Supporting Information

Melting of Hemoglobin in Native Solutions as measured by IMS-MS

Daniel W. Woodall,[a] Christopher J. Brown,[a] Shannon A. Raab,[a] Tarick J. El-Baba,[a] Arthur Laganowsky,[b] David H. Russell,[b] and David E. Clemmer[a,*]

[a]*Department of Chemistry, Indiana University, Bloomington, Indiana, 47405*

[b]*Department of Chemistry, Texas A & M University, College Station, TX 77843*

*Corresponding author:

David E. Clemmer

clemmer@indiana.edu

Table S1. SYNAPT G2 Instrument Settings:

Polarity	ES+
ESI Capillary (kV)	0.85
Source temperature (°C)	50 °C
Sampling cone	25
Extraction cone	2.0
Source gas	N/A
Desolvation temperature	N/A
Trap collision energy	6.0
Transfer collision energy	4.0
Trap gas flow (mL/min)	9.00
He gas flow (mL/min)	180.00
IMS gas flow (mL/min)	90.00
Detector	3000
Trap DC Entrance	2.1
Trap DC Bias	40.0
Trap DC	1.0
Trap DC Exit	2.0
IMS DC Entrance	25.0
Helium Cell DC	35.0
Helium Exit	-5.0
IMSBias	3.0
IMS DC Exit	0.0
Transfer DC Entrance	4.0
Transfer DC Exit	15.0
Source Wave Velocity (m/s)	100
Source Wave Height (V)	0
Trap Wave Velocity (m/s)	311
Trap Wave Height (V)	5.9
IMS Wave Velocity (m/s)	650
IMS Wave Height (V)	40.0
Transfer Wave Velocity (m/s)	191
Transfer Wave Height (V)	4.0
Pressures	
Backing	7.30e0
Source	5.12e-3
Sample Plate	1.00e-6
Trap	4.15e-2
Helium Cell	1.07e3
IMS	2.92e0
Transfer	4.72e-2
TOF	1.58e-6
IMS gas temperature (°C)	28

Far UV Circular Dichroism experiments

For comparison to the MS melting experiments, temperature dependent circular dichroism (CD) experiments were performed. CD experiments were performed using a Jasco J-715 spectrometer (Jasco Inc., Easton, MD). A 1 mm path length quartz cuvette was used to hold the sample. The sample preparation methods used in the CD experiments were identical to those used in the IMS-MS experiments (15 uM HbA in 150 mM ammonium acetate, pH 7.4).

Data Normalization

Mass spectral peaks of each protonated ion charge state were integrated and normalized to the sum of all HbA, and aggregate signals observed at each temperature. For the purposes of normalization, aggregate signals are defined as the area of all peaks in the *m*/*z* range from (*m*/*z* 500 – 1950). At low temperatures, the signal in this *m*/*z* range is near baseline (~1.5% of the total signal). At higher temperatures (≥55 °C), the signal intensity of the peaks corresponding to HbA species begins to decrease, and this region from *m*/*z* = 500 – 1950 becomes populated by numerous peaks, that are not readily identified by *m*/*z* as HbA monomers or dimers. We interpret these signals as is likely originating from various mixed oligomers of apo and holo monomers, possibly having varying numbers of oxidation modifications, and nonspecific metal adducts. While these signals do not constitute a bonafide identification of protein aggregates, we treat them as such for the sake of normalization purposes. The same normalization procedure was used in the analysis of drift time spectra, using the extracted drift time area of *m*/*z* 500 – 1950, rather than *m*/*z* peak area. Example mass spectra and normalized abundance curves of the aggregate species are shown below in Figure S1.

