Protein Conformation

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Detection of a Protein Conformational Equilibrium by Electrospray Ionisation-Ion Mobility-Mass Spectrometry**

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Ion mobility spectrometry (IMS) is emerging as a promising technique for providing low-resolution protein-structure information, particularly in combination with electrospray ionization (ESI) and mass spectrometry (MS).[1] There is currently much debate on the structure of protein ions in the gas phase, as summarized by Breuker and McLafferty.[2] Whilst it appears probable that some structural collapse occurs within picoseconds of dehydration, the onset of gross structural rearrangement may require tens of milliseconds.[3] This time provides a potential window for the observation of "near-native" structures that may retain some elements of the solution structure, with the ability to provide biologically relevant information. A number of recent applications have used IMS to study the conformation and stoichiometry of proteins and their complexes. Structural changes to amyloid and prion proteins, as well as steps in amyloid fibril assembly, have been detected by using this approach,[4] and the calcium-dependent conformational change in calmodulin has been probed by IM-MS,[5] as has the relationship between tertiary structure and chemotactic activity in antibacterial peptides.[6] Insights into the structures of large multiprotein complexes, such as the RNA-binding TRAP protein, GroEL, and the 20S proteasome have also been provided by ion mobility measurements.[7]

We postulated that IM-MS may be used to study the dynamic equilibrium between well-characterized conformations of a monomeric multidomain protein. To test this hypothesis, we have examined NADPH-cytochrome P450 reductase (CPR) using ESI-IM-MS. CPR is a 76 kDa membrane bound flavoprotein that catalyses the transfer of electrons from NADPH to a number of oxygenase enzymes.[8] CPR consists of three folded domains,[9] an FAD- and NADPH-binding domain, an FMN-binding domain, and a linker domain which may serve to orient the other two domains. The FMN-binding domain is connected to the rest of the protein by a 14-residue "hinge", thus providing the flexibility that is thought to be important for the function of the protein. An N-terminal 57 amino acid peptide is responsible for anchoring CPR to the endoplasmic reticulum membrane; recombinant CPR, which lacks this N-terminal peptide, is both soluble and functional, thus facilitating detailed structure–activity studies.

The CPR-mediated electron transfer from NADPH to cytochrome P450 proceeds in a stepwise fashion: NADPH→FAD→FMN→P450. Interflavin electron transfer requires spatial proximity of the two prosthetic groups, and the X-ray crystal structure of CPR (PDB file: 1AMO[9]) confirms this is the case (closest approach of the FAD and FMN methyl groups: 3.85 Å (C–C)).[9] However, in this compact or "closed" conformation, the FMN cofactor appears to be inaccessible to the large cytochrome P450 molecule, and so the need for domain movement as an essential part of the catalytic cycle has been widely assumed.[10] Recently, NMR spectroscopy, small-angle X-ray scattering (SAXS), and crystallographic evidence for this movement has been obtained,[10] thus suggesting that in solution, CPR exists in an equilibrium between a compact conformation appropriate for interflavin electron transfer and an extended conformation appropriate for electron transfer to P450 (Figure 1).

Herein we show that two major conformations of wild-type CPR are present in the gas phase, and that their relative abundance can be influenced by the ionic strength of the solution from which they are electrosprayed, by removal of key intramolecular ionic interactions, and, crucially, by the redox state of the flavin groups. This study demonstrates the ability of ESI-IMS-MS to detect a protein conformational

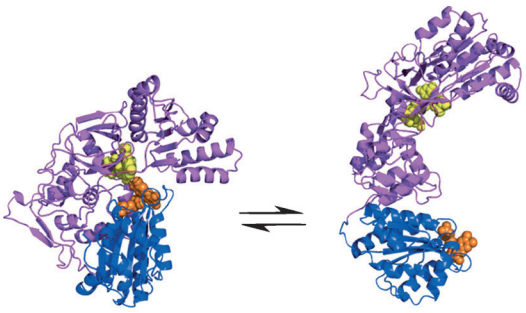

Figure 1. Compact and extended forms of CPR showing the FMN binding domain (blue), the FAD binding and linker domains (magenta), FMN (orange), and FAD (yellow). NADPH is omitted for clarity. FAD = flavin adenine dinucleotide, FMN = flavin mononucleotide, NADPH = nicotinamide adenine dinucleotide phosphate.

