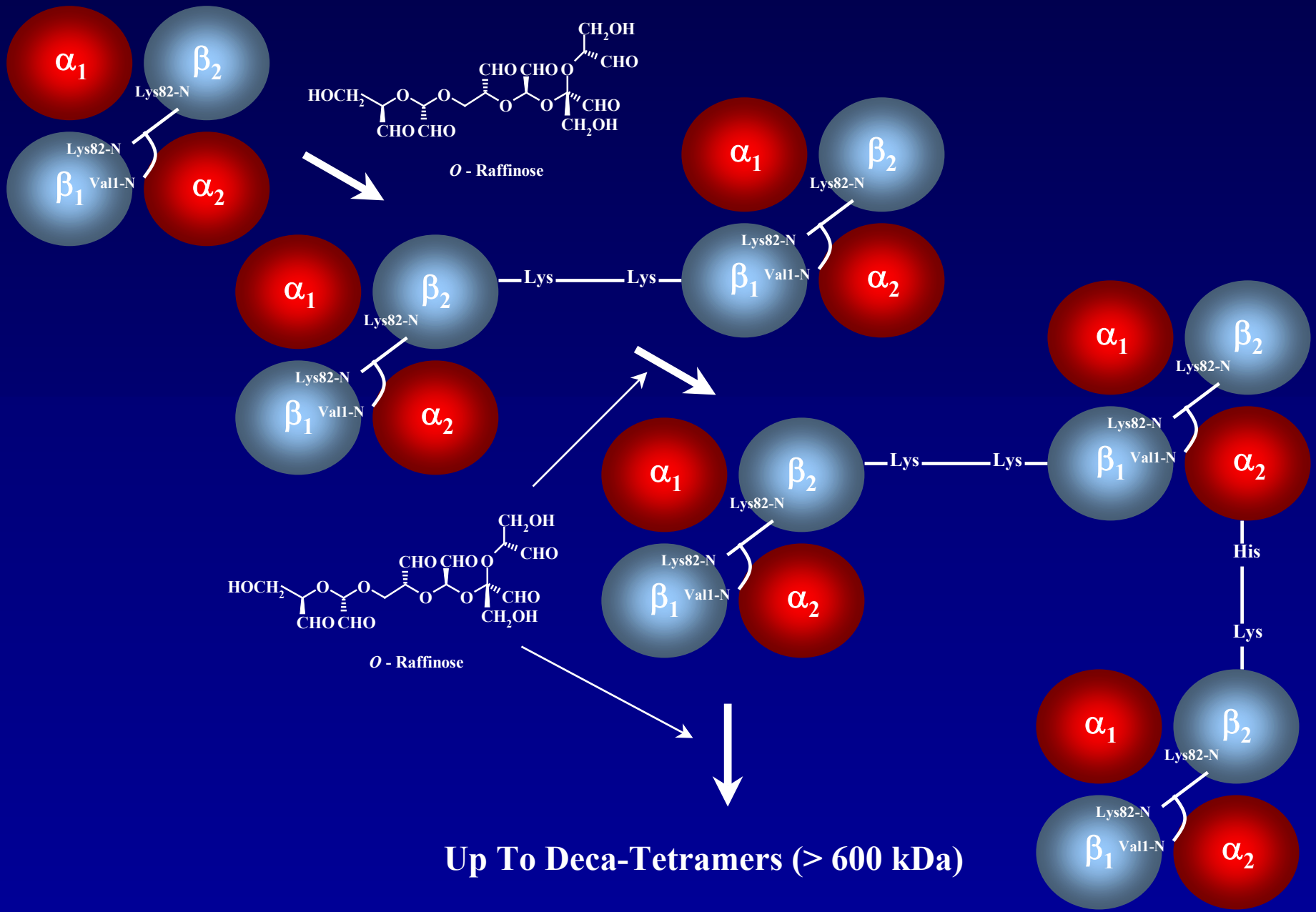
O-Raffinose Cross-linked Hemoglobin (Hemolink ™): Structural and Functional Consequences of a Non-site Specific Chemistry

Robert A. Boykins – Staff Scientist
Laboratory of Biophysics
Division Bacterial, Parasitic and Allergenic Products
Office of Vaccines
CBER-FDA

Hemoglobin



(Proposed Sites of Inter-molecular Cross-linking)



Hemolink ™ (O-R-PolyHbAo): Background and Evidence of Dysfunction

Purpose of Current Investigation

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Polymer Fractionation

↓

Functional Oxygen Binding Characteristics

↓

Structural Modification Analysis

↓

(a) Intra-molecular Cross-linking

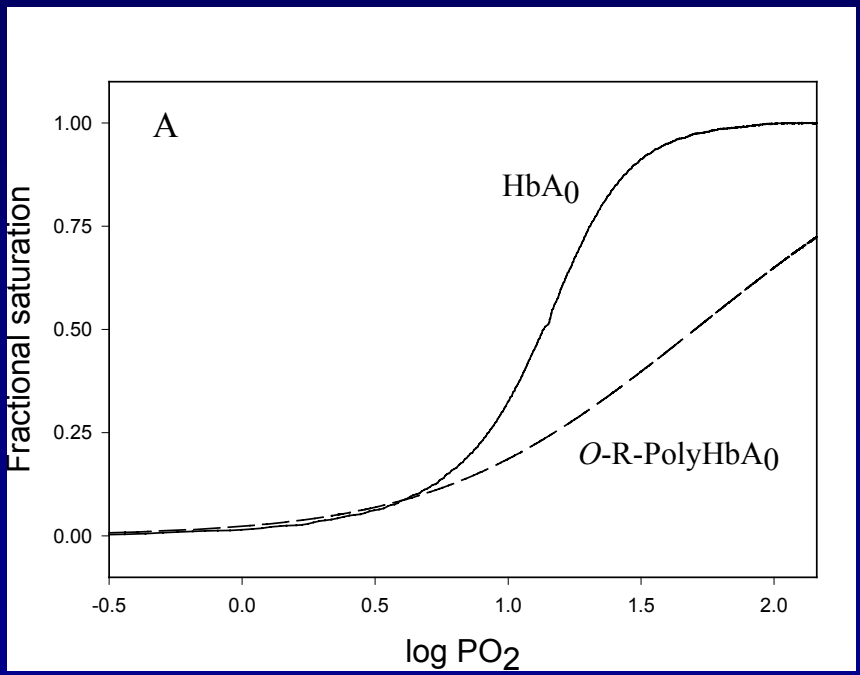
(b) Inter-molecular Cross-linking

(c) Alternative Modification

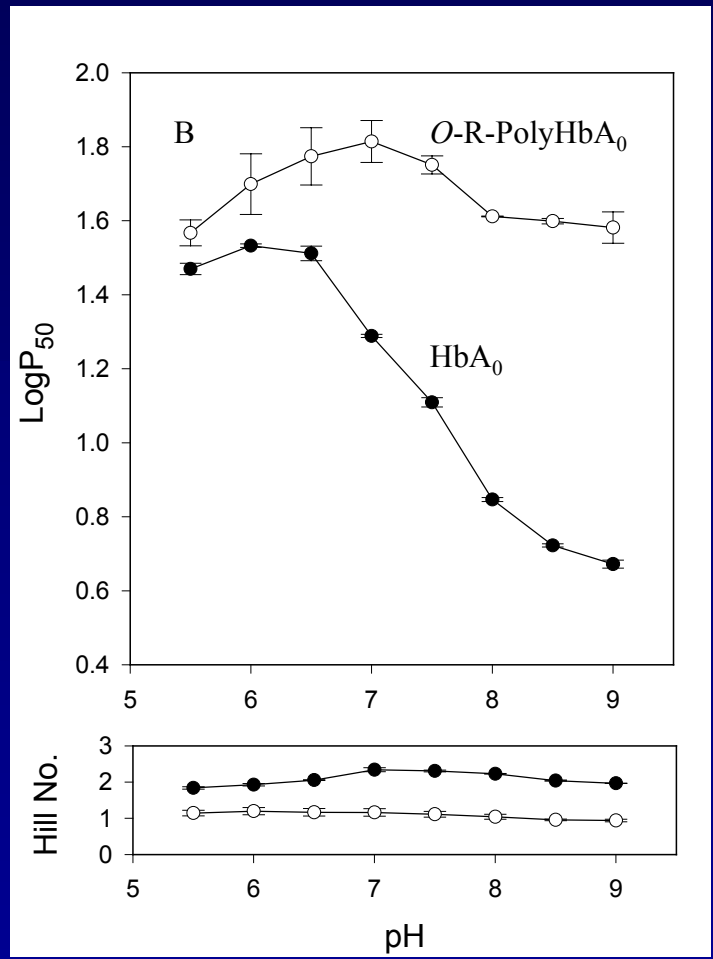
(d) Molecular Modeling

Hemolink™ (*O*-R-PolyHbA$_0$): Evidence of Dysfunction
(Oxygen Binding Characteristics)

Oxygen Equilibrium



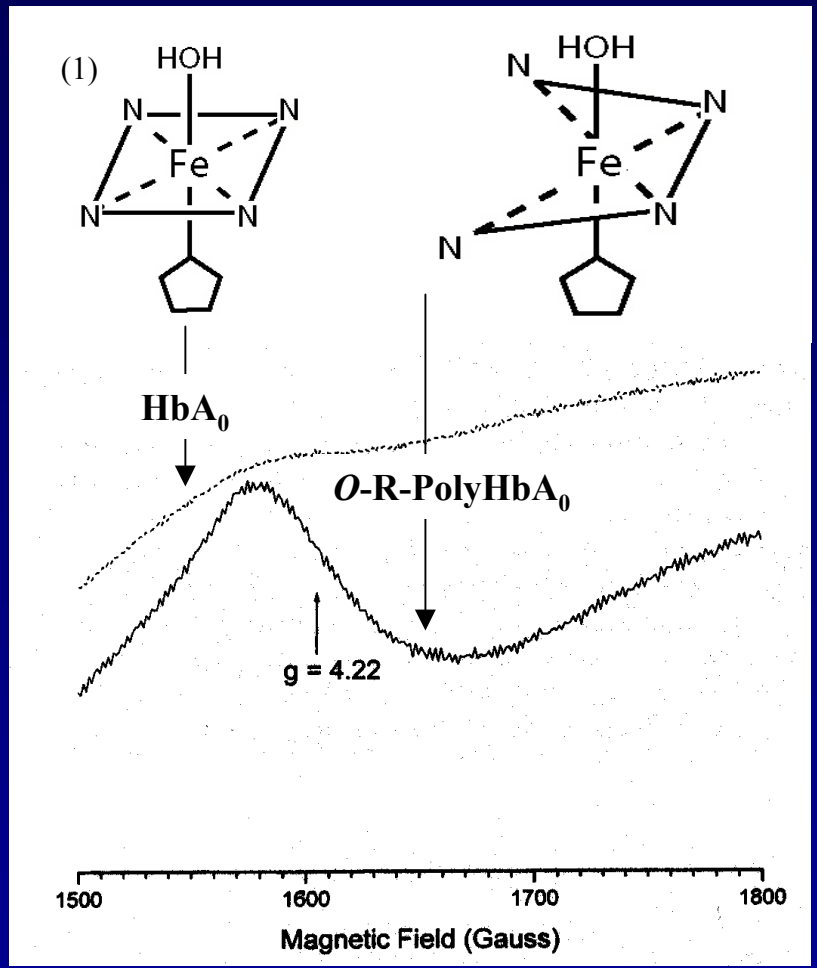
Bohr Effect (Sensitivity to pH)



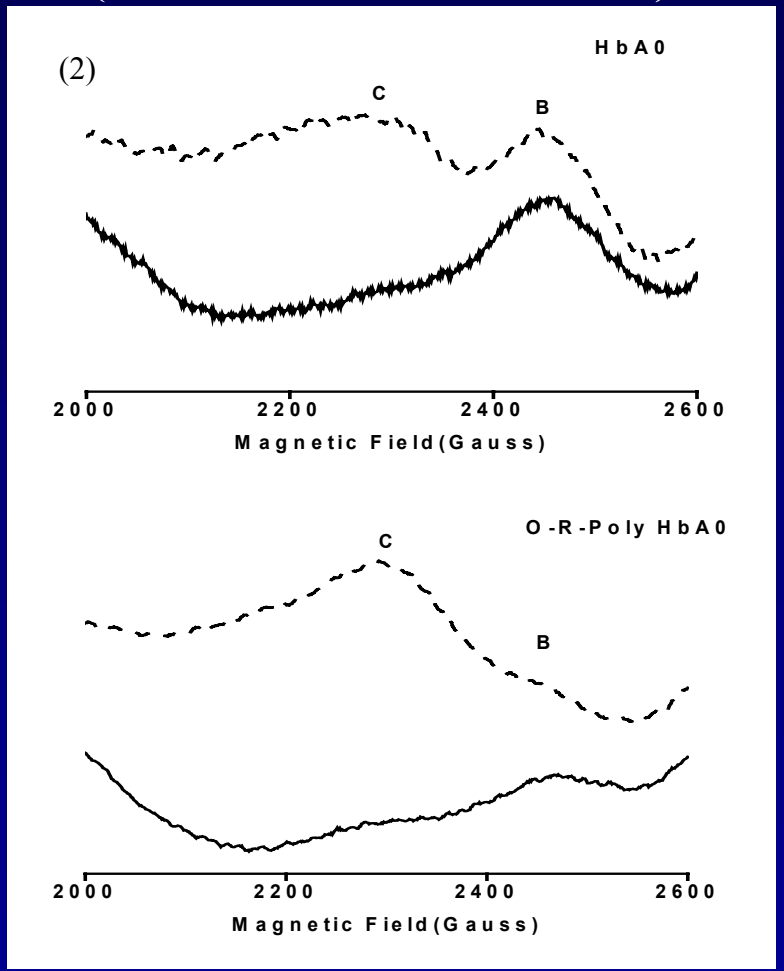
Jia ,Y., Ramasamy, S., Wood, F., Alayash, A.I. and Rifkind, J.M.(2004) *Biochem. J.* **384**, 367-375

