

# Surface-Induced Dissociation of Anionic vs Cationic Native-Like Protein Complexes

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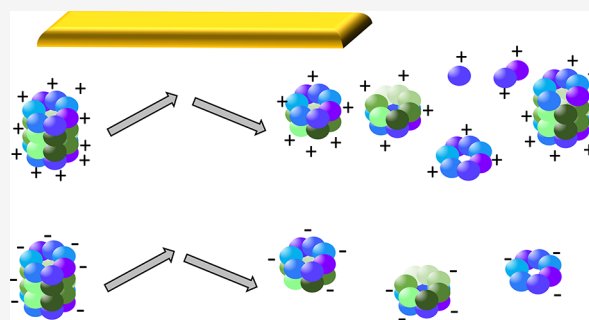
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**ABSTRACT:** Characterizing protein–protein interactions, stoichiometries, and subunit connectivity is key to understanding how subunits assemble into biologically relevant, multisubunit protein complexes. Native mass spectrometry (nMS) has emerged as a powerful tool to study protein complexes due to its low sample consumption and tolerance for heterogeneity. In nMS, positive mode ionization is routinely used and charge reduction, through the addition of solution additives, is often used, as the resulting lower charge states are often considered more native-like. When fragmented by surface-induced dissociation (SID), charge reduced complexes often give increased structural information over their “normal-charged” counterparts. A disadvantage of solution phase charge reduction is that increased adduction, and hence peak broadening, is often observed. Previous studies have shown that protein complexes ionized using negative mode generally form lower charge states relative to positive mode. Here we demonstrate that the lower charged protein complex anions activated by surface collisions fragment in a manner consistent with their solved structures, hence providing substructural information. Negative mode ionization in ammonium acetate offers the advantage of charge reduction without the peak broadening associated with solution phase charge reduction additives and provides direct structural information when coupled with SID. SID of 20S human proteasome (a 28-mer comprised of four stacked heptamer rings in an  $\alpha\beta\beta\alpha$  formation), for example, provides information on both substructure (e.g., splitting into a  $7\alpha$  ring and the corresponding  $\beta\beta\alpha$  21-mer, and into  $\alpha$  dimers and trimers to provide connectivity around the  $7\alpha$  ring) and proteoform information on monomers.



## INTRODUCTION

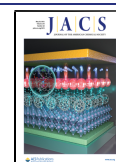
Within the cell, a host of biological processes are mediated through multicomponent protein complexes.<sup>1</sup> Characterization of these protein complexes, including the stoichiometry, topology, identification of all binding partners, and classification of posttranslational modifications, is essential to better understand how these complex systems carry out their function and how misfolding or alterations in architecture can lead to malfunction.<sup>2,3</sup> While classical techniques, such as X-ray crystallography, NMR, and, more recently, cryo-EM, can provide high-resolution structures, these techniques have limitations; often high concentrations and high purities are required, and conformationally dynamic systems can still pose challenges.<sup>4–6</sup> Since the advent of electrospray ionization, mass spectrometry has emerged as a powerful tool to study large proteins and protein complexes.<sup>7–10</sup> Using appropriate electrolyte solutions, typically ammonium acetate (AmAc), and gentle desolvation and instrumental conditions, intact complexes, including protein:protein and protein:ligand complexes, can be transferred into the gas phase, kinetically trapped in native-like structures.<sup>11–13</sup> Once transferred into the gas phase, information on their stoichiometry, substructure, and conformations can be obtained using dissociation techniques or ion mobility measurements.<sup>14–16</sup> While being

lower resolution than traditional structural characterization methods, mass spectrometry offers advantages in low sample consumption (typically  $\mu\text{L}$  of sample at  $\mu\text{M}$  concentrations) and can be used to study dynamic and heterogeneous systems.<sup>17</sup>

Most protein MS studies have been conducted on protein cations. Several studies have, however, utilized negative ionization mode.<sup>18–21</sup> Interestingly, these studies reported that the protein anions have lower average charge states than the corresponding cations which has resulted in discussions regarding the process by which these molecules are ionized.<sup>19,20</sup> Several electrospray mechanisms have been previously proposed, with discussion focusing on positive ionization mode, and are thought to depend on the nature of the molecule.<sup>22–24</sup> In positive mode electrospray, it has been proposed that globular proteins follow the charged residue

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model, producing ions with a composition close to  $[M + z_R H]^{z_R+}$ , where  $z_R$  is the Rayleigh charge of protein-sized water droplets. Previous studies have shown that for proteins and protein complexes, positive charge states observed are approximately 90% of the Rayleigh model predicted charge; however, observed negative charge states are only approximately 70% of the predicted charge.<sup>19</sup> This difference has been attributed to the emission of small charge-carrier ions in the final stages of desolvation, in which there are lower-energy charge-carrier emission processes available to anions than cations.<sup>19,20</sup> The identities of the charge carriers include ammonium and acetate but could also include other ions as well. A reviewer suggested that the difference may be partially attributable to negative charge sites being more basic than positively charged basic sites are acidic; this would result in lower charge states for negative mode. Interestingly, despite their lower charge states, ion mobility has shown that cations and anions have similar collisional cross sections (CCS).<sup>20</sup> The similar CCS suggest that similar conformational families might be sampled in each case, especially for multi-subunit complexes held together at protein:protein interfaces, and hence negative mode ESI could be beneficial in studies when lower charge states are desired.

