Gaseous Conformational Structures of Cytochrome *c*

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Abstract: Solution folding of a protein removes major sections of it from their aqueous environment. Complete removal, by forming water-free gaseous protein ions with electrospray ionization/mass spectrometry, profoundly changes the conformation of cytochrome *c*. Of these ions' exchangeable hydrogen atoms, gaseous D_2O replaces 30% to 70% in distinct values indicative of at least six conformational states. Although this is increased to >95% by colliding ions with D_2O, colliding instead with N_2 and subsequent D_2O exposure gives the same H/D exchange values, although in different proportions; on solvent removal, denatured ions spontaneously refold. Deuterated State I, II, and V ions of a range of charge values up to 17+ when charge stripped to 9+ ions do not fold appreciably, even though their cross section decreases by 20%, confirming that each has a characteristic conformational structure insensitive to electrostatic repulsion; the charge solvation of an added protonated side chain also protects additional exchangeable sites. Dramatic temperature effects on H/D exchange also support unique State I, II, IV, and V conformers with a variety of charge values. Despite extensive H/D scrambling, dissociation to locate D sites of State I, II, IV, and V ions indicates that four small α-helical regions are maintained even in the most open ionic conformations; these regions are consistent with salt bridge stabilization. In the more open conformers the α-helical regions could be partially converted to either β-sheet or denatured structures. No close similarities were found between the gaseous conformer structures and those in solution, a cautionary note for the use of ESI/MS gas-phase data to characterize noncovalent interactions in solution.

Introduction

A protein's sequence determines its unique self-assembly in water to produce its biologically active folded form; understanding this process continues to attract intensive research interest.[1−9] Folding involves intramolecular noncovalent binding to remove substantial portions of the protein molecule from its aqueous environment, suggesting studies on the effect of anhydrous conditions on protein conformation. For example, substituting acetonitrile for water can severely reduce enzymatic activity, even though the α-helix content and crystalline enzyme structure are little changed.[8] Even with the complete removal of water and its concomitant hydrophobic stabilization, gaseous protein cations can exist in several conformational states that can be folded and unfolded.[10,11] In a critical mechanistic examination of such data for cytochrome *c*,[10b] Wolynes pointed out[2] that van der Waals packing forces would have to be unusually important for native structures to survive in vacuo, without solvent stabilization, so that many other structures should be competitive energetically. Although the absence of water should increase the stability of classical secondary structures such as the α-helix and β-sheet, the apolar vacuum could even stabilize hydrophobic residues on the exterior to produce an "inside out" conformation.[2] Understanding gaseous conformations is also important because the stabilities of noncovalent enzyme−substrate complexes in solution have been shown to have both substantial similarities[12] and differences[13] in comparison to those of their gaseous ions formed by electrospray ionization (ESI).[14] A recent definitive review states: "These weakly bound systems reflect, to some extent, the nature of the interaction found in

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the condensed phase".[13c] The reliability of such gas-phase data as a diagnostic for combinatorial screening of potential substrates in solution[15] is dependent on the structural integrity of ESI; the intramolecular noncovalent bonding of conformational structures should provide a unique test.

Extensive studies of the cytochromes *c* (~104 amino acids)[4] include H/D exchange to measure amide H sites exposed by unfolding in aqueous solution. This demonstrates that its N- and C-terminal α-helices are the last to unfold;[5a] their interaction through the highly conserved Leu[94] is stabilized by hydrophobic and van der Waals forces.[5,6] This interaction of the terminal helices provides a similar degree of stabilization for the A state conformer formed in acidic (pH 1) solutions of cytochrome *c*.[5b] For our previous experiments with this protein,[10] gaseous ESI cations of charge values 6+ through 17+, trapped (10^{-9} Torr) in a Fourier transform (FT) mass spectrometer,[16] were subjected to H/D exchange with D_2O (10^{-7} Torr). From their unit-resolution mass spectra,[17] all but the 9+ charge value exhibited more than one discrete level of exchange; in all, five levels involving 30−70% (none denatured) of the 198 solution-exchangeable hydrogens were assigned as gaseous conformational states I to V.[10a,b] Less open states could be formed by charge stripping of more open states. State II, the most open, appeared to be unusually stable, being formed for charge values 8+ through 17+ by ESI and/or by unfolding with infrared (IR) laser heating[18] or charge stripping.[10]