(Heme Disorientation and Heme Pocket Rigidity)

Heme Disorientation and Instability



Distal Heme Pocket Rigidity (Locked T-State Conformation)

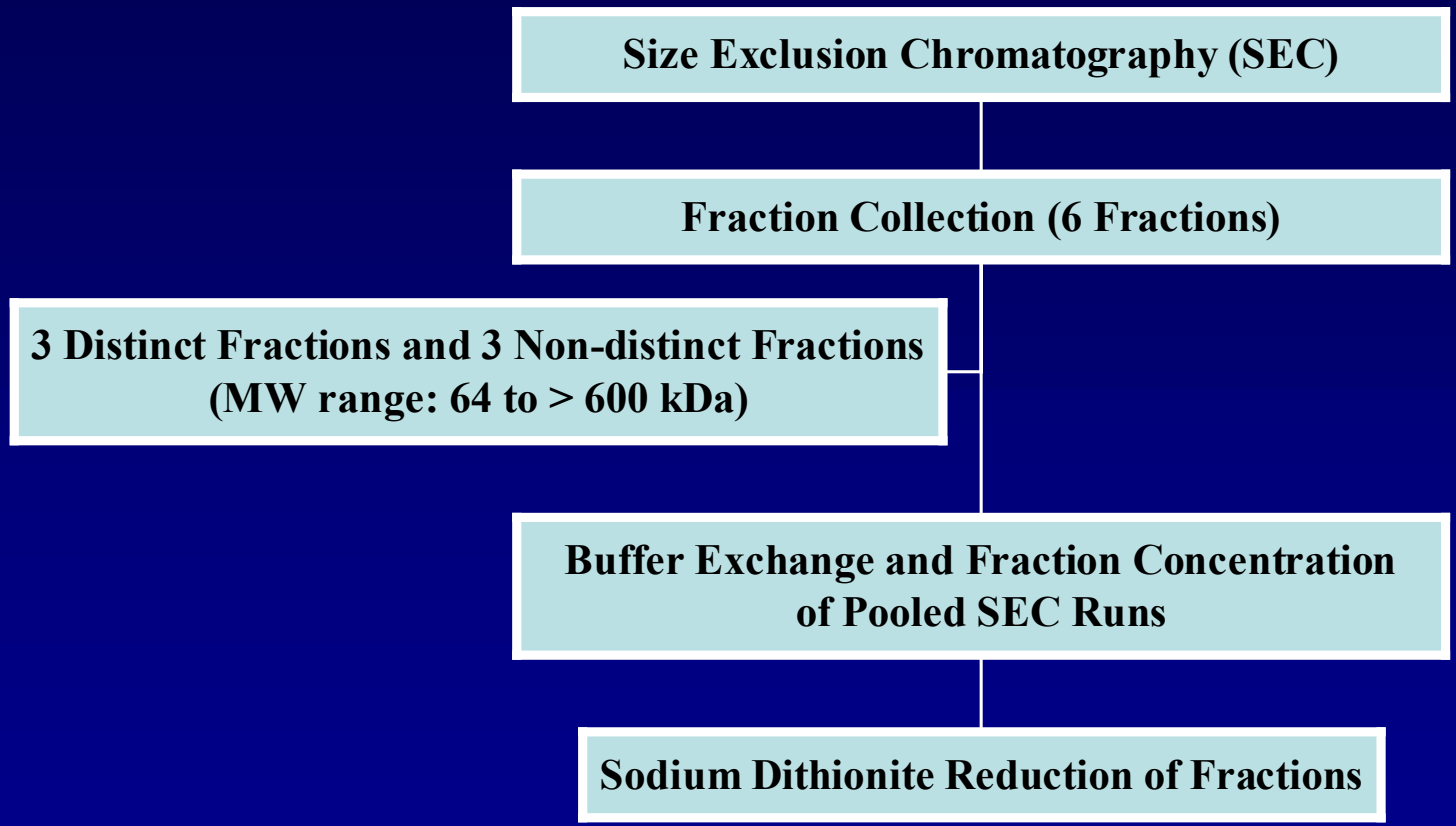


(1) Nagababu, E., Ramasamy, S., Rifkind, J.M., Jia, Y. and Alayash, A.I. (2002) Biochemistry **41**, 7407-7415. (2) Jia ,Y., Ramasamy, S., Wood, F., Alayash, A.I. and Rifkind, J.M.(2004) *Biochem. J.* **384**, 367-375

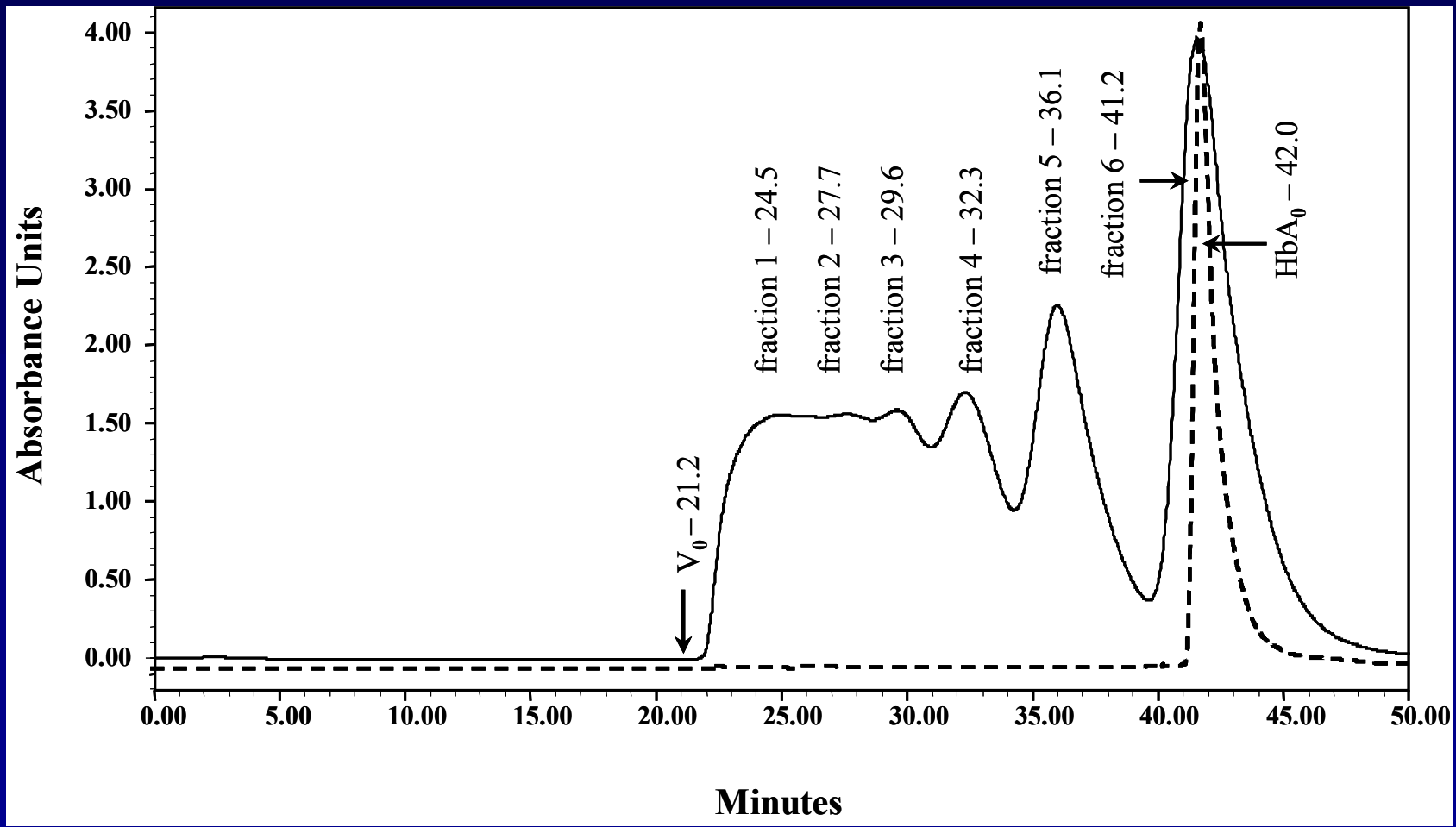
Purpose of the Current Investigation

- Fractionate the heterogeneous polymeric mixture of Hemolink™ (*O*-R-PolyHbA_0) and determine if any fraction(s) contribute to the unusual oxygen binding properties of the mixture as a whole.
- Stringently evaluate the proposed sites of intra- and inter-molecular cross-linking given the potential "non-specific" reactivity of oxidized raffinose with proteins such as HbA_0.
- Determine the presence of additional sites of modification to HbA_0 which may contribute to the previous observation of a locked T-state conformation.
- Suggest potential explanations for our findings using amino acid accessible surface area (ASA) and distance calculations.

Hemolink™ (*O*-R-PolyHbA_0) Polymer Fractionation



SEC Indicating (6) Fractions Collected



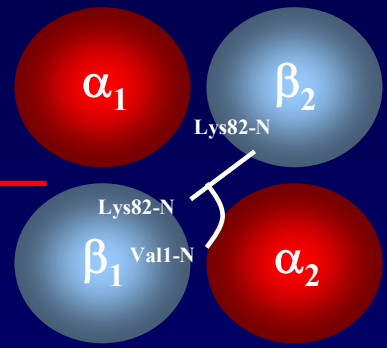
Results of Oxygen Binding Studies

Equilibrium and kinetic binding parameters of HbA_0 , *O*-R-$PolyHbA_0$ and fractions (F1 – F6)

	P_{50}	n	$k_{off}(s^{-1})$	$k_{off}(s^{-1})$ + IHP (150 μM)	k_{on} x10^{-5} ($M^{-1}sec^{-1}$)	k_{on} x10^{-5} ($M^{-1}sec^{-1}$) + IHP (100 μM)
HbA_0	14.7	2.16	31 ± 0.06	49.8 ± 0.2	1.70 ± 0.08	0.71 ± 0.01
$polyHbA_0$	47.3	1.00	130 ± 3.5	125 ± 1.3	1.20 ± 0.04	1.14 ± 0.06
$polyHbA_0$F1	44.4	1.06	126 ± 1.5	126 ± 3.3	1.25 ± 0.01	1.43 ± 0.008
$polyHbA_0$F2	47.3	1.00	134 ± 2.1	139 ± 2.3	1.58 ± 0.003	1.32 ± 0.007
$polyHbA_0$F3	39.3	1.00	123 ± 1.9	123 ± 2.0	1.64 ± 0.009	1.18 ± 0.019
$polyHbA_0$F4	44.0	1.00	133 ± 2.0	138 ± 1.7	1.16 ± 0.001	1.29 ± 0.003
$polyHbA_0$F5	40.4	1.00	127 ± 4.1	135 ± 2.6	1.21 ± 0.006	1.19 ± 0.006
$polyHbA_0$F6	40.0	1.08	128 ± 2.2	124 ± 2.3	1.34 ± 0.006	1.29 ± 0.005

The P_{50} and n values were derived from oxygen equilibrium binding curves as described in the text and represent the mean of three separate runs (SEM < 10% for each mean value and group). Oxygen binding kinetics in the presence and absence of IHP is represented as the mean +/- SEM for at least three curve fitting calculations.

