Supplemental Text and Supplemental Figures

Salt Bridge Rearrangement (SaBRe) Explains the Dissociation Behavior of Noncovalent Complexes

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Is Coulomb Repulsion Always Significant when 16 Charges are Placed on a Natively-Folded Myoglobin-Like Molecule?

Recall that *holo*-myoglobin sprayed from "native" solution conditions tends to yield positive ESI mass spectra with peaks for 8+-10+ ions. It is generally assumed that like-charge repulsion will be significant in the gas phase if we deposit 6 additional charges onto that "native" structure, but because multiply charged ions exhibit reactivities akin to singly charged ions when charge separations exceed 10 Å [1], it is reasonable to explore how difficult it would be to *distribute 16 charges such that none is within 10 Å of another*.

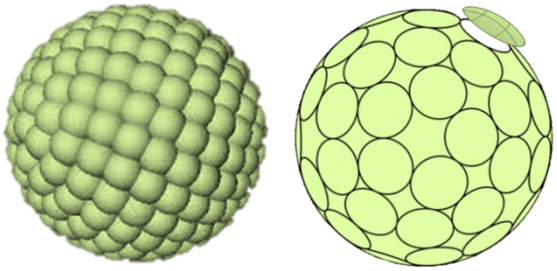
Myoglobin has a collision cross-section of 1761 $Å^2$ [2]. Let us simplify calculations by assuming (i) that that the molecule is spherical with radius r_c = 23.68 Å (such that $\pi\ r_c^{\ 2}$ = 1761 $Å^2$) and (ii) that point charges may be pinned anywhere on the surface.

We calculate surface area (A). $A = 4\pi\ r_c^{\ 2} = 7044\ Å^2$

If, for each charge, we crudely reserved a 10 Å x10 Å square on a planar surface of 7044 $Å^2$, <u>the 16 charges would require 1600 $Å^2$, less than ¼ of the available surface area</u>.

We note that the surface area estimated above differs little from the 6656 $Å^2$ surface area that one could calculate employing Kendrew's [3] x-ray diffraction-based 25 x 34 x 42 Å size estimation of sperm whale myoglobin molecules; nor does it differ from the 6710 $Å^2$ estimated solvent accessible surface area [4]. Calculations based on the 18 Å radius of gyration, R_g [5], yield a similar surface area of 6786 $Å^2$ assuming a spherical structure [$r^2 = (5/3)\ R_g^2$]. At an extreme limit, one could assume that the 18 Å radius of gyration applied to an infinitely thin disk ($r^2 = 2\ R_g^2$) to yield a surface area of $\pi r^2 = 2\pi R_g^2$ = 2036 $Å^2$, accommodating, at most, 20 charges.

We might also inquire, *"What is the maximum number of 10 Å diameter circles that could be placed without overlap on the surface of a 7044 Å^2 sphere?"*



If we assume that 10 Å diameter circles are packed on a planar surface at maximum density (hexagonal packing), then 90.69% of available surface area would be covered by circles. The maximum number of circles that can be accommodated, without overlap, would be $[(7044 \bullet 0.9069)/(\pi\ r^2)] = 81$ circles. This treatment does not account for surface curvature, as illustrated above, thus overestimating slightly the maximum number of circles that can be accommodated. Calculations addressing surface curvature rarely yield exact solutions, but available tabulations of the "best" packings obtained by numerical methods yield a 74 circle estimate [6].

These calculations, based on ratios of charge/surface area, demonstrate that *the quantities of charge deposited on proteins by electrospray ionization, even under supercharging conditions, would not necessarily yield significant repulsive energies.* That repulsive interactions are important cannot be assumed without considering the location of point charges.