Recent ion mobility measurements[12d,19] of gaseous bovine cytochrome *c* ions[20] indicated multiple cross-section values for both the 7+ and 8+ species, consistent with our earlier H/D exchange studies.[10a,b] Cross sections for the 5+ and 9+ ions corresponded to those calculated for the native structure and an α-helix, respectively. Each of the 9+ through 20+ ion species showed only one ion cross section value (in contrast to multiple H/D exchange values for 10+ to 17+ ions),[10b] possibly by unfolding to our State II induced by collisional heating in these high-pressure experiments.[10b] However, the cross-section values increased by 20% from the 9+ to 17+ species,[20] explained by "the protein unfolds as the charge increases";[20b] this is in direct conflict with our proposal that State II of the 9+ to 17+ species is a singular basic structure.[10] Further evidence for the singularity of the five proposed[10] and additional conformational states, and for their structures as contrasted to those in solution, is presented here for the 6+ to 19+ ions.

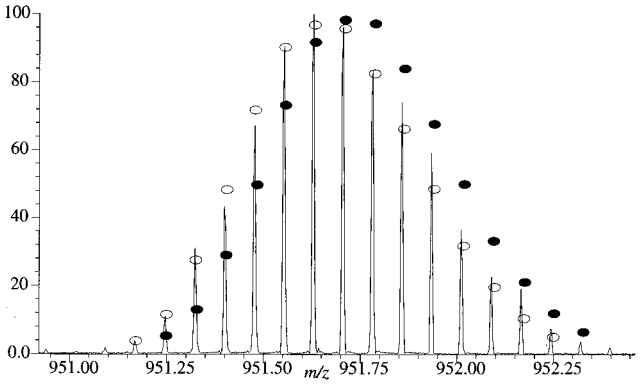

Figure 1. The $(M + 13H)^{13+}$ ions from ESI of the ascorbic-acid-reduced cytochrome *c*; isotopic peak abundances predicted for (●), Fe(II) and (○), Fe(III).

Experimental Section

Equine cytochrome *c* (95% Fe^{3+}, Sigma) in a variety of solutions (e.g., 20 μM in methanol/water/acetic acid, 76:22:2 (v/v/v), and in pure water) were ionized (ESI) and the ions trapped (10^{-9} Torr) at ~26 °C (unless noted otherwise) in a modified FT/ICR mass spectrometer (6 T, Finnigan FTMS, Madison, WI),[17] as described previously.[10b,c] For deuteriation, ions of the desired charge and D content were isolated by SWIFT[21] to prevent contamination by D_2O charge stripping of higher charge states; these ions were exposed to D_2O (10^{-7} Torr) for 30 min (~98% completion; 90−95% completion at 130 °C). Quadrupolar axialization[10c,22] used D_2O or N_2 (5×10^6 Torr) as the collision gas. Ion charge stripping used NH_3 or a small amine ($\sim 5 \times 10^{-8}$ Torr) for 2−10 s, and MS/MS ion dissociation utilized SORI.[23] Spectra were mass calibrated by using ubiquitin, M_r (most abundant isotopic peak) = 8564.63-*5*; the italicized final digit denotes the mass difference (units of 1.0034 Da, the $^{13}C/^{12}C$ difference) between the most abundant isotopic peak and the monoisotopic peak.[17]

Results and Discussion

ESI Oxidation. The driving force for folding the Fe(II) form of cytochrome *c* in solution is greater than that for the Fe(III) form.[24] The heme fragment ion from ESI of cytochrome *c* appears to be in the Fe(II) form, in contrast to the Fe(III) form for these ions from ESI of hemoglobin and myoglobin.[12c] The corresponding molecular ions should be distinguishable by MS, as the reduced form has an extra hydrogen atom (calculated M_r: Fe(II), 12359.34-*9*; Fe(III), 12358.33-*9*). Reduction of the brownish Fe(III) cytochrome *c* solution with ascorbic acid gave a clear reddish solution, but its ESI gave molecular ion isotopic abundances (13+, Figure 1) consistent with Fe(III) as the dominant (80 ± 20%, measured M_r = 12358.43-*9*) form. ESI of the Fe(III) solution gave abundances indicating a negligible Fe(II) content: $M_r(13^+)$ = 12358.39-*9*. Oxidation during ESI has been observed to produce oxygenated $(M + H + 16)^+$ ions from peptides[25a] and higher charge states from cytochrome *c*.[25b]

