Collisional Cooling Enhances the Ability to Observe Non-Covalent Interactions within the Inducible Nitric Oxide Synthase Oxygenase Domain: Dimerization, Complexation, and Dissociation

Jeffrey C. Smith and K.W. Michael Siu

Department of Chemistry and Centre for Research in Mass Spectrometry, York University, Toronto, Ontario, Canada

Steven P. Rafferty

Chemistry Department, Trent University, Peterborough, Ontario, Canada

The investigation of protein quaternary structure, protein-cofactor, and protein–ligand interactions by mass spectrometry is often limited by the fragility of such interactions under experimental conditions. To develop more gentle conditions of perhaps general use, we used as a model for study the oxygenase domain of murine inducible nitric oxide synthase (iNOS), which is homodimeric, binds heme and tetrahydrobiopterin H_4B cofactors, and the substrate L-arginine. The energetics of the collisions in q_2 and in the lens region of the mass spectrometer were manipulated for varying the degree of solvation around the non-covalently bound ions. Furthermore, the number of low-energy collisions in the collision cell of the instrument was varied, focusing and dampening the ion beam. Under gentle source collision conditions, and using multiple low-energy collisions in the collision cell of the mass spectrometer, dimers of the iNOS oxygenase domain containing heme, H_4B , and arginine were observed intact after electrospraying at pH values near neutrality; a mutant of this protein (Trp188 $\rightarrow$ Phe) was monomeric and did not bind cofactors. The pH dependence of the iNOS oxygenase domain under acidic conditions was also studied; while heme remained bound to the protein between pH 2.5 and 4.0, the dimeric structure was disrupted. Our findings confirm that non-covalently bound macromolecular complexes are retained and observable using electrospray mass spectrometry under the appropriate experimental conditions. (J Am Soc Mass Spectrom 2004, 15, 629–638) © 2004 American Society for Mass Spectrometry

The enzyme nitric oxide synthase (NOS) is an appropriate focus for studying non-covalent interactions as few proteins rival it for the richness in variety in which it partakes. These include the binding of four cofactors (two flavins, a heme, and a pterin), three substrates (L-arginine, NADPH, and oxygen), and at least two protein–protein interactions (between subunits of this homodimeric enzyme and interactions with the small calcium-sensing protein calmodulin, which regulates enzyme activity). In spite of the many functional differences, the NOS isotypes produce NO by the same mechanism:

This similarity in mechanism is a consequence of structural homology within the NOS isotypes. All isotypes have a common domain architecture consisting of an oxygenase domain that binds heme and tetrahydrobiopterin (H_4B) linked by a short calmodulin binding site to a carboxy-terminal reductase domain which, in turn, binds flavin cofactors [1]. Electrons are transferred from NADPH via the reductase domain to the oxygenase domain, which is the site of oxygen binding and NO production. Furthermore, NOS is active only as a dimer; dimerization requires the presence of the heme cofactor and is stabilized further by binding of H_4B and arginine [2]. NOS dimerization occurs primarily through interac-

Published online February 27, 2004

Address reprint requests to Dr. K. W. M. Siu, Department of Chemistry, York University, 4700 Keele Street, Toronto, ON M3J 1P3, Canada. E-mail: kwmsiu@yorku.ca

tions between the oxygenase domains on the two subunits [3].

The organization of NOS into distinct structural domains lends itself to their study as independent species, obtained either by limited proteolysis of the complete protein or by recombinant expression. For example, the independently expressed oxygenase domain of NOS retains the capacity to bind cofactors, substrate, and to dimerize, and thus reflects the structural and functional properties of this domain in the intact protein [4]. Establishing a gas-phase representation of the equilibrium between oxygenase monomers and dimers will allow a standard to be set to which future modifications of the protein (i.e., point mutations) may be compared. As dimerization directly correlates to the protein's ability to be active, studying factors that affect this equilibrium may allow the elucidation of key stabilizing residues or inhibitory substances.

Mutational studies commonly allow insight into the intra-physiological processes and key residues within a protein. Recently, it has been reported that a mutant form of the inducible NOS (iNOS) oxygenase domain has been created, and that this mutant lacks the ability to bind the cofactor heme [5]. In this mutant, the 188th amino acid residue, tryptophan, is replaced by phenylalanine. It is thought that this tryptophan residue in the wild type plays a major role in stabilization of the heme in the heme-binding pocket; it has been shown that, through a mutation to a phenylalanine residue, the heme binding becomes severely destabilized despite the protein remaining folded [5]. The present study also examines this mutant (known as the W188F) in order to observe its gas-phase behavior and any non-covalent interactions that may be present.