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equilibrium of biological relevance, and shows that important features of protein conformation are maintained for at least several milliseconds in the gas phase.

The ESI mass spectrum of CPR (7 μM) from ammonium acetate (250 mM, pH 7, see the Supporting Information) exhibited charge states in the range 16+ to 19+, which correspond to a mass of 71105 Da (Figure 2). The binding of both FAD (M_r = 785.3 Da) and FMN (M_r = 456.2 Da) to the protein was demonstrated by measurement of CPR under denaturing conditions to give a mass of 69862 Da for the polypeptide, as well as signals for the ions arising from protonated FAD and FMN (Figure S1 in the Supporting Information). Addition of NAD^+, NADH, or NADPH (3–10 μM) to CPR (7 μM) resulted in detection of nicotinamide cofactor binding by ESI-MS (Figure S2).

Travelling wave ion mobility spectrometry (TWIMS)[11] was performed on CPR under conditions that ensure minimal unfolding of CPR through gas-phase activation processes (see the Supporting Information). When sprayed from ammonium acetate (using electrospray, nanospray, or electrosonic spray ionization), CPR produced two major series of peaks in the TWIMS drift trace (Figure 3a for 18+ charge state). The relative intensity of the two peaks was found to be charge-state-dependent (Figure S4), with the 19+ charge state tending to show a slightly more intense peak at the later arrival time (lower mobility), thus indicating a Coulombic contribution. Care was taken to ensure that this observation was not an artefact of gas-phase activation by using instrument conditions well below the threshold required to induce unfolding, and by use of numerous control proteins (see the Supporting Information). The higher-mobility peak (drift time t_D = 5.1 ms, collisional cross-section (CCS) = 3926 Å^2 for 18+ charge state) possessed a similar arrival time to that theoretically predicted for the compact structure seen in the crystal structure by using the projection approximation (PA) method for calculating ion mobility and collisional cross-section (Figure 3).[12] The lower-mobility peak (t_D = 6.4 ms, CCS = 4261 Å^2 for 18+ charge state) suggested the presence

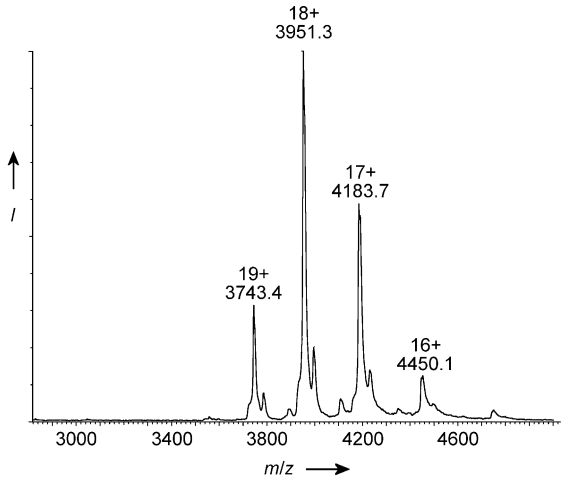

Figure 2. ESI mass spectrum of CPR sprayed from ammonium acetate (250 mM, pH7) showing the 16+ to 19+ charge states. The mass of 71105 Da corresponds to the protein with FAD and FMN prosthetic groups bound.

