

1Analyzing slowly exchanging protein conformations by ion mobility mass 2spectrometry: study of the dynamic equilibrium of prolyl oligopeptidase

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15Keywords: protein dynamics, ion mobility mass spectrometry; conformational
16equilibrium, native mass spectrometry, gas-phase protein ions

17

18Abstract

19Ion mobility mass spectrometry (IMMS) is a biophysical technique that allows the
20separation of isobaric species on the basis of their size and shape. The high separation
21capacity, sensitivity and relatively fast time scale measurements confers IMMS great
22potential for the study of proteins in slow (μ s-ms) conformational equilibrium in
23solution. However, the use of this technique for examining dynamic proteins is still not
24generalized. One of the major limitations is the instability of protein ions in the gas
25phase, which raises the question as to what extent the structures detected reflect those in
26solution. Here we addressed this issue by analyzing the conformational landscape of
27prolyl oligopeptidase (POP)-a model of a large dynamic enzyme in the μ s-ms range-by
28native IMMS and compared the results obtained in the gas phase with those obtained in
29solution. In order to interpret the experimental results, we used theoretical simulations.
30In addition, the stability of POP gaseous ions was explored by charge reduction and
31collision induced unfolding experiments. Our experiments disclosed two species of POP
32in the gas phase, which correlated well with the open and closed conformations in
33equilibrium in solution; moreover, a gas-phase collapsed form of POP was also
34detected. Therefore, our findings support the potential of IMMS for the study of
35multiple co-existing conformations of large proteins in slow dynamic equilibrium in
36solution, but also stress the need for careful data analysis to avoid artefacts.

37

38Introduction

39Mass spectrometry (MS) is becoming an established methodology in structural biology¹.
40One of the most important breakthroughs of MS in this field was the development of
41soft ionization techniques, namely electrospray ionization² (ESI) and nanoflow

42electrospray ionization³ (nanoESI). Soft ionization techniques allowed native MS,
43which pushed the limit of MS beyond mass analysis and quantification^{4,5}. In particular,
44native MS was crucial for the application of ion mobility MS (IMMS) in structural
45biology. Ion mobility (IM) is based on the time that analyte ions take to cross a cell
46filled with a buffer gas under the effect of an electric field (which is known as drift time,
47 t_d)^{6,7}. The mobility of ions strongly depends on their collision cross-section (CCS),
48which is defined as the orientationally averaged area in which the protein ion collides
49with buffer gas molecules. Hence, coupled IMMS devices allow the simultaneous
50separation of gaseous protein ions not only on the basis of their mass but also on their
51size and shape⁸. IMMS has been successfully applied in the structural characterization
52of proteins and non-covalent protein complexes⁹⁻¹¹, in the study of the self-aggregation
53of pathogenic proteins¹²⁻¹⁴, and in the detection of non-canonical DNA secondary
54structures^{15,16}, among others¹⁷.

55Conformational dynamics is crucial for the biological function of proteins¹⁸. In
56particular, the slow dynamic motions of proteins in the time scale of μ s-ms are closely
57linked to enzyme catalysis¹⁹, allosteric regulation²⁰, and protein-protein interactions²¹.
58However, elucidating protein structures that fluctuate as a function of time requires
59biophysical techniques with fast experimental time scales that are sensitive to minor and
60transiently populated species. In this regard, several methods have been developed, for
61instance, solution nuclear magnetic resonance²² (NMR) and small-angle X-ray
62scattering²³ (SAXS).