With the advent of soft ionization techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), large biomolecules are now accessible to investigation using mass spectrometry (MS). It has been stated that nanospray, a low-flow subclass of ESI, is even softer [6, 7] and has a greater tolerance for non-volatile salts [8] than conventional electrospray, thereby making it even more suitable for the study of non-covalent interactions. There are now a number of reports that detail the observation of non-covalent interactions using mass spectrometry, and this topic has been recently reviewed by several groups [9–13]. Most of these studies dealt with rather strong, electrostatic interactions that are arguably enhanced in the absence of solvent, [14] e.g., protein/DNA complexes [15], DNA/ligand complexes [16], heme/protein and other interactions [17–19]. Extensive time and effort have also gone into studying protein/protein interactions through mass spectrometry [14, 20–26]. A few groups have reported the ability to observe hydrophobic non-covalent interactions by mass spectrometry including leucine zipper peptides [27] as well as acyl CoA derivatives to acyl CoA binding protein [28]. Both studies reported a very low relative

abundance of non-covalently bound species (~10–20% or less), which was somewhat expected as hydrophobic interaction is likely to diminish in the absence of solvent. The degree of hydrophobicity of a particular non-covalent interaction was rarely discussed, perhaps because it is not fully known, or due to the fact that, upon transfer to the gas phase, hydrophobic stabilization effects diminish, while electrostatic effects are generally enhanced. It has been reported that, in the endothelial NOS isotype, 55% of the dimer interface consists of hydrophobic residues, thereby showing that dimer stability relies heavily on entropic effects of the surrounding aqueous solvent [29]. Due to the high degree of homology between the NOS isoforms, it may be inferred that hydrophobic effects likely play a significant role in the dimerization of both the inducible and neuronal isoforms as well. However, in the gas phase, one would expect the electrostatic interactions along the dimer interface to be the major contributor to dimer stability. Deciphering the exact nature of the forces that contribute to gas-phase protein/protein interactions, including that in the NOS dimer, is currently intractable.

Mass spectrometric sampling from an electrospray source results in cooling of the ions due to gas expansion and subsequent heating via downstream collisions with sampled gas molecules in the presence of an electric field. Collisional heating is generally required in order to adequately desolvate the ions, and can be “harsh” when the pressure drop is large, allowing the ions to acquire significant energy from the electrostatic field in between collisions (because of the long mean free path). Alternatively, it can be “gentle” when, under the same electrostatic field, the pressure differential is relatively small [30]. It has been shown that “collisional cooling”, i.e., a large number of gentle collisions, helps preserving weak interactions, and allows for sufficient resolution of non-covalently bound macromolecules without significant fragmentation [30–33]. Several recent studies have described deliberate increases of the pressure in the sampling hexapole (equivalent to q_0 in our instrumental setup) that was upstream to the first mass analyzer [30, 31, 33]. The many low-energy collisions were believed to be sufficiently energetic to shed the attached solvent molecules from the macromolecular complex; however, they were not energetic enough to cause fragmentation of the complex itself [30, 31]. In addition, these low-energy collisions served to dampen ion motion in the radial direction of the multipole and concentrate the ions onto the central axis, thereby improving ion transmission and sensitivity [32, 34–36]. In our present study, we employ a collisional cooling strategy that utilizes an additional region for implementation. The q_0 continues to allow for a degree of collisional cooling due to the conventional pressure gradient in that region; however, here, the collision cell (or second quadrupole, q_2) is employed as an additional region where multiple, low-energy collisions can take place. It has previously been shown that the

optimal pressure for ion transmission and sensitivity through collisional cooling, and focusing, over a distance of about 15 cm, is 10 mTorr [37]. Therefore, we endeavour here to optimize the relative abundance of NOS dimer ions by performing experiments in this pressure range as well as with gas pressures that exceed this value.

We also report MS/MS results of the iNOS oxygenase domain. Several groups have studied the effects of conducting MS/MS on full-length proteins, specifically citing the trends associated with ferri- and ferro-heme loss from various heme proteins [38–41]. The presence of some cofactors may be determined within a protein through their loss from the complex. If the cofactor is charged, e.g., it is a ferri-heme, its loss will be evidenced by the presence of the cofactor ion and a lower-charge as well as lower-mass protein ion [38].

Experimental

Recombinant oxygenase domain was expressed and purified as described previously [42]. The protein was purified in the presence of H_4B (100 μM) and L-arginine (5 mM). The calculated isoelectric point (pI) of the NOS oxygenase domain is 6.3. Due to the aggregation observed at pH = 6.3, two buffer exchange techniques were employed to produce samples suitable for electrospraying. About 100 μL of purified iNOS oxygenase domain was brought to the desired pH by either dialysis against two 500 mL volumes of 0.001–0.34% formic acid for a total of three hours in the case of acidic samples, or through size exclusion chromatography (SEC) (P10 Sephadex, Qiagen, Mississauga, Canada) into 10 mM ammonium acetate adjusted to pH 7.0 in the case of neutral and basic samples. For these solutions, no aggregation state was observed as the pH was never near or passed through 6.3; SEC provided the quickest method for exchanging the buffer. Speedy sample preparation reduced the risk of disrupting the non-covalent interactions due to non-physiological salt concentrations. For acidic solutions, SEC could not be used, as the protein aggregated and stuck to the column; dialysis was used instead, allowing the protein to aggregate and then dissolve back into the solution once the pH inside the dialysis chamber fell below ~ 6.3 . In some experiments, the ammonium acetate was replaced by 10 mM ammonium bicarbonate or 10 mM triethylammonium bicarbonate for testing the effect of changing the buffer; in others, ammonium hydroxide or triethylamine was added to solutions to raise the pH to between 7 and 10 for evaluating the effect of changing the pH. The protein samples were diluted to 5 μM before analysis.

