Ionic charge manipulation using solution and gas-phase chemistry to facilitate analysis of highly heterogeneous protein complexes in native mass spectrometry

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Abstract

Structural heterogeneity is a significant challenge complicating (and in some cases making impossible) electrospray ionization mass spectrometry (ESI MS) analysis of non-covalent complexes comprising structurally heterogeneous biopolymers. The broad mass distribution exhibited by such species inevitably gives rise to overlapping ionic signals representing different charge states, resulting in a continuum spectrum with no discernable features that can be used to assign ionic charges and calculate their masses. This problem can be circumvented by using limited charge reduction, which utilizes gas-phase chemistry to induce charge-transfer reactions within ionic populations selected within narrow *m/z* windows, thereby producing well-defined and readily interpretable charge ladders. However, the ionic signal in native MS typically populates high *m/z* regions of mass spectra, which frequently extend beyond the precursor ion isolation limits of most commercial mass spectrometers. While the ionic signal of single-chain proteins can be shifted to lower-*m/z* regions simply by switching to a denaturing solvent, this approach cannot be applied to non-covalent assemblies due to their inherent instability under denaturing conditions. An alternative approach explored in this work relies on adding supercharging reagents to protein solutions as a means of increasing the extent of multiple charging of non-covalent complexes in ESI MS without compromising their integrity. This shifts the ionic signal down the *m/z* scale to the region where ion selection and isolation can be readily accomplished, followed by limited charge reduction of the isolated ionic population. The feasibility of the new approach is demonstrated using non-covalent complexes formed by hemoglobin with structurally heterogeneous haptoglobin.

Introduction

Native mass spectrometry (MS) enjoyed a steady growth in popularity in the past two decades, and its ability to produce ionic signals representing intact biomolecular assemblies is now routinely used to address a wide range of problems in biochemistry, structural biology, biophysics and biotechnology.[1] However, applications of native MS remain limited in areas where non-covalent complexes are comprised of highly heterogeneous biopolymers, such as extensively glycosylated proteins, protein-polymer conjugates, and heparin-related products. Such species typically exhibit broad mass distribution, a feature that inevitably leads to extensive overlap of ionic signals corresponding to different charge states in electrospray ionization (ESI) MS. The resulting mass spectra frequently display a near-continuum signal with little-to-none discernable features to assign ionic charges and calculate the masses, making ESI MS impractical vis-à-vis intact mass measurements[2] even for single-chain biopolymers exhibiting a high degree of structural heterogeneity, not to mention their non-covalent assemblies. This problem can be ameliorated to some extent by using chromatography or electrophoresis as a front-end to ESI MS, with both denaturing LC modalities (such as reversed-phase LC[3] and HILIC[4]) and non-denaturing ones (size exclusion[5,6] and ion exchange[7]) enjoying increasing popularity in intact-mass analysis of proteins carrying multiple post-translational modifications (PTMs), as well as other biopolymers. However, extensive heterogeneity frequently involves variation of multiple structural characteristics (*e.g.*, in the case of proteins this includes a combination of enzymatic PTMs, such as glycosylation and sequence variants, and non-enzymatic ones, such as oxidation, glycation and deamidation; an additional layer of structural heterogeneity may involve the so-called “designer” PTMs, such as PEGylation or protein/drug conjugation). Therefore, the complexity reduction afforded by a specific front-end separation technique is frequently insufficient to enable meaningful MS analysis of biopolymers exhibiting extreme degrees of structural heterogeneity.[8]

This problem can be circumvented by manipulating the charge of biomolecular ions using the tools of gas-phase ion chemistry, such as charge transfer reactions.[9,10] In particular, limited charge reduction, a technique that utilizes polycation/electron or polycation/anion interactions to induce charge-transfer processes within ionic populations selected within narrow *m/z* windows, had been shown to generate well-defined and readily interpretable charge ladders.[11] This approach proved very useful in a range of demanding applications, enabling meaningful analyses of systems as complex as intact unfractionated heparin and its complexes with heparin-binding proteins.[12] However, ionic signal in native MS populates high *m/z* regions of mass spectra,[13] which may be problematic vis-à-vis precursor ion selection for charge transfer reactions. While few types of mass analyzers allow the precursor ion selection to be carried

out at *m/z* values exceeding 10,000 (enabling limited charge reduction analyses on 0.5 MDa macromolecular complexes[14]), most instruments currently used for native MS rely on quadrupole mass filters or linear quadrupole ion traps as a means of precursor ion selection in tandem MS measurements,[15] which typically provide much less generous *m/z* limits for ion isolation.