63Interestingly, IMMS is a promising MS-based technique for the study of proteins in
64slow conformational equilibrium^{24,25}. The main advantage of this method with respect to
65amide hydrogen-deuterium exchange coupled to MS²⁶ is its capacity to obtain direct
66measurements of the CCS of transient species co-existing in solution. IMMS has
67allowed the detection of minor populated conformational ensembles of intrinsically
68disordered proteins in dynamic equilibrium in solution. Hence, different conformational
69populations are observed in the gas phase for the intrinsically disordered proteins high
70mobility group A (HMGA)²⁷ and some domains and constructs of p53 protein²⁸. This
71technique also allowed the detection of transiently populated oligomeric species of β_2 -
72microglobulin^{12,13}, α -synuclein^{29,30} and the amyloid peptides A β 40/42^{14,31,32}, thus
73providing further insights into the self-aggregation mechanisms of these IDPs. Fewer
74examples are found in the literature concerning structured dynamic proteins analyzed by
75IMMS. For instance, the outward and inward conformations of P-glycoprotein were
76detected in the gas phase by IMMS³³, as well as the two well-characterized
77conformations of the multidomain protein NADPH-cytochrome P450 reductase³⁴. In the
78case of the ICL₁₂ ATPase subcomplex, the high conformational heterogeneity caused by
79the rotation of subunit I was manifested as increased peak broadening in the ion
80mobilograms³⁵. However, the application of IMMS to proteins in conformational
81equilibrium remains controversial. The stability of protein ions in the absence of solvent
82is a major concern when studying native protein structures^{10,36}, questioning the extent to
83which structures in dynamic exchange in solution are transferred to the gas phase^{37,38}.
84Here we used prolyl oligopeptidase (POP), an 81-KDa serine peptidase, as a model of a
85large protein populating various well-defined conformations in slow equilibrium in
86solution.

87POP is formed by two domains, namely the α/β -hydrolase and the β -propeller³⁹ (Figure 881 A). *In vitro*, this enzyme hydrolyzes short proline-containing peptides in the carboxyl 89side of proline⁴⁰. The *in vivo* role of POP involves protein-protein interactions⁴¹. In 90particular, recent studies have revealed that POP is involved in the clearance of α - 91synuclein and α -synuclein aggregates⁴²⁻⁴⁴, which are the toxic species responsible for 92Parkinson's disease. POP is likely to benefit from conformational dynamics in order to 93execute this function^{45,46}. In this regard, using relaxation dispersion NMR and SAXS 94experiments, we have recently demonstrated that POP is in a slow ms equilibrium 95between open and closed conformations. This equilibrium arises from the hinge 96separation between the α/β -hydrolase and the β -propeller domains occurring in 97solution⁴⁷ (Figure 1 B).

98In the present study we tested the capacity of native IMMS to examine multiple co- 99existing protein conformers in slow equilibrium in solution using a commercial Synapt 100G1 mass spectrometer⁴⁸ and POP as a molecule of interest. The results obtained in the 101gas phase were correlated with our previous results obtained in solution⁴⁷ by means of 102computational simulations performed under different conditions. Moreover, the effect of 103the Coulomb repulsion and the stability of native structures of POP in the gas phase 104were examined by performing charge reduction and collision induced unfolding (CIU) 105experiments, respectively. Remarkably, the IMMS experiments detected two species of 106POP that correlated well with the open and closed conformations in dynamic 107equilibrium. Therefore, our findings emphasize the value of IMMS for the study of 108multiple co-existing conformations of large proteins in slow equilibrium in solution.

109

110Materials and methods

111Chemicals and protein expression

112All chemicals used for the preparation of MS buffers were of analytical grade and were 113purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used in buffers was 114LC-MS grade and was purchased from the same company. Cells were provided by 115Novagen (Merck-Millipore, Darmstadt, Germany). Affinity and size exclusion 116chromatography columns were purchased from GE Healthcare (Little Chalfont, UK). 117Buffer exchange columns were from Bio-Rad (Hercules, CA, USA).