Mutational studies using the W188F variant were also conducted in 10 mM ammonium acetate. The mutant was created as described previously [5], and was purified in a procedure identical to the wild type protein. SEC was also used for the W188F species; however, it was noted that below pH 7.5, the protein would aggregate. Therefore, the minimum pH in which

the mutant was placed was 7.5. Solutions of 5 μM concanavalin A and human hemoglobin were also prepared in 10 mM ammonium acetate, pH 7.5. Unless otherwise indicated, all reagents were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO).

The mass spectrometer used for all initial experiments was an MDS Sciex Centaur (Concord, ON), a prototype version of MDS Sciex' commercially available QSTAR hybrid quadrupole time-of-flight (QqTOF) tandem mass spectrometer. Nanospray (MDS Proteomics, Odense, Denmark) was used for sample introduction using custom-made "short" gold/palladium alloy-coated silica capillaries. Nitrogen was used both as the curtain and the collision gas. The Centaur was modified in-house for higher-pressure operations in q2. By increasing the collision gas inlet pressure, the maximum collision-gas pressure was approximately doubled. An upstream pressure regulator was installed for precise pressure control. The working pressure in q2 was regulated by a series of four unique flow-limiting orifices that are controlled in a binary fashion by four solenoid valves. The actual pressure in the q2 was not measurable in the current setup. However, previous measurements of the original instrument configuration at Sciex allow us to estimate that at the maximum setting, the pressure in the q2 is approximately 12 mTorr. Lower pressures are accessible by opening or closing the appropriate binary configuration of the four solenoid valves to create 12 levels of differing q2 pressure in a range of approximately 1 to 12 mTorr. To maintain proper operating pressure in the analyzer regions of the mass spectrometer, a fourth turbomolecular pump was added to evacuate the analyzer region, i.e., the vicinity of Q1 and q2. The optimal source voltages varied with each experiment, allowing for various degrees of desolvation and resolution. Nanospray voltages ranged from 800–1200 V. Orifice and curtain plate voltages ranged from 11–50 V for a low degree of desolvation, and 100–140 V for a high degree of desolvation. Ring voltages varied proportionately to the orifice voltages at 120–175 V for the former, and 200–275 V for the latter condition. The skimmer voltage was typically held at 26 V. Individual voltages were optimized for each experiment in order to maximize the relative abundance of non-covalently bound species observed and to attain the desired level of desolvation.

Most experiments were repeated on two QSTAR XL prototype QqTOF mass spectrometers at MDS Sciex (designated as XL 1 and XL 2 for differentiation in this manuscript). The two XL instruments are functionally identical to each other with respect to their ion sources, ion optics hardware, and operating pressures (albeit different in design details). They differ from the Centaur as follows: First, the XL instruments have LINAC II collision cells, whereas the Centaur does not. LINAC II collision cells provide an axial potential gradient along the collision cell [43], whereas the older model collision cell in the Centaur does not. Second, the XL instruments have lower frequency Q1 rods, meaning they are able to

mass analyze ions of greater mass to charge ratio (≤ 8000 Th); the Centaur is restricted to a maximum limit of 3000 Th. Third, as described above, the Centaur was modified to allow greater pressures in its collision cell than the other two instruments without causing the instrument to lose its operating pressure. After modification, the pressure in the q2 of the Centaur could reach ~ 24 mTorr, approximately 1.5 times as great as the maximum pressure allowed in the XL instruments. Finally, although both the XL and Centaur instruments were run on a Macintosh platform, their versions of TOFMA (spectral attaining and displaying software) were different. The significance in this is that the relative ion abundances impinging upon the four detector plates of the MCP detector was easily observable for the XL instruments, but not so for the Centaur. It will be shown that, for the same type of iNOS solutions, the relative distribution of monomeric and dimeric ions under various pressure conditions in q2 is dependent on the instrument type (i.e., XL versus Centaur).

Results and Discussion

General Observations for Neutral iNOS Solutions

Observation of the dimeric iNOS oxygenase domain required careful optimization of lens voltages and gas flows. However, once these conditions were found, they were generally applicable for both the XL and Centaur instruments, with some minor adjustments needed between experiments due to spraying variation. As a control, both concanavalin A and hemoglobin were sprayed in order to confirm the optimized instrument parameters and trends noted herein; in both cases a majority of tetrameric species was observed (data not shown) in accordance with previous observations [21, 22]. It was quickly discovered that increasing the q2 pressure in the Centaur beyond its original limit did not offer any advantage. The ion intensity decreased at a pressure ≥ 12 mTorr, presumably due to scattering [30]. In general, the trends are similar in all three instruments, although there are exceptions. In all three instruments, introduction of the collision gas into q2 (within the 0–12 mTorr limit) increases the abundances of protein ions. Figure 1 shows the relationships between absolute dimer abundance and q2 pressure for the three instruments. It should be noted that the intended comparison is the change in abundance within a given instrument with q2 pressure, and not the change in absolute abundance of a given q2 setting among the three instruments; the experiments were carried out with different nanospray capillaries and hence differing spray conditions. Introducing the collision gas increases the absolute abundance of dimer ions. This increase is approximately linear for the XL 1 and XL 2 instruments throughout the pressure range from 0 to 16 mTorr, but levels off at approximately 8–10 mTorr for the Centaur and then reverses itself (i.e., the abundance decreases beyond this range). The effect of q2 pressure on the