Sequence Mutations Affect Charge Allocation

The ion pairing model rationalizes observations that (i) sequence mutations affect the proportion of charge dispensed from a dissociating homomer to the ejected monomer, and (ii) that the direction of the mutation effect (increase or decrease in dispensed charge) depends on whether complexes are positively or negatively charged [7]. Sinelnikov, *et al.* [7] compared the charge states for monomers released from dissociating Shiga toxins Stx1 and Stx2. These B_5 complexes have nearly identical higher order structures and homologous primary sequences. Nevertheless, decomposing Stx2 B_5^{12-} precursors by black body infrared dissociation (BIRD) tended to eject monomers bearing more charge than decomposing Stx1 assemblies (average charge -4.84 *vs*. -4.52, respectively), but dissociating Stx1 B_5^{12+} complexes expelled monomers with more charge than Stx2 complexes (4.46 *vs*. 4.15, respectively). Charge polarity-related differences in charge partitioning are intriguing, yet they are easily rationalized from an ion pairing view. Stx1 has 8 basic residues and 8 acidic residues. Stx2 has 7 basic and 10 acidic residues. If dissociation of a complex requires the heterolytic cleavage of many subunit-spanning salt bridges to be energetically feasible, then Stx1 pentamers would have a small advantage in their ability to retain positive charge after heterolytic cleavage, simply because they have more basic sites for potential involvement. Stx2 pentamers, however, have the advantage over Stx1 in negative ion mode, due to 3 additional acidic sites. Of course, these arguments do not consider whether the pentamer's transition state structure would be compatible with salt bridge formation at any one residue and/or spanning subunits; they only illustrate that salt bridges explain how the primary sequence of the leaving subunit, independent of the rest of the complex, could exert control over the partitioning of charge during dissociation. Results from Stx1 site-selected

mutants also follow our expectations for salt bridge involvement [7]. Mutant R69D and R69A monomers were expelled from homogeneous B_5^{12+} pentamers with less positive charge than the monomers expelled from non-mutated pentamers, but with still more than borne by the double mutants R69A/K8A [7]. In negative ion mode, R69D departed with more average charge than non-mutants, R69A mutants, and R69A/K8A double mutants.

Equally Charged Sequence Variants Differ in Cross-Section by an Amount that Increases Monotonically with Charge (Ω and ΔΩ are functions of Z) Consistent with Eliminating a Restraining Salt Bridge

Gas phase collision cross-section measurements of hemoglobin tetramers $(\alpha^h\beta^h)_2$, from normal (HbA) and sickle cell (HbS) variants provide another example with which to ponder an inverse relationship between charge and number of ion pairs. Scarff, *et al.* [8] found that HbS cross-sections for all measured charge states (15+ - 18+) were 2-7% larger, respectively, than those obtained from *equally* charged HbA. Although smaller than the 10% difference predicted from projection approximation calculations employing crystal coordinates, the size difference was in the appropriate direction; *i.e.*, HbS > HbA. An increase in CCSs with increasing charge is a common observation, but it is very interesting that the CCS *difference* (ΔΩ) between HbS and HbA also became *larger with increasing charge*, consistent with HbS losing one salt bridge per ß-subunit, due to its ß6 Glu → Val mutation. A structure with less reinforcement would deform more easily at *increasing lab frame energies*.

If HbS ß-subunits indeed possess one less salt bridge, we might then guess that the number of gas phase salt bridges in 17+ and 18+ HbA might approach the number present in 15+ and 16+ HbS, respectively, because two subunits are mutated. CCS values in Fig. 4 of Scarff, *et al*. [8], indicate that the 17+ HbA and 15+ HbS collision cross-sections are within ~1% of each other, as are the 18+ HbA and 16+ HbS, consistent with our naive guess.

Divalent Cations and Subunit Interfaces

Recent studies explored the stabilization of complexes to CID provided by divalent cations, demonstrating that bound Ca^{2+} or Mg^{2+} ions reinforce hemoglobin tetramers with multidentate ion bridges; *e.g.*, R′-COO^- Ca^{2+} $^-$OOC-R″ [9-11]. Asymmetric dissociation of Ca^{2+}- or Mg^{2+}-bound hemoglobin was proposed to proceed by (1) unfolding a subunit containing fewer than the average number of carboxylate-bridging metal ions while (2) migrating protons simultaneously relocate from peripheral subunits to the unraveling one. By hypothesizing that divalent metal ion "staples" conformationally constrained individual subunits to inhibit their unfolding, Liu and Konermann [11] rationalized the experimental observation that *ejected monomers carried less than the average metal load*. Because unraveling metal-stapled regions would require more energy, the subunits most susceptible to deformation were argued to be the ones possessing fewer staples.