Structural Modification Analysis



Intra-molecular Cross-linking

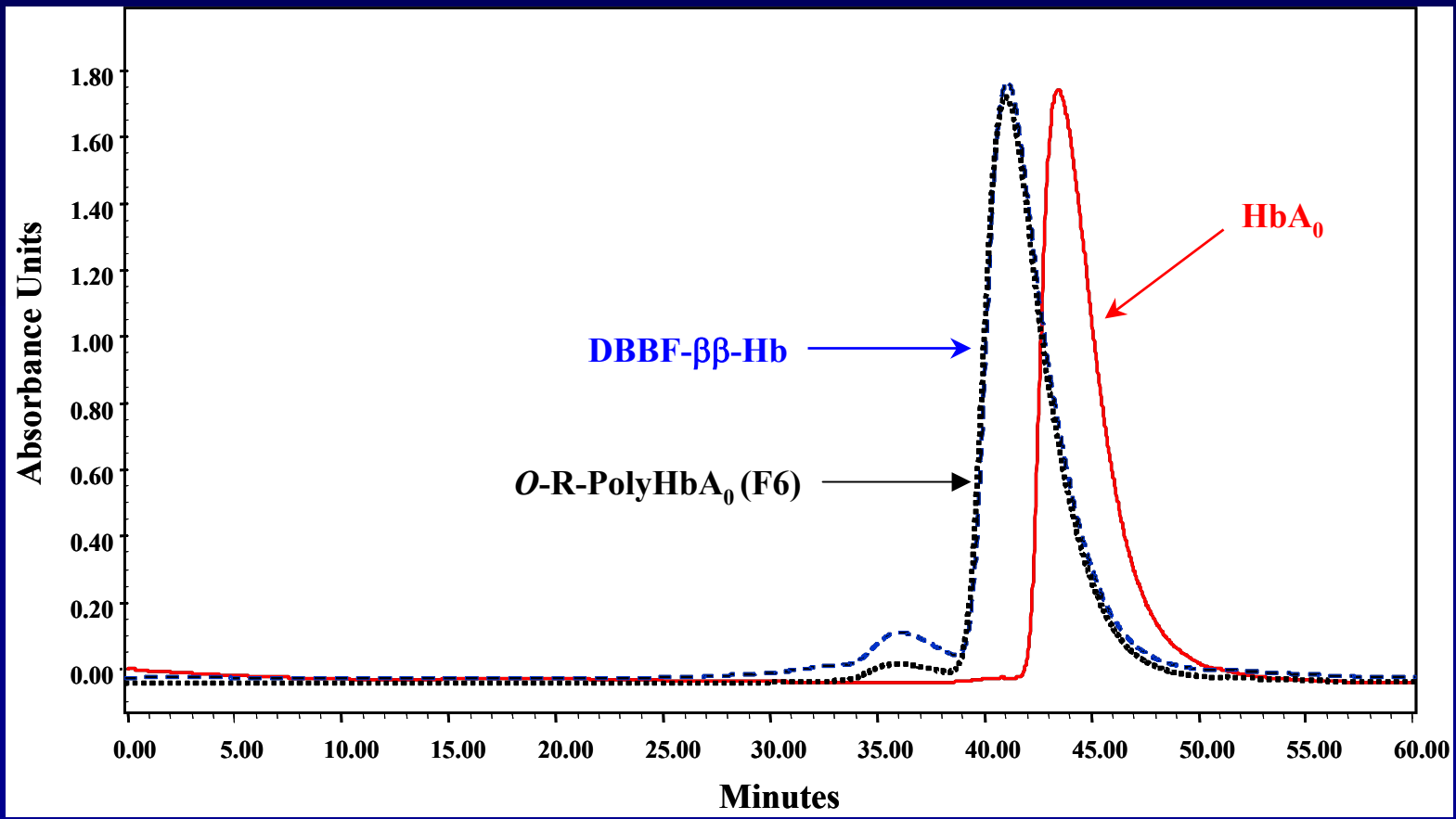
SEC Under Dissociating / Non-Denaturing Conditions
(0.5 M MgCl2)

Matrix Assisted Laser Desorption/Ionization -Mass Spectrometry
(MALDI-MS)

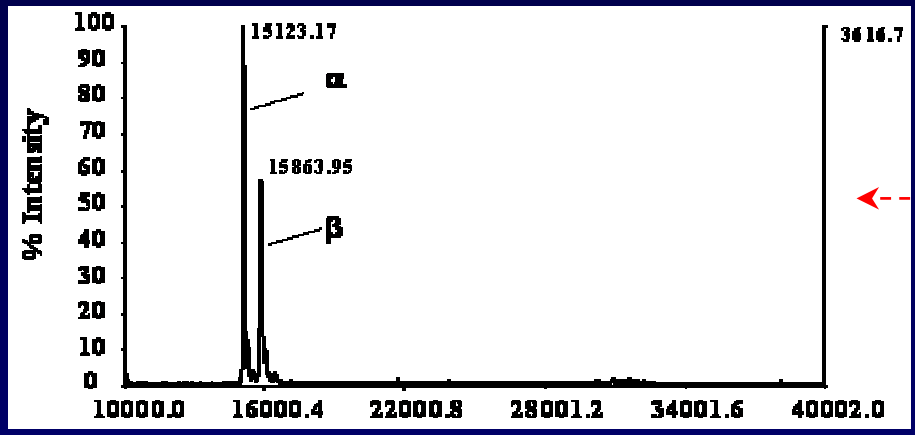
Enzymatic Digestion (Trypsin, CNBr and Glu-C)

MALDI-MS and LC-MS/MS of Digests

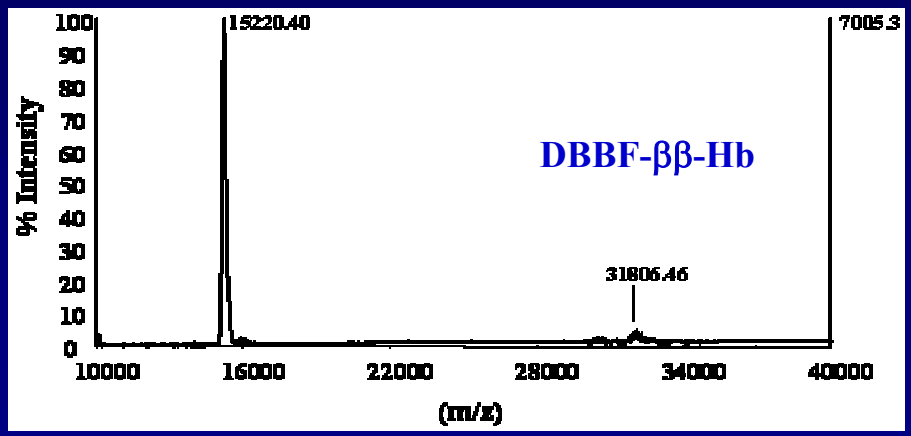
SEC Performed Under Dissociating Conditions (0.5 M $MgCl_2$)

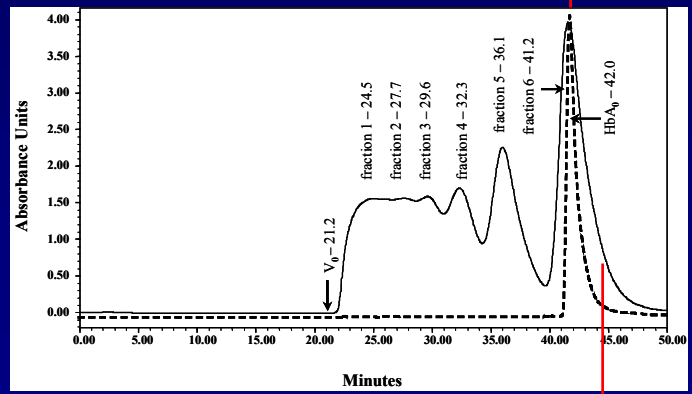


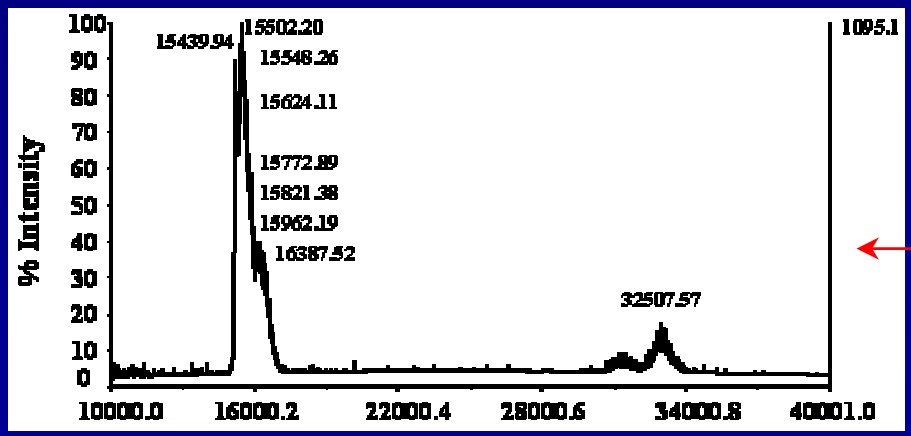
MALDI-MS: HbA_0, Fraction (6) and DBBF-ββ-Hb



HbA_0

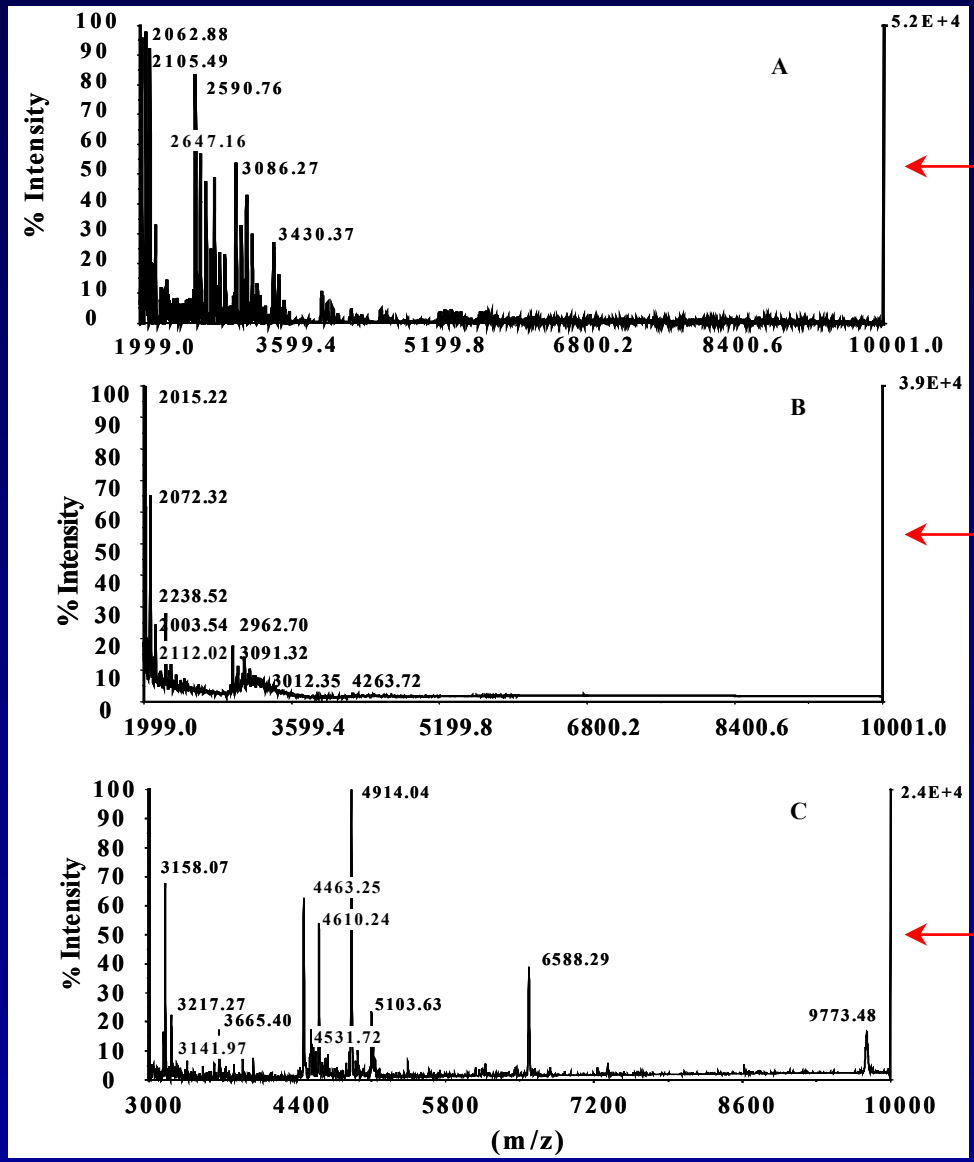






O-R-PolyHbA_0 (F6)

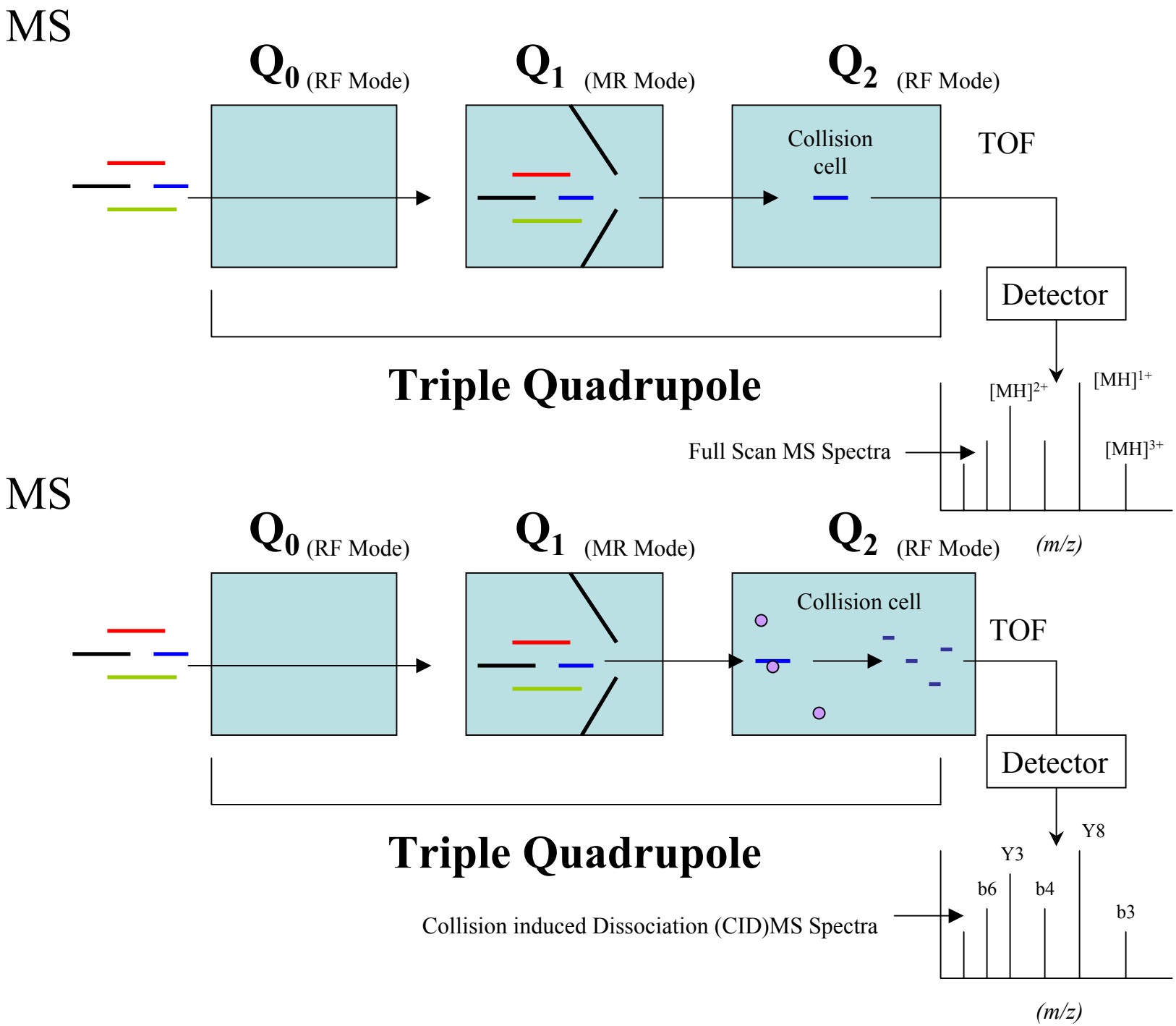
MALDI-MS of Trypsin Digested: HbA_0, Fraction (6) and DBBF-ββ-Hb