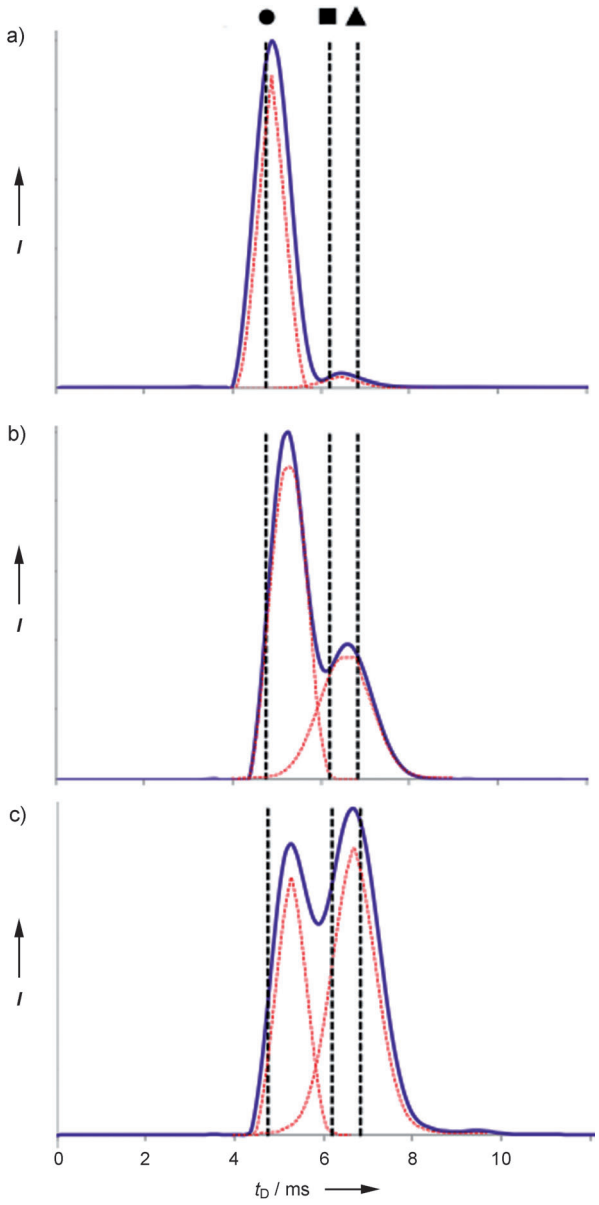

Figure 3. TWIMS drift traces for CPR (18+ charge state) electrosprayed from a) 250 mM ammonium acetate, and b) 1 M ammonium acetate. c) Drift trace: D352N/E354Q double-mutant of CPR (18+ charge state, 1 M ammonium acetate). FAD and FMN are bound in all cases. Red dotted traces represent individual Gaussians. ● theoretical PA t_D for compact CPR[9] (CCS 3818 Å^2); ■ theoretical PA t_D for extended CPR (CCS 4199 Å^2);[10b] and ▲ theoretical PA t_D for extended CPR (CCS 4338 Å^2).[10c] Measured CCS values of 18+ CPR: 3926 Å^2 (compact) and 4261 Å^2 (extended).

of a second conformation of CPR in the gas phase. It was postulated that this conformation may be related to the extended form of CPR believed to exist in solution.[10] Calculation of the expected t_D value for the model of the extended conformation of CPR based on NMR and SAXS data[10b] provided a value that was in good agreement with the measured range (Figure 3). It should be noted that the PA calculation is believed to underestimate the true CCS of ions.[13] Theoretical PA values are, therefore, presented as a guide only. Trajectory method (TM) calculations are thought

to provide a more reliable measure of theoretical protein ion CCS. Using MOBCAL,[12] TM CCS values were determined to be 4779 and 5167 Å^2 for the compact (PDB: 1AMO) and extended (PDB: 3ES9) CPR structures, respectively. These cross-sections are significantly higher than our measured values, a finding that is consistent with reports for many other proteins.[13] This result suggests a degree of structural collapse upon desolvation, and gas-phase molecular dynamics simulations performed on the compact and extended structures reveal a reduction in CCS of 9 and 14% respectively over 100 ps (see the Supporting Information). It is interesting to note that results from SAXS experiments[10b] indicate a cross-section of 3817 Å^2 for the compact form of CPR in solution, a value extremely close to the measured CCS value of 3926 Å^2. Comparison with the extended form seen by SAXS in solution is less straightforward because of its irregular shape. The IM-MS data strongly indicate that CPR exists as a mixture of two states, namely a family of compact structures and a population of extended structures, both of which are reasonably well-defined conformations; this result is entirely consistent with the analysis of solution SAXS data.[10b]