The ionic signal of single-chain proteins can be shifted to lower-*m/z* regions simply by switching to a denaturing solvent;[13] however, this approach cannot be applied to non-covalent assemblies due to their inherent instability under denaturing conditions. In this work we explore an alternative approach to increasing the charge density of protein ions, which uses supercharging,[16] a process that relies on solvent modifiers as a means of changing the physical properties of the ESI-generated charged droplets to significantly shift the extent of multiple charging of the protein ions. While certain supercharging agents can cause the proteins to lose their higher order structure in solution,[17] there is considerable evidence that it is possible to achieve the supercharging effect in ESI MS without triggering protein denaturation.[18-20] Therefore, a judicious use of supercharging reagents may result in a dramatic shift of the ionic charge state distribution to populate the low *m/z* region of the mass spectrum (where ion selection can be readily made) without compromising the integrity of non-covalent complexes. The subsequent application of limited charge reduction to such "supercharged" ionic populations selected within narrow *m/z* windows should allow their charges and masses to be readily calculated based on the resulting charge ladders.

The feasibility of the new approach is tested in this work using complexes formed by hemoglobin (Hb) with haptoglobin (Hp), an acute phase plasma glycoprotein, which captures free Hb in circulation during intravascular hemolysis and transports it to macrophages.[21] In addition to being an important anti-oxidant, Hp may also act as a modulator of inflammation,[22] a property that still awaits careful exploration vis-à-vis its relevance to inflammation accompanying severe COVID-19. In fact, significant elevation of Hp levels was observed in response to respiratory coronavirus infections in both animal models[23] and humans,[24] where it was suggested to act as an important factor in lung tissue repair following the inflammatory (neutrophil-mediated) damage.[24] Hp is a multi-meric protein incorporating two types of polypeptide chains, non-glycosylated light chains (L) and extensively glycosylated heavy chains (H), which are cross-linked by disulfide bonds.[25] In humans, Hp is expressed in two allelic forms, HP1 and HP2, which differ by size of the light chain.[26] The larger light chain (which we will term L*) incorporates three additional cysteine residues, two of which participate in formation of an internal disulfide bond (see ***Supplementary Material*** for more detail) while the third leads to formation of external disulfide bonds and, as a result, extensive multimerization of the protein. Out of three major phenotypes (termed Hp 1-1, Hp 2-1, and Hp

2-2), only Hp 1-1 lacks the L* unit, leading to formation of a well-defined tetrameric Hp (with the linear connectivity H-L-L-H).[21] Hp 2-1 contains both L and L*-type light chains, giving rise to polymeric chains of HL* subunits (connected via L*-chains) terminated with HL units at both ends of the $(HL^*)_n$ oligomer (see ***Supplementary Material*** for more detail). The absence of the L-type light chain (phenotype Hp 2-2) means that the HL polymerization can be terminated only via cyclization, which gives rise to circular $(HL^*)_n$ oligomers.

Each H-chain of a Hp molecule can bind a single Hb dimer α*β*,[27] although accommodation of a single globin chain is also possible.[28] Therefore, even the simplest Hp/Hb complexes (formed by Hp 1-1) exhibit heterogeneity at multiple levels (*e.g.*, due to the extensive glycosylation – four N-linked glycans per single H-chain – and due to the varying numbers of accommodated globin ligands). Variation in the size of the $(HL^*)_n$ oligomers present in both Hp 2-1 and 2-2 phenotypes introduces an additional layer of structural heterogeneity, which makes their characterization particularly difficult. Not surprisingly, the high level of heterogeneity exhibited by Hp 2-1 and 2-2 prevented their characterization by means of X-ray crystallography, with stain electron microscopy[25] and mass spectrometry[29-31] being the only biophysical techniques capable of producing structural information on mixed-type Hp and their complexes with Hb. Native MS in particular shows a great promise vis-à-vis characterization of such complexes, but the wide mass distributions exhibited by Hp oligomers (particularly, the species containing multiple H-chains) give rise to near-continuum ionic signals extending to *m/z* region well above 5,000.[30,31] In this work we take advantage of these properties of Hp to test the feasibility of the new approach combining supercharging in solution and limited charge reduction in the gas phase to obtain meaningful information on the masses of large non-covalent complexes formed by highly heterogeneous biopolymers. We demonstrate that a dramatic increase of the extent of multiple charging (sufficient to enable ion selection with commonly used quadrupoles) can be achieved using *m*-nitrobenzyl alcohol (*m*NBA) without compromising the integrity of the non-covalent complexes. Subsequent application of limited charge reduction to the *m/z*-selected populations of supercharged ions produces well-resolved charge ladders, from which masses of Hp/Hb complexes containing up to four H-chains can be readily determined.

Experimental

Materials. Human serum transferrin (*h*Tf), Hb and *m*NBA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Human Hp (both 1-1 and 2-1 phenotypes) were purchased from Athens Research & Technologies, Inc. (Athens, GA). All buffers, solvents and salts were of analytical grade or higher. All protein samples were reconstituted in 150 mM ammonium acetate (pH 6.9) to a final concentration of 1-5 μM prior to MS analyses.