118POP was expressed in *E.coli* cells using pET-11 plasmid containing the human POP 119gene, following our standard protocol⁴⁷. In order to eliminate interferences with the CCS 120measurements, HisTag was removed by digestion with TEV protease. Gel filtration 121chromatography in the last step of protein purification was critical to achieve 122satisfactory quality and reproducibility of the results. Given that POP is sensitive to pH 123and ionic strength⁴⁹, we used 50 mM NH₄AcO pH = 8 as native MS buffer. POP showed 124adequate stability and retained the enzymatic activity in this buffer. Moreover, NH₄AcO 125favored the stability of native structures in the gas phase⁵⁰ and prevented the formation 126of adducts. In order to perform buffer exchange, HisTag-cleaved POP samples in 127storage buffer (50 mM Tris, 20 mM NaCl, pH = 8) were doubly exchanged to MS buffer 128with Micro Bio-Spin P-30 columns immediately prior to the experiments; similarly, 129protein standards used for calibration were doubly buffer-exchanged with Micro Bio-

130 Spin P-6 columns. Typically, a protein concentration of 10-15 μM was used in all
131 experiments. For charge reduction experiments, sample preparation was identical, but
132 using 50 mM NH_4AcO with 0.05 % imidazole pH = 8 as buffer. All experiments
133 consisted of triplicate protein batches expressed and purified separately.

134 Optimization of the instrumental conditions

135 All experiments were carried out with a Synapt G1 spectrometer (Waters, Manchester,
136 UK)⁴⁸. A chip-based Triversa Nanomate nanoESI source (Advion Inc., NY, USA)
137 operating in the positive mode was used. In order to obtain native POP ions and avoid
138 unfolding, instrumental conditions were optimized near threshold values. The capillary
139 potential, trap collision energy (CE) and trap DC bias were crucial to ensure the stability
140 of native POP structures⁵¹. These parameters were therefore kept at the minimum. In
141 particular, we chose a capillary potential of 1.75 kV, a trap CE of 6 V, and a trap DC
142 bias of 15. In turn, backing pressure was minimized to 3 mbar to prevent structural
143 alterations⁵². The remaining parameters were the following: 20 V for the sampling cone;
144 1 V for the extraction cone; and 6 V for transfer collision energy. Automatic trap release
145 was used in all experiments. In the case of CIU experiments, the trap CE voltage was
146 incremented in a step-wise fashion while maintaining other instrumental values⁵¹. In
147 particular, trap CE voltages were sampled at 6 (native), 15, 20, 25, 30 and 35 V. For the
148 IM separation we used N_2 as buffer gas, at a pressure of 0.46 bar and flow of 24
149 ml/min⁵²; in turn, the IM resolution was optimized by tuning simultaneously the
150 travelling wave height and velocity⁵². Hence, the T-wave velocity was tuned at 300 m/s,
151 while triplicate experiments at various T-wave heights (8, 9 and 10 V) were
152 systematically performed in order to prevent electric field inhomogeneities^{52,53}. The TOF
153 mass analyzer was tuned and calibrated between 500 and 8000 m/z, in a V reflector
154 mode. For data processing, MassLynx v.4.1 SCN 704 and Driftscope v.2.4 programs
155 were used. In order to perform Gaussian fitting, ion mobility traces were extracted
156 manually from Driftscope and exported as numerical lists. Afterwards, Gaussian
157 functions were adjusted with the GraphPad Prism 4 software (La Jolla, CA, USA).

158 Extraction of experimental and theoretical CCSs

159 Experimental CCSs were extracted following the native calibration protocol described
160 by Bush and co-workers⁵⁴ (see Supporting information). Transthyretin tetramer, bovine
161 serum albumin, and concanavalin A were the native standard proteins used in the
162 calibration of POP ions. Calibration plots were created using the tabulated CCSs of
163 native protein standards in N_2 . CCSs were extracted from the experimental t_d with the
164 calibration plot. Afterwards, results were averaged for all replicates, and the error was
165 given as the standard error of the mean. The use of N_2 must be considered when
166 comparing experimental and theoretical CCSs. The calculations used He as *in silico*
167 buffer gas⁵⁴, which yields smaller values than those determined experimentally in N_2 ⁵⁵.
168 The theoretical determination of CCSs was performed with the MOBCAL program^{56,57}
169 using the atomic coordinates of POP structures. The trajectory method was used, and a
170 representative charge of +20 was assigned for the calculations.