Bottom up LC-MS/MS Identification of Post-translational Modifications

LC-MS/MS sequencing of HbA post-translational modifications were performed as follows. A 2.0 mg sample of lyophilized hemoglobin was resuspended in 100 µℓ of 100 mM ammonium bicarbonate with 8 M urea. Free thiols were alkylated with 5 mM iodoacetamide in the dark for 45 minutes at room temperature. Protein solution was diluted in 100 mM ammonium bicarbonate to decrease the urea concentration to 1 M, and digested using sequencing grade trypsin (Promega, Madison, WI) in a 1:100 ratio of trypsin to substrate and incubated at 37 °C overnight. Tryptic peptides were desalted using C18 ZipTips (MilliporeSigma, Burlington, MA) and dried using a Centrivap concentrator (Labconco, Kansas City, MO). Peptides were resolubilized in 0.1% aqueous formic acid (buffer A) and loaded onto a reversed phase trap column (Acclaim PepMap 100, 75µm × 2 cm, nano viper, C18, 3 µm, 100 Å, ThermoFisher, Waltham, MA) by an easyNanoLC 1200 (ThermoFisher, Waltham, MA) at a flow rate of 5 µℓ/min for 10 µℓ. Peptides were separated on an analytical reversed phase C18 column (Acclaim PepMap RSLC, 75 µm × 25 cm, 2 µm, 100 Å, ThermoFisher, Waltham, MA) over a 24 min linear gradient from 7% to 40% buffer B (buffer A: 0.1% aqueous formic acid, buffer B: 80% acetonitrile, 20% H_2O, Fisher Scientific, Hanover, NH). Peptides eluting from the analytical column were electrosprayed into an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher, Waltham, MA). Precursor ions

(scan range: 400-2000 *m*/*z*) were monitored with a resolving power of 120,000 (@ 200 *m*/*z*). Precursor ions having intensity greater than 2.5×10^4, and charge states between z = 2 and z = 9 were selected for tandem mass analysis (MS/MS). Selected precursor ions were isolated in the quadrupole with an offset from the monoisotopic mass by 0.5 *m*/*z* with a window of 2 *m*/*z*. Peptides were subjected to both higher energy collision dissociation (30 ± 5%) and supplemental activation electron transfer dissociation (EThcD; calibrated charge dependent ETD parameters, SA HCD energy 20%) and fragment ion masses were measured with a resolving power of 50,000 (@ 200 *m*/*z*). The AGC target was set to 5.0×10^4 or a fill time of 86 ms. The MS method cycle time was set to 3.0 sec, and precursor ion dynamic exclusion window was 30 s.

Database searching was performed using Proteome Discoverer 2.1.1.2 (Thermo Fisher, Waltham, MA) from the SwissProt database (downloaded:January 2019). Precursor and fragment ion tolerances were set to 10 ppm and 0.02 Da, respectively. For peptide identification, two missed cleavages sites were allowed, as well as up to three variable modifications on each peptide. Variable peptide modifications searched included oxidation and dioxidation of methionine; carbamidomethylation, S-nitrosylation, oxidation, dioxidation, and trioxidation of cysteine. Additionally, glutamine conversion to pyro-glutamic acid at the peptide terminus, and N-terminal acetylation, methionine loss, and N-acetylmethionine loss were all included as variable modifications.