Methods. All MS measurements were carried out with a Synapt G2Si (Waters, Milford, MA) hybrid quadrupole/time-of-flight mass spectrometer equipped with a nanospray ion source and an ion mobility analyzer. The following set of parameters was used in order to minimize collisional activation of ions in the ESI interface region and preserve the non-covalent complexes: capillary voltage, 1.5 kV; sampling cone voltage, 100 V; source offset, 80 V. Isolation of ionic populations for limited charge reduction in the trap cell was performed by setting the quadrupole LM resolution in the range of 4.3 - 4.7. Limited charge reduction of polycationic ions was triggered by introducing 1,3-dicyanobenzene anions after setting the trap wave height to 0.8-1.5 V and the discharge current as 8-20 μA. MS data were analyzed by using Mass Lynx 4.1 (Waters, Milford, MA).

Results and discussion

The new approach to ionic charge manipulation has been initially tested using human serum transferrin (*h*Tf), a protein that retains a compact conformation in solution at both neutral and mildly acidic pH.[32] Indeed a mass spectrum of *h*Tf acquired under near-native conditions exhibits a narrow distribution of the ionic signal at *m/z* > 3,500 with the charge states ranging from +19 to +22 (see ***Supporting Information*** for more detail). As expected, addition of a supercharging reagent to *h*Tf solution (6% *m*NBA) results in a dramatic shift of the ionic signal to the lower *m/z* region, with the charge states as high as +35 becoming visible in the mass spectrum. Selection of a narrow ionic population representing a subset of these super-charged *h*Tf species (charge state +29) followed by their brief exposure to 1,3-dicyanobenzene anions in the gas phase gives rise to a charge ladder extending all the way to a charge state +21 (see ***Supporting Information***). The protein mass calculated on the basis of the charge ladder produced by the limited charge reduction is 79,649 Da, a number that is in agreement with the mass calculated based on the original mass spectrum acquired prior to adding the supercharging reagent to the protein solution (79,651 Da). However, *h*Tf is a single-polypeptide chain protein, and even if its unfolding were to occur in solution in the presence of the supercharging reagent, it would not affect the mass measurements. In order to determine the feasibility of using the new approach to determine the masses of non-covalent complexes, a similar procedure has been applied to a Hp/Hb mixture.

A mass spectrum of Hp 1-1 incubated with a slight molar excess of Hb is dominated by the ionic signal of the 1:1 Hb/Hp complex (Figure 1A), consistent with the notion of each single H-chain of Hp accommodating an α*β* dimer,[27] the so-called semi-hemoglobin. The mass assignment for the major ionic species in this spectrum was verified by carrying out limited charge reduction on a population of ions selected within a 6,340-6,390 *m/z* window; the resulting charge ladder yields the mass value of 159±3.2 kDa, consistent with the (α*β*)HLLH(α*β*) composition of the complex. In addition to the Hb/Hp complex, the mass

spectrum reveals the presence of ionic species representative of free Hb in solution. These are Hb tetramers $(\alpha^*\beta^*)_2$, semi-hemoglobin dimers α*β* and single globin chains (Figure 1A); the presence of the latter species reflects transient dissociation of Hb tetramers in solution when the protein concentration is low.[33,34] No evidence of gas phase dissociation of Hb tetramers can be seen in the mass spectrum, as the ionic species produced in the course of such a process (*e.g.*, the Hb trimers[35]) are clearly absent. Likewise, the absence of ions representing free Hp molecules indicates that the Hp/Hb complexes remain stable in the gas phase.

Addition of 0.7% *m*NBA to the Hp/Hb solution resulted in a dramatic shift of the appearance of the mass spectrum (Figure 1B). The average charge state of ions representing semi-hemoglobin dimers increased from +10 to +13/+14, and the average charge state of Hb tetramers increased from +15/+16 to +18/+19. The mass spectrum is dominated by a poorly-resolved ionic signal distributed over *m/z* range 4,500-6,000; processing this signal using limited charge reduction (an example is shown as a red trace in Figure 1B) yields the mass of 160±2.9 kDa, confirming that these ions represent intact complexes formed by association of two Hb dimers with a single Hp molecule. Therefore, the mass spectrum contains ionic signals of all species that were detected in solution prior to addition of the supercharging reagent; no ionic signal corresponding to either free Hp ions or Hp associated with a single semi-Hb dimer were observed. The presence of the supercharging reagent in solution has not triggered dissociation of the non-covalent Hb/Hp complexes; furthermore, our attempts to increase the extent of multiple charging of the Hb/Hp complexes by c of the ionic signal to the lower *m/z* region of the mass spectrum. Indeed, the most abundant charge state of the Hb/Hp complex ions remained +33, and the highest charge state detected was +35 when the amount of *m*NBA was elevated to 1% (by volume). Further increase of the *m*NBA content of the protein solution has led to a noticeable deterioration of the ionic signal, at which point the Hb/Hp complex remained intact. Nevertheless, the extent of supercharging that can be achieved without compromising the integrity of the non-covalent complexes and significantly reducing the spectral signal-to-noise ratio is sufficient to shift the ionic signal of Hb/Hp complexes down the *m/z* scale by more than 1,500 *m/z* units.