Many native mass spectrometry (nMS) studies are conducted using “charge-reducing” reagents, as lower charge states can give more stable, native-like structures and fragmentation products.<sup>25–27</sup> Charge reduction is typically achieved using solution-phase additives such as triethylammonium acetate (TEAA), which is an efficient charge reduction reagent; however, TEAA can lead to peak broadening due to increased adduction on the ion.<sup>28</sup> Here we seek to determine if the lower charge state protein complex anions generated through negative ESI can be used as an alternative to solution-phase charge reduction in nMS protein structural studies, offering the advantage of lower charge states without the increased adduction.

nMS studies commonly employ gas-phase dissociation methods to obtain substructural information. The most commonly used method of dissociation is collision-induced dissociation (CID).<sup>29</sup> In CID, ions are accelerated into a neutral buffer gas, and the analytes undergo multiple collisions with the very low mass collision gas. For protein complexes, the large number of low energy collisions means that CID typically proceeds via an unfolding or restructuring mechanism producing unfolded, highly charged monomer and the complementary  $N-1$  mer.<sup>30–32</sup> Hence CID provides limited direct information on substructure and assembly ( $N$ -mers of different structure/connectivity produce monomer and  $N-1$  mer). An alternative method of dissociation used in nMS studies is surface-induced dissociation (SID). In SID, analytes are accelerated toward, and collide against, a surface, a collision target that is more massive than the complex. This energy deposition process results in a sudden energy jump, a number of studies have been conducted to investigate the energy deposition process in SID,<sup>33,34</sup> with one such study suggesting that the energy deposition efficiency may increase with mass up to 68%.<sup>35</sup> Following surface collision, dissociation typically occurs with less unfolding than CID and in a manner that is consistent with the solved structure.<sup>14,36–38</sup> Note, however, that products of the same charge state produced by CID or SID tend to have the same CCS values but SID tends to produce lower charged products and CID higher charged products.<sup>39</sup> Recently, it was shown that the SID spectra

produced from complexes analyzed under gentle instrumental conditions vs intentional preactivation are different, and hence SID can be used to assess structure and structural rearrangement.<sup>40</sup> Here we use negative mode nano-ESI (nESI) and SID to study the substructure of several model protein complexes, highlighting the utility of this method for structural characterization. Comparisons with positive mode spectra are also provided.

## METHODS

**Materials.** C reactive protein was purchased from Sigma-Aldrich. TRAP was expressed in *Escherichia coli* BL21(DE3) cells, purified, and refolded as previously described.<sup>41,42</sup> Samples were buffer exchanged into 200 mM AmAc or 400 mM AmAc, respectively, using Biorad micro P6 spin columns. Human proteasome was purified by FLAG affinity purification from HEK293 cells as previously described, and free FLAG peptide was present in the analyzed samples.<sup>43</sup> Human proteasome was received from the lab of Michal Sharon in 500 mM AmAc and diluted to 150 mM AmAc before analysis. Samples were run at approximately 700 nM–1  $\mu$ M protein complex concentration. AmAc concentrations for individual samples were based on previously optimized conditions.<sup>42,44,45</sup>