SaBRe offers an alternative explanation for divalent ion reinforcement: rather than stapling only intra-subunit, divalent metal ions can also staple across subunits. Monomeric units linked to other units by fewer metal bridges would be easier to dislodge than subunits extensively metal-stapled to others. Because the number of *intra*-subunit staples does not necessarily affect stability in the SaBRe model, the observation that ejected monomers tend to carry less metal appears to imply that Ca^{2+} and Mg^{2+} bridges enrich at interfaces.

The implication above must be rationalized, especially because the metal binding is non-specific. The loss of interfacial water molecules during transfer from solution to the gas phase, in concert with activation, would likely drive some rearrangement, relocating metal ions to interfaces [12]. Pre-activation applied to detach extensive Cl^- ions in those experiments would

additionally facilitate charge rearrangements [11]. Recall our earlier mention of how protein interfaces, stabilized *in vivo* by bound water molecules [12], tend to be less highly optimized than the structural folds within subunits. The stability lost upon desolvation could be recovered by salt bridge formation between the metal ions and opposing charges enriched at the interfaces of activated gas phase complexes. Hence, the SaBRe explanation for divalent ion stabilization and for the reduced metal loads of expelled monomers is reasonable.

Because asymmetric charge partitioning is necessary to obtain dissociation products, given the limited energy available to the reaction coordinate, some interface-spanning salt bridges (preferably monovalent acidic/basic residue linkages (organic staples), rather than the stronger divalent metal-bridged linkages (metal staples)) must cleave heterolytically to enrich charge density in the departing monomer.

Ultimately, two models attempt to explain how divalent metal ions stabilize non-covalent complexes and why the monomers ejected from such complexes carry less than the average metal load. (i) Metal staples conformationally constrain individual subunits to prevent unfolding. Folded subunits retain enough of their initial non-covalent bonds and higher order structure to remain associated in complexes. (ii) Metal and organic staples can bind subunits to one another and stabilize them against dissociation. Collisional activation can drive staple rearrangements inter- and intra-subunit, recovering and even adding to stabilization lost when interfacial water molecules were detached during ESI. Because metal staples are stronger than organic staples, the monomers dissociated from such complexes tend to have been attached by fewer metal staples and, thus, are expelled with less metal than the average subunit. Monomers are expelled with more than the average charge per subunit, because additional charge was acquired when interfacial organic staples cleaved heterolytically.

Does Correspondence between the CCSs Measured for Proteins Delivered by Native ESI to that Calculated from Crystal Structure Coordinates Necessarily Mean that Structural Arrangements (Folds) are Preserved in the Gas Phase?

If salt bridges provide stabilization from distortion to the CCSs of gas phase proteins and protein complexes, must the solution phase arrangement of hydrogen bonds and van der Waals interactions (the conformation) necessarily also be retained? Does correspondence between the CCSs measured for proteins delivered by native ESI and the CCSs calculated from crystal structure coordinates necessarily mean that structural arrangements (folds) are preserved in the gas phase?

A challenge in answering these questions is that the term "folded" is rarely defined for gas phase proteins. In so far as experimental cross sections are generally compared to those calculated from crystal structures, it would seem reasonable to *define "folded" as "to assume a single gas phase structure that maintains atoms within 3 Å of their relative positions in crystals."*

With this definition, then, one should ask *is it possible for many atoms in a gas phase complex to be more than 3 Å distant from their relative positions in the crystal structure, yet yield the same value for the calculated CCS?* If so, more evidence (higher resolution?) will be needed to establish whether or not solution phase *folds* continue into the gas phase.