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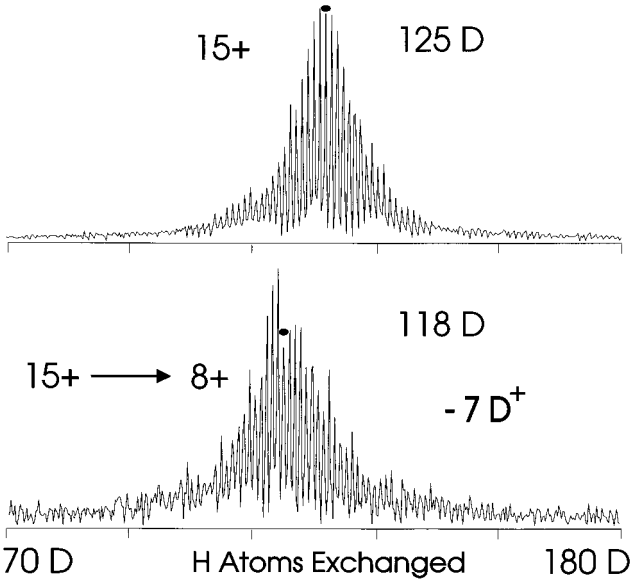

Figure 2. ESI/FTMS spectrum of 15+ cytochrome *c* ions (a) after exposure to 10^{-7} Torr of D_2O for 30 min and (b) after exposure of these ions to $C_4H_9NH_2$ to yield 8+ ions.

The "Inside-Out" Structure[2] vs Exterior Protonation. Electrosprayed 15+ ions from equine cytochrome *c* exposed at room temperature to D_2O (10^{-7} Torr) exchange, on average, 125 H atoms to D atoms in 30 min (~98% complete).[10] Its isotopic distribution is only 4 Da wider (half-height, Figure 2A) than that of Figure 1, indicative of a single conformer; this was designated as State V.[10b] When these D-labeled ions are charge stripped in the ion cell by exposure to butylamine, the 8+ product ions contain 118 D atoms (Figure 2B). Within experimental error, only D^+, not H^+, species are removed for 15+ → 8+, as would be expected from the far higher reactivity of ionic sites. Extensive evidence, in particular that of Williams,[11] indicates that the most basic residues are the protonation sites; thus they do *not* provide the interior hydrogen bond stabilization of an "inside-out" structure, consistent with Wolynes' predictions.[2] This also shows that the number of *protein* H atoms exchanged is the observed value of added D atoms minus the number of charges. In preparing Figure 3, the previous data[10] have been adjusted for this and combined with new measurements discussed below.

Kinetic Control of Conformer Formation. As previously observed,[10] the relative abundance of the conformational states and their formation specificity for a particular charge value are sensitive to experimental conditions (e.g., solution, temperature, apparatus). With many repeated measurements over a 4-year period, this was found to be especially true for charge values below 9+. For example, the lowest exchanging conformers (~57 protein sites) for 6+ and 7+ could originally be formed directly by ESI (Figure 3 values corrected for ~90% D_2O purity),[10a] but with the new instrument these have only been formed by charge stripping. Also, measurements of 5+ to 8+ ions formed by charge stripping often show overlapping isotopic distributions and are poorly reproducible; relative exposure to the slowly desorbing stripping agent is difficult to duplicate, but also isomerization barriers appear to be lower at lower charge states. To delineate this problem, over 50 separate H/D exchange measurements of 7+ ions formed by ESI and charge stripping were made, varying the ESI solvent from pure water to 76:22:2 methanol/water/acetic acid. Although the abundances indicated for specific conformers were highly variable, the spectra are consistent with as many as seven stable conformations for the 7+ ions, and further measurements support these conformational states for the 8+ ions (and States I, III, VI, and VII for 6+ ions). Four of these states (I, III, IV, V) were identified in our previous measurements.[10] Also, note that the cross-section values reported recently by Jarrold and coworkers[20b] indicated five conformers for both the 7+ and 8+ charge values.