The Hb/Hp 2-1 complex presents a significantly more challenging case vis-à-vis native ESI MS measurements, as in addition to the well-resolved ions representing free Hb and the (α*β*)·HLLH·(α*β*) complex, an abundant ionic signal populates the high *m/z* region of the mass spectrum (above 7000, see Figure 2A). The shape of this convoluted distribution suggests the presence of at least two additional distinct species, but only one of them generates ionic signal that falls within the *m/z* range where isolation of precursor ions for subsequent limited charge reduction is possible. The well-defined charge ladder produced

upon exposure of the ionic population selected within the 7600-7650 *m/z* window (the blue trace in Figure 2A) allows the mass of this species to be determined as 236±3.6 kDa, which corresponds to an HL(L*H)LH isoform of Hp accommodating three semi-Hb dimers α*β*. However, the rest of the ionic signal extends beyond the upper *m/z* limit (8,000 *m/z*) for precursor ion isolation, preventing the identification of this species using limited charge reduction directly. The latter likely represents Hb-bound $HL(L^*H)_2LH$, but the stoichiometry of Hb binding cannot be established (all attempts to deconvolute the poorly resolved peaks in this region had failed).

In order to enable application of limited charge reduction to the Hp/Hb complex giving rise to the ionic signal above *m/z* 8,000, the protein solution was modified with 0.7% *m*NBA. The addition of the supercharging reagent resulted in a significant shift of the ionic signal to lower *m/z* values (Figure 2B). The increase in the extent of multiple charging for all ions resulted in a notable crowding of the mass spectrum: no distinct ion peaks could be observed for the ionic signal representing the Hb/Hp complexes. However, the three abundant spectral features could be discerned above *m/z* 4,500, suggesting that ions representing different Hb-bound isoforms of Hp ($HL(L^*H)_nLH$) were still confined to distinct regions of the mass spectrum (*m/z* 4,500-6,000, 6,000-7,500 and 7,500-9,000). Indeed, limited charge reduction of ions within the first range (*m/z* 4,500-6,000) yields the mass of 154±2.5 kDa, corresponding to the simplest isoform of Hp (HLLH) accommodating two semi-Hb dimers (these ions were confined to the *m/z* region 5,500-7,000 prior to supercharging, see Figure 2B). Likewise, limited charge reduction of supercharged ions confined to the second distinct region of the mass spectrum shown in Figure 2B (*m/z* 6,000-7,500) yields a mass of 241±3.9 kDa, corresponding to the HL(L*H)LH isoform of Hp accommodating three semi-Hb dimers α*β* (the species whose ionic signal was localized within *m/z* region 7,000-8,500 prior to supercharging).

Most importantly, supercharging succeeded in shifting the third distinct spectral feature (that was previously postulated to represent the Hb-bound isoform $HL(L^*H)_2LH$, but remained out of reach of the limited charge reduction analysis) below the precursor ion isolation threshold. This allowed ions within the *m/z* window centered at the apex of this spectral feature to be isolated and subjected to limited charge reduction, yielding a well-defined charge ladder (the purple trace in Figure 2B), from which the ionic mass was calculated. This measured value (336±4.3 kDa) indeed corresponds to the $HL(L^*H)_2LH$ isoform accommodating four dimeric Hb subunits (α*β*). Therefore, despite the noticeable decrease in the spectral quality (as judged by the closer spacing between ionic peaks representing different charge states), supercharging provides a clear advantage of being able to “move” the ionic signal of larger complexes to the spectral regions below the threshold for ion isolation, thereby enabling limited charge reduction analysis of species that were out of reach of this technique in

"common" native MS. Remarkably, such shifts of the ionic signal across the *m/z* scale can be accomplished without compromising the integrity of large non-covalent complexes.

In this proof-of-concept work we used one of the most common supercharging reagents (*m*NBA), but there are a variety of reagents (small organic and inorganic compounds) that can induce this effect while preserving the non-covalent complexes existing in solution[36] and, therefore, would be good candidates for further enhancing the experimental strategy described in this work. Particularly promising in this respect is a recent report of inducing the supercharging effect in ESI MS using divalent metals:[37] we anticipate that using such cationizing agents would be a particularly gentle method of producing high charge-density ions without compromising the integrity of non-covalent assemblies. Another attractive option for further refining the methodology presented in this report is using elevated pressure in the ESI source, which was shown recently to produce a supercharging effect,[38] while unlikely to trigger dissociation of non-covalent assemblies in the ESI interface. Eventually, the application scope of this new experimental startegy will be defined by the physical limitations of the supercharging phenomenon,[39,40] placing a premium on the on-going studies of its mechanism.[41,42]