Figure 1. Plot of the ratio of absolute dimer abundance versus the pressure within the q2 for the Centaur, XL 1, and XL 2 instruments. The line with the filled diamonds represents the trend for the Centaur, the line with the filled circles represents the trend for the XL 1, and the line with the filled squares represents the trend for the XL 2. The asterisks indicates that the abundance for the XL 1 instrument has been multiplied by (1×10^{-2}) to allow easier comparison between the instruments. In each experiment, the source collision conditions were gentle, (orifice potential was set to 30 V), and the collision energy was low ($E_{\text{lab}} = 7.5$ V). LINAC voltages in the XL 1 and 2 were both set to 50 V.

relative abundance between the dimer and the monomer ions, however, is quite different between the Centaur and the XL instruments (Figure 2). On the Centaur, a substantial increase in the relative abundance of dimer ions is observed as the q2 pressure is increased up to approximately 10 mTorr (above this pressure it decreases), whereas on the XL the relative abundance is

Figure 2. Plot of the ratio of relative dimer to monomer abundance versus the pressure within the q2 for the Centaur and XL 1 instrument (the XL 2 instrument showed similar results to the XL 1). The line with the filled diamonds represents the trend for the Centaur, and the line with the open triangles represents the trend for the XL 1. In each experiment, the source collision conditions were gentle, (orifice potential was set to 30 V), and the collision energy was low ($E_{\text{lab}} = 7.5$ V). LINAC voltages in the XL 1 and 2 were both set to 50 V.

Figure 3. Typical iNOS oxygenase domain mass spectra obtained from a 5 μ M solution at a pH of 7.0 (10 mM ammonium acetate) and at a q_2 pressure of 10 mTorr on (a) the Centaur and (b) XL 1. Source collision conditions were harsh (orifice potential (OR) of 140 V). Monomers (M) and dimers (D) are labeled, followed by their charge state (i.e., $D^{21+} = [2M + 21H]^{21+}$). In (b), species are seen with no heme loss (filled circle), with one heme molecule lost (open circle), and with two heme molecules lost (asterisk).

essentially independent of q_2 pressure. The increase in absolute abundance with increasing q_2 pressure is likely an effect of collisional focusing [30, 32] and the decrease is in accordance with the expectation of increasing scattering due to increasing numbers of collision [30]. The different behaviors between the Centaur and the XL instruments are puzzling but are likely a consequence of different q_2 designs (*vide infra*).

Figure 3 shows typical iNOS mass spectra obtained from a 10 μ M solution at a pH of 7.0 and at a q_2 pressure of 10 mTorr on (Figure 3a) the Centaur and (Figure 3b) the XL 1. The maximum charge states observed in the dimer and monomer ion envelopes are slightly different on the two instruments. These differences are within the variations observed within a given instrument, and are attributable to different optimal instrumental conditions (different nanospray voltages, curtain gas pressures, etc.). Changing the nature of the buffer salt produced little change to the spectra, except that, with everything else being equal, there was a shift towards lower charge states, when triethylammonium bicarbonate was used. This shift is in line with the higher proton affinity of triethylammonia than that of ammonia; the former is expected to be much more efficient than the latter in competing with iNOS for the protons [44]. Furthermore, raising the solution pH from 7 through 10 appeared also to have little effect on the spectra. Figure 3b also shows existence of dimer ions that have lost one or both heme groups. This is most

likely a gas-phase phenomenon as heme is knocked out via collisional activation and dimer disruption does not occur on the time scale of the MS experiment. Note that in Panel a, a greater degree of solvation is observed and no heme-free peaks are seen. This concept was also examined by conducting experiments varying the source collision conditions. Under harsher conditions, heme-free dimers became more apparent; the gas-phase nature of these reactions was confirmed by collision-induced dissociation (CID) experiments (*vide infra*).

Acidic iNOS Solutions

Acidic solutions of the iNOS oxygenase domain were also examined. Lowering the pH from 7.0 results in aggregation of the protein at $\sim$ pH 5.5–6.5. Further lowering of the pH results in protein re-dissolution. The nanoelectrospray mass spectra of iNOS oxygenase domain solutions at pH 2.5–4.0 show the presence of only monomers. Under mild source conditions (Figure 4a showing pH = 3.5), the monomers are all heme-bound; under harsher source conditions (Figure 4b showing pH = 3.5), both heme-free and heme-bound monomers are observed. Decreasing the pH shifts the centroid of the charge state distribution towards increasing charge; in addition, it increases the relative ratio of heme-free to heme-bound monomers. These observations are in

Figure 4. Mass spectra of 10 μ M iNOS oxygenase domain at pH 3.5 at (a) low (OR = 50 V) and (b) high (OR = 140 V) source collision conditions obtained on the Centaur. Each peak in the spectra represents a difference charge state of the monomeric protein [labeled M, followed by charge state (i.e., $M^{34+} = [M + 34H]^{34+}$)] with zero (open circle), one (filled circle), or two (asterisk) heme molecules bound. Heme proteins have been shown previously [45] to behave in this manner (i.e., bind two heme molecules per monomer) in the gas phase.

qualitative agreement with the expectation that, at a lower pH, more protons are available for attachment (therefore, more highly charged protein ions are formed) and more extended is the iNOS conformation (therefore, the heme is more loosely bound).