There are reasons to believe that the solution phase folding/crystal structure arrangement of atoms might not be recapitulated after gentle activation from desolvation. Once energy is imparted by a collision, two factors affect the energy flow/redistribution and bond rearrangements/scissions across a macromolecule. The first factor is the energy of any single

bond, whether high or low. Individual hydrogen bonds (H-bonds) are weaker than salt bridges and covalent bonds, making them more susceptible to cleavage/alteration. The second factor is the availability of alternative bond arrangements similar in energy; *e.g.*, options to H-bond or ion pair elsewhere, recouping the energy lost in breaking the original bond. Proteins present a vast number of opportunities to form and rearrange H-bonds and the cost to reposition *any one* bond is relatively low. From this consideration, collisional activation seems likely to rearrange many H-bonds. Salt bridged-bonds are rarer and stronger, making their rearrangements seemingly harder to accomplish. For example, successfully rearranging the *intersubunit* bridge Glu-x1/Arg-y2 (where*, e.g.*, x1 and y2 correspond to the xth residue of subunit 1 and the yth residue of subunit 2, respectively) to an *intrasubunit* bridge such as Glu-x1/Arg-z1, would recoup the large Coulomb energy, *but can only occur if an available opposite charge is nearby*.

These considerations suggest that activation should readily rearrange H-bonds, while requiring more energy to rearrange salt bridges. Still more energy would be needed to *break* a salt bridge without forming a new bridge to compensate for lost Coulomb energy. This view may explain the discrete features present in "collision induced unfolding" plots (CCS *versus* activation energy), namely, the gradually rising slopes, plateaus, step increases and the widths (spread in CCSs at a given activation energy). If ion mobility is to be interpreted as measuring the extent to which solution phase folding is maintained in the gas phase, we must ultimately ask, if salt bridge "staples" restrain the extent of gas phase protein expansion, how much other rearrangement; *e.g.* in H-bonds, could theoretically occur without significantly increasing the measured CCS, and is this amount small enough to conclude that an atomic arrangement from solution has been maintained in the gas phase?

Does Manipulating Charge State Distributions with Gas or Solution Phase Additives also Manipulate the Ratio of Charges Paired-to-Unpaired with Opposite Charges?

Previously, we described the three-regime model [13], a view of the electrospray process in which the elongated filament protruding from distorted, decomposing droplets expels high charge density *progeny droplets* and high charge density *protein ions*. The model attributes charge disposition on ejected proteins to solution and gas phase properties along with the properties of an intermediate regime, transitioning from solution to gas phase. An important feature of the three-regime model is that it allows for opposite charges to be abundantly present within ionized proteins and predicts the impact of solution additives on charge state distributions and on those opposite charges.

The three-regime model predicts two means by which ESI supercharging agents can increase charge on protein and non-protein analytes [13]. Additives can (1) reduce the amount of excess charge lost to solvent ions, thus leaving more charge for analyte (*e.g.*, additives that disrupt hydrogen-bonding networks reduce H_2O basicity) and/or (2) reduce the number of opposing charges on analytes by reducing ionization in bulk solution. To understand charge manipulation and partitioning in complexes, we begin with precursor ions as described by the three-regime model.

Solvents that are very weak bases *and* very weak acids in their neutral form are known to reduce analyte ionization *in bulk solution* [13]. Despite its ionization status in solution, however, an analyte will accrue excess charge of the spray's polarity when electrohydrodynamically emitted from disintegrating ESI droplets [14], traversing the intermediate regime to the gas

phase. Initially less ionized within solvents *modestly* supplemented by *m*-NBA or sulfolane, an analyte can thus become supercharged when the high electric fields of the intermediate regime deposit excess electrospray charge onto it. Interestingly, analytes exposed to low volatility additives that *reduce solution ionization* should have *fewer opposing charges*, because any opposing charges persisting in the gas phase regime *must have been transferred from solution.* Thus, charge-manipulated ions can differ subtly from equally-charged ions direct from ESI, providing predictions by which to test theories.