Conclusions

Attempts to analyze large and extensively glycosylated macromolecules and their non-covalent complexes using conventional native MS frequently fail due to the significant overlap of ionic signals representing different charge states. Limited charge reduction had been successfully applied in the past to a range of structurally heterogeneous proteins and other biopolymers to address this deficiency. However, the reach of this technique is limited to ionic species that fall within the *m/z* range where isolation of ionic populations can be readily accomplished, a hardware parameter that cannot be adjusted on most commercial MS instruments. An approach explored in this work allows this limitation to be circumvented by combining supercharging of ESI-produced ions with limited charge reduction in the gas phase. The former allows the ionic signal of large non-covalent assemblies to be shifted to *m/z* regions where ion isolation can be readily accomplished, while the latter produces well-defined charge ladders enabling straightforward measurements of the ionic masses. The ongoing mechanistic studies of the supercharging phenomenon will be critical vis-à-vis further enhancing the utility of the new technology by enabling a rational (rather than empirical) selection of supercharging reagents that give rise to the highest possible increase of the ionic charge density without compromising the stability of large non-covalent assemblies.

Supporting Information

Supporting information contains (*i*) amino acid sequences of the L-, L*-, and H-chains of human Hp and schematic representation of the quaternary organization of the Hp 1-1 and Hp 2-1 isoforms; and (*ii*) ESI mass spectra of human *h*Tf before and after addition of *m*NBA to the protein solution, and the mass spectrum of ions produced upon limited charge reduction of the supercharged *h*Tf ions.

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Authors' contributions: I.K. and Y.Y. designed the study; C.N., C.B. and Y.Y. planned the experimental work; Y.Y. acquired and interpreted the data; I.K. and Y.Y. analyzed the experimental data and wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Figure legends

Figure 1. ESI mass spectra of a Hb/Hp 1-1 mixture (0.16 and 0.13 mg/mL, respectively) in 150 mM ammonium acetate before (**A**) and after addition of 0.7% *m*NBA to the protein solution (**B**). The red traces show examples of the limited charge reduction work on (α*β*)·HLLH·(α*β*) complexes. The gray-shaded trace in panel (**A**) represents a mass spectrum of free Hp 1-1.

Figure 2. ESI mass spectra of a Hb/Hp 2-1 mixture (0.32 and 0.20 mg/mL, respectively) in 150 mM ammonium acetate before (**A**) and after addition of 0.7% *m*NBA to the protein solution (**B**). The red traces show examples of limited charge reduction work. The red and blue traces show the results of the limited charge reduction analyses of ions representing the (α*β*)·HLLH·(α*β*) and (α*β*)·HL[L*H·(α*β*)]LH·(α*β*) complexes, respectively. The purple trace represents the results of the limited charge reduction analysis of ions representing the (α*β*)·HL[L*H·(α*β*)]$_2$LH·(α*β*) complexes. No ion isolation can be achieved in the shaded regions of the mass spectra due to the hardware limitations.