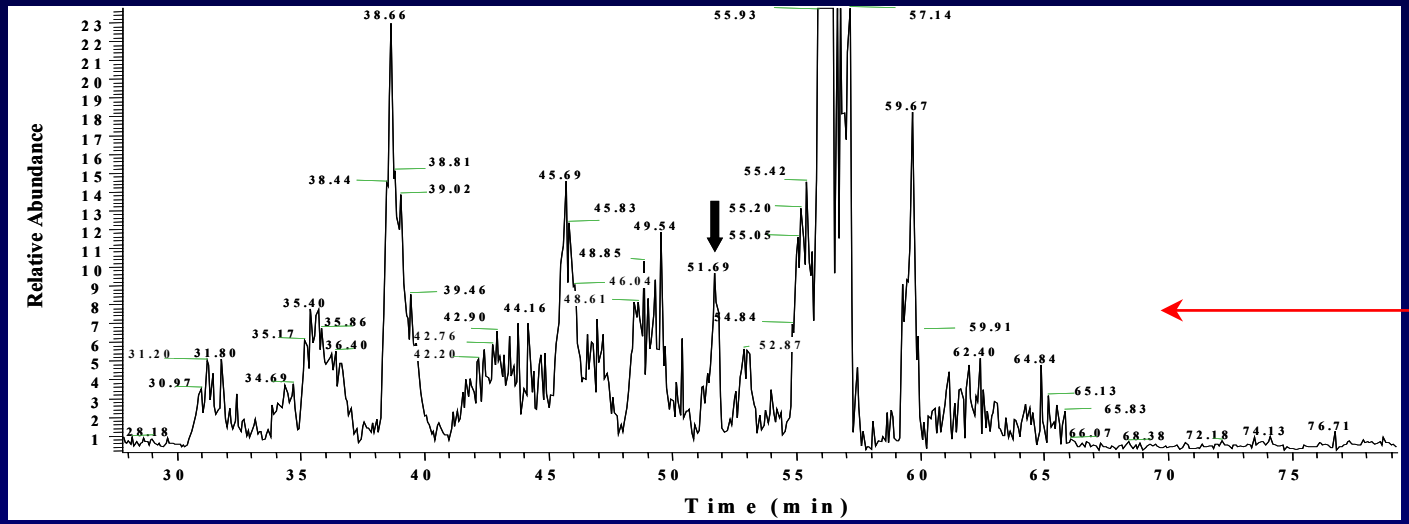
HbA_0

O-R-PolyHbA_0 (F6)

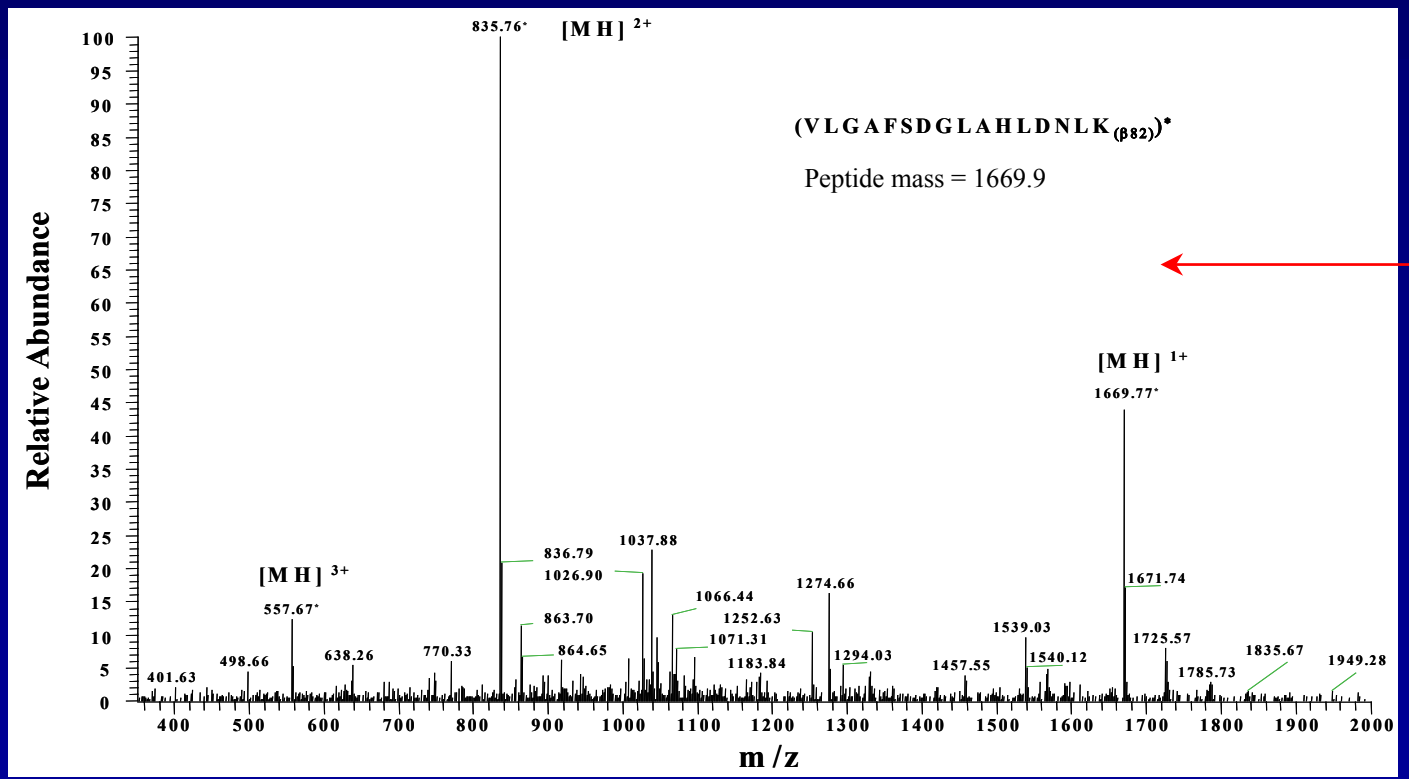
DBBF-ββ-Hb


MS
Q0 (RF Mode)
Q1 (MR Mode)
Q2 (RF Mode)
Collision cell
TOF
Detector
Triple Quadrupole
Full Scan MS Spectra
[MH]2+
[MH]1+
[MH]3+
MS
Q0 (RF Mode)
Q1 (MR Mode)
Q2 (RF Mode)
(m/z)
Collision cell
TOF
Detector
Triple Quadrupole
Collision induced Dissociation (CID)MS Spectra
Y8
Y3
b6
b4
b3
(m/z)

Tryptic Digest of βLYS82 (O-R-PolyHbAo) Peptide

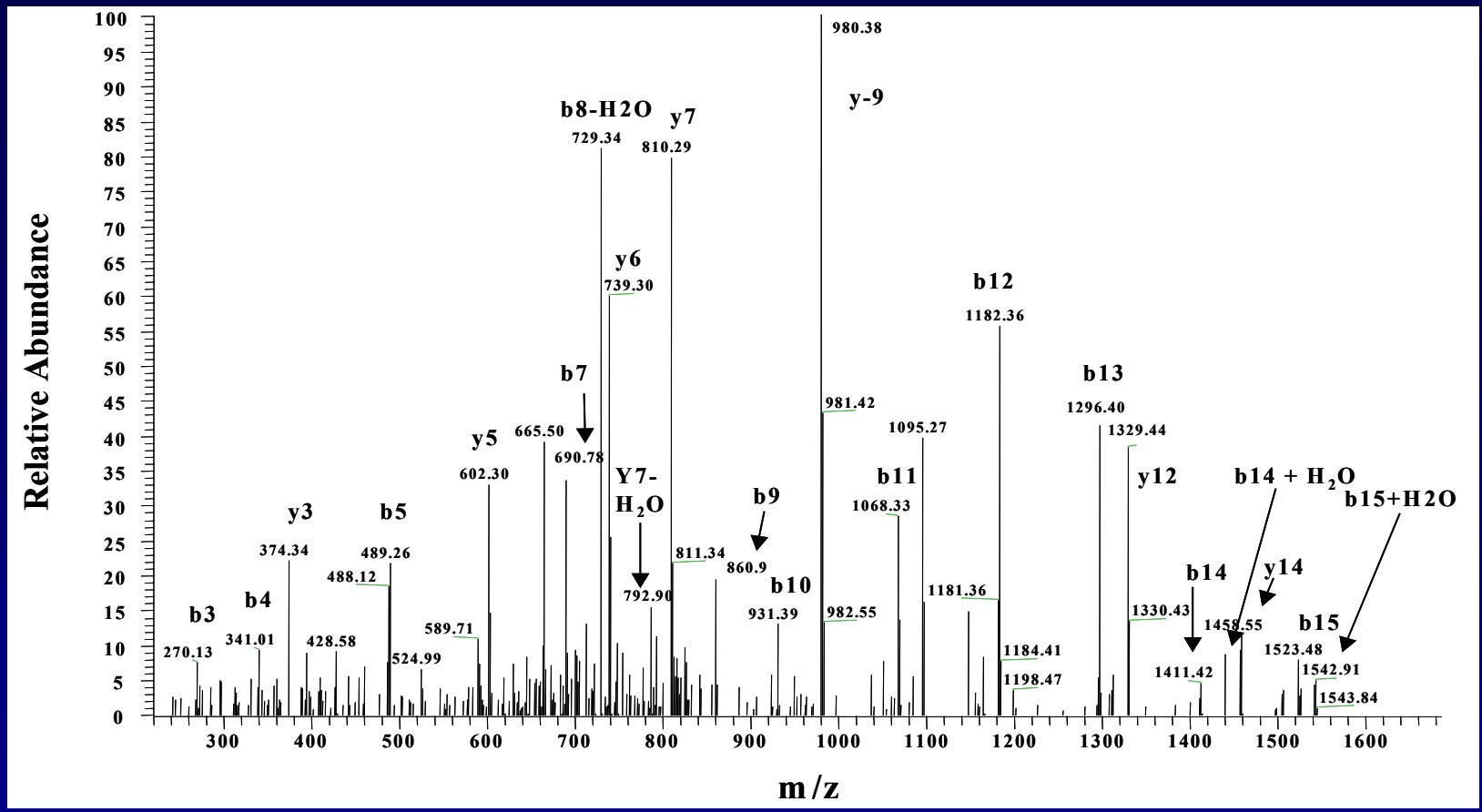


Total Ion Current (TIC)



Full Scan MS Spectrum of Peak 51.69 in TIC

Collision Induced Dissociation (CID) of the βLys82 Peptide

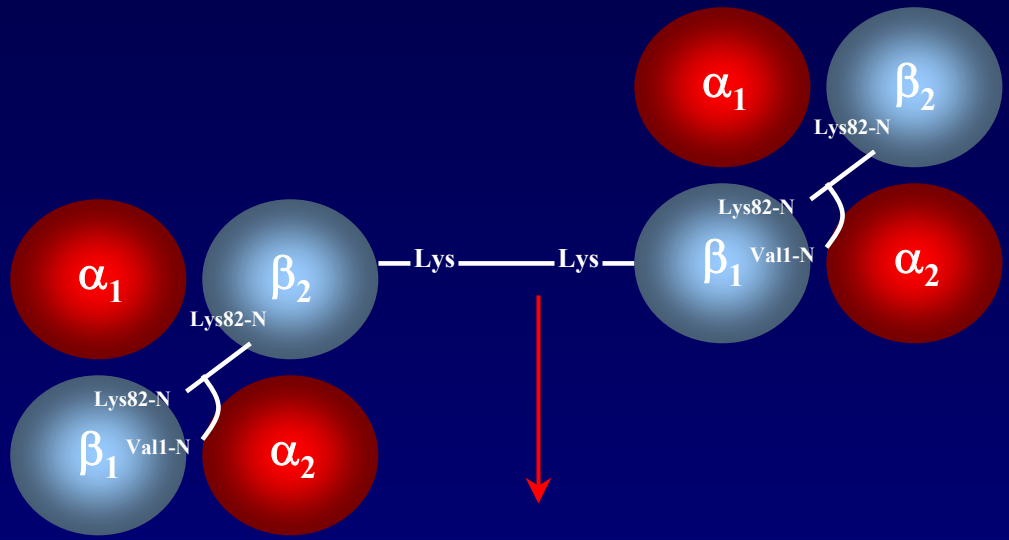


Collision induced mass ions from the tryptic peptide (β67-β82)