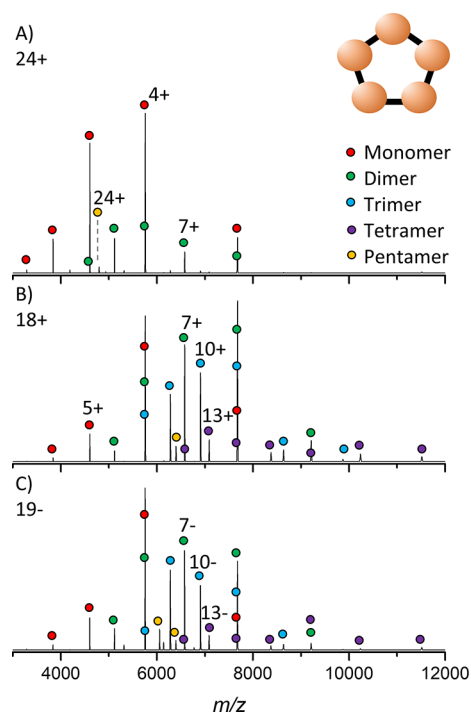
**Instrumentation.** All experiments were conducted on a Thermo Q Exactive Ultra High Mass Range (UHMR) Orbitrap MS (Bremen, Germany), which has been modified to include SID, as described previously.<sup>46,47</sup> In brief, a 4 cm SID device is inserted in place of the transfer multipole between the selection quadrupole and the C-trap. Voltages were supplied to the SID cell via external dc power supplies (Ardara Technologies, Ardara, PA) and controlled through the accompanying Tempus Tune software (Ardara Technologies, Ardara, PA). As described previously,<sup>46</sup> in order to increase the SID voltage, and hence energy range, the C-trap offset voltage is also supplied with an external power supply. In positive mode this involves setting the C trap offset voltage low relative to upstream optics and in negative mode, setting the C trap offset voltage high relative to upstream optics. The surface was prepared as follows; a 17 mm  $\times$  13 mm  $\times$  0.5 mm gold surface slide, 1000 Å of Au on 50 Å of Ti on glass (EMF Corp.), was cleaned for 15 min in a UV cleaner (Model 135500; Boekel Scientific). The cleaned surface was then incubated in  $\sim$ 3 mL of 1 mM solution of alkanethiol perfluorinated on the final 10 carbons of the 12 carbon chain (FC<sub>12</sub>) in ethanol in the dark. FC<sub>12</sub> was synthesized using a previously reported protocol.<sup>38</sup> Following incubation, the surface was cleaned by sonicating in  $\sim$ 3 mL of ethanol for 1 min; this cleaning process was repeated six times with fresh aliquots of ethanol.

All samples were ionized via static nanospray ionization using in-house pulled borosilicate capillaries with the electrospray voltage applied to a platinum wire in contact with the solution. A capillary temperature of 200 °C was applied and a trap gas flow rate of 5–9 was used, depending on the size of the system studied with higher gas flows used for larger systems. The resolution, as defined at  $m/z$  400, was set to 3,125 for all complexes studied here. In negative mode, the protein complex signal can sometimes be lower in intensity than in positive mode, and for these studies we found that obtaining sufficient signal required higher voltages to be applied across the source optics. Positive mode data were acquired using the same source settings as negative to enable comparisons to be made between polarities using the same instrumental settings. Typical settings used here for transmission mode and SID can be found in the Supporting Information (Table S1). SID is reported here as SID energy (voltage $\times$ charge state). For comparisons of different charge states of the same protein, different SID voltages were chosen in order for similar energies to be compared.

## RESULTS AND DISCUSSION

We first considered C-reactive protein (CRP), a 115 kDa model cyclic homopentameric protein complex which has been previously studied with SID.<sup>39,40,45</sup> We studied CRP ionized in

positive mode and prepared under both “normal” charge conditions (200 mM ammonium acetate (AmAc)), and “charge-reduced” conditions (160 mM AmAc + 40 mM triethylammonium acetate (TEAA)).<sup>25,48</sup> Charge-reducing conditions result in a lowering of the average charge state from  $+23.38 \pm 0.58$  to  $+17.47 \pm 0.16$  (Figure S1). For SID studies under each condition we selected the most abundant charge state, namely 24+ for normal charge and 18+ for the charge reduced complex. The lower average charge states observed in TEAA are commonly exploited in structural studies, particularly using SID. Lower charged protein complexes can be more compact and less likely to be restructured; they also have a lower propensity to unfold or expand upon collision and can therefore retain a greater number of subunit contacts after dissociation, and hence increased structural information can be obtained from SID of the lower charged species, as discussed previously and demonstrated in Figure 1.<sup>38,39</sup> Low energy SID (1152 eV) of