171 Computational simulations of POP under different conditions

172 Replica exchange molecular dynamics (REMD) simulations of POP were performed
 173 with explicit TIP3P water molecules⁵⁸. The protonation state of residues corresponding
 174 to the default protonation state determined by GROMACS^{59,60} at neutral pH was used in
 175 REMD I. In order to promote conformational rearrangements in REMD II, the
 176 protonated state of Asp, Glu and His residues was used. An ionic strength of 0.1 M of
 177 NaCl was used, and periodic boundary conditions were applied in a system of about
 178 $10 \times 10 \times 10$ nm³. First, the system was equilibrated by means of 100 ps of a MD
 179 calculation with protein heavy atoms restrained. REMD simulations were performed
 180 using 18 replicas distributed over range from 300 to 327 °K, where multiple replicas of
 181 identical systems were simulated in parallel at various temperatures. The frequency of
 182 exchange was selected to have an average accepted exchange every 400 ps for each
 183 temperature. REMD I and II simulations were performed for a simulation time of 8.4
 184 and 20 ns, respectively. The structure with a detached loop A obtained in the replica
 185 with highest temperature of REMD II (see Results and discussion section) was used as a
 186 starting structure for an additional 20-ns REMD simulation at neutral pH (REMD III).
 187 We used 75 and 187 structures of REMD I and III simulations, respectively, to calculate
 188 the theoretical CCSs as described previously (see Supporting information).

189 Classical MD simulations in vacuum were also performed with 10 distinct protonation
 190 states of a +20 charge state of POP. All simulations were performed using GROMACS
 191 software package version 4.5.5⁵⁹ with the AMBER03 force field in the NVT ensemble.
 192 An integration time step of 2 fs was used. A cut-off value of 1.0 nm was fixed for Van
 193 der Waals interactions; electrostatic interactions were treated with the particle mesh
 194 Ewald method, applying a real-space cut-off of 1.0 nm. No cut-off was used for either
 195 electrostatic or Lennard-Jones interactions in the gas phase. A representative charge
 196 state of +20 was chosen for in vacuum MD simulations. A semi-grand canonical Monte
 197 Carlo procedure was designed to provide a pool of microstates with a protonation
 198 distribution of charged residues compatible with this overall charge.
 199 Protonation/deprotonation processes of ionizable residues in vacuum depend on gas-
 200 phase basicities. We estimated these basicities from pKa values obtained in solution,
 201 assuming that this approximation will not significantly affect the final protonation states
 202 in the gas phase. The Monte Carlo algorithm considered the free energy associated with
 203 the protonation/deprotonation process of each charged residue according to the
 204 following equation (1)^{61,62}:

$$205 \quad \Delta G = \pm k_B T (\ln(10)) (pH - \log(K_i)) \quad \text{Equation 1}$$

206 A positive sign was used for protonation, whereas a negative sign was used for
 207 deprotonation. K_i is the protonation constant of each charged residue i , obtained from
 208 the PropKa program through the web server⁶³. Monte Carlo simulations were performed
 209 using a pH value of 5, in a total of 100000 steps. Then, for the 10 selected microstates,
 210 MD simulations were carried out for 6 ns. In order to reproduce the conditions in the IM
 211 cell, an effective temperature of 313°K was used in the calculations. This microscopic
 212 temperature was obtained by adapting the Mason-Schamp equation⁶ in order to take into
 213 account field heating⁶⁴ (Equation 2):

$$214 \quad T_{eff} = T_{gas} + T_{field} = 298 + \frac{M_N}{3 k_B} \left[\frac{2 v_d s^2}{s + v_d} \right] \quad \text{Equation 2}$$

215 Where M_N is the mass of a molecule of buffer gas (in kg), k_B is the Boltzmann constant,
216 s is the TWIMS wave velocity (300 m/s), and v_d is the drift velocity of the analyte ion
217 (which was estimated as 26.4 m/s). Structures were sampled at 11 time points during the
218 production run, from 1.5 to 6 ns in 500-ps intervals (see Figure S3 of the Supporting
219 information). Finally, the CCS was averaged for all 10 simulations (110 final
220 structures).