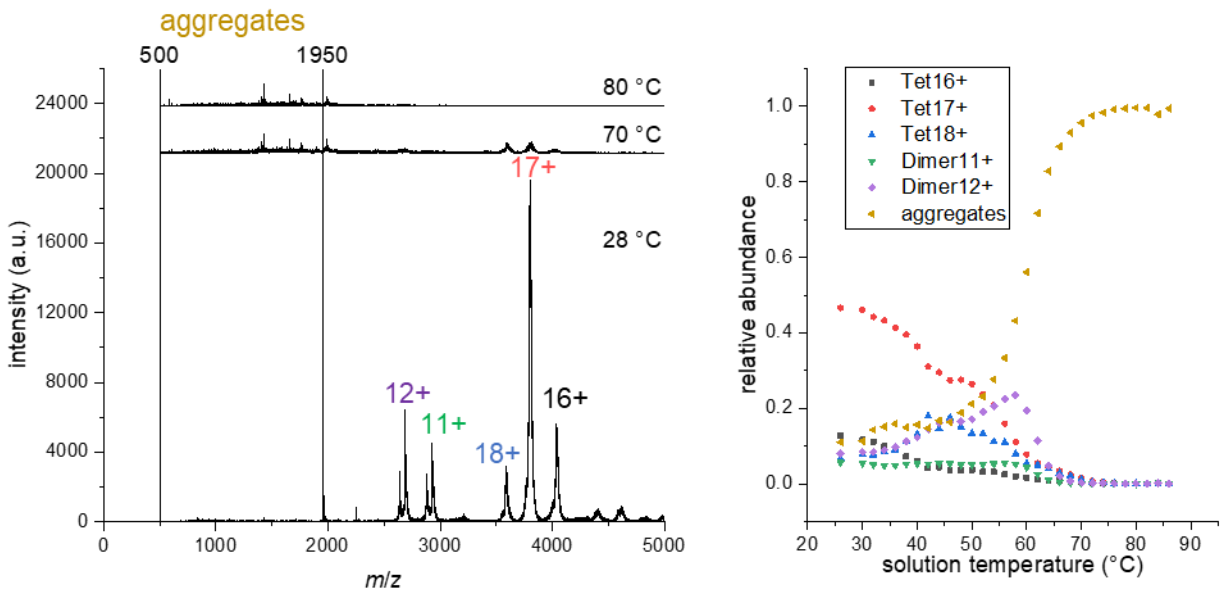

Figure S1. Representative mass spectra acquired at solution temperatures 28, 70, and 80 °C (left). The integrated *m*/*z* region treated as aggregates for data normalization is indicated by the two vertical lines (*m*/*z* = 500 – 1950). Relative abundance plot showing normalized abundances of HbA protein signals, and those assumed to be aggregates (right)

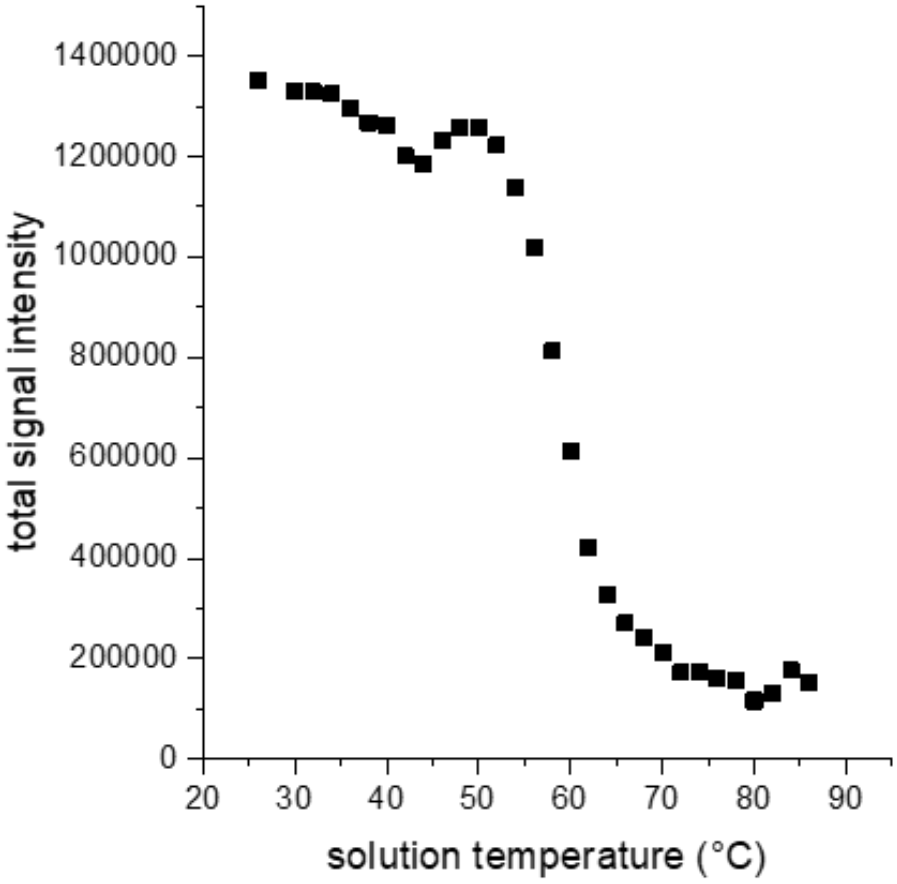

Figure S2. Plot of the total signal intensity for all mass spectral peaks as a function of solution temperature. At temperatures above ~55 °C, the signal intensity begins to decrease likely due to the formation of insoluble protein aggregates.