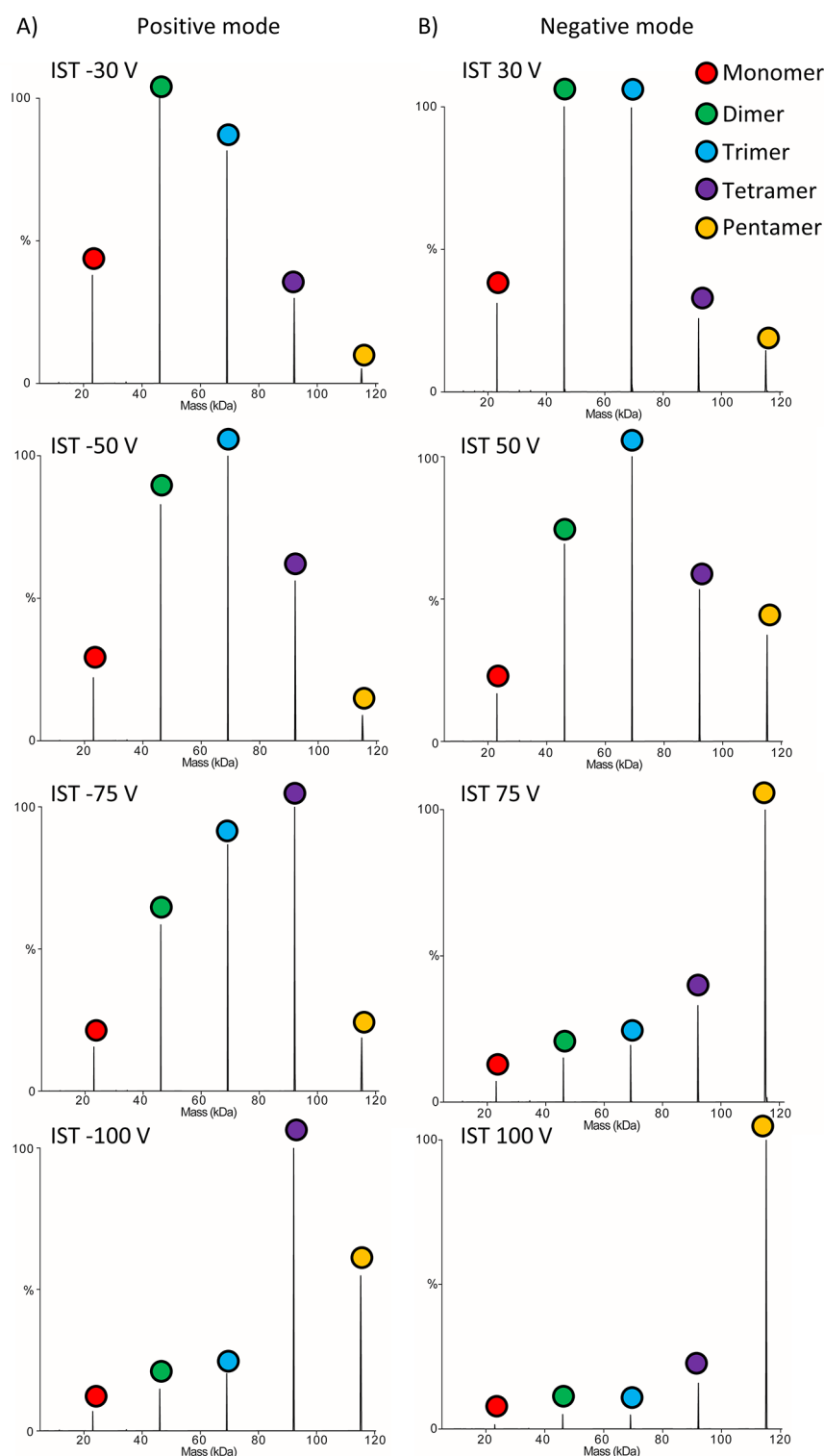


**Figure 1.** Low-energy SID of CRP, acquired with an in-source trapping voltage of  $\pm 30$  V. (A) 24+ CRP from 200 mM AmAc acquired at an SID energy of 1152 eV, (B) 18+ CRP from 160 mM AmAc + 40 mM TEAA, acquired at an SID energy of 1152 eV, and (C) 19- CRP from 200 mM AmAc, acquired at an SID energy of 1140 eV. Inset is cartoon representation of structure (PDB 1GNH).<sup>49</sup> Deconvoluted data are shown in Figure S2.

the 24+ CRP pentamer in AmAc produces primarily monomer and dimer with some low intensity trimer also observed, Figure 1A and Figure S2A. However, the lower-charged CRP pentamer (18+) fragments to monomer+tetramer and dimer+trimer with low energy SID (1152 eV) as shown in Figure 1B and Figure S2B, consistent with the cyclic structure of this complex.<sup>49</sup> Hence, the addition of TEAA and dissociation of lower-charge states is advantageous in SID studies, allowing all subcomplexes to be observed consistent with the lower charge species retaining greater subunit contacts upon dissociation than their AmAc counterparts for which only the smaller subcomplexes are observed. However, the addition of TEAA

can often result in increased adduction and hence increased peak width,<sup>28</sup> which can be problematic in ligand binding studies when high mass accuracy is required. Given the previous reports of lower average charge states for proteins when analyzed in negative ionization mode, we next studied CRP prepared in 200 mM AmAc but ionized in negative ionization mode. In negative mode, nESI the average charge state of CRP is significantly lower than when analyzed in positive mode ( $-18.98 \pm 0.08$  vs  $+23.38 \pm 0.58$ , Figure S1). Interestingly, the average charge state is only slightly higher in negative mode than when analyzed under charge reducing conditions in positive mode ( $-18.98 \pm 0.08$  vs  $+17.47 \pm 0.16$ ). To determine if the same level of structural information could be obtained from CRP anions as was obtained for charge-reduced cations, we subjected the most abundant charge state (19-) to low energy SID (1140 eV), Figure 1C and Figure S2C. SID of 19- CRP produced monomer+tetramer and dimer+trimer, consistent with the solved pentameric ring structure, suggesting that the lower charge states obtained from negative mode ionization can be exploited in protein structural studies.