Sequence	Val	Leu	Gly	Ala	Phe	Ser	Asp	Gly	Leu	Ala	His	Leu	Asp	Asn	Leu	Lys
Mass	99.1	113.2	57.0	71.1	147.2	87	115.1	57	113.2	71.1	137.1	113.2	115.1	114.1	113.2	128.2
b			269.3	341.0	489.26		690.78	729.34[a]	860.9	931.39	1068.33	1182.36	1296.40	1411.42	1523.48	
y			1458.55		1329.33			980.38		810.29	739.3	602.30		374.34		

[a] b8 – H2O

Structural Modification Analysis (Continued)



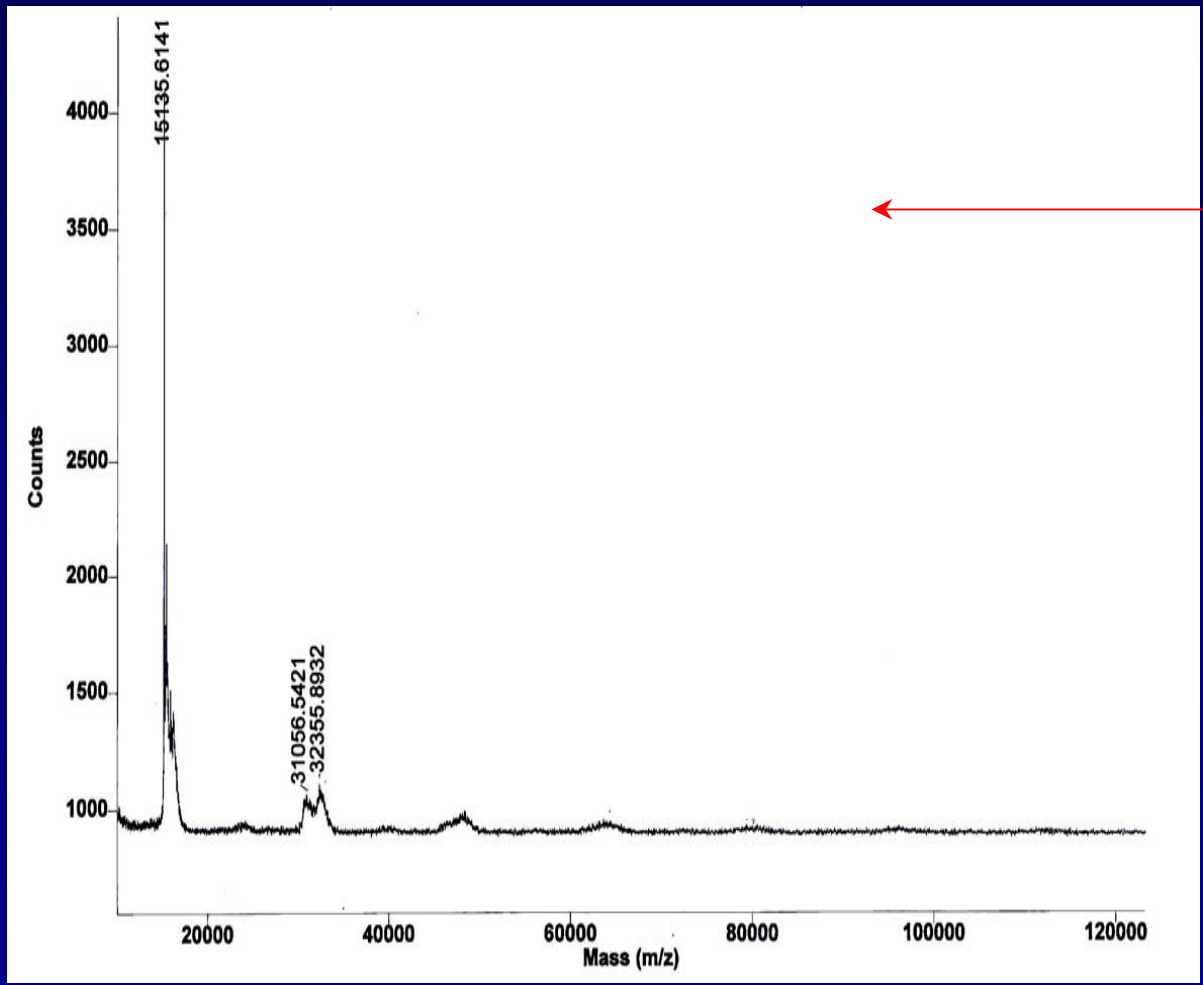
Inter-molecular Cross-linking

MALDI-MS of Largest Molecular Weight Fraction (F1)

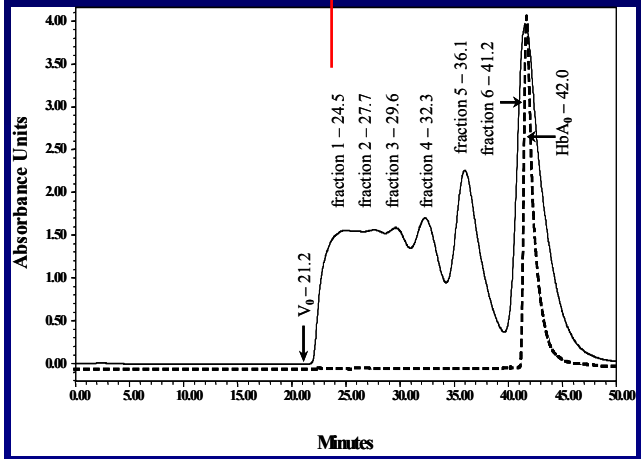
Enzymatic Digestion and Analysis by MALDI-MS and LC-MS/MS

Amino Acid Hydrolysis (5.7 N HCl) and Ion Exchange Chromatography (Focus on reported surface reactive A.A.s: LYS, HIS, TYR and CYS)

MALDI-MS of Large Molecular Weight Fraction



***O*-R-PolyHbA$_0$ (F1)**



Peptides Identified by MALDI-MS and LC-MS/MS

Unidentified peptides in HbA_0 and *O*-R-$PolyHbA_0$

Modified peptides in *O*-R-$PolyHbA_0$

O-R-$PolyHbA_0$ peptide fragments from theoretical and actual tryptic digestions.

Fragment residues	Sequence	Theoretical MW	MALDI-TOF	LC-MS/MS
α (1-7)	VLSPADK	728.8	nmwo	nmwo
α (8-11)	TNVK	460.5	nmwo	nmwo
α (12-16)	AAWGK	531.6	nmwo	nmwo
α (17-31)	AHAGEYGAEALER	1529.6	1529.65	1499.11[a]
α (32-40)	MFLSFPTTK	1071.4	1072.52	1071.46
α (41-56)	TYFPHFDLSHGSAQVK	1834.0	1832.93	1833.02
α (57-60)	GHGK	397.4	nmwo	nmwo
α (61)	K	146.2	nmwo	nmwo
α (62-90)	VADALTNAVAHVDDMPNALSALSDLHAHK	2997.3	2293.93	2997.22
α (91-92)	LR	287.4	nmwo	nmwo
α (93-99)	VDPVNFK	817.9	nmwo	nmwo
α (100-127)	LLSHCLLVTLAAHLPAEFTP AVHASLDK	3025.5	nmwo	nmwo[b]
α (128-139)	FLASVSTVLTSK	1252.5	1233.68[c]	1252.57
α (140-141)	YR	337.4	nmwo	nmwo
β (1-8)	VHLTPEEK	952.1	nmwo	nmwo
β (9-17)	SAVTALWGK	932.1	931.03	932.48
β (18-30)	VNVDEVGGEALGR	1314.4	1314.90	1314.53
β (31-40)	LLVVYPWTQR	1274.5	1274.24	1274.52
β (41-59)	FFESFGDLSTPDAVMGNPK	2059.3	2057.87	2058.70
β (60-61)	VK	245.3	nmwo	nmwo
β (62-65)	AHGK	411.5	nmwo	nmwo
β (66)	K	146.2	nmwo	nmwo
β (67-82)	VLGAFSDGLAHLDNLK	1669.9	1669.36	1669.2
β (83-95)	GTFATLSELHCDK	1478.6	nmwo	nmwo[d]
β (96-104)	LHVDPENFR	1126.2	nmwo	nmwo[d]
β (105-120)	LLGNVLVCVLAHHFGK	1777.1	1775.05	1777.63
β (121-132)	EFTPPVQAAYQK	1378.5	1377.90	1378.59
β (133-144)	VVAGVANALAHK	1149.1	1151.43	1148.62
β (145-146)	YH	318.2	nmwo	nmwo

N-Terminal Modification

Cys104

N-Terminal Modification

Trypsin Missed Cleavage — Cys93

(nmwo) = no molecular weight observed

[a] (-28 = CO)

[b] Several MS/MS ions were found for this peptide, however, no $[MH]^{1+}$ was identified at 3025

[c] (-18 = H_2O)

[d] βLys95 could not be cleaved in repeated tryptic digests

Amino Acid Hydrolysis (5.7 N HCl)

(Represents the mean values for minimum of 10 hydrolysates)

Hydrolysis and analysis of amino acids in HbA_0 and *O*-R-PolyHbA_0

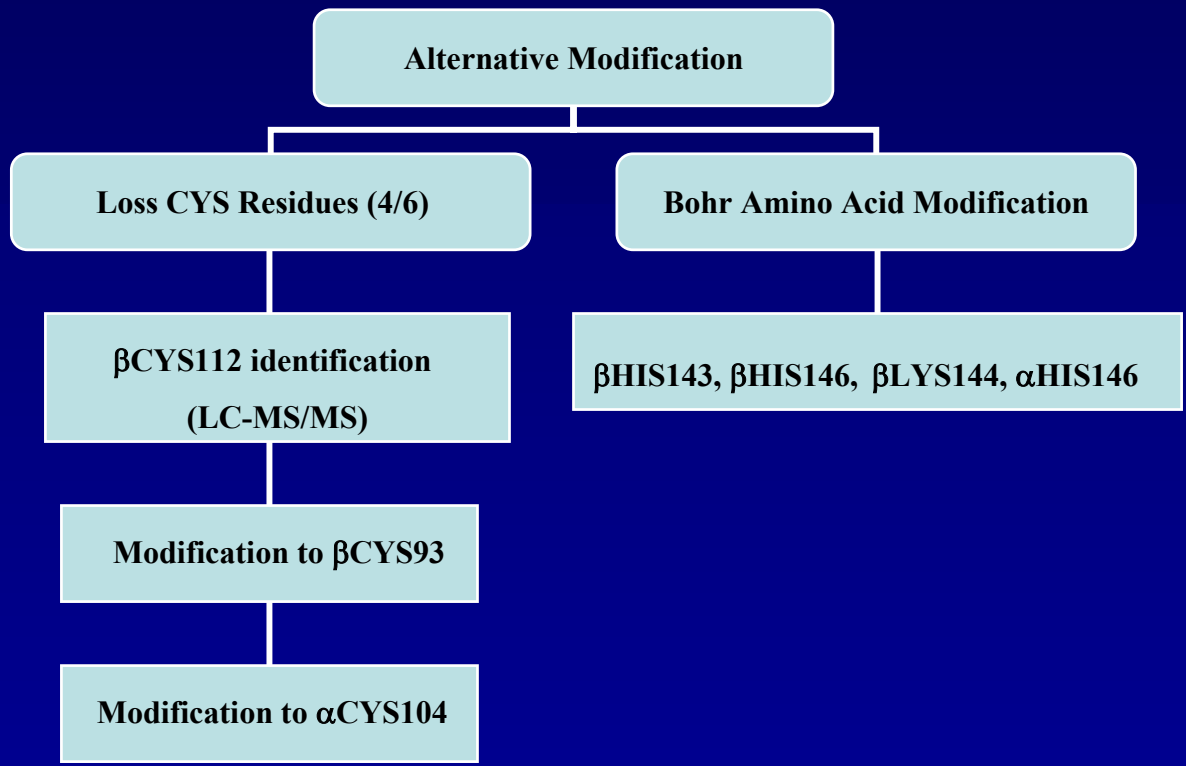
Amino acid	Theoretical HbA_0	Experimental HbA_0	Experimental *O*-R-PolyHbA_0
Lys	44	45.1 (103)	41.1 (93.4)
Cys	6	5.60[a] (93.0)	2.04[a] (34.0)
Tyr	12	12.6 (105)	11.3 (94.2)
His	38	38.3 (100)	34.4 (90.5)

[a] Represents recovery of cysteic acid following performic acid oxidation. Parentheses denote % recovery relative to theoretical values. Data represent the mean value of at least 10 separate analyses (SEM < 10%).