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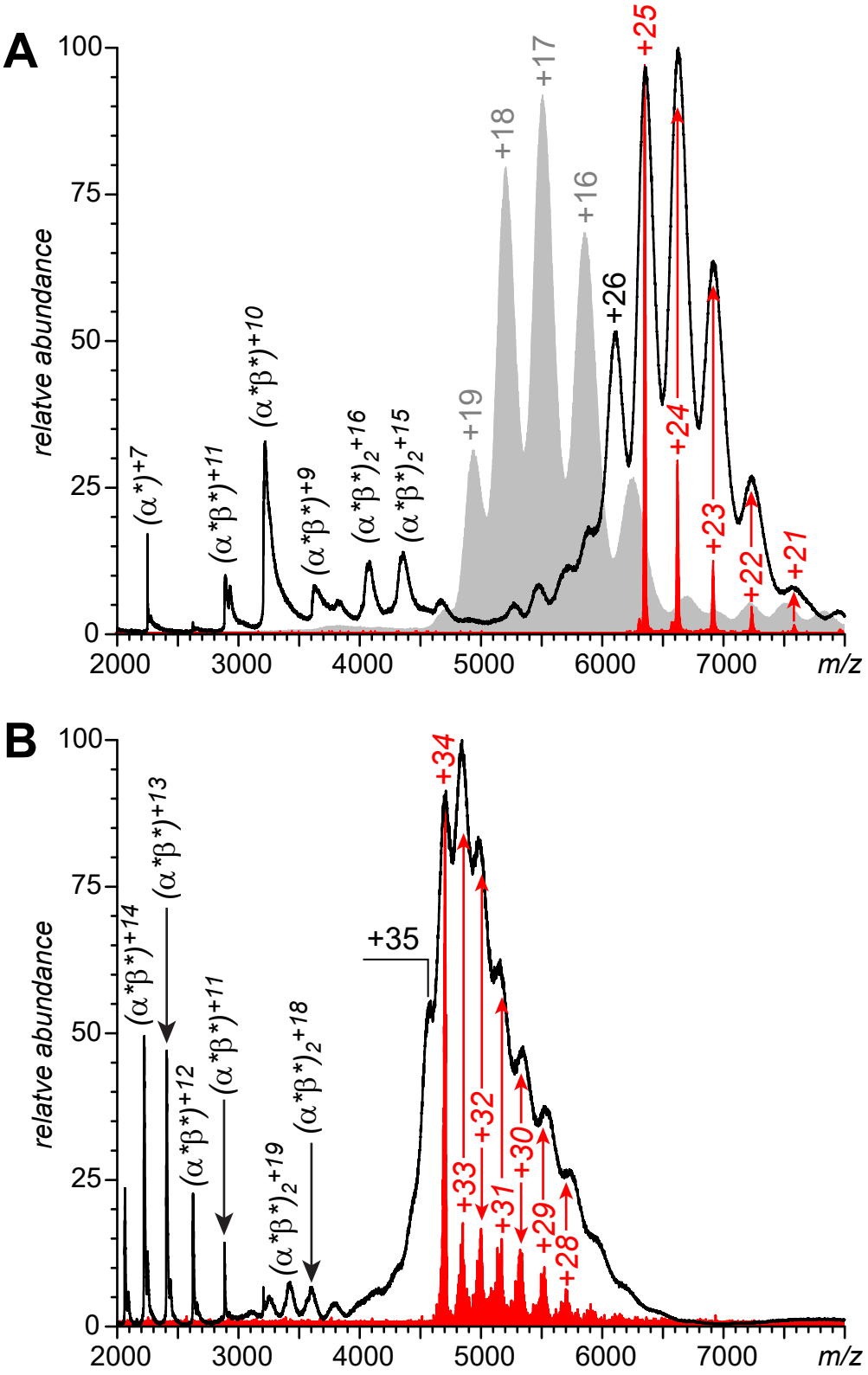
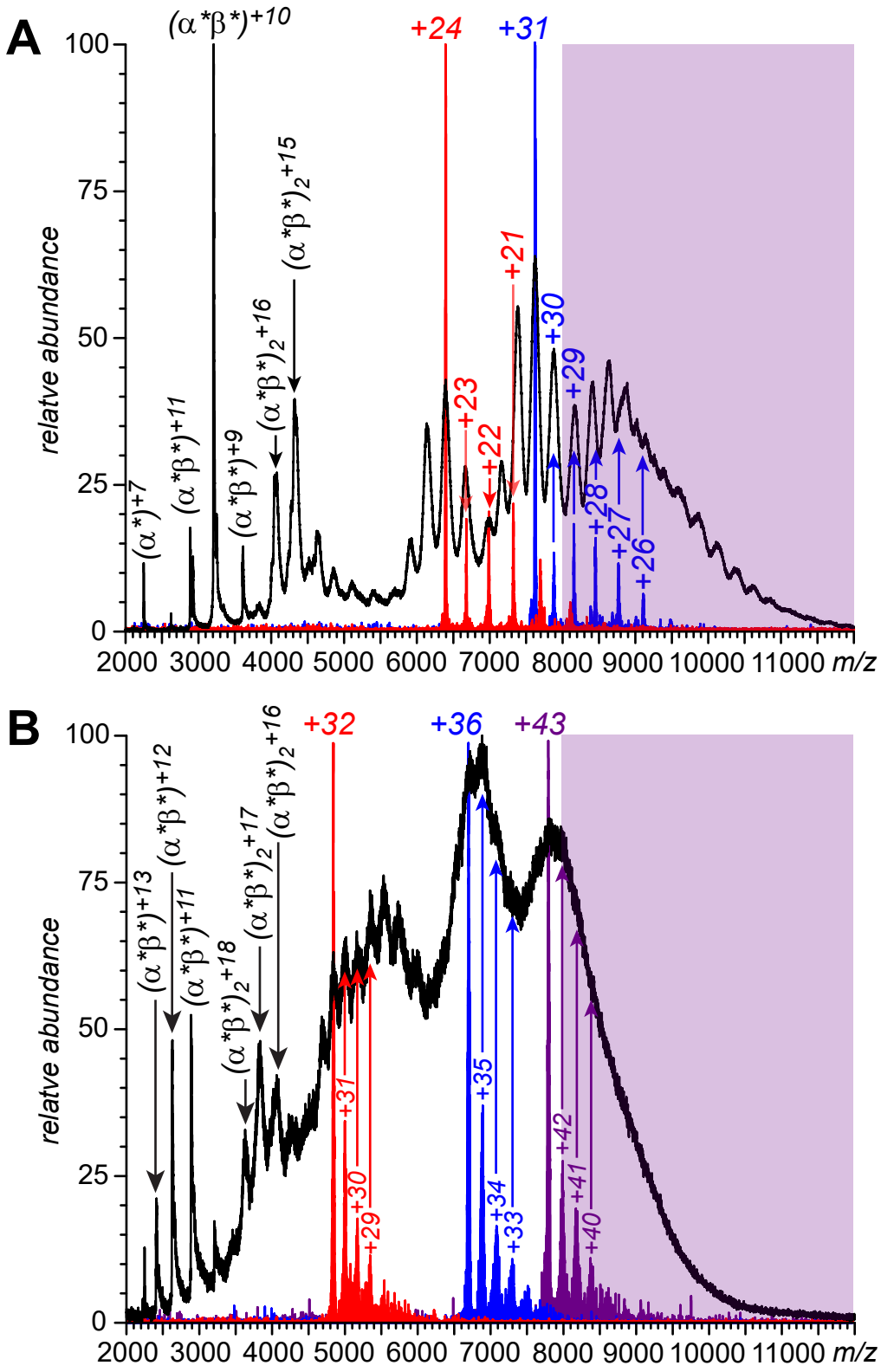