221

222 Results and discussion

223 To date, the study of dynamic proteins by IMMS has focused mainly on intrinsically
224 disordered proteins. These proteins populate a broad conformational space in fast
225 equilibrium, thus challenging IM separation. Therefore, in order to evaluate the capacity
226 of IMMS to characterize protein conformations in dynamic equilibrium, two main
227 issues must be considered. First, the need of reference data based on solution-based
228 experimental techniques, which allows the interpretation and comparison of the results
229 obtained in the gas phase with those obtained in solution. Second, the compatibility
230 between the time scale of the conformational exchange and the time resolution of the
231 IM separation, which typically lies in the range of several ms⁶⁵. For these reasons, here
232 we analyzed the slow conformational equilibrium of POP by IMMS and established the
233 correlation between the results obtained in the gas phase with those obtained with
234 solution-based techniques⁴⁷.

235 Native POP ions in the gas phase

236 Native mass spectra of POP showed +17 to +21 charge states, the most populated being
237 +18, +19 and +20 (Figure 2 A). Extracted ion mobilograms disclosed various peaks,
238 corresponding to partially resolved species of POP in the gas phase (Figures 2 A and B).
239 As shown, the populations of these species depend on the charge state. In order to
240 quantify these species, the ion mobilograms were individually fitted to Gaussian
241 functions following Pujol-Pina *et al.*¹⁴ (Figure 2 A). In the case of +17 charge state, a
242 single major population was present, namely species A. In the case of the +18 charge
243 state, two additional peaks were observed at higher t_d , named as species B and C.
244 Hence, the ion mobilogram was adjusted to three Gaussian functions corresponding to
245 species A, B and C. The fitting to three Gaussian functions was also performed for +19
246 and +20 charge states. Finally, in the case of the +21 charge state, only two Gaussian
247 functions corresponding to species B and C were fitted. In order to monitor the
248 dependence of the occurrence of species on the charge state, the relative population was
249 calculated for each i specie according to the following formula (Equation 3):

$$250 \text{ Relative population}(i) = \frac{A_i}{A_A + A_B + A_C} \text{ Equation 3}$$

251 Where A_X is the area of the fitted Gaussian function of a given species. The plot of the
252 relative populations as a function of the charge state is shown in Figure 3 A (upper
253 panel). The relative population of species A decreased as a function of the charge state,
254 while that of species B was relatively constant between +18 and +21 charge states.
255 Conversely, the relative population of species C increased with the charge state.

256Next, the experimental CCSs of species A, B and C were calculated from t_d values using
257the native calibration protocol⁵⁴, as described in the Materials and methods section
258(Figure 3 A, lower panel). In all species, the CCSs increased steadily as a function of the
259charge state. This phenomenon is attributed to the higher Coulomb repulsion
260experienced by multiply charged gaseous ions in the absence of solvent, which causes
261an expansion of the tertiary structure^{10,66}. The experimental CCSs summarized in Table 1
262(left panel) reveal large differences between species (approximately 7% between species
263A and B, and 10% between species B and C). According to these values, the three
264species of POP in the gas phase can be assigned to different conformations, probably
265originated by large-scale rearrangements. Overall, these results correlate with the charge
266state distribution: the increased population of species C at higher charge states reflects
267the higher exposure of protonated residues in conformations with a larger CCS^{9,67}.