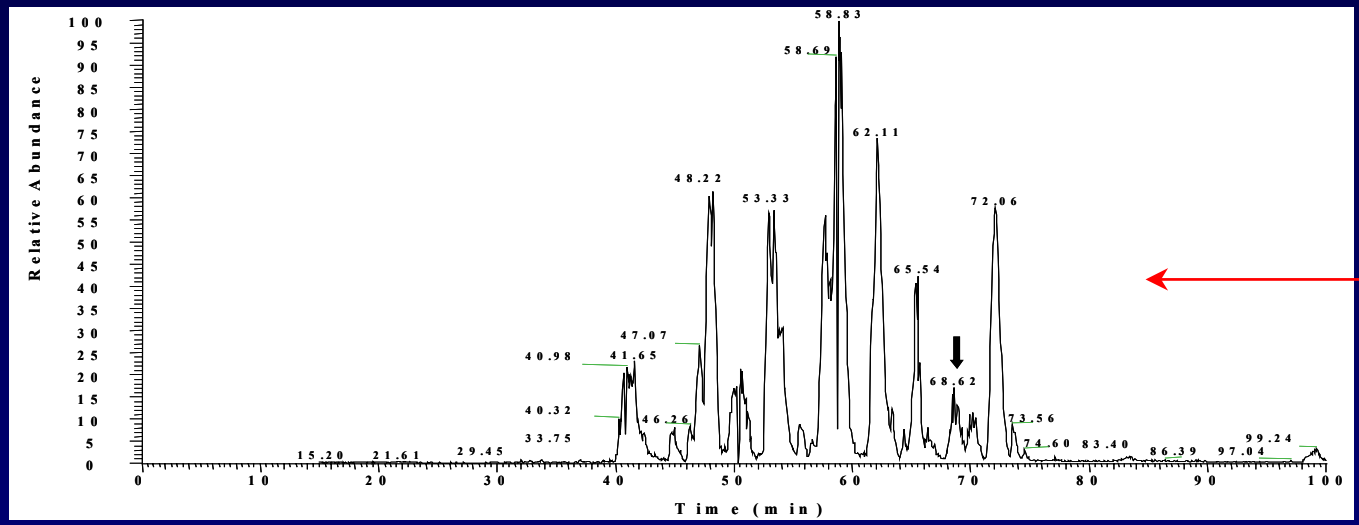
Brief Summary:

- **LYS, HIS and TYR are minimally modified with *O*-R-PolyHbA_0.**
- **No apparent or extensive inter-molecular cross-linking (polymerization) exists in *O*-R-PolyHbA_0.**
- **Extensive CYS modification was found confirming our previous data assessing thiol content.**

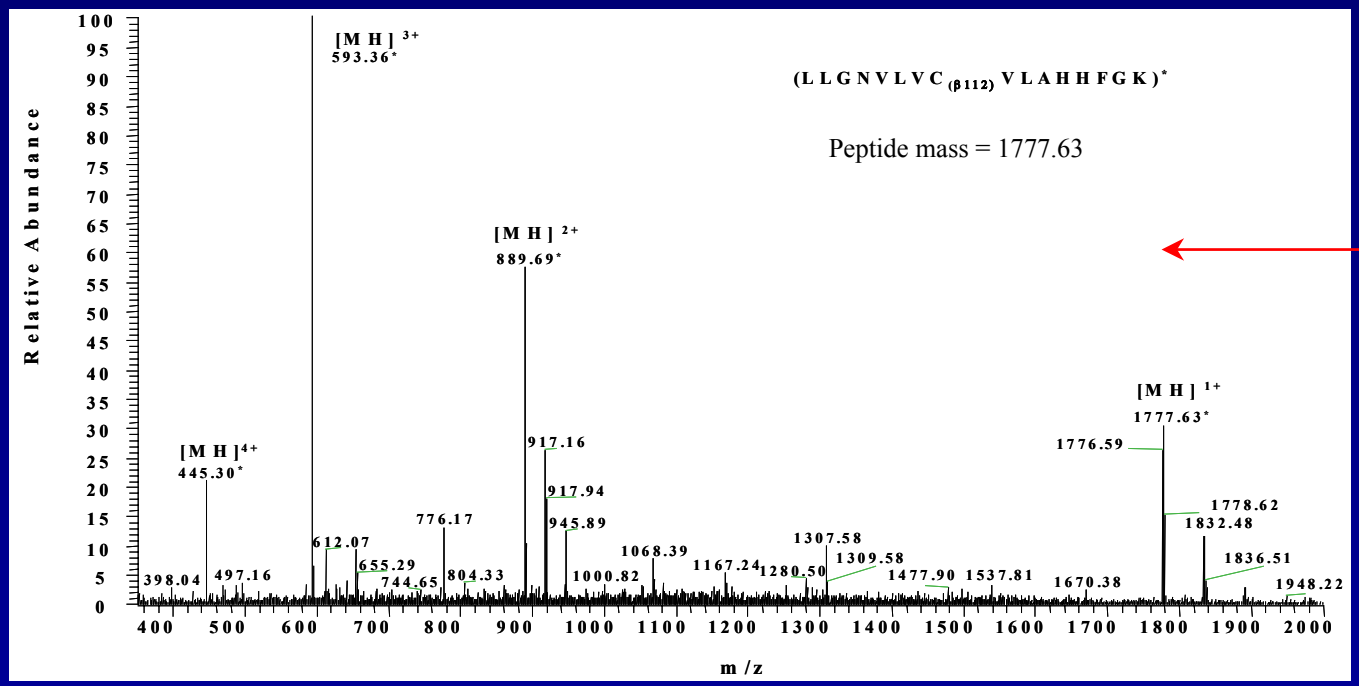
Structural Modification Analysis (Continued)



LC-MS/MS: Identification and Sequencing of the βCYS112 Peptide

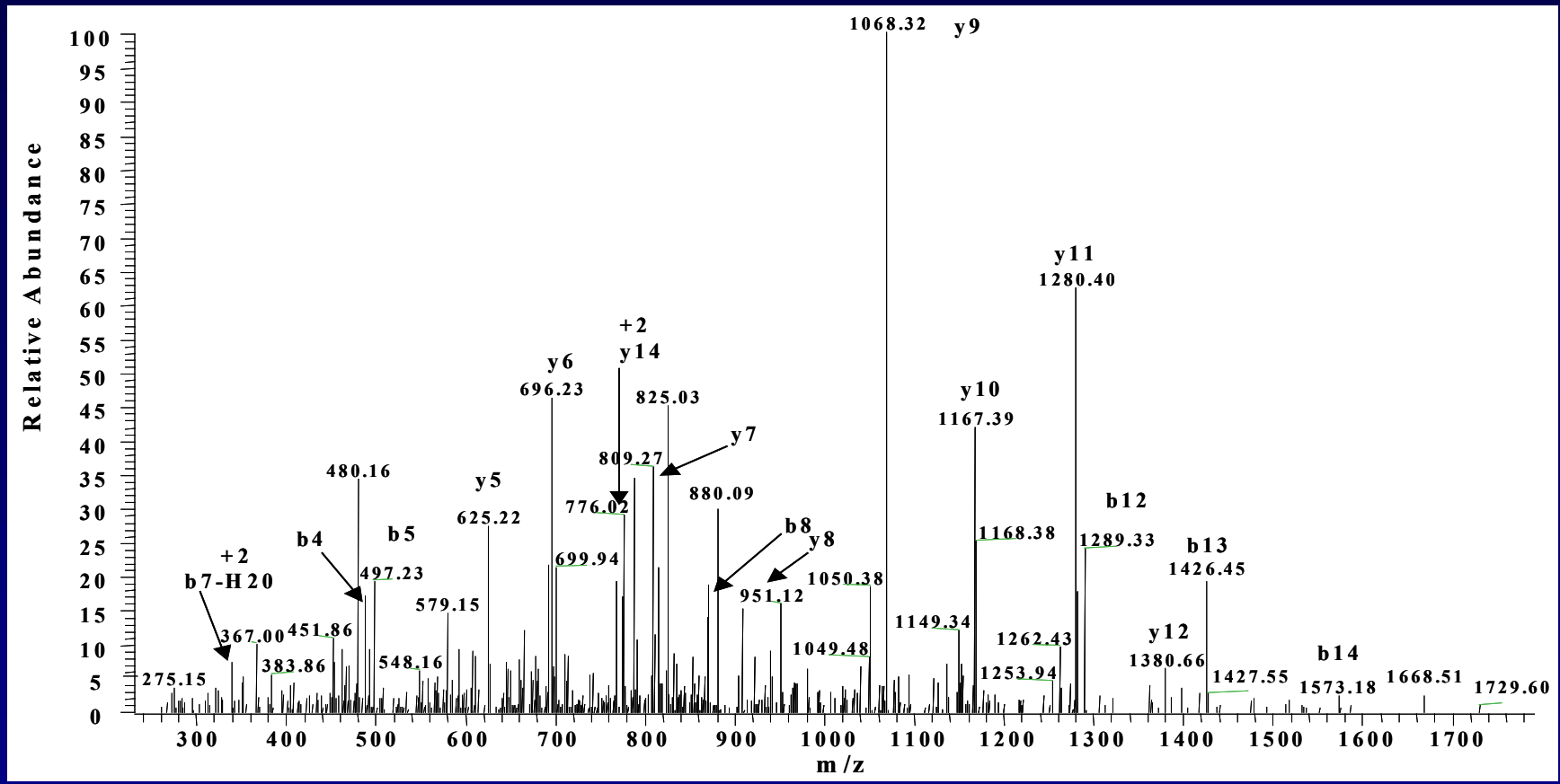


Total Ion Current (TIC)



Full Scan MS Spectrum of Peak at 68.62 in TIC

Collision Induced Dissociation (CID) of the βCYS112 Peptide



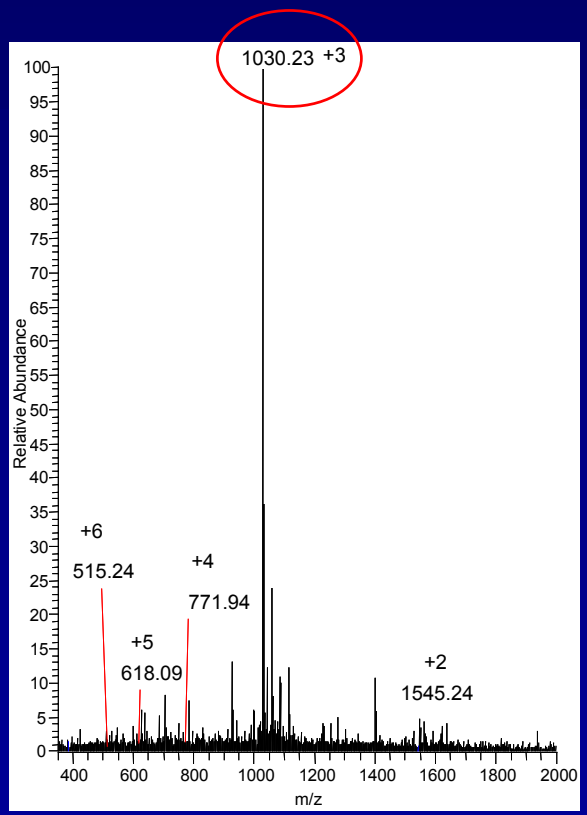
Collision induced mass ions from the tryptic peptide (β105-β120)

Sequence	Leu	Leu	Gly	Asn	Val	Leu	Val	Cys	Val	Leu	Ala	His	His	Phe	Gly	Lys
Mass	113.2	113.2	57	114.1	99.1	113.2	99.1	103.1	99.1	113.2	71.1	137.1	137.1	147.2	57.0	128
b				397.5	497.23		336.77[a]	812.63				1289.33	1426.45	157.18		
y			776.02[b]		1380.66	1280.40	1167.39	1068.39	951.12	809.27	696.23	625.22				

[a] $b7^{2+}$-H_2O
[b] $y14^{2+}$

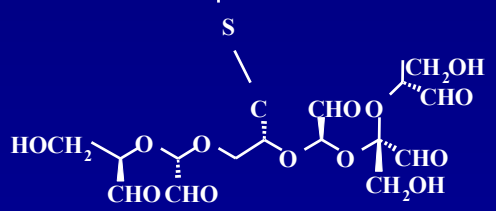
Modification to βCYS93

- The βCYS93 residue is contained in the non-cleavable β83-β104 (GTFATLSELHC$_{(β93)}$DKLHVDPENFR) peptide.
  - G$_{(β83)}$TFATLSELHC$_{(β93)}$DK$_{(β95)}$ = 1478.6 mass units
  - L$_{(β96)}$HVDPENFR$_{(β104)}$ = 1126.2 mass units
  - *O*-raffinose = 483 mass units
  - Total mass = 3087.8