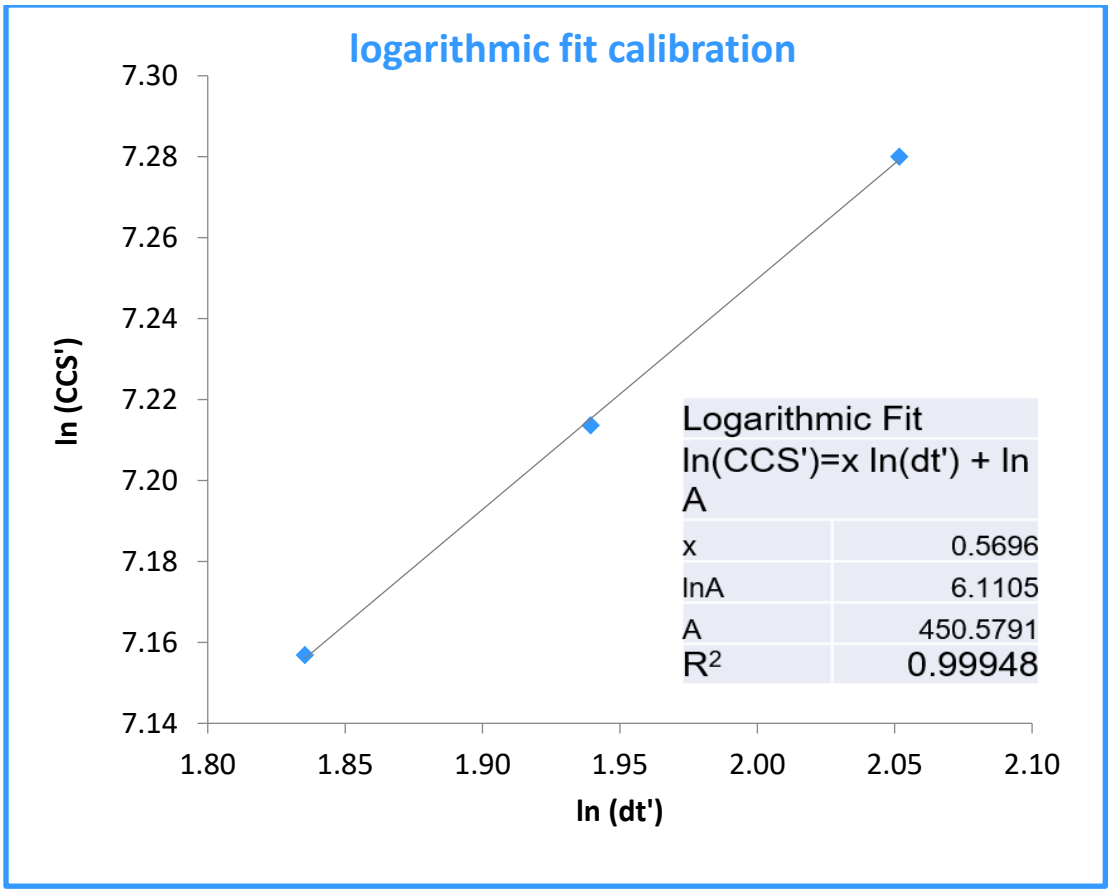

Figure S3. Travelling wave IMS calibration curve generated using 5 µM human transthyretin in 200 mM ammonium acetate as a calibrant. Wave height and wave velocity used were 40 V, and 650 m/s respectively.