268Structural properties of gaseous POP species

269Analysis of the structural features of POP species in the gas phase requires the
270combination of experimental and simulated data. For this reason, we explored the
271conformational space of POP in solution and in the gas phase by performing simulations
272under different conditions. The theoretical CCSs of the simulated structures were
273calculated with the MOBCAL program^{56,57} (see Materials and methods). This procedure
274allowed us to establish correlations between the simulated structures of POP and the
275experimental CCS values.

276First, we considered the open conformations of POP in solution described in our
277previous work⁴⁷. Since these structures were obtained by a hybrid approach combining
278SAXS experiments and MD simulations, we directly used the coordinates for the
279calculation of the theoretical CCSs. In particular, the three structures displaying larger
280interdomain angles (measured as the angle formed by residues 582-71-177) were
281selected. As seen in Figure 3 A and Table 1, the theoretical CCS of the open
282conformation obtained with this approach was in good agreement with that of species C
283in the gas phase. Given the significant broadening of the IM peak, it can be speculated
284that species C correspond to an ensemble of open conformations of POP featuring an
285interdomain separation similar to that found in solution. It cannot be ruled out that the
286broad distribution also includes several structures originated by gas-phase
287rearrangements during the IM experiments, especially considering that the population of
288these species increases under collisional activation (see below). According to the
289literature, compact states of proteins have been found to undergo gas-phase transitions
290towards extended forms during tens of ms^{37,68-70}, but the extent of these transitions is
291minimized in the case of using gentle instrumental conditions⁶⁷. Hence, gas-phase
292transitions are expected to be low due to the relatively fast experimental time scale (25.6
293ms) and the stability of native POP structures under the mild conditions used here.
294Nevertheless, in order to discard extensive gas-phase transitions occurring during the
295IM separation, different experiments were performed in which the drift times of POP
296species were varied (see Supporting information and Figure S4). The absence of large
297population changes confirms that species observed in the gas phase mainly reflect the
298conformational heterogeneity in solution.

299In the case of the closed conformation, we explored the conformational space of POP in
300solution by REMD simulations under different conditions, using the X-ray structure
3011H2W as starting point. First, we performed a short REMD simulation with explicit
302solvent molecules at neutral pH (REMD I). Since we did not observe major
303conformational rearrangements in this simulation, the flexible loops surrounding the
304active site (inset of Figure 1 A) were detached by performing a REMD simulation at
305acidic pH (REMD II). At low pH, the replica with the highest temperature showed that
306loop A spontaneously detached and opened a small cavity (Figure 3 B). Afterwards, the
307structure of POP with loop A detached was stabilized by running an additional REMD
308simulation at neutral pH (REMD III). As seen in Figure 3 A and Table 1, the CCSs
309obtained by REMD I and III simulations showed a good correlation with those of
310species B. Moreover, the theoretical CCSs obtained by the two simulations showed only
311small variations ($< 3\%$), indicating that the structural fluctuations of loop A would not
312be resolved from the IM peak of species B. Hence, we can establish a correlation
313between the closed conformation of POP in solution and species B in the gas phase,
314which probably has a certain degree of loop flexibility. In addition, according to our
315previous study using solution-based biophysical methods⁴⁷, the open and closed
316conformations of POP are almost equally populated in solution. Similarly, the relative
317populations of species B and C at the intermediate charge states +19 and +20 are 0.40
318vs. 0.34, respectively (the contributions of the open and closed structures in these charge
319states are expected to be similar).