If, in a protein complex, the same charge state is examined by direct ESI and by supercharging (*e.g.*, comparing the highest charge state obtained without a supercharging agent to one of the lowest charge states in a CSD shifted by sulfolane) the supercharged ion *may* behave differently. Decomposing complexes could, in principle, be more likely to allocate charge symmetrically if they are supercharged, a consequence of fewer ion pairs. It has been demonstrated that CID of 30+ SAP pentamers (obtained with either *m*-NBA or sulfolane) yielded primarily 6+ monomers (symmetrically partitioned charge), while CID of the 25+ complex (obtained without additives) released 12+ monomers (asymmetrically partitioned charge) [15]. However, it is desirable to compare identical charge states; fortunately, results are available for the SP-1 protein complex sprayed from 100 mM ammonium acetate and from 6.25% sulfolane solutions [16]. CID-MS/MS spectra of 31+ dodecamers (Suppl. Fig. 2 in Erba, *et al*. [16]) dissociated to yield the same product ions: 10-, 9-, 8-, 6-, and 4-mers, along with dimers and monomers. The spectra were very similar, although 31+ complex from sulfolane solutions appeared somewhat less likely to dissociate by monomer expulsion or by decomposing to dimer/10-mer, suggesting a slight preference towards symmetric-like dissociation pathways, consistent with a reduction in salt bridges. Further exploration of whether different extents of ion

pairing can exist in the identical charge states of gas phase complexes sprayed from different non-denaturing solutions is needed.

Gas phase bases have been employed to reduce the charge deposited on electrospray-generated protein ions for over two decades [17-19], including proteins sprayed from native solutions [20]. Base exposure has also been applied to native mass spectrometry to extend the range of low charge states available for study. With *gas phase* delivered-bases, we expect transfer of protons from cationic sites un-involved in salt bridges. Consequently, a 13+ ion generated by charge reducing a 17+ species may possess fewer ion pairs than the 13+ ion produced directly by ESI of that *same* solution. Indeed, data was reported a decade ago in which gas-phase proton transfer from amines to non-specific cytochrome *c* dimers was employed to compare identical charge state precursors of different provenance [21]. Dissociating 13+ dimers produced directly by ESI partitioned charge asymmetrically, in contrast to the symmetrically-charged products released by 13+ dimers of charge-stripped 17+, consistent with our hypothesis that the former is capable of more ion pairs.

In principle, charge-reduced analytes prepared by proton or electron transfer in ion-ion reactions should behave similarly to those prepared by ion-molecule reactions, as long as contributions from the reaction exothermicity are not important. The situation is more complicated when neutral amines and other bases are delivered as solution additives, a practice favored for its experimental simplicity. Solution administration of base can reduce protonation of basic sites both in solution and in the gas phase (due to its volatility and gas phase basicity), but it can also affect charging during ion evaporation, and its conjugate acid (*e.g.*, ammonium) can bind to carboxylates in solution to reduce salt bridging. If carboxylate-paired ammonium is dislodged as the neutral base (NH_3), the number of salt bridges could be lower than in the

absence of NH_4^+. If dislodged as a cation (conjugate acid), it will strip charge. The complex interactions associated with solution-phase base introduction may make it hard to predict the impact on dissociation behavior. We suggest that the dissociation behavior of n+ complexes may well differ, depending on whether they are produced directly by ESI, by exposure to amines in the gas phase (or ion-ion reaction-induced proton transfers), or by amine exposure in solution.

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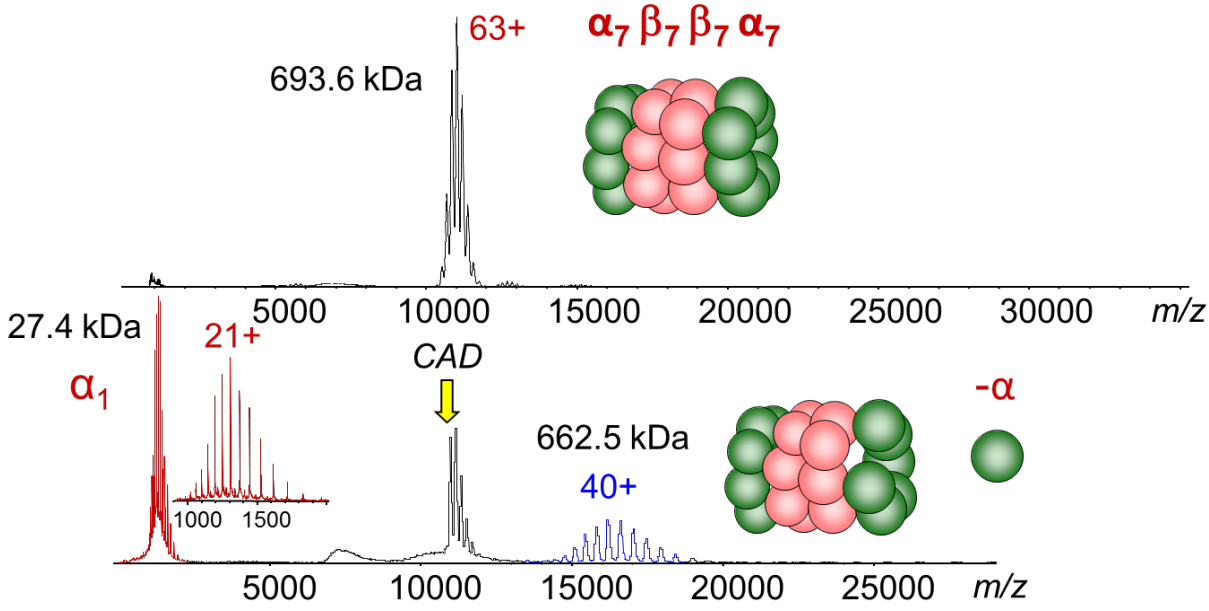
Supplemental Figure Captions