Previous reports have shown that SID can probe structural rearrangement in addition to native structures.<sup>40,50</sup> One such study intentionally activated CRP using increasing voltages in the source region and then measured the unfolding/rearrangement with ion mobility and SID.<sup>40</sup> To determine if negative mode SID can also be used to probe structural rearrangements, we performed a similar experiment in this case utilizing increasing in-source trapping voltages on the UHMR instrument. We considered CRP prepared under charge reducing conditions in positive mode and in AmAc in negative mode, both of which were able to provide structural information with SID with gentle in-source trapping (Figure 1). In both cases, increasing the in-source trapping voltage from  $\pm 30$  V to  $\pm 100$  V appeared to have only a minor effect on the full MS (MS1), with the pentamer remaining the dominant product with only minor shifts in the charge state distribution observed and the 18+ and 19- charge states remaining the most abundant (Figure S3). When SID was performed on the dominant charge state (18+ or 19-) at low in-source trapping voltages, we see fragments consistent with a cyclic pentamer, monomer+tetramer, and dimer+trimer (Figure 2 and Figure S4). However, as the in-source trapping voltage is increased we begin to see decreased dissociation by SID, with higher abundance of the precursor remaining, and a shift in the dissociation products produced in both positive and negative mode. The more abundant remaining precursor could be due to either the restructured form being more stable or, as a recent study has suggested, the fact that there is reduced energy deposition in SID following unfolding due to the decreased protein-surface collisional cross section.<sup>35</sup> Because different product ion distributions are noted after in-source trapping (or in-source activation in general), for both CID and SID, we favor the explanation that the restructured form has a different stability.<sup>40</sup> At high in-source trapping voltages, the dimer+trimer products decrease in intensity and the majority of pentamer dissociates to monomer+tetramer by SID. This shift in the observed SID dissociation pathway, as in-source trapping voltage is increased, is consistent with the protein restructuring/unfolding during in-source trapping activation and SID measuring the altered structure. It should be noted that different charge states are studied in negative vs positive mode (19- vs 18+); this could, in-part, explain why the shift in



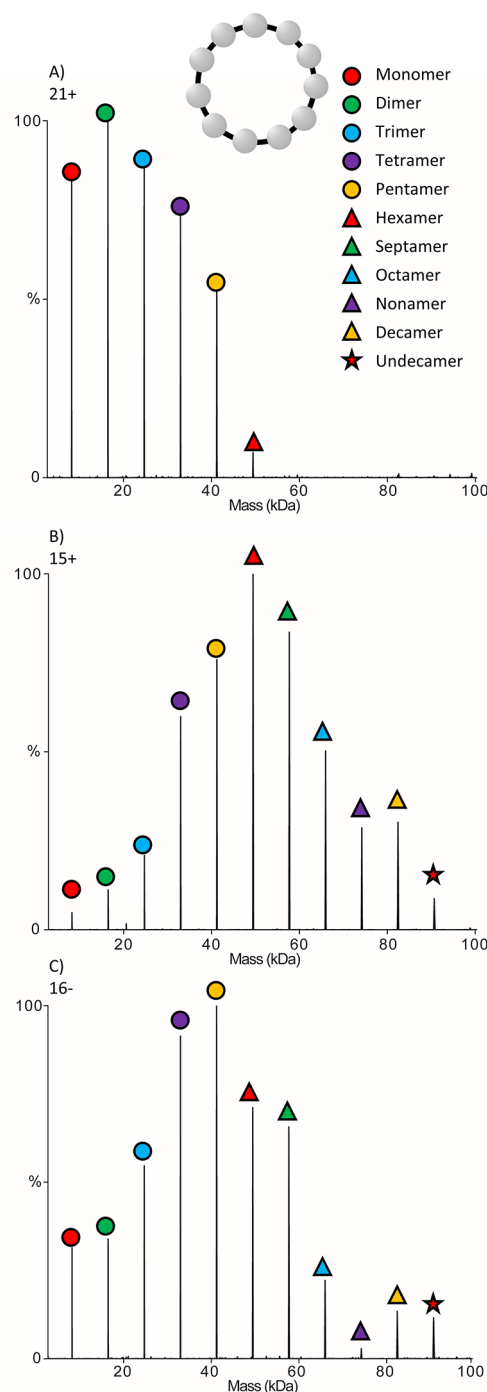
**Figure 2.** Deconvolved (mass-only domain) low-energy SID of CRP, acquired with increasing in-source trapping voltages. (A) 18+ CRP from 160 mM AmAc + 40 mM TEAA, acquired at an SID energy of 1152 eV and (B) 19- CRP from 200 mM AmAc, acquired at an SID energy of 1140 eV. Data deconvolved using UniDec;<sup>51</sup> raw data can be found in Figure S4.

dissociation patterns occurs at lower in-source trapping voltages in negative mode compared with positive mode. It is also possible that the different location of charges on the anions vs cations could result in gas-phase structural differences and different patterns of restructuring during in-source trapping. In future work, collision-induced unfolding may help characterize these differences. The fact that the

expected fragmentation pattern without source activation and altered fragmentation pattern after in-source trapping are observed in both positive mode and negative mode suggests that under gentle desolvation and instrumental conditions native protein structures can be preserved and studied in nMS using either polarity and that, like positive mode, negative mode SID can be used to probe structural changes.