W188F Mutant

The W188F mutant solutions were electrosprayed under conditions that were optimal for observation of the dimeric iNOS oxygenase domain. Figure 5 shows a typical mass spectrum. It is evident that no dimer ions are visible. The monomers are heme-free. These observations are in accordance with the expected absence of dimeric mutant in solution, and poor heme binding of the mutant monomer.

More Detailed Analysis of the iNOS Oxygenase Domain

The molecular weight of one monomeric iNOS oxygenase domain polypeptide chain is 50548.6 Da. The loss of the N-terminal methionine residue (a common occurrence) results in a total mass of 50417.4 Da, adding heme (MW 616.2) gives a mass of 51033.6 Da. The dimeric form of this protein is, therefore, expected to have a mass of 102067.2 Da. However, H_4B has been

shown to provide stability to the dimeric structure; one or two molecules of H_4B (MW 241.1 Da) would result in

Figure 5. Mass spectrum of 5 μ M iNOS oxygenase domain mutant protein, W188F, in 10 mM ammonium acetate solution, pH 7.5 obtained on the XL 1. The orifice voltage was set to 11 V, and the pressure in the q2 was set to 11 mTorr. Peaks are labeled as M (monomeric) followed by the charge state (i.e., $M^{15+} = [M + 15H]^{15+}$). The spectrum shows only a monomeric species that is devoid of the heme cofactor, thus confirming that this mutant species does not dimerize, nor bind heme.

Figure 6. CID mass spectra of the (a) $[2M + 21H]^{21+}$ dimer ion, and (b) $[M + 15H]^{15+}$ monomer ion from a $5 \mu\text{M}$ iNOS oxygenase domain in 10 mM ammonium acetate solution, pH 7.5 obtained on the XL 1. The dimer ion peak was subjected to soft source collision conditions (OR = 30 V), and a collision energy of $E_{\text{cm}} = 0.338$ eV (corresponding to a potential difference between q_0 and q_2 of 60 V). The monomer ion peak was subjected to harsh source collision conditions (OR = 140 V), and a collision energy of $E_{\text{cm}} = 0.675$ eV (corresponding to a potential difference between q_0 and q_2 of 120 V). Low mass peaks are labeled on the inset spectra; H_4B = tetrahydrobiopterin, heme^+ = ferri-heme, **a** = protonated tetrahydrobiopterin with a neutral mass loss of 76 Da, **b** = protonated arginine, and **c** = sodiated tetrahydrobiopterin with a neutral mass loss of 76 Da. The loss of neutral heme in (b) is clearly seen through the appearance of the heme free peak (labeled with an open circle) from the parent, which contains heme (labeled with a filled circle). Monomers (M) and dimers (D) are labeled, followed by their charge state (i.e., $D^{21+} = [2M + 21H]^{21+}$).

a dimeric mass of 102308.8 Da and 102549.4 Da, respectively. Furthermore, the protein isolation protocol involved purification in the presence of the substrate arginine (MW 174.1 Da); each subunit in a dimer may bind this molecule giving total masses of 102723.5 Da for one, and 102897.6 Da for two arginine molecules binding to the protein.

Deconvolution of spectra obtained at low orifice potentials (30 V or less) reveals monomers with an average mass of 51245 ± 3 Da, and dimers with an average mass of 103174 ± 6 Da. These masses might indicate that the monomer contains heme and possibly a molecule of arginine as well; however, further experiments revealed that arginine is in fact not bound, and that the additional mass is due to solvent and salt molecules (vide infra). The mass of the dimer in these conditions is sufficiently large for the postulation that two molecules of H_4B as well as two molecules of arginine are bound to the dimer, in addition to two heme molecules and some solvent molecules. As orifice potentials were raised, monomers shed adducted solvent molecules to reveal an average mass of 51057 ± 2 Da, (the difference between this value and 51033.6 Da

indicates there is residual adduction of water or sodium); a small amount of heme loss was also observed. Dimer ions also lost mass as the orifice potential was increased, revealing an average mass of 102644 ± 10 Da at a moderate potential (75 V), and 102143 ± 4 Da at a high potential (140 V). The former of these two mass values lies 96 Da above the expected mass of a dimer containing two molecules of heme and H_4B ; the latter above the expected mass of a dimer containing only two molecules of heme plus the mass of some unknown adduct or solvent molecule(s) equalling 77 Da. To characterize the iNOS components that were lost with increasing potential, CID experiments were conducted on both monomers and dimers on the XL 2 instrument.