Figure S1. ESI-MS of the 63+ charged α7ß7ß7α7 28-mer 20S proteasome from M. thermophila. (Reprinted from J. Am. Soc. Mass Spectrom., Vol. 16, J. A. Loo, B. Berhane, C. S. Kaddis, K. M. Wooding, Y. Xie, S. L. Kaufman, and I. V. Chernushevich, 998-1008 (2005), with permission from American Society for Mass Spectrometry.)

Figure S2. **(A)** Stacked hexameric rings of torroidal complex HSP16.9. (Left) Charge (32+) distributed relatively uniformly amongst 12 subunits. (Right) Charge migration from 11 subunits to rationalize release of a 14+ monomer (pink subunit in upper ring) and 18+ charge-stripped undecamers. **(B)** Illustrating only one of the two hexameric rings from the HSP16.9 torroidal dodecamer. (Left hand side) A distribution of positive and negative charges yielding an excess charge of 16+ on the hexameric ring. White charges indicate interface-spanning ion pairs. (Right hand side) Filled white ovals indicate homolytically cleaved (neutralized) ion pairs, while hollow ovals with interior charges reflect heterolytically cleaved ion pairs. These indicated cleavages would create a 9+ monomer and a 7+ pentamer from an initial 16+ hexamer. Likewise, one can imagine that a second (stacked) hexameric ring contacting the illustrated ring and heterolytically cleaving 4 more ion pairs contacting the rose-colored subunit would release 13+ monomer and 19+ undecamer.

Figure S3. (top) Highly charged complexes, tending to bear the fewest opposite charges (and fewest potential subunit-spanning salt bridges) dissociate by CID along a symmetric pathway, illustrated here as a 30+ tetramer decomposing to a pair of 15+ dimers. (middle) Less highly charged complexes, possessing somewhat more opposite charges (subunit-spanning salt bridges), dissociate by CID *via* an asymmetric pathway, here as a 15+ tetramer decomposing by releasing a charge/mass "enriched" product (6+ monomer) and a charge-stripped (9+) trimer. (bottom) Complexes with little charge (most opposite charges) may be so heavily reinforced with subunit-spanning salt bridges that they decompose by cleaving covalent bonds in preference to noncovalent bonds.