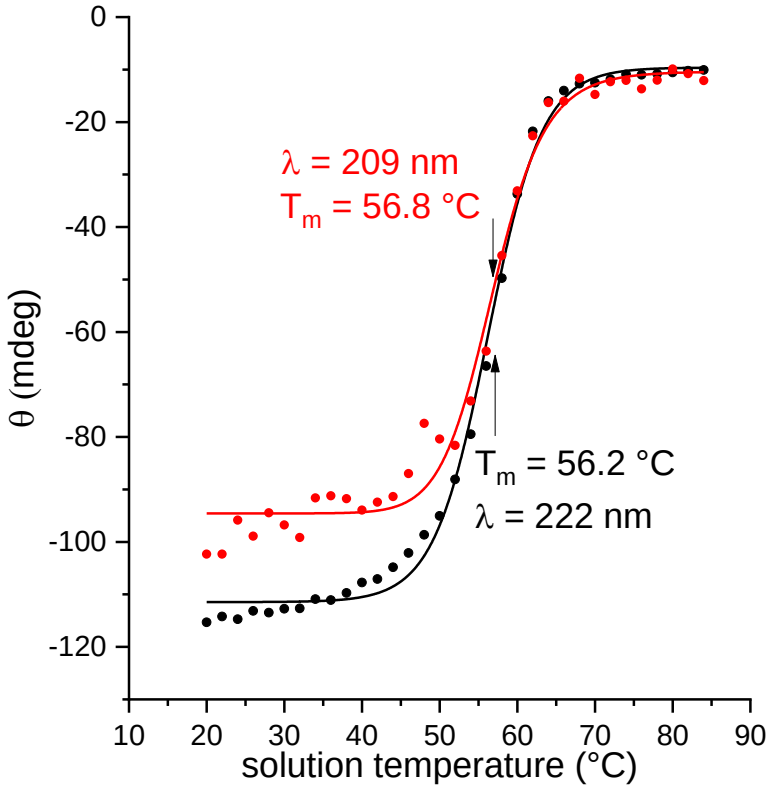

Figure S4. Circular dichroism spectroscopy melting curves of 15 µM HbA in 150 mM ammonium acetate solution (pH 7.4). Bands corresponding to alpha helical content - ellipticity (θ) at wavelengths 209 (red), and 222 (black) nm are plotted as a function of solution temperature.