Figure 6 shows MS/MS spectra of (Figure 6a) a dimer ion, and (Figure 6b) a monomer ion. The dimer ion subjected to MS/MS (Figure 6a) is the $[2M + 21H]^{21+}$ ion at m/z 4975, corresponding to a dimer mass of 104454 Da, significantly larger than the mass of a dimer containing two heme, two H_4B , and two arginine groups. The spectrum reveals very little heme loss, but considerable losses of H_4B and arginine (see the inset). Furthermore, four ions of lower charge states (+17 to

+20) are observed. Analysis of their m/z values reveals that they are dimeric iNOS oxygenase domain that are heme-bound but have lost their adducted H₄B and arginine molecules as ions that are seen in the inset. In addition, losses of neutral H₄B, arginine, and heme from the precursor ion ($[2M + 21H]^{21+}$) have also been observed; these are revealed by shoulders and partially resolved details in expanded windows of the dimeric ions, (not shown). It is interesting that there is a distinct lack of monomeric species generated from these experiments. As alluded to before, this could possibly be due to the kinetics of the dimer separation requiring more time than the analysis time of the experiment.

Under energetic collision conditions, the loss of heme from the monomer was observed (Figure 6b); H₄B or arginine ions were not observed at any collision energy, in accordance with the expectation that they are not bound by the monomer. The loss of neutral ferri-heme from the precursor ion (both monomers and dimers) was also observed (not shown).

As a control, fragmentation studies were conducted on dimer ions with masses lower than 102,308.3 Da (generated by increasing the orifice potential to 140 V). The $[2M + 19H]^{19+}$ ion at m/z 5380 (corresponding to a mass of 102,220 Da) was fragmented (data not shown). This ion is not expected to contain H₄B or arginine; its fragmentation was considerably simpler, showing predominantly the loss of ferri-heme.

The dimeric form of iNOS oxygenase domain has been shown through crystallography studies to contain a lone zinc ion coordinated by four cysteine residues along the dimer interface; this structure has been suggested to contribute to the stability of the complex [46]. There was no indication, however, of the presence of zinc in any of the experiments; no adduct of zinc was ever observed, nor was zinc lost as an ion in any CID experiments. Inductively-coupled plasma mass spectrometric analyses reveal an abundant concentration of approximately 8 mM of zinc in the samples and buffer solutions, consistent with background levels from contamination of the buffers and solvents. The apparent lack of zinc in the iNOS could indicate that it is either weakly bound within the complex itself, therefore being easily lost in the transition to the gas phase, or it may not bind at all in the solution conditions that are used.

Experiments were also conducted at different collision energies, for evaluating the relative barrier to fragmentation. The loss of heme requires higher collision energy relative to H₄B and arginine. Lower energy collisions ($E_{cm} = 0.338$ eV for the first collision, corresponding to a potential difference between q0 and q2 of 60 V) produced more abundant H₄B and arginine ions than the heme ion. These observations were confirmed through deconvolution of the protein ions of lower charge; no heme loss was evident at low collision energies. As collision energy was raised, the relative abundance of the heme ion increased dramatically, accompanied by obvious losses of both the ferri- and ferro-heme from the protein ions. At higher energies

Figure 7. Plot of the ratio of relative monomer to dimer abundance versus the voltage applied to the LINAC II axial gradient within the q2 for the XL 1 instrument (the XL 2 instrument showed similar results to the XL 1). The line with the filled diamonds represents a q2 pressure of 6.3 mTorr, and the line with the filled squares represents a q2 pressure of 16.7 mTorr.

($E_{cm} = 0.675$ – 0.844 eV for the first collision, corresponding to a potential difference between q0 and q2 of 120–150 V), the abundance of H₄B ions did not increase nearly as much as the heme ion; this could be due to fragmentation of the former to smaller products, or that these higher energy conditions favoured other fragmentation channels.

As a final confirmation that the iNOS oxygenase domain mutant W188F did not bind cofactors, CID experiments were performed on the $[M + 16H]^{16+}$ ion at m/z 3312. This ion has a mass of 52976 Da, indicating that it is excessively solvated and/or has cofactors bound to it. The absence of charge loss under collision conditions of 100 eV was taken as proof that no cofactors were bound (data not shown).

Additional Discussion regarding Instrumental Discrimination

The Centaur uses a conventional quadrupole rod set in q2, whereas the XL employs a LINAC II rod set that imposes an axial electric field [43]. It is expected that, all other conditions being equal, ions injected into the q2 of the Centaur will spend a longer time within the quadrupole than those ions injected into the q2 of the XL instruments. To investigate the effect of this residence time, the axial electric field gradient in the XL 1 instrument was varied. Figure 7 shows that as the density of the collision gas in q2 increased, the effect of this potential gradient was more dramatic. At lower gradients (0– 0.005 V·cm^{−1}, corresponding to LINAC voltages of 0 to 10 V), monomer ions were relatively less intense than the dimer ions; as gradient increased, the relative abundance of the monomer ions increased approximately logarithmically. It was also noted that the near logarithmic curve became less steep as the q2

gas density was increased. The Centaur lacks an axial electric field in its q2, its behavior as seen in Figure 2 mirrors that of the XL instruments operated under non-optimal electric field gradient. The XL instruments, however, maintain an optimal gradient near the limit of the logarithmic slope (i.e., at an operating LINAC voltage of 50 V in all experiments), and hence the relative abundances of the dimers and monomers did not change appreciably as the q2 gas density was raised. The fact that the ratio of monomer to dimer ion levels off at high LINAC voltages is in accordance with the expectation that the monomer ions are not created by the presence of the LINAC voltage (otherwise the trend would continue to greater relative monomer abundance as the voltage increased), but are released from the collision cell by this axial potential gradient. These results suggest that longer residence times within the q2 discriminates against the lighter monomer ions.