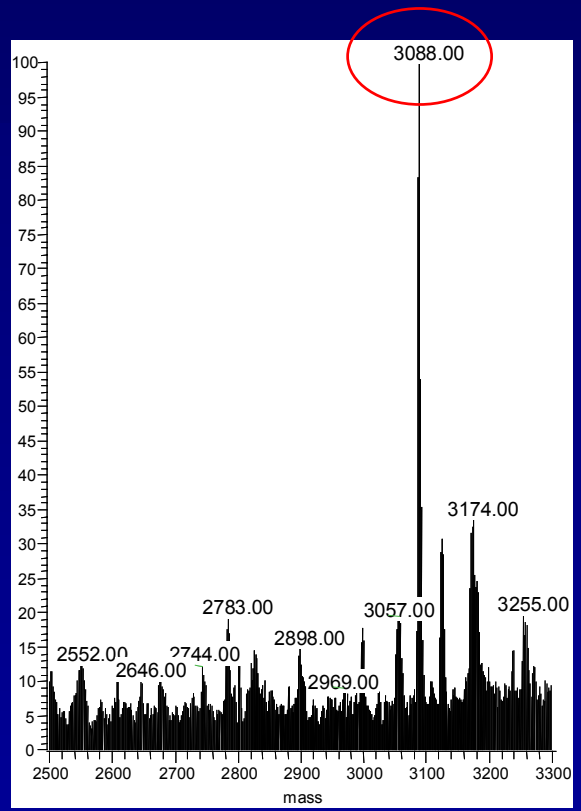


Deconvolution

GTFATLSELHC$_{(β93)}$DKLHVDPENFR

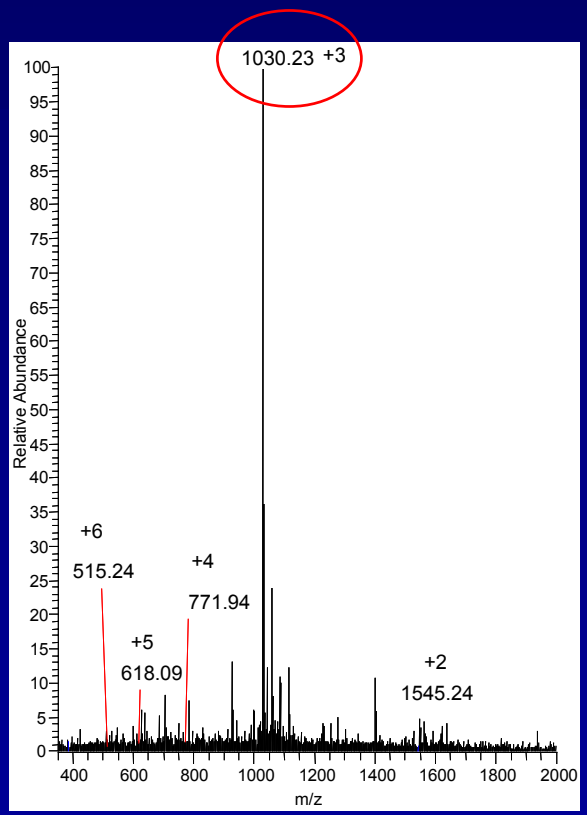


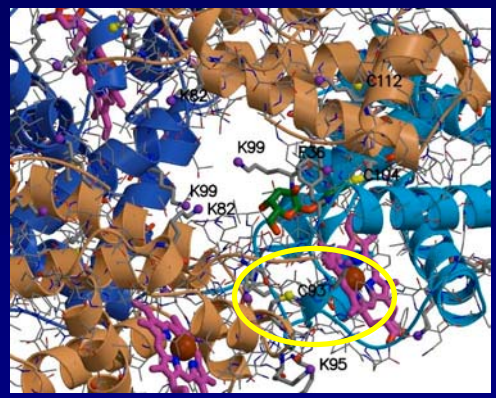
O - Raffinose



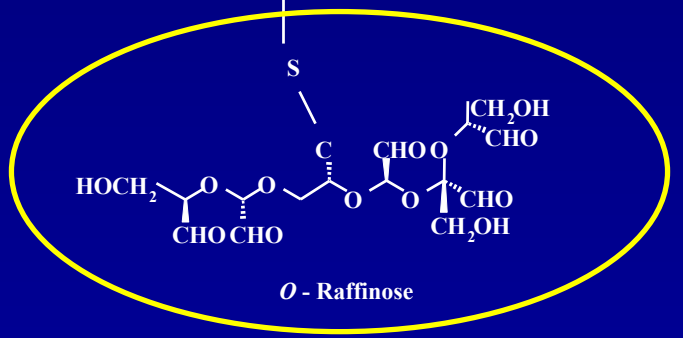
Identification of Chemical Modification Sites on Hemolink™ by Mass Spectrometry

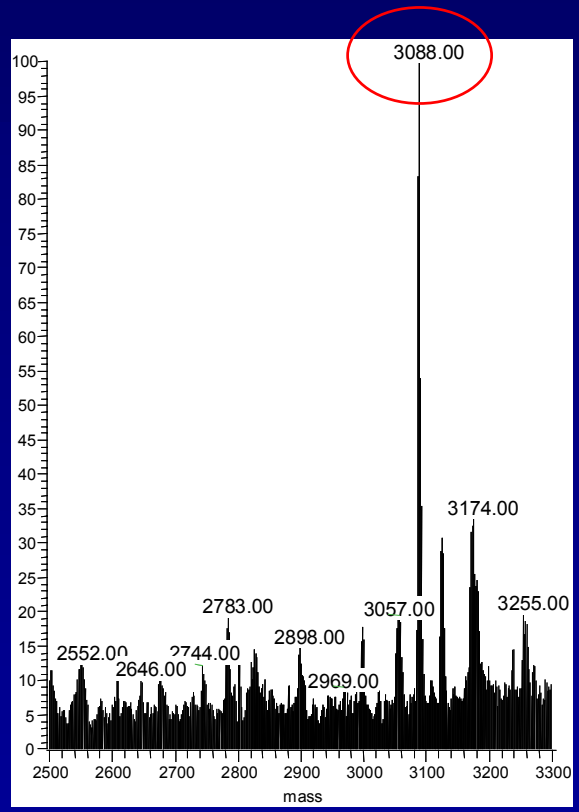
- Unmodified peptide mass = 2064.9 mass units
- *O*-raffinose = 483 mass units
- Total mass of *O*-raffinose modified peptide = **3087.9** (2064.9 + 483)





GTFATLSELHC$_{(\beta 93)}$DKLHVDPENFR





Modification to αCYS104

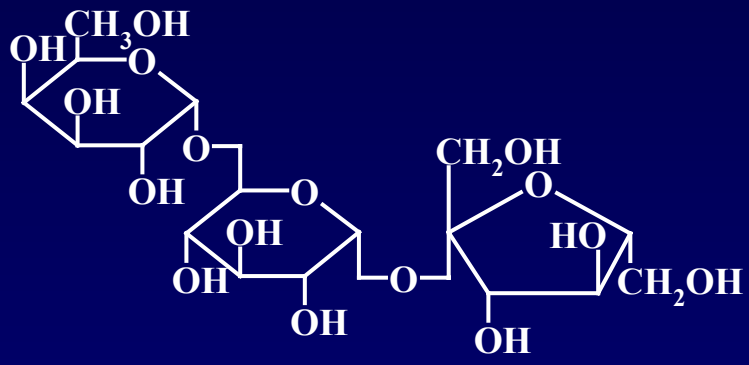
- **The αCYS104 residue is contained within the α100- α127 (LLSH$C_{(\alpha 104)}$LLVTLAAHLPAEFPAVHASLDK) peptide (Theoretical mass = 2968).**

- **Within the CNBr/tryptic digest mixture a $[MH]^{3+}$ ion *m/z* = 1059.07 was detected and several internal fragment ions consistent with the αCYS104 peptide were observed. Deconvolution gave a monoisotopic mass of 3177 suggesting an increase in the peptide mass by 227 mass units (3177 – 2968).**

- **This data suggests a modification to αCYS104 with a partially oxidized product (Smith degradation product) in the staring material known to occur during the oxidation sugars such as raffinose*.**

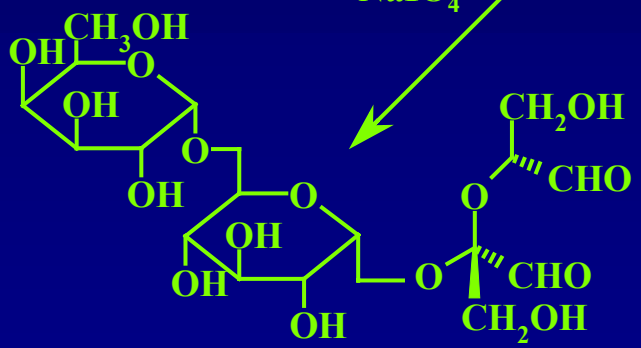
* Goldstein, I.J., Hay, G.W., Lewis, B.A., and **Smith, F**. Controlled degradation of polysaccharides by periodate oxidation, reduction, and hydrolysis. (1965) *Methods in Carbohydrate Chem.* **5**, 361-370.

Oxidation of Raffinose with $NaIO_4$



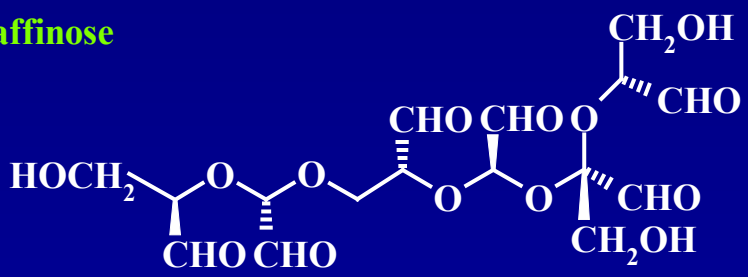
Raffinose

$NaIO_4$



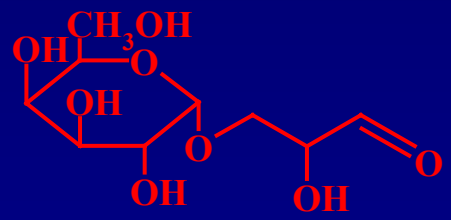
Partial *O* - Raffinose

$NaIO_4$



O - Raffinose

$NaIO_4$



Raffinose Degradation Product

Likely the Source of Modification to αCYS104

M.W. = 225 mass units

Hemoglobin Amino Acid Sequence

Alpha chain

VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPH

FDLSHGSAQVKGHGKKVADALTNAVAHVDDMPNALSALSDLHAHK

LRVDPVNFKLLSHCLLVTLAAHLPAEFTPAVHASLDKFLASVSTVLT

SKYR

104

Beta chain

VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQ

82

RFFESFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKGTFATLSELH

CDKLHVDPENFRLLGNVLVCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHK

93

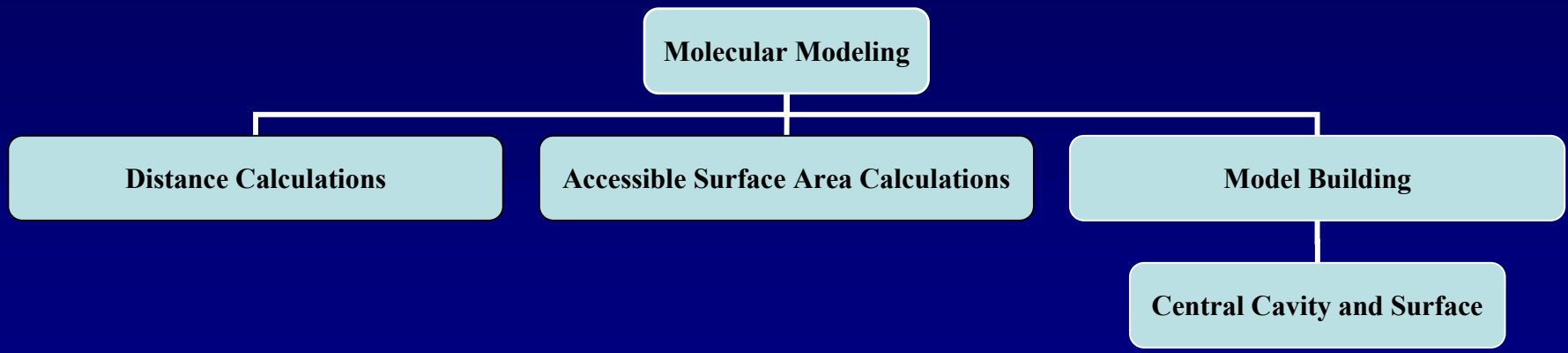
YH

Bohr Amino Acid Modification

βHIS143, βLYS144, βHIS146, and αHIS122:

- **Two of the four classic Bohr amino acids were readily identifiable by LC-MS/MS (βHIS143, βLYS144) in the β(133-144) tryptic peptide (VVAGVANALA$H_{(143)}K_{(144)}$).**
- **The βHIS146 residue is the C-terminal amino acid of the β chain and contained in the β(145-146) tryptic peptide (YH) and was not identifiable based on the size of the fragment.**
- **The αHIS122 residue is in the tryptic peptide also containing αCYS104. The mass of the peptide was increased by a modification consistent with a *O*-Raffinose degradation product and thus suggests no modification to αHIS122.**

Structural Modification Analysis (Continued)



Distances Between LYS Within and Between Globin Chains

Distance between Lysine residues within and between globin chains

Subunit 1	Residue 1	Subunit 2	Residue 2	Distance (Å)
α_1	Lys7	α_1	Lys11	7.44
α_1	Lys7	α_1	Lys127	9.95
α_1	Lys56	α_1	Lys60	10.8
α_1	Lys60	α_1	Lys61	10.5
α_2	Lys7	α_2	Lys11	6.50
α_2	Lys7	α_2	Lys127	10.1
α_2	Lys60	α_2	Lys61	9.02
α_2	Lys61	α_2	Lys90	12.0
β_1	Lys17	β_1	Lys120	10.5
β_1	Lys65	β_1	Lys66	8.90
β_1	Lys82	β_1	Lys144	9.77
β_2	Lys17	β_2	Lys120	10.2
β_2	Lys65	β_2	Lys66	10.7
β_2	Lys82	β_2	Lys144	11.2
$^*\alpha_1$	Lys40	$^*\beta_1$	Lys132	10.9
$^*\alpha_1$	Lys99	$^*\alpha_2$	Lys99	7.47
$^*\alpha_2$	Lys40	$^*\beta_2$	Lys132	10.5
$^*\beta_1$	Lys82	$^*\beta_2$	Lys82	9.33