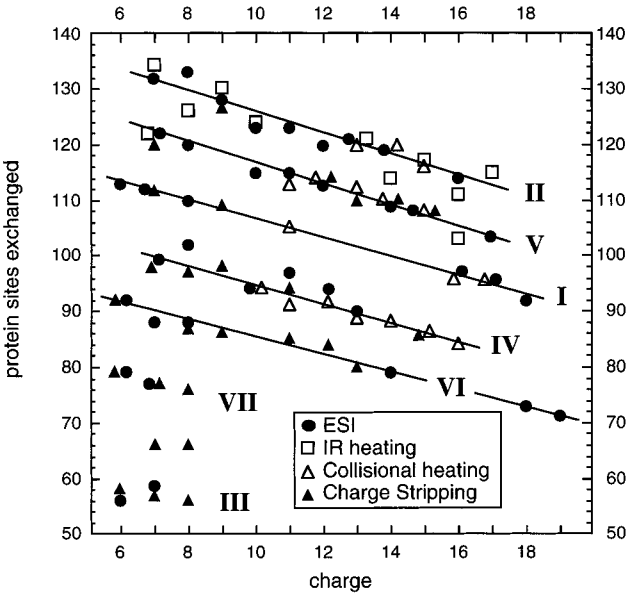

Figure 3. Number of H atoms of cytochrome *c* ions (excluding H^+ added by ESI) exchanged by 10^{-7} Torr of D_2O in 30 min (~98% complete) for ions formed by (●) l, ESI, (□) IR laser heating of ions of the same charge value, (▲), charge stripping, and (Δ) quadrupolar axialization with N_2 followed by ion cooling.

For this H/D exchange study, much more specific values were found for charge states 9+ and larger, so that the interpretation for these values is emphasized below. For these charges, States I, II, IV, and V were identified previously,[10b] with two lower values for 13+ and 14+ ions; further measurements, including those for ESI of 18+ and 19+ ions, now indicate these as State VI (Figure 3). Note again that these values are the number of *protein* H atoms exchanged, subtracting the number of charges from the exchange total.

Charge Insensitivity of Conformational Structure. Although the ion mobility measurements at atmospheric pressure gave qualitative support for the multiplicity of gaseous conformers for the 7+ and 8+ ions, for each of the charge states above 9+ only one mobility value was observed.[20] This could result from collisional heating, in the same way that IR heating (Figure 3) converts most such conformers to State II. However, the *decrease* in cross section with decreasing charge, an expected effect of reducing electrostatic repulsion,[20] is in apparent conflict with the Figure 3 data that show a linear *increase* in the number of exchangeable sites with decreasing charge for all of the proposed conformational states. In fact, the lowest charge states (7+ to 9+) show the highest number of protein H atoms exchanged, almost double that of the 19+. The State II conformer undergoes a 13% *increase* in H/D exchange from lowering the charge from 17+ to 9+ (Figure 3), while mobility values indicate a 20% *decrease* in cross section with decreasing ion charge for values from 17+ to 9+, which was thought to indicate a tighter conformer of *decreased* exterior exposure.[20b,c] The linear correlation of H/D exchange values for the distin-