The interdomain interfaces seen in the crystal structure of CPR include a substantial number of ionic interactions, and changes in ionic strength are well known to affect the kinetics of the enzyme.[14] Increasing the ionic strength by increasing the ammonium acetate concentration had a remarkable effect on the TWIMS results for CPR. When electrosprayed from 250 mM ammonium acetate, the more compact form of CPR was dominant ((85 ± 5) % of the total for the 18+ charge state, Figure 3a). A buffer concentration of 1M resulted in a significant and reproducible increase in the more extended form of CPR (to (45 ± 7) % of total for the 18+ charge state, Figure 3b), without changing the overall charge-state distribution detected. It should be noted that CPR has higher catalytic activity at high salt concentrations ($k_{cat} = 49\ s^{-1}$ for reduction of cytochrome c in 1M ammonium acetate vs. $24\ s^{-1}$ in 250 mM ammonium acetate), and that the increase in ionic strength had no effect on the conformer populations of numerous control proteins measured by TWIMS-MS under identical conditions (Figures S6 and7). SAXS experiments in solution also show that a high ionic strength leads to a more extended form of CPR (97% compact at 200 mM NaCl; 94% extended at 500 mM NaCl)[15]. The fact that the distribution of enzyme conformers observed in the gas phase by ESI-IM-MS was dependent upon solution ionic strength suggests that the distribution reflects the equilibrium between compact and extended forms in solution. However, it should be noted that, given the highly polar nature of the interdomain interactions, the compact population might be expected to be over-represented by gas-phase measurements relative to the situation in solution.

Examination of interdomain contacts within CPR revealed, in the compact form, a closely situated pair of salt bridges between K75 and E354, and R78 and D352 (Figure 4a). As both K75 and R78 are located on the FMN-binding domain, the two salt bridges must be broken for the enzyme to adopt the extended form (Figure 4b). On this basis, it was postulated that a D352N/E354Q double mutant might decrease the population of the compact form of CPR by removing the ionic component of the two salt bridges whilst

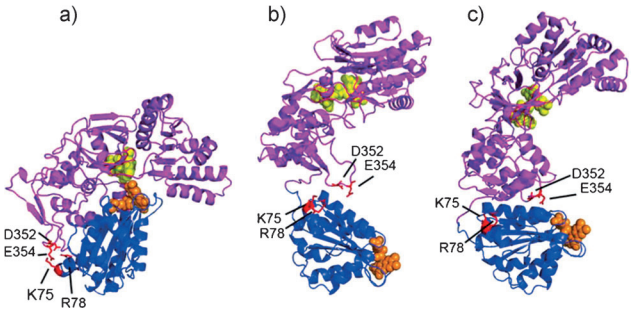

Figure 4. Structures of CPR, illustrating a) D352-R78, and E354-K75 salt bridges in the compact form (modified from PDB file 1AMO[9]); and the absence of these interactions in the extended form of the enzyme b) based on molecule B of PDB 3ES9;[10c] c) from the model in Ref [10b].

maintaining hydrogen-bond interactions. D352N/E354Q-CPR did indeed show a significantly increased population of the extended conformation upon ESI-IM-MS analysis (to (65 ± 8) %, Figure 3c). The significance of this finding is twofold: firstly it strongly suggests that this double salt bridge is in place in desolvated wild type-CPR; and secondly it indicates that this interaction is a major stabilizing force for the compact form of the protein.

SAXS studies have shown that the proportion of compact and extended conformations of wild type-CPR is dependent upon the redox state of the two flavin prosthetic groups.[10b] To establish whether the distribution of gas-phase conformers could also be affected by the redox state of the protein, CPR was incubated with either excess NADPH (under anaerobic conditions) or $NADP^+$. In the presence of excess NADPH, the FAD and FMN prosthetic groups of CPR are fully reduced to $FADH_2$ and $FMNH_2$, whereas, as a control, no change in flavin oxidation state is induced when $NADP^+$ is added to CPR. The CPR–NADPH complex under anaerobic conditions showed an almost completely compact conformation ((93 ± 4) %) for the 18+ charge state when sprayed from 1M ammonium acetate (Figure 5a).[16] In contrast, the CPR-$NADP^+$ complex exhibited both compact ((67 ± 11) %) and extended forms in the IM drift trace for the 18+ charge state (Figure 5b), with the cofactor and prosthetic groups oxidized. This result is in good agreement with SAX values for conformer populations of CPR, with reported values of 85% compact (reduced) and 50% compact (oxidized).[10b] These results show that the population of CPR conformers seen by IM-MS is dependent upon the oxidation state of the flavins. This behavior provides compelling evidence that important aspects of the solution structure and conformational equilibria of CPR are retained upon desolvation and that IM-MS is able to provide structural information of biological relevance.