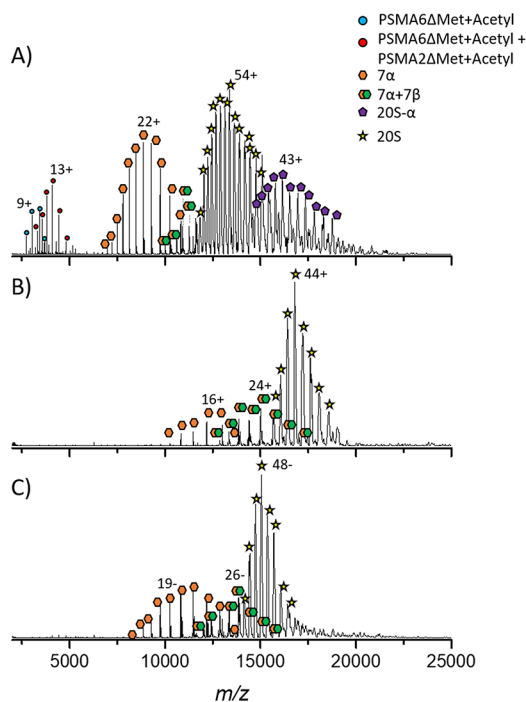
We next considered Trp RNA binding attenuation protein (TRAP), which is a 91 kDa cyclic homo 11-mer. Like CRP, TRAP presents with a lower average charge state in negative mode in 400 mM AmAc compared to positive mode ( $-16.17 \pm 0.08$  vs  $+21.08 \pm 0.25$ ) and with a slightly higher average charge in negative mode than when prepared in charge reducing conditions, 320 mM AmAc + 80 mM TEAA ( $+15.27 \pm 0.10$ ), as shown in Figure S5. Under each of the three conditions studied here we selected the most intense charge state for SID studies, 21+, 15+, and 16-. TRAP is a homomeric cyclic 11-mer, with equal interfaces between subunits (see inset in Figure 3), and therefore by SID we would expect it to dissociate to all possible subcomplexes (monomer to decamer). When TRAP is prepared in AmAc and subjected to low energy SID, multiple dissociation products are observed from monomer to hexamer, but the larger multimers expected for this complex are not observed (Figure 3A and Figure S6A), consistent with previous studies for non-charge reduced samples; this may reflect secondary dissociation of the initial higher order oligomeric products.<sup>39</sup> Under charge reducing conditions in positive mode, all expected oligomers can be observed from monomer to undecamer, consistent with the cyclic structure of this complex (Figure 3B and Figure S6B). When the lower charge state TRAP anions are studied by SID, like CRP, structural information can also be obtained with all expected subcomplexes observed (Figure 3C and Figure S6C). A shift to the lower oligomeric states is observed for the 16- TRAP compared to the 15+ TRAP which could be due to the higher charge state species studied in negative mode. This once again highlights that it is possible to utilize the lower charge state anions for structural studies using SID, without having to add any solution additives.

Finally, we wanted to see if this approach could be extended to larger, hetero-oligomers so we also considered human 20S proteasome. The 20S proteasome is composed of 28 subunits (14  $\alpha$ -type subunits and 14  $\beta$ -type subunits), which are arranged in a cylindrical structure consisting of four heptameric rings: the two outer rings are comprised of  $\alpha$ -type subunits, and the two central rings are comprised of  $\beta$ -type subunits ( $\alpha_7\beta_7\beta_7\alpha_7$ ).<sup>52</sup> As expected based on the results above, the average charge state decreases for proteasome when analyzed in 150 mM AmAc in negative mode compared to positive mode (centered around 49- vs 60+) and is slightly higher than when analyzed in 120 mM AmAc + 30 mM TEAA (centered around 49- vs 44+), Figure S7. In addition, under the soft instrumental conditions employed here, the average measured mass is similar when prepared in AmAc and analyzed in positive or negative mode ( $724\,056 \pm 301$  Da vs  $724\,317 \pm 267$  Da) and slightly larger than the theoretical mass of 716 224 Da due to adducting and non-covalent binding of the FLAG peptide (1012.97 Da) present in solution from purification. An increase in the measured mass is observed when the sample is prepared in AmAc+TEAA and analyzed in positive mode ( $727\,788 \pm 382$  Da), demonstrating one of the challenges of using charge reducing reagents. For human 20S proteasome a single charge state was not selected for SID studies; instead a wide isolation window was applied to include the whole charge state distribution, and average energies listed are determined from the average charge state. Under all three conditions studied here (AmAc positive mode, AmAc+TEAA positive mode, and AmAc negative mode) at low voltage SID, both the  $\alpha_7$  ring and  $\alpha_7+\beta_7$  half proteasome are observed,