320Finally, we examined the effect of the solvent on the stability of POP ions in the gas
321phase by performing MD simulations in vacuum. In order to obtain representative
322results, a net charge state of +20 was assumed. Ten microstates with different
323protonation states compatible with +20 overall charge were generated and used as
324starting structures for MD simulations (see Materials and methods). Analysis of the
325tertiary structures obtained by these simulations disclosed no large-scale structural
326rearrangements in vacuum. However, the averaged RMSD of 3.2 Å with respect to the
327X-ray structure 1H2W indicated certain variations of POP structures in vacuum. The
328overlay of the gaseous structures with the X-ray structure showed that some of the β -
329propeller blades and β -turns experienced tighter packing around the central tunnel of the
330 β -propeller; in addition, certain parts of the N- and C-terminal regions also adopted a
331more compact conformation (Figure 3 C). Interestingly, the average CCS of these
332simulated POP structures in the gas phase were in good correlation with species A
333(Figure 3 A and Table 1). This result indicates that species A is originated by the
334structural collapse of gaseous POP ions in the absence of the stabilizing effect of the
335solvent.

336Charge reduction of POP ions

337In order to minimize the Coulomb repulsion in gaseous POP ions, we performed IMMS
338experiments using charge-reducing strategies. In particular, we applied the ion cooling
339mechanism via addition of imidazole in order to explore charge-reduced ions of POP
340(see Materials and methods)^{38,71}. Hence, in the presence of 0.05 % imidazole, the charge
341state distribution of POP was expanded from +11 to +20 charge states (Figure 4 A). Due
342to calibration limitations, it was only possible to analyze the ion mobilograms from +15
343to +20 charge states. Ion mobilograms were adjusted to Gaussian functions as described

344previously in order to quantify the species present at each charge state. As shown in
345Figure 4 B, +15 and +16 charge states exclusively revealed species A, while the + 17
346charge state indicated the presence of species A and B. In turn, +18 to +20 charge states
347consisted of species A, B and C.

348Analysis of the relative populations as a function of the charge state (Figure S5, upper
349panel) revealed that decreasing Coulomb repulsion favors species A. Hence, it can be
350hypothesized that the interplay between attractive intramolecular interactions in vacuum
351and Coulomb repulsion prevents the structural collapse of POP gaseous ions at higher
352charge states, thereby favoring the native B and C species¹⁰. However, the presence of
353imidazole resulted in high CCS for all species at high charge states (+19 and +20,
354Figure S5, lower panel). This expansion is attributed to the destabilization of gaseous
355protein ions at high charge states caused by charge-reducing agents, as described by
356Bornschein *et al*²².

357Stability and unfolding of gaseous POP species

358After addressing the effect of Coulomb repulsion on gaseous POP ions, we monitored
359the stability of species A, B and C by forcing unfolding in the gas phase. To do this, we
360performed CIU experiments (see Materials and methods)^{73,74}, in which the kinetic
361energy of ions in the trap region is increased by raising the trap CE voltage. As a
362consequence of the increased collisional activation of the ions with the gas molecules,
363ions undergo structural transitions in the gas phase and show less folded forms.
364Afterwards, ions are injected to the IM cell and analyzed normally. Hence, the
365collisional activation required for gas-phase transitions reflects the thermodynamic
366stability of gaseous ions. Figure 5 A and B shows the ion mobilograms and relative
367populations of the species present in the representative charge state +19 as a function of
368the trap CE voltage, respectively. The relative populations of species A and B decreased
369as a function of voltage, and disappeared at 25 and 30 V, respectively. In contrast, the
370relative population of species C reached a maximum at 25 V. We hypothesized that
371species A and B experience a separation between the α/β -hydrolase and β -propeller
372domains in the gas phase upon collisional activation, thereby leading to a new
373distribution of open states of POP with CCSs similar to that of species C.

374New highly extended species D and E were detected at high trap CE voltages (25 and 30
375V, respectively). The occurrence of these extended species implies a striking decrease in
376the relative population of species C. The CCS of species D was $65.8 \pm 0.4 \text{ nm}^2$ and is
377expected to be much higher for species E (the limitations of the calibration protocol
378prevented the calculation of the CCS of this species). Hence, species D and E
379correspond to ensembles of denatured forms of POP, which arose by the unfolding of
380certain domains or regions of native POP ions at high collisional activation⁷⁴. In
381particular, the high collisional activation required to unfold native POP ions indicates
382the stability of species B and C in the gas phase and discards the existence of artefactual
383species in the mild experimental conditions used in our experiments.