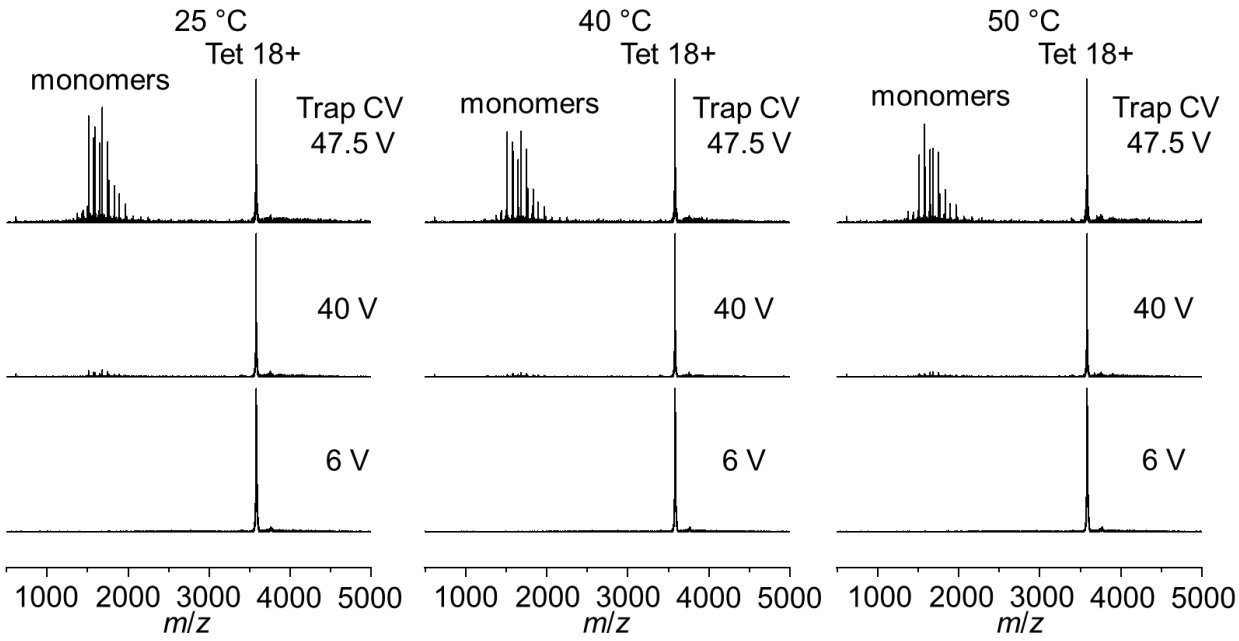

Figure S5. Comparison of CID mass spectra of the 18+ tetramer species from solution temperatures 25 °C, 40 °C and 50 °C. Collisional activation was performed by increasing the voltage (6, 40, and 47.5 V) in the trap collision cell. 40 V was observed to be the threshold voltage for dissociation of the tetramer into monomers. The threshold voltage for dissociation appears to be the same, or slightly lower at increased solution temperatures.

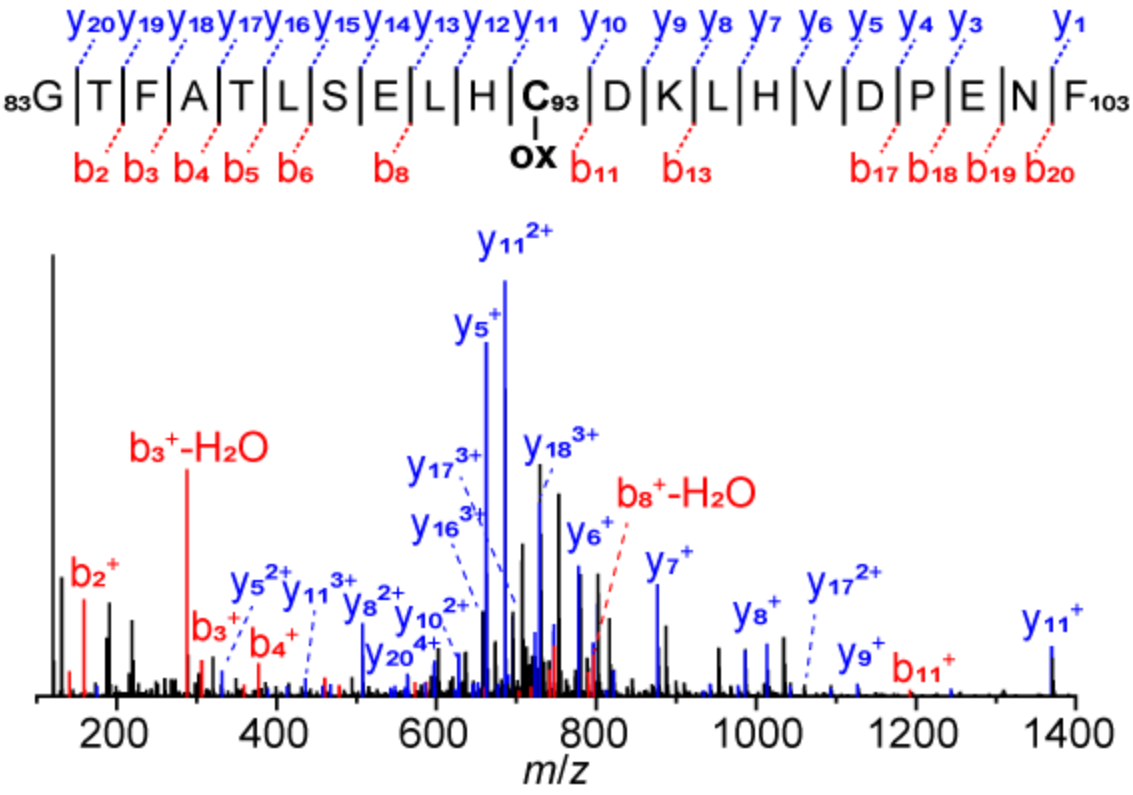

Figure S6. HCD MS/MS scan of the highest intensity tryptic peptide with a dioxidation modification. Modification site is identified as to Cys93 of the β-subunit. Sequence coverage and fragment ion assignments are indicated on the peptide sequence.