In summary, we have shown, by using ESI-TWIMS-MS, that two major conformations of CPR exist in the gas phase; the effects of ionic strength and changes in redox state indicate that this result reflects a conformational equilibrium in solution. Ion mobility studies on the effect of the flavin oxidation state show that CPR exhibits an increase in the population of the compact form upon FAD/FMN reduction, thus mirroring the results obtained in solution by SAXS. Thus, even though desolvation is expected to perturb the structure

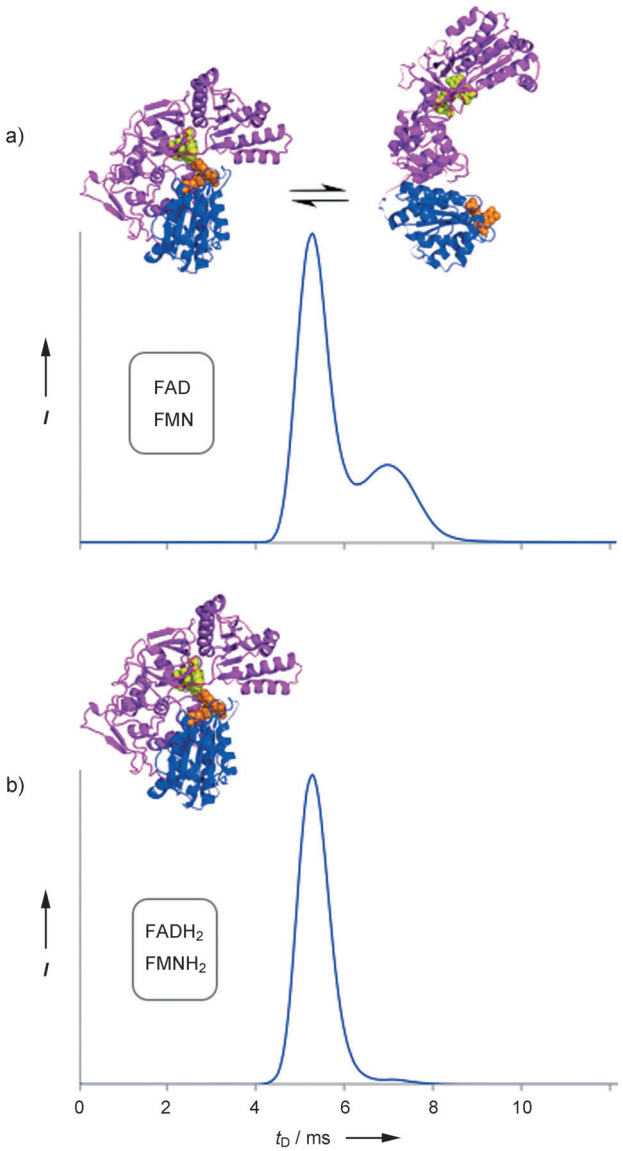

Figure 5. TWIMS drift traces for CPR (18+ charge state) in the presence of a) $NADP^+$ and b) NADPH (anaerobic conditions) showing the dependence of the conformational equilibrium on the redox state of FAD and FMN (bound to CPR in both cases).

of CPR, elements of its distinct compact and extended conformations appear to survive dehydration and are sufficiently long-lived (tens of milliseconds) to be measured by IM-MS. These results provide a compelling demonstration that gas-phase ion mobility spectrometry is a rapid, sensitive, and robust probe of protein conformation and has wider potential to provide important insights into the dynamic conformational equilibria of other proteins in solution.

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[16] That the system was under reducing conditions was evidenced by MS detection of excess free NADPH in solution ($[M+H]^+ = m/z$ 746, Figure S7). It may be concluded that both FAD and FMN are in their fully reduced forms for the duration of this measurement.