384

385Conclusions

386 Here we evaluated the capacity of IMMS to analyze the different conformations of large
387 proteins in slow equilibrium in solution. For this purpose, we analyzed POP, a large
388 bidomain enzyme in a slow open/close conformational equilibrium, by IMMS. Our
389 experiments disclosed three species of POP in the gas phase. Two of these species
390 correlated well with the co-existing open and closed conformations in equilibrium in
391 solution, while the third corresponded to a gas-phase collapsed form. In order to transfer
392 the native conformational equilibrium in solution to the gas phase and minimize gas-
393 phase derived artefacts, we carefully optimized the instrumental conditions. Theoretical
394 simulations under different conditions allowed the interpretation of the results. In
395 particular, the conformational space of POP in solution and in vacuum was simulated in
396 order to evaluate the stabilizing effect of the solvent. We also used charge-reducing
397 strategies and CIU experiments to provide qualitative insights into the Coulomb
398 repulsion and the thermodynamic stability of gaseous ions, respectively. Hence, we
399 conclude that IMMS is a highly versatile biophysical technique for the study of multiple
400 protein conformers in slow exchange in solution, overcoming the limitations associated
401 with other biophysical techniques. The relatively fast time scale, the high sensitivity and
402 resolution, and the tolerance of a wide range of molecular sizes and complexity confers
403 IMMS great potential for the analysis of protein conformations co-existing in slow
404 equilibrium in solution. This work provides additional support for the previous IMMS
405 studies performed on a wide variety of biomolecules. Our findings are coherent with the
406 fact that the multiple co-existing protein species detected by this technique can be
407 effectively correlated to dynamic processes of biological relevance occurring in
408 solution. In particular, previous studies detected different populations of IDPs arising
409 from the fast exchange between conformational ensembles^{27,28}, as well as the
410 conformational heterogeneity of large proteins and protein complexes originated by
411 structural flexibility³³⁻³⁵. In the case of self-aggregating proteins, our results sustain that
412 transient conformations or oligomeric species detected by IMMS can be closely related
413 to those occurring in solution during the complex and dynamic aggregation process<sup>12-
414 14,29-32</sup>. However, we stress the importance of careful optimization of the experimental
415 conditions in order to avoid artefacts. In this regard, standardization of methodological
416 procedures, as well as development of stabilizing buffer molecules or ions in MS^{73,75,76}
417 would help to ensure transferring native protein structures to the gas phase. Finally, we
418 also emphasize the need to use theoretical approaches to analyze the data and to validate
419 the results.

420

421 Acknowledgements

422 This work was supported by the Institute for Research in Biomedicine, MINECO-
423 FEDER (Bio2013-40716-R), the Spanish Ministry of Education and Science (Projects
424 CTM2012-39183) and the *Generalitat de Catalunya* (XRB and Grup Consolidat
425 2014SGR521 and 2014SGR1017). AL received funding from the Instituto de Salud
426 Carlos III. The IRB Barcelona Mass Spectrometry Core Facility is a ProteoRed
427 laboratory, part of PRB2-ISCI. It is supported by grant PT13/0001 and is active group
428 participant in the European COST Action BM 1403 (*Native Mass Spectrometry and
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579**Tables**

580Table 1: Experimental (left panel) and theoretical (right panel) CCSs of POP species.
581Errors in the experimental CCS are expressed as the standard deviation.

EXPERIMENTAL CCS		THEORETICAL CCS		
Species	CCS (nm ²)	Simulation	Average (nm ²)	σ
A	50.4 ± 0.3	Gas phase MD	48.41	0.57
B	53.8 ± 0.4	REMD I	54.05	0.30
		REMD III	55.51	0.35
C	59.3 ± 0.7	Open conformation	58.53	0.15

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