In addition, dimer discrimination was observed in the TOF section of the XL instrument at q2 pressures lower than ~6 mTorr. Presumably, this also occurs in the Centaur; however, the older software used in the Centaur to monitor the internal processes of the instrument makes it difficult to confirm this. At pressures lower than ~6 mTorr, dimers were observed to be biased towards the fourth quadrant (furthest from q2) in the MCP detector of the XL, indicating that these ions entered into the TOF region of the instrument with residual kinetic energy in the axial direction of the quadrupoles; this potential problem has been noted before [33, 34]. This excess energy caused the dimer ion beam to not be centered on the detector, and a percentage of these ions might have missed the detector altogether after time-of-flight. This phenomenon was not observed with the monomers, which were well centered. As suggested by Chernushevich and Thomson [36] in their studies of large non-covalent clusters, this indicates that at lower q2 pressures (less than 6 mTorr in our case), the dimer ions do not undergo enough collisions in q2 to fully dampen their kinetic energy in the axial direction. Monomers, however, because of their larger cross-section per unit mass, have their axial kinetic energy more efficiently dampened, and enter the TOF section at the optimum energy for detection.

Our results support the notion that some of the solution-phase chemistry associated with non-covalently bound proteins is retained in the gas phase. In every case, solutions containing proteins known to non-covalently associate with each other showed this behavior in our gas-phase experiments. Furthermore, those species that do not associate with each other in solution retained this behavior in the gas phase, as seen with the W188F iNOS oxygenase domain mutant. Additionally, CID experiments revealed that cleaving of the heme required a higher energy than cleaving of tetrahydrobiopterin or arginine. In solution, binding of the heme occurs first to monomeric units, and then the two other cofactors bind during or after the process of

dimerization. It is interesting that this order of reaction correlates with the order of decreasing CID energy.

Conclusion

The iNOS oxygenase domain dimer was observed intact and partially solvated using three hybrid quadrupole time-of-flight mass spectrometers. In general, observation of abundant dimer ions requires relatively high pressure in q2. Furthermore, binding of heme, H₄B, and arginine was demonstrated via CID of the dimer ion.

iNOS oxygenase domain was also run in acidic solutions. The results showed that although the heme is not lost from the protein between pH 2.5 and 4.0, the dimeric structure is disrupted. Finally, solutions of the iNOS oxygenase domain mutant W188F were also run under optimal conditions for the dimer ions, confirming that this species neither dimerizes, nor binds heme.

Acknowledgments

The authors thank Drs. Igor Chernushevich, Bruce A. Thomson, and Vladimir Baranov of MDS Sciex for access to their instruments and for their helpful suggestions and discussion on the details of this work. Financial support of the research from NSERC, MDS Sciex, and York University is gratefully acknowledged. JCS thanks NSERC for a postgraduate scholarship.

References

1. Sheta, E. A.; McMillan, K.; Masters, B. S. S. *J. Biol. Chem.* **1994**, 269, 15147–15153.
2. Abu-Soud, H. M.; Loftus, M.; Stuehr, D. J. *Biochemistry* **1995**, 34, 1167–1175.
3. Venema, R. C.; Ju, H.; Zou, R.; Ryan, J. W.; Venema, V. J. *J. Biol. Chem.* **1997**, 272, 1276–1282.
4. Gachhui, R.; Ghosh, D. K.; Wu, C.; Parkinson, J.; Crane, B. R.; Stuehr, D. J. *Biochemistry* **1997**, 36, 5097–5103.
5. Wilson, D. J.; Rafferty, S. P. *Biochem. Biophys. Res. Commun.* **2001**, 287, 126–129.
6. Ens, W.; Standing, K. G.; Chernushevich, I. V. *New Methods for the Study of Biomolecular Complexes*; Kluwer Academic Publishers: Boston, MA, 1998; pp 103–104.
7. Chung, E. W.; Henriques, D. A.; Renzoni, D.; Morton, C. J.; Mulhern, T. D.; Pitkeathly, M. C.; Ladbury, J. E.; Robinson, C. V. *Protein Sci.* **1999**, 8, 1962–1970.
8. Juraschek, R.; Dulcks, T.; Karas, M. *J. Am. Soc. Mass Spectrom.* **1999**, 10, 300–308.
9. Loo, J. A. *Mass Spectrom. Rev.* **1997**, 16, 1–23.
10. Veenstra, T. D. *Biophys. Chem.* **1999**, 79, 63–79.
11. Maleknia, S. D.; Downard, K. *Mass Spectrom. Rev.* **2001**, 20, 388–401.
12. Rusconi, F.; Guillonnet, F.; Praseuth, D. *Mass Spectrom. Rev.* **2002**, 21, 305–348.
13. Winston, R. L.; Fitzgerald, M. C. *Mass Spectrom. Rev.* **1997**, 16, 165–179.
14. Huang, E. C.; Pramanik, B. N.; Tsarobopoulos, A.; Reichert, P.; Ganguly, A. K.; Trotta, P. P.; Nagabhushan, T. L.; Covey, T. R. *J. Am. Soc. Mass Spectrom.* **1993**, 4, 624–630.
15. Veenstra, T. D. *Biochem. Biophys. Res. Commun.* **1999**, 257, 1–5.
16. Little, D. P.; McLafferty, F. W. *J. Am. Soc. Mass Spectrom.* **1996**, 7, 209–210.
17. Hunter, C. L.; Mauk, A. G.; Douglas, D. J. *Biochemistry* **1997**, 36, 1018–1025.