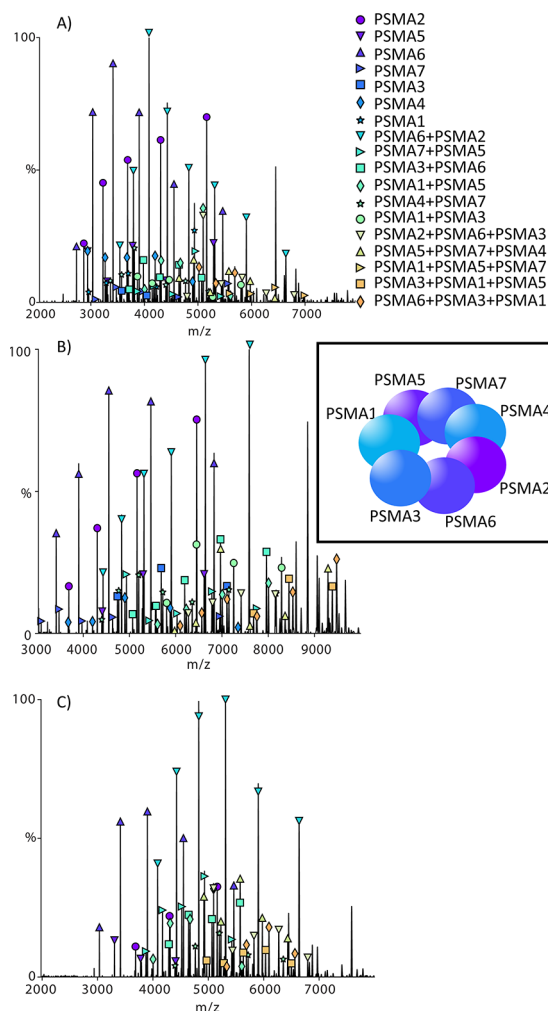
**Figure 3.** Low-energy SID of TRAP, acquired with an in-source trapping voltage of  $\pm 5$  V. (A) 21+ TRAP from 400 mM AmAc acquired at an SID energy of 1050 eV, (B) 15+ TRAP from 320 mM AmAc + 80 mM TEAA, acquired at an SID energy of 1050 eV, and (C) 16- TRAP from 400 mM AmAc, acquired at an SID energy of 1056 eV. Data deconvolved using UniDec;<sup>51</sup> raw data can be found in Figure S6.

consistent with the structure and previous SID studies (Figure 4 and Table S2).<sup>32</sup> PISA interfacial analysis reveals that the  $\alpha$ - $\beta$  interface is smaller and, therefore, can be considered weaker than the  $\beta$ - $\beta$  interface; therefore, we would expect low energy SID to cleave the  $\alpha$ - $\beta$  interface producing a free  $\alpha_7$  ring and, if enough energy is input, to also cleave the  $\beta$ - $\beta$  interface, consistent with products observed.<sup>44,52,53</sup>



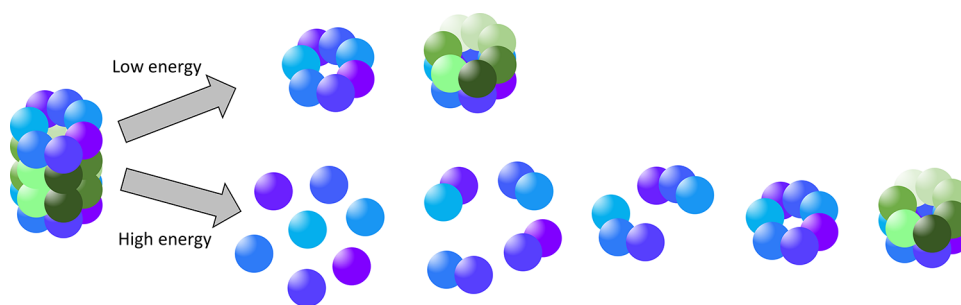
**Figure 4.** Low-energy SID of human proteasome, acquired with an in-source trapping voltage of  $\pm 50$  V. (A) Proteasome in 150 mM AmAc in positive mode (average precursor charge state +60.9) acquired at an SID voltage of 90 V (5481 eV), (B) proteasome in 120 mM AmAc + 30 mM TEAA in positive mode (average precursor charge state +44) acquired at an SID voltage of 130 V (5720 eV), and (C) proteasome in 150 mM AmAc in negative mode (average precursor charge state  $-49.1$ ) acquired at an SID voltage of 110 V (5401 eV). Note that experimental masses are higher in TEAA due to increased adducting on the precursor and are given in Table S2. Average charge states of precursors were determined from UniDec.<sup>51</sup>