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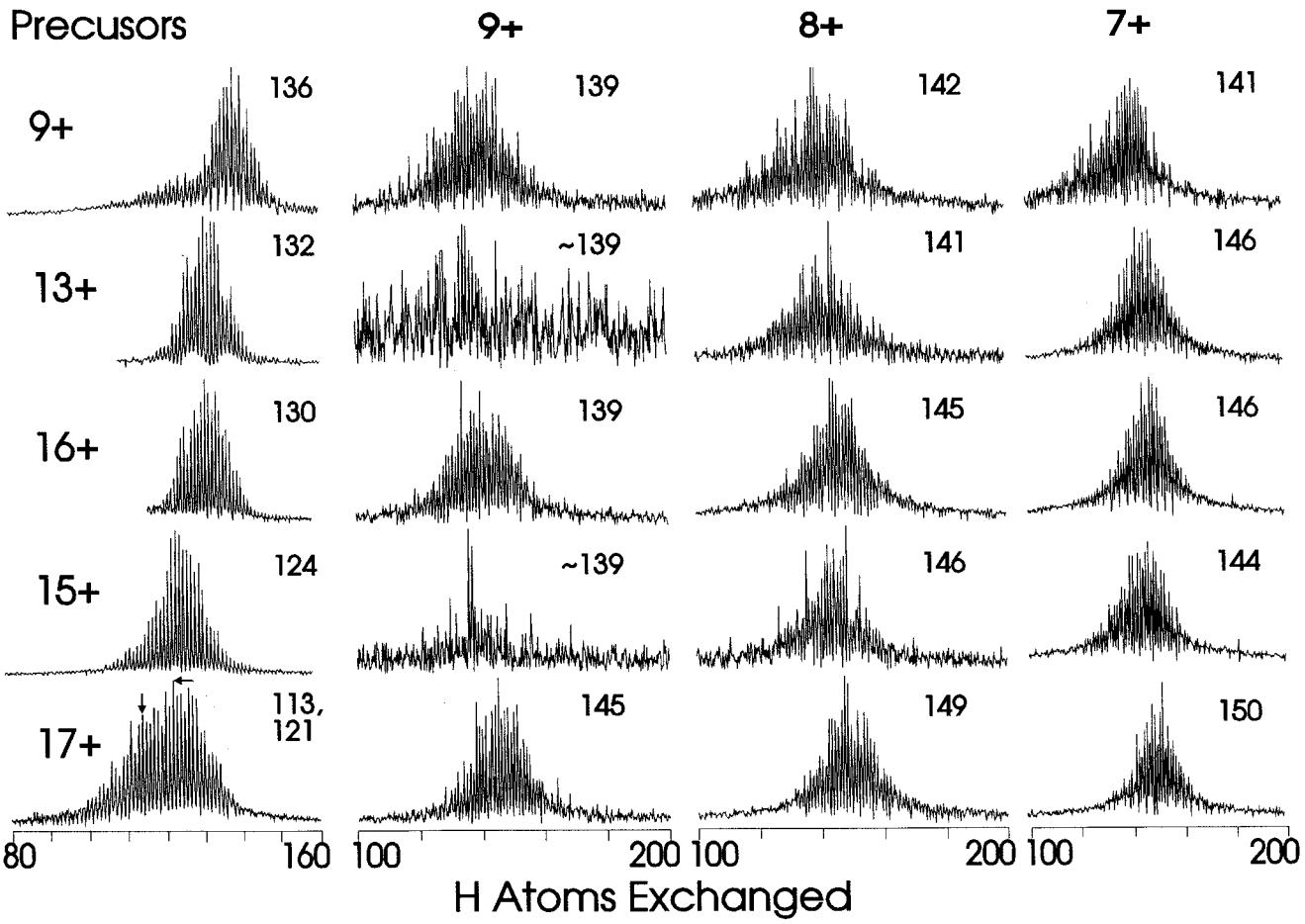

Figure 4. ESI/FTMS partial spectra from 30 min of D_2O exchange (column I), then charge stripping to form 9+, 8+, and 7+ ions, and then an additional 30 min of D_2O exchange (columns II−IV). Data for 6+, although of much poorer signal/noise, are similar to that for the 7+ ions (column IV).

guishable five conformational States I, II, IV−VI suggests instead that decreasing only causes a gradual nonspecific opening of each conformational structure, unaccompanied by local folding to form a new structure. To check this, deuterated ions of higher charge values were charge stripped to 9+ and exchanged again with D_2O (Figure 4); if any of the more highly charged ions have regions that are more open than the 9+ State II conformer formed from them, those deuterated sites would be protected after charge stripping to 9+, so that additional deuteriation would give a higher total exchange than 9+ ions formed directly (Figure 3 shows that charge stripping can also form a minor amount of 9+ ions of the more folded States I, IV, and VI).[26] The ref 9+ ions initially exchanged a total of ~136 D atoms (~98% complete, 127 protein H atoms exchanged); exposure to triethylamine and again to D_2O gave ~139 D (>99% complete) for the remaining 9+ ions. The slightly higher values for 8+ and 7+ ions (and similar 6+ values with lower signal/noise) correspond to those of the State II conformers formed by ESI (Figure 3), consistent with State II 9+ ions as a major intermediate in charge stripping to the more folded 6+ to 8+ ions.[26]