Within Globin Chains

Between Globin Chains

* Between globin chains

Accessible Surface Area (ASA) of CYS Sulfhydryl groups

Cysteine sulfhydryl accessible surface area (ASA) in deoxyHbA$_0$

Intact Tetramer

Subunit	Residue	ASA
α1	Cys104	0.000
β1	Cys93	4.149
β1	Cys112	0.362
α2	Cys104	0.000
β2	Cys93	4.259
β2	Cys112	0.854

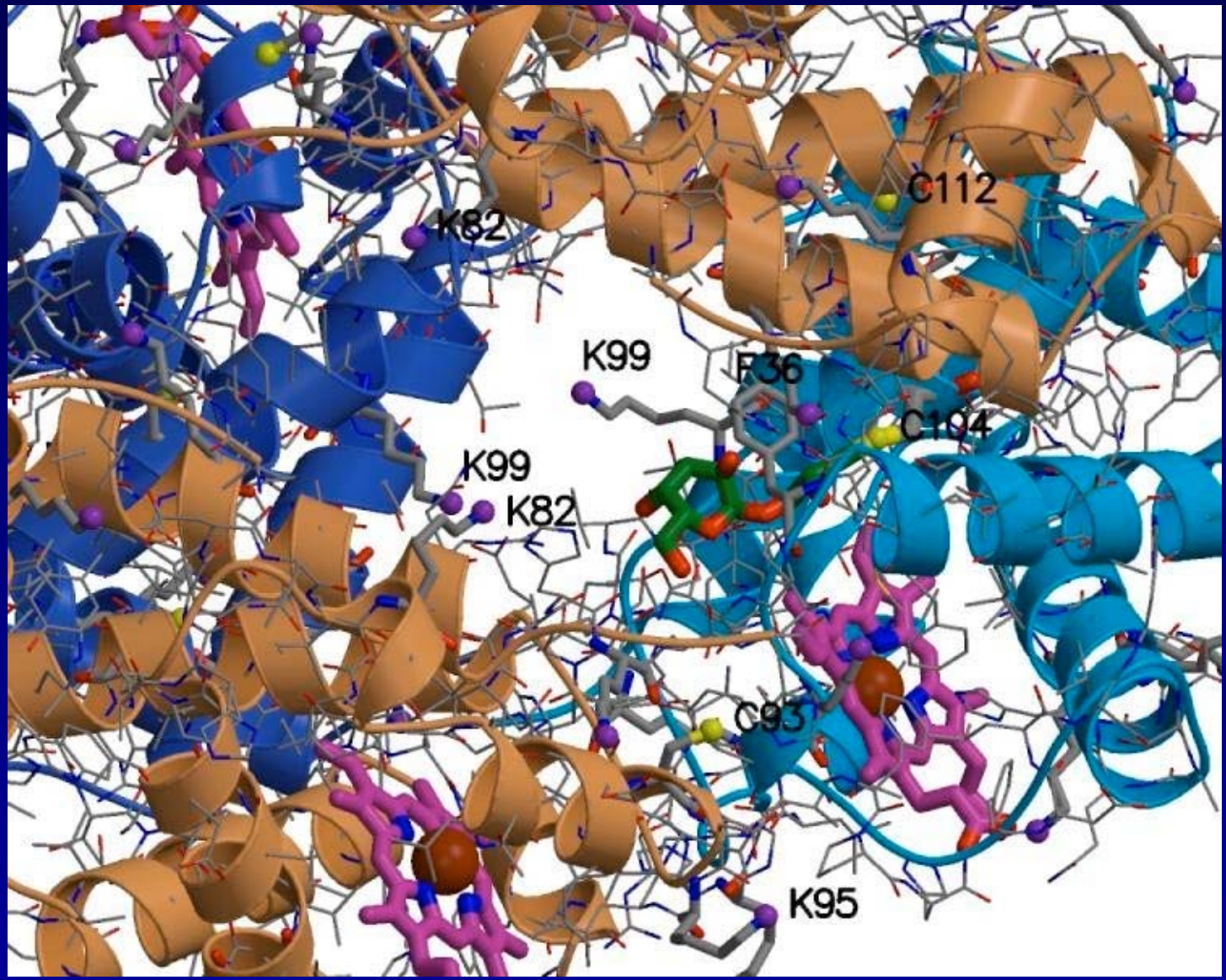
Separated Globin Chains

Subunit	Residue	ASA
α1	Cys104	0.9046
β1	Cys93	7.869
β1	Cys112	59.20
α2	Cys104	0.7478
β2	Cys93	8.266
β2	Cys112	60.53

Molecular Model of the Central Cavity

α2

β1

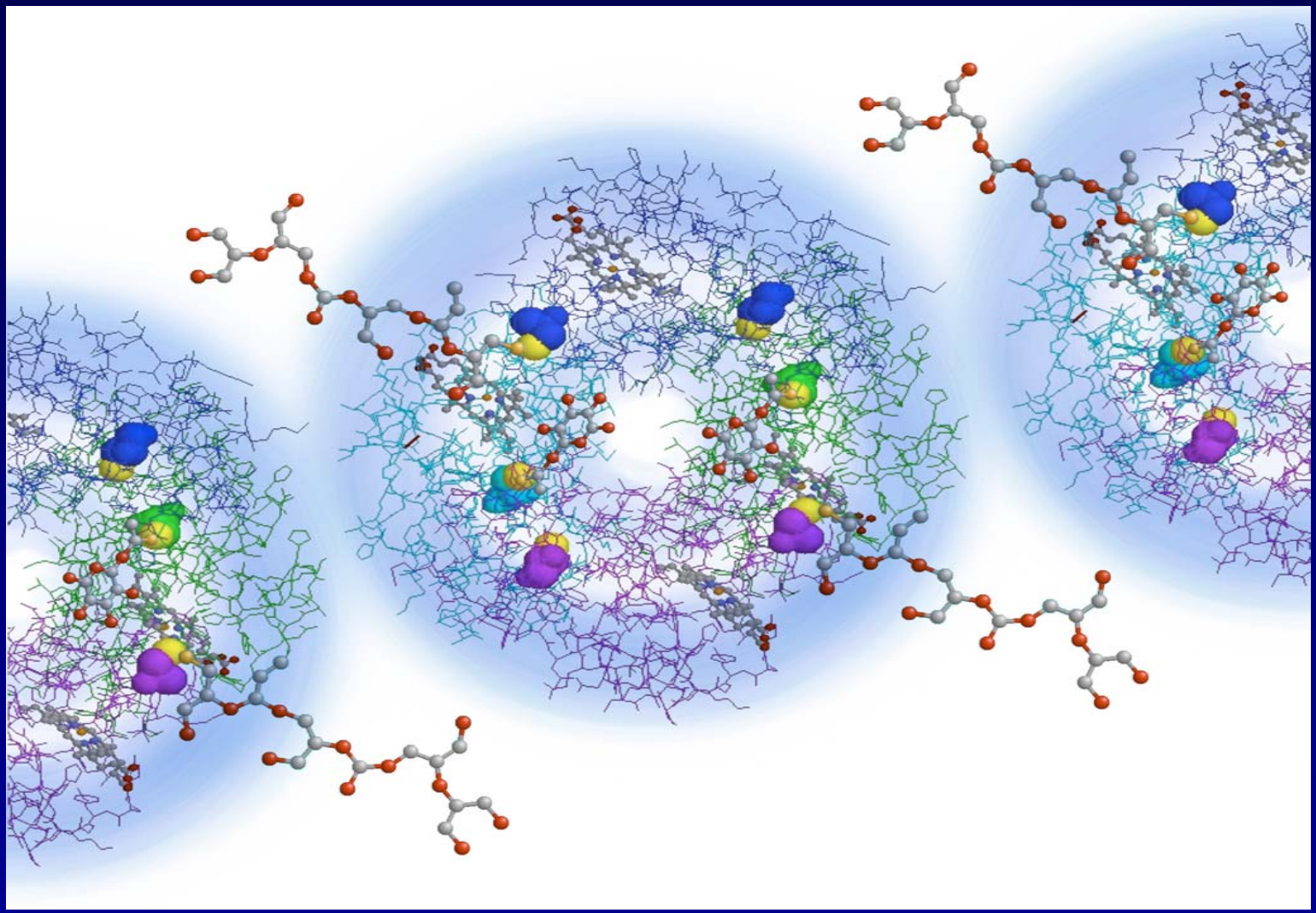


β2

α1

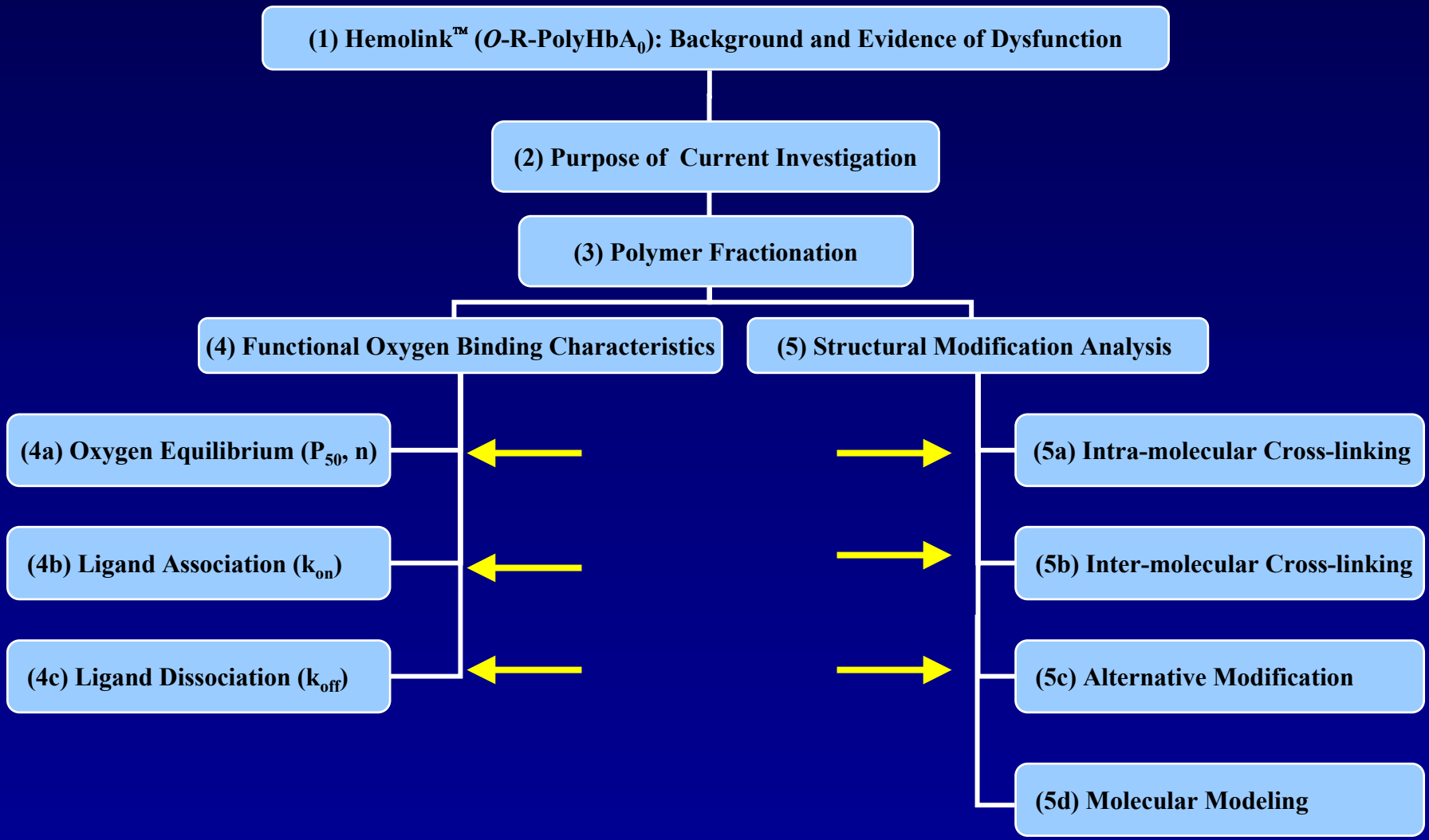
A critical question remains: **How can we reconcile the inability to detect covalent cross-linking (inter-molecular) sites of abundance when SEC data suggests a wide range of M.W. distribution (64 to > 600 kDa)?**

Hypothetical Model for Experimental Observations Suggesting the Absence of Inter-Molecular Cross-linking



oxyHb (R) $\xrightarrow[-\ 64\ H_2O]{N_2\ gas}$ DeoxyHb (T) $\xrightarrow[\text{Additional } H_2O \text{ loss (amount ?)}]{\text{O-raffinose}}$ Locked T-State

Summary of Experimental Observations



Summary of Claims vs. Experimental Findings

Sponsor's Claim	Our Findings
Complete oxidation of raffinose	Multiple oxidation products of raffinose, including degradation products
Intra-molecular cross-linking: β1Lys82, β1Val1, β2Lys82	Intra-molecular cross-linking: β1Lys82 and β2Lys82 not modified. No abundant reproducible sites of linking
Inter-molecular cross-linking: e-amino groups of surface lysines	Inter-molecular cross-linking: No significant modification to lysines, yet SEC data indicates increased M.W.
No Alternative sites of modification noted	Abundant modification to βCys93 and αCys104

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