In addition, some free  $\alpha$  monomer and dimers are observed when the 20S proteasome is analyzed in positive mode from AmAc (Figure 4A); this is not observed under the other conditions despite SID being performed at similar energies. The observation of monomer and dimer from low energy SID for 20S in AmAc may be due to higher charge state precursors being restructured before collision or more susceptible to unfolding upon surface collision, enabling these products to be observed. While it is possible that the higher charge states could be Coulombically less stable, previous SID studies for dendrimers demonstrated that intramolecular Coulomb repulsion within multiply protonated species did not result in easier fragmentation.<sup>54</sup> While the production of  $\alpha$  monomer does not provide structural insight, it does provide information on the proteoforms present in the sample and, hence, could provide insight in functional studies. Increasing the SID voltage allows higher intensity  $\alpha$  monomer, dimers, and trimers to be observed; therefore information on the proteoforms present for  $\alpha$  monomers can be obtained (Figure 5, Figure S8, and Table S3). In this case we find that positive mode provides the most extensive coverage of proteoforms, with AmAc having the greatest coverage as is expected given that under these conditions the highest charge states and most extensive dissociation of the 20S proteasome are observed. Therefore, for full  $\alpha$  subunit proteoform characterization of the 20S proteasome by SID, AmAc in positive mode would be preferred. However, further structural information can be



**Figure 5.** High energy SID of human 20S proteasome (A) in 150 mM AmAc in positive ionization mode (average charge state +60.9), 170 V SID (10, 353 eV), (B) in 120 mM AmAc + 30 mM TEAA in positive ionization mode (average charge state +44.2), 240 V SID (10, 608 eV), and (C) in 150 mM AmAc in negative ionization mode (average charge state  $-49.1$ ), 210 V SID (10,311 eV). Shown here is a zoom in of the low mass region, highlighting  $\alpha$ -monomers, dimers, and trimers; the full MS range is shown in Figure S8. Inset is cartoon representation of the  $7\alpha$  ring, with connectivity determined from the crystal structure.<sup>52</sup> The average charge states of precursors were determined from UniDec.<sup>51</sup>

obtained by considering the  $\alpha$  dimers and trimers observed (Figure 5 and Table S4), as these can be used to confirm connectivity in the  $7\alpha$  ring.<sup>44</sup> In all three conditions, sufficient coverage of the dimers and trimers are observed to enable the full connectivity of the  $7\alpha$  ring to be determined. It should be noted, however, that generally higher mass errors are observed for the precursor and products when prepared in TEAA, consistent with increased adducting under these conditions. The combination of low and high energy SID allows more complete characterization of the 20S proteasome, with low energy SID giving substructural information and high energy SID providing proteoform information, as demonstrated in Figure 6. This demonstrates that negative mode SID can be used to obtain substructural information on protein complexes of various sizes and complexity and has the advantage of generating lower charge states without the need for additives that cause peak broadening and a corresponding decrease in



**Figure 6.** Cartoon representation of the major fragment ions produced for the 20S proteasome at low and high energy.

spectral quality. Therefore, in the future, preparing samples in AmAc and analyzing by both positive and negative mode SID could provide increased structural and proteoform characterization.

## CONCLUSIONS

Understanding how proteins assemble into multicomponent complexes and how subunits within the complex interact remains a cornerstone of structural biology. It has been previously demonstrated that surface-induced dissociation is a useful tool for studying protein complex substructure and connectivity. When SID studies are performed on proteins prepared in AmAc in positive mode ionization, the larger substructural fragments are often missing with higher intensity monomer, dimer, etc. often being observed instead. Therefore, SID studies are routinely performed under charge reducing conditions, typically through the addition of TEAA, to obtain the most native-like fragmentation and hence substructural information; however, the addition of TEAA can often lead to increased adducting and peak broadening. Lower charge states can, however, be generated from negative mode ESI without the need for additives. We demonstrated here that those lower charge state anions fragment in a manner consistent with the native structure and can be used to obtain substructural information for protein complexes. For the human 20S proteasome, preparing the sample in AmAc and analyzing in positive mode (highest charge states) allowed for in-depth proteoform characterization, whereas, charge reduced conditions can provide substructural information namely predominantly  $7\alpha$  at low energies. Therefore, for future studies it may be advantageous to prepare samples in AmAc and perform structural SID studies in negative mode and proteoform characterization in positive mode, all from the same sample solution. The results presented here demonstrate that nMS and SID can be used for structural characterization at multiple levels from stoichiometry and subunit connectivity to identification of posttranslational modifications. The ability to do this in a single experiment enables rapid and detailed structural characterization to be performed on low sample amounts and is applicable to even heterogeneous samples.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.1c00855>.

Additional figures (PDF)

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### Notes

The authors declare no competing financial interest.

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