Next the 13+ and 16+ ions that had added 132 and 130 D atoms, respectively, were studied. These have exchanged 8 and 11, respectively, fewer protein H atoms than the 9+ ions. If these 13+ and 16+ ions have the same basic State II conformational structure as the 9+ ions, only differing in the fact that 8 and 11 sites, respectively, are more protected in the 13+ and 16+ ions, they should not have open sites containing D atoms that are closed on conversion to State II 9+ ions. After charge stripping these 13+ and 16+ ions to produce 9+ to 7+ ions, their further H/D exchange yields the same or only slightly higher values than those found in treating the 9+ ions (Figure 4). Any 9+ ions of States I, IV, and VI would also give such values, but deuteriation after charge stripping shows these are only minor products.[26] Thus, despite their higher cross sections,[20] these 13+ and 16+ State II ions have a negligible number of open (and thus deuterated) sites that are closed in the 9+ to 7+ State II ions due to decreased coulombic repulsion, as these additional D atoms are not found in the charge-stripped State II ions.

Of other conformers, the deuterated State V 15+ ions (124 total D, 109 protein H exchanged) on charge stripping to 9+ to 7+ ions and D_2O exposure give (Figure 4) nearly the same values as the State II ions formed from 9+, 13+, and 16+ ions; the State V → State II transformation only involves unfolding. The 17+ ions, which appear to be a mixture of State I and V conformers (96 and 104 protein H atoms exchanged), in the same experiment are charge stripped and deuterated to produce 9+ to 7+ ions with possibly six more D atoms than do the State II ions. Assuming that the State V 17+ ions behave like the 15+ (State V) ions, State I appears to have approximately six open sites that are closed in the 9+ State II ions. The State I (17+) → State II (9+) conversion exposes 127 − 96 + 6 =

(26) The folding accompanying the formation of deuterated charge-stripped products with tighter conformations was probed by their back-exchange with H_2O; the results were inconclusive because efficient removal of the D_2O background requires extensive pump out.

37 protein exchangeable sites while folding six. Thus converting these 17+ and 15+ State I and V conformers to the more open 9+ State II conformer ions also involves essentially no folding for the State V ions, and little folding for the State I, despite the decreased electrostatic repulsion.

The data for the 7+ to 19+ ion conformers all fit well with a linear *decrease* of nearly two exchangeable hydrogen atoms per decrease in unit charge (Figure 3; States II and V, 1.87; States I and IV, 1.67; State VI, 1.53). This minimal effect of charge on conformation is also inconsistent[11] with an "inside out" structure[2] for which the extra charges would be added to the polar interior. Further, this minimal conformational effect is seen in solution, where the native cytochrome *c* conformer is stable over a wide range of hydrogen ion concentration, pH 9 (carrying a net charge of 5+) to 4.5 (10+),[4,27] with even the A state (pH 1) of similar conformational structure.[5b] The very recent Clemmer study also finds no increase in the total H/D exchange with increasing charge for exposure of the cytochrome *c* ions to D_2O at atmospheric pressure.[20c,f]

Local Structural Effect of an Added Proton. For gaseous ions without aqueous solvation, Williams[11] has presented persuasive evidence that the protonated side chains are solvated instead by other functional groups of the protein. A recent report attributes dissociation of 15+ cytochrome *c* ions by low-energy electrons to their capture by protons partially bonded to backbone carbonyl groups; this causes cleavage of 63 of the 103 backbone bonds.[28] After protonation, a basic side chain (Lys, His, Arg) thus tends to fold in on the gaseous protein ion and hydrogen bond to sites that would otherwise undergo H/D exchange. Such a folded side chain could reasonably protect several exchange sites (Figure 3), but only if its proton does not transfer to another site during the D_2O exposure. This should be true of the most probable site of first protonation (that of highest basicity) after several other protons have been added; with each additional proton, the number of protonated side chains that remain protected during D_2O exposure should increase by one, on average. Even if this side chain solvation with increasing charge decreases the ion's H/D exchangeable sites (van der Waal's forces will also bind side chains to the core),[29] the increased Coulombic repulsion could still cause the observed[20] overall increase in the ionic cross section by incremental extension of side chains and/or of the chain length without changing the basic conformational structure.