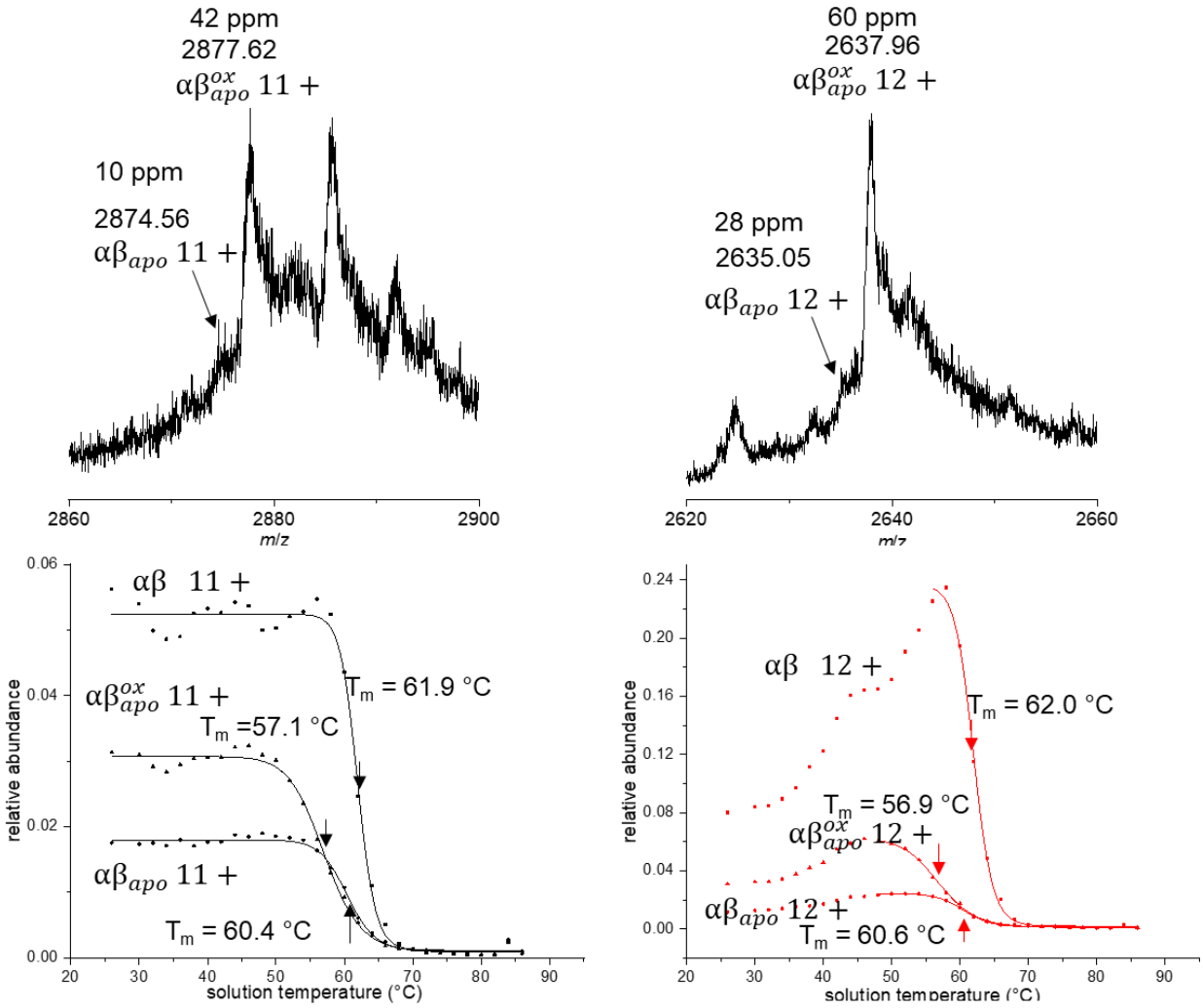

Figure S7. Zoomed mass spectra of the +11 charge state of the dimer species that is missing a heme, and containing an oxidation PTM ($\alpha\beta^{ox}_{apo}$) (top left). A low intensity peak corresponding to the dimer missing a heme without the oxidation modification ($\alpha\beta_{apo}$) is also detected at *m/z* 2874.56. The same species in the +12 charge state are shown in the top right panel. Mass error in ppm