Yang, et al. **Figure 1**



Yang, et al. **Figure 2**

Supplementary Information for

Ionic charge manipulation using solution and gas-phase chemistry to facilitate analysis of highly heterogeneous protein complexes in native mass spectrometry

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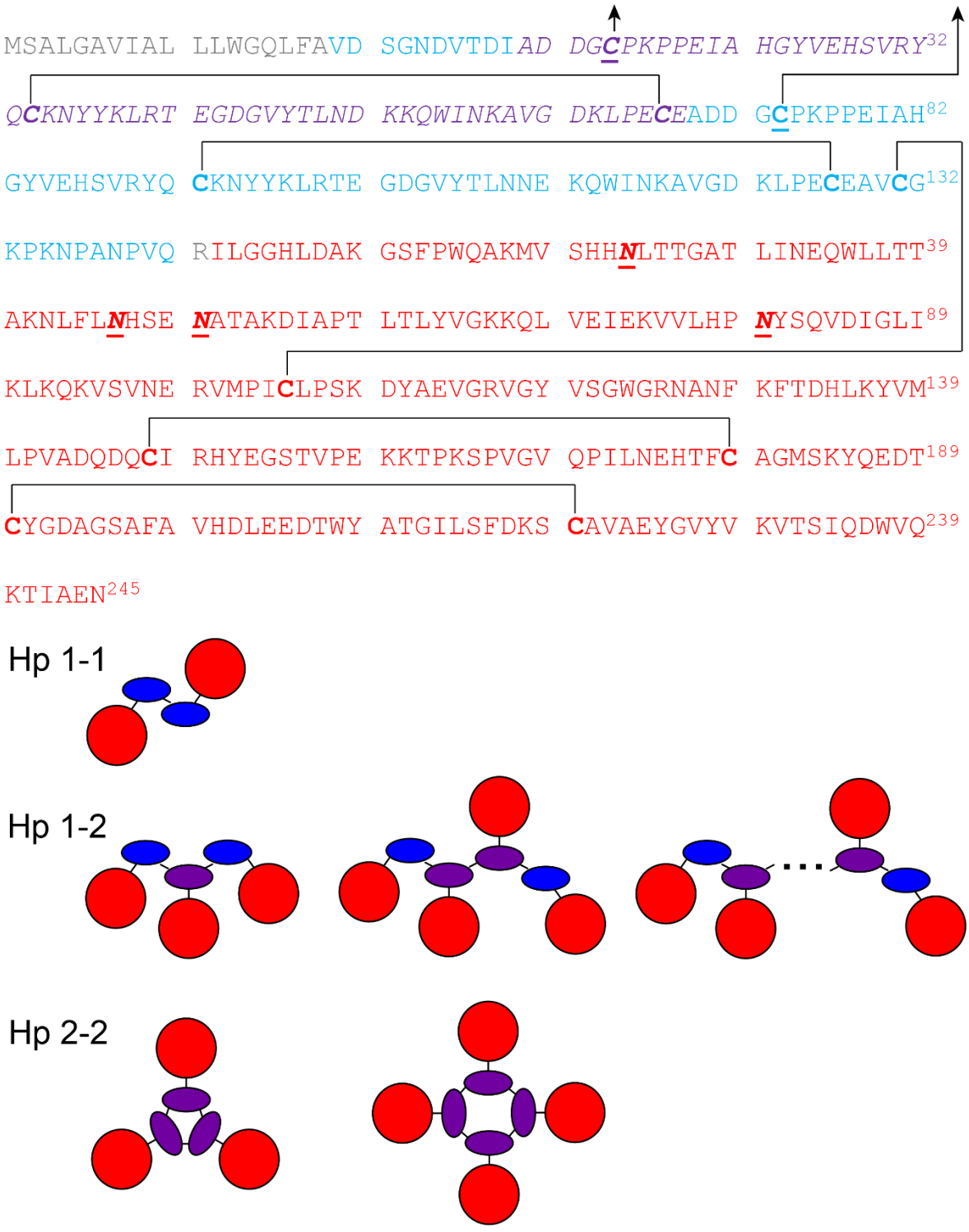
Contents

Figure S1. Top: the amino acid sequence of human haptoglobin pre-protein (P00738). The sequences of the light (L) and heavy (H) chains are highlighted in blue and red, respectively. The segment highlighted in purple corresponds to the additional insert present in the light chain L*. The black brackets show the disulfide bonds; arrows indicate the external disulfide bonds (L-L, L-L* and L*-L*). Bottom: schematic representations of the quaternary organization of haptoglobin oligomers.