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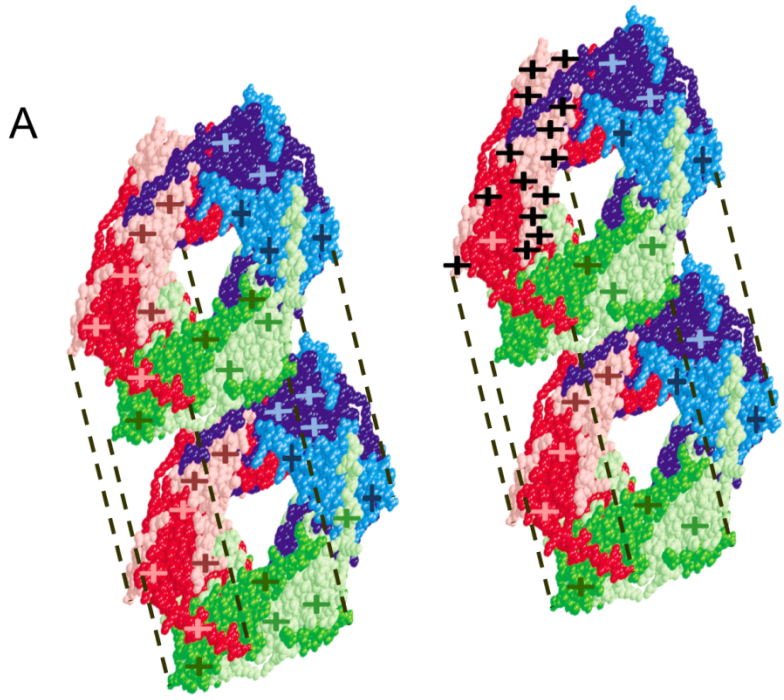


Figure S2A. Stacked hexameric rings of torroidal complex HSP16.9. (Left) Charge (32+) distributed relatively uniformly amongst 12 subunits. (Right) Charge migration from 11 subunits to rationalize release of a 14+ monomer (pink subunit in upper ring) and 18+ charge-stripped undecamers.