18. Konermann, L.; Rosell, F. I.; Mauk, A. G.; Douglas, D. J. *Biochemistry* **1997**, *36*, 6448–6454.
19. Li, Y.-T.; Hsieh, Y.-L.; Henion, J. D. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 631–637.
20. Fitzgerald, M. C.; Chernushevich, I.; Standing, K. G.; Whitman, C. P.; Kent, S. B. H. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 6851–6856.
21. Light-Wahl, K. J.; Winger, B. E.; Smith, R. D. *J. Am. Chem. Soc.* **1993**, *115*, 5869–5870.
22. Light-Wahl, K. J.; Schwartz, B. L.; Smith, R. D. *J. Am. Chem. Soc.* **1994**, *116*, 5271–5278.
23. Tolic, L. P.; Harms, A. C.; Anderson, G. A.; Smith, R. D. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 510–515.
24. Schwartz, B. L.; Bruce, J. E.; Anderson, G. A.; Hofstadler, S. A.; Rockwood, A. L.; Smith, R. D. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 459–465.
25. Douglas, D. J.; Collings, B. A.; Numao, S.; Nesatyy, V. J. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 89–96.
26. Pramanik, B. N.; Bartner, P. L.; Mirza, U. A.; Liu, Y. H.; Ganguly, A. K. *J. Mass Spectrom.* **1998**, *33*, 911–920.
27. Li, Y.-T.; Hsieh, Y.-L.; Henion, J. D.; Senko, M. W.; McLafferty, F. W.; Ganem, B. J. *Am. Chem. Soc.* **1993**, *115*, 8409–8413.
28. Robinson, C. V.; Chung, E. W.; Kragelund, B. B.; Knudsen, J.; Aplin, R. T.; Poulsen, F. M.; Dobson, C. M. *J. Am. Chem. Soc.* **1996**, *118*, 8646–8653.
29. Raman, C. S.; Li, H.; Martasek, P.; Kral, V.; Masters, B. S.; Poulos, T. L. *Cell* **1998**, *95*, 39–50.
30. Schmidt, A.; Bahr, U.; Karas, M. *Anal. Chem.* **2001**, *73*, 6040–6046.
31. Sobott, F.; Hernandez, H.; McCammon, M. G.; Tito, M. A.; Robinson, C. V. *Anal. Chem.* **2002**, *74*, 1402–1407.
32. Douglas, D. J. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 101–113.
33. Tahallah, N.; Pinkse, M.; Maier, C. S.; Heck, A. J. R. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 596–601.
34. Krutchinsky, A. N.; Chernushevich, I. V.; Spicer, V. L.; Ens, W.; Standing, K. G. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 569–579.
35. Covey, T.; Douglas, D. J. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 616–623.
36. Chernushevich, I. V.; Thomson, B. A. *Proceedings of the 51st ASMS Conference on Mass Spectrometry and Allied Topics*; Montreal, Canada, June, 2003.
37. Douglas, D. J.; French, J. B. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 398–408.
38. Chrisman, P. A.; Newton, K. A.; Reid, G. E.; Wells, J. M.; McLuckey, S. A. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 2334–2340.
39. Engel, B. J.; Pan, P.; Reid, G. E.; Wells, J. M.; McLuckey, S. A. *Int. J. Mass Spectrom.* **2002**, *219*, 171–187.
40. Versluis, C.; Heck, A. J. R. *Int. J. Mass Spectrom.* **2001**, *210/211*, 637–649.
41. Schmidt, A.; Karas, M. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 1092–1098.
42. Rafferty, S. P.; Boyington, J. C.; Kulansky, R.; Sun, P. D.; Malech, H. L. *Biochem. Biophys. Res. Commun.* **1999**, *257*, 344–347.
43. Loboda, A.; Krutchinsky, A.; Loboda, O.; McNabb, J.; Spicer, V.; Ens, W.; Standing, K. *Eur. J. Mass Spectrom.* **2000**, *6*, 531–536.
44. Lemaire, D.; Marie, G.; Serani, L.; Laprévote, O. *Anal. Chem.* **2001**, *73*, 1699–1706.
45. Lee, V. W.; Chen, Y. L.; Konermann, L. *Anal. Chem.* **1999**, *71*, 4154–4159.
46. Li, H.; Raman, C. S.; Glaser, C. B.; Blasko, E.; Young, T. A.; Parkinson, J. F.; Whitlow, M.; Poulos, T. L. *J. Biol. Chem.* **1999**, *274*, 21276–21284.