Figure S2. ESI mass spectra of 5 μM aqueous (150 mM ammonium acetate) solution of *h*Tf before (the blue trace) and after modifying the solvent with of 6% mNBA (the red trace). The "supercharged" protein ions at charge state +29 were isolated (the black trace) and subjected to limited charge reduction producing a well-defined ladder extending to the charge state +21 (the purple trace).



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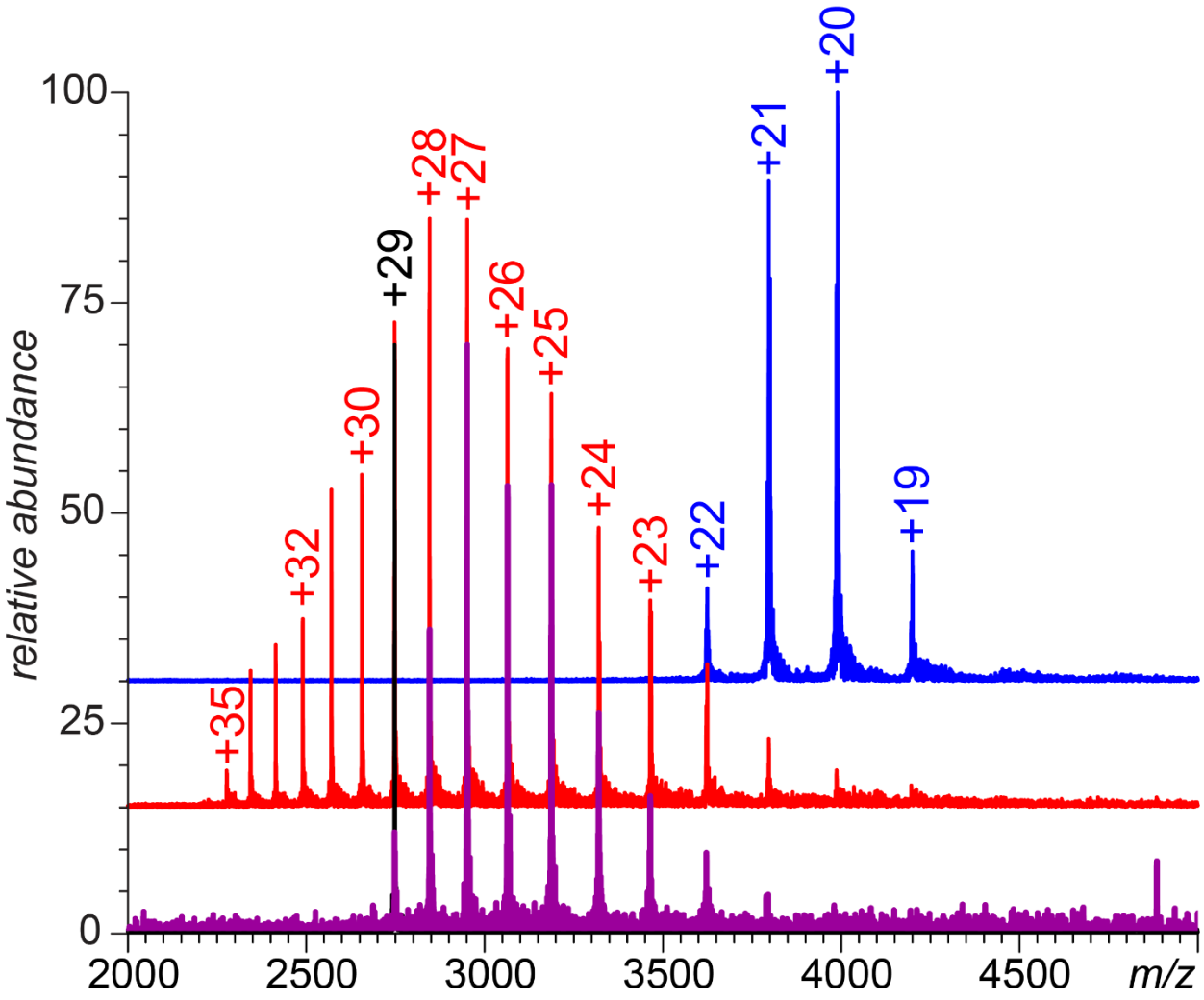

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