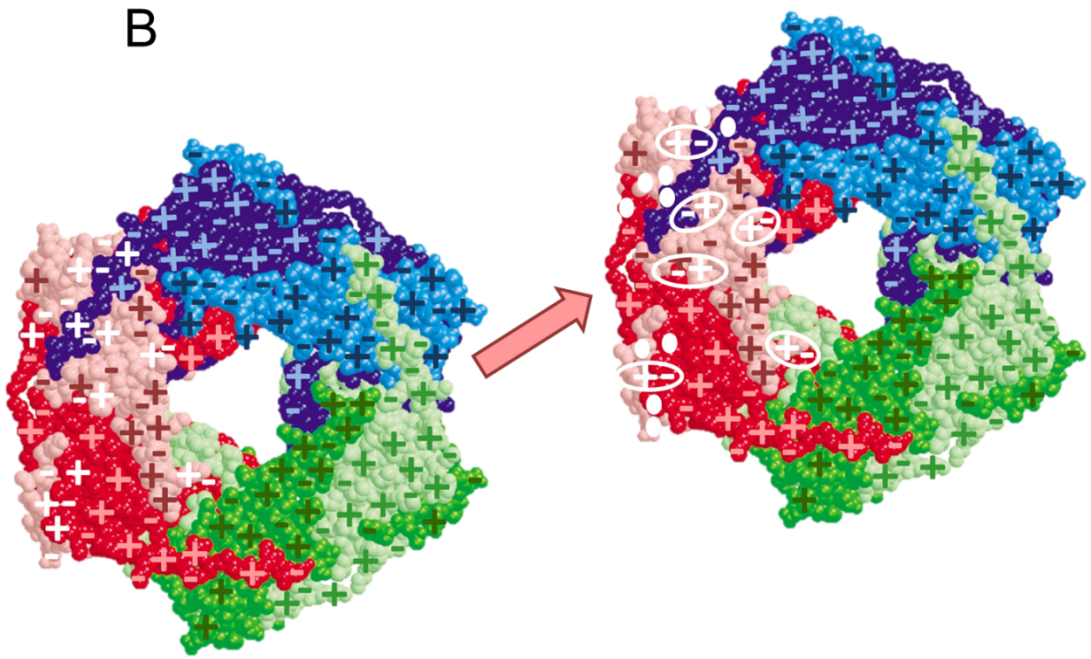
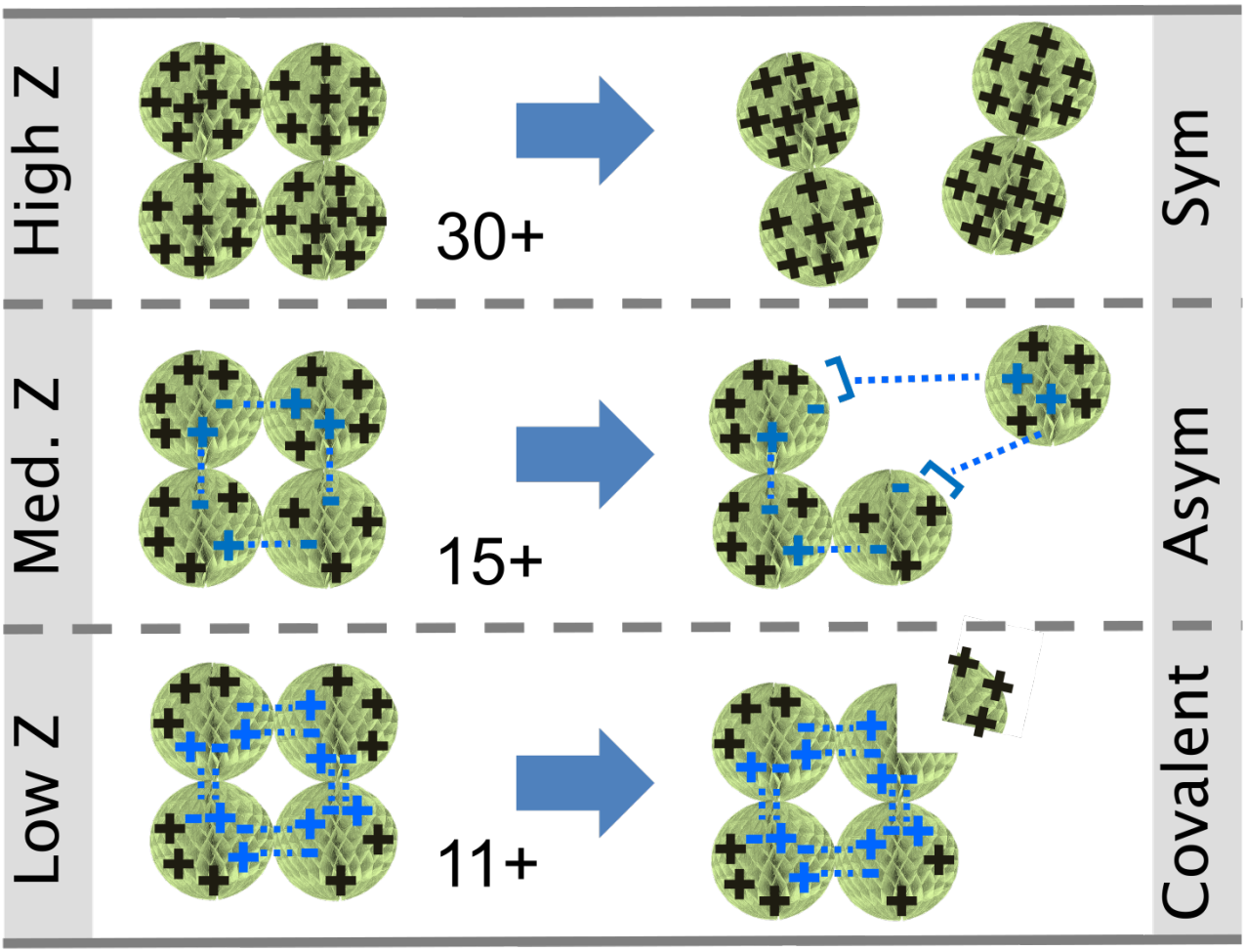


Figure S2B. Illustrating only one of the two hexameric rings from the HSP16.9 torroidal dodecamer. (Left hand side) A distribution of positive and negative charges yielding an excess charge of 16+ on the hexameric ring. White charges indicate interface-spanning ion pairs. (Right hand side) Filled white ovals indicate homolytically cleaved (neutralized) ion pairs, while hollow ovals with interior charges reflect heterolytically cleaved ion pairs. These indicated cleavages would create a 9+ monomer and a 7+ pentamer from an initial 16+ hexamer. Likewise, one can imagine that a second (stacked) hexameric ring contacting the illustrated ring and heterolytically cleaving 4 more ion pairs contacting the rose-colored subunit would release 13+ monomer and 19+ undecamer.



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