is displayed above the labeled peaks. Relative abundance curves of the $\alpha\beta^{ox}_{apo}$ and $\alpha\beta_{apo}$ species are compared to the normal αβ dimer species (bottom). T_m values are indicated on the melting curves by arrows.

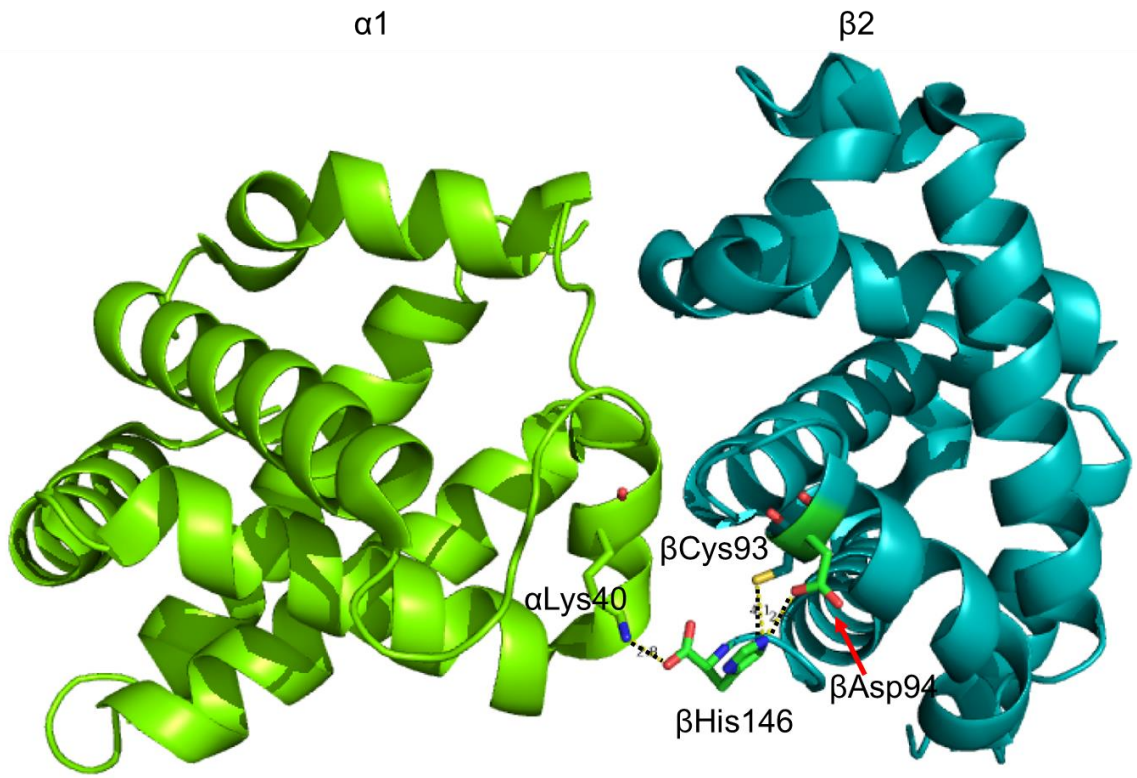

Figure S8. Cartoon representation of the α1β2 subunit interface indicating interface contacts, and the proximity of βCys93 to the residues involved in the binding interaction.