Effect of Temperature. The tendency for these conformations to be denatured by heat also appears to be characteristic of the conformational state and surprisingly independent of charge value. For example (Figure 5), the 13+ State II ions exchange 33 more protein H atoms than the 13+ State IV ions at room temperature, but at 90° and 110° they exchange only 17 and 21 more, respectively. In contrast, the 16+ State II ions exchange 19 more H atoms than the 16+ State I ions at room temperature, but this difference has *increased* to 34 and 38 H atoms at 90° and 110°, respectively. States II and V have the most similar temperature behavior, with State V exchanging ~10 fewer H atoms at most temperatures; this is consistent with the Figure 4 data, indicating that the State V → II conversion involved only opening of the State V conformation. The temperature effect on 8+ State II ions (not shown) is similar to that of 9+ ions, although other 8+ conformational states are indicated. Obviously these differing behaviors caused isotopic overlaps that made conformer assignment difficult in some cases, as indicated by larger symbols in Figure 5. At 130 °C the isotopic distributions of the 16+ and 9+ deuterated ions are sufficiently narrow to show that water loss (−18 Da) is <10% and <20%, respectively.

At room temperature, the local structural effect of an added proton (slopes of the Figure 3 correlations) is to protect an additional 1.5−1.9 H atoms; at 100 °C this is nearly doubled, at least for the State II conformer. Surprisingly, all four States show decreasing exchange at the highest temperatures; the State I 16+ value is even lower at 130 °C than at room temperature. Despite the evidence above for low isomerization barriers between conformers, these barriers appear to be maintainted at 130 °C. Further studies to explain this unexpected behavior, such as location of D exchange sites (vida infra), are in progress.

Metastable Denaturated States. Extensive IR laser heating[18] (even that sufficient for dissociation) of the 7+ to 17+ cytochrome *c* ions *followed* by D_2O treatment exchanged no more than 130 protein H atoms,[10] yet using quadrupolar axialization (QA)[22] to heat the ions by collisions *with* D_2O gave a far higher exchange.[10c] Extending this, extensive (30 min) QA in D_2O (Figure 6a) exchanged as many as ~193 protein H atoms, consistent with collisional denaturation to expose nearly all the 198 sites exchangeable in solution. QA in D_2O for intermediate times (Figure 6, upper right) indicated metastable conformer states corresponding to the exchange of ~158 (as observed previously)[10b] and ~175 protein H atoms. Recent ion mobility experiments in D_2O (10^{10} higher pressure than that used here) are consistent with 9+ (State II) and 5+ (State III) conformers that exchange only 54 and 48 protein H atoms, respectively, but exchange completely (~198H) at 110 °C.[20f]

However, using N_2 instead as the collision gas for 10 min QA activation, with time for ion relaxation during N_2 pump out, the D_2O exchange again is incomplete (Figure 6b). In contrast to the denaturation to State II from IR heating,[10] now the collisionally excited and relaxed 10+ to 16+ ions produce conformers whose exchange levels correspond primarily to those of States I, IV, and V. Again,[10] the proportion of conformers formed at a specific charge value depends on the method of preparation, supporting relatively similar free energies for States I, II, IV, and V, with strong entropic control on their formation. Although IR irradiation (Figure 3) of 7+ to 10+ and 13+ to 17+ ions produced primarily State II, the low H/D exchange values after IR found previously[10b] for 14+ and 16+ ions could be due to overlapping formation of State V. In a subsequent measurement, ESI 16+ ions with IR irradiation and 30 min ion equilibration exchanged only 103 protein H atoms (Figure 3).

Conformational Effects of Adducts. In preliminary investigations, $(M + 1K + 14H+)^{15+}$ State V ions of cytochrome *c* exchanged 20 less D atoms than the $(M + 15H)^{15+}$ ions. The possibility that this additional protection is due to strong K^+ bonding between acidic residues is not supported by the fact the 2K and 3K adducts also exchange ~20 less D atoms. Further, the +1Na, +2Na, and +3Na adducts of the 15^+ ion exchange 10, 9, and ~7 D, respectively, *more* D atoms than the all-protonated ion. It is possible that the K^+ adducts represent State IV and the Na^+ adducts represent State II, but more experiments are necessary.

ESI after addition of 0.02% H_3PO_4 to the cytochrome *c* solution gave H_3PO_4 adducts (+98n Da) that could be "boiled off"[10c] by exposure to blackbody infrared radiation[30] (e.g., 50 s at 125 °C cell temperature) or even to some extent by SWIFT.[21]

(27) Theorell, H.; Akesson, A. *J. Am. Chem. Soc.* **1941**, *63*, 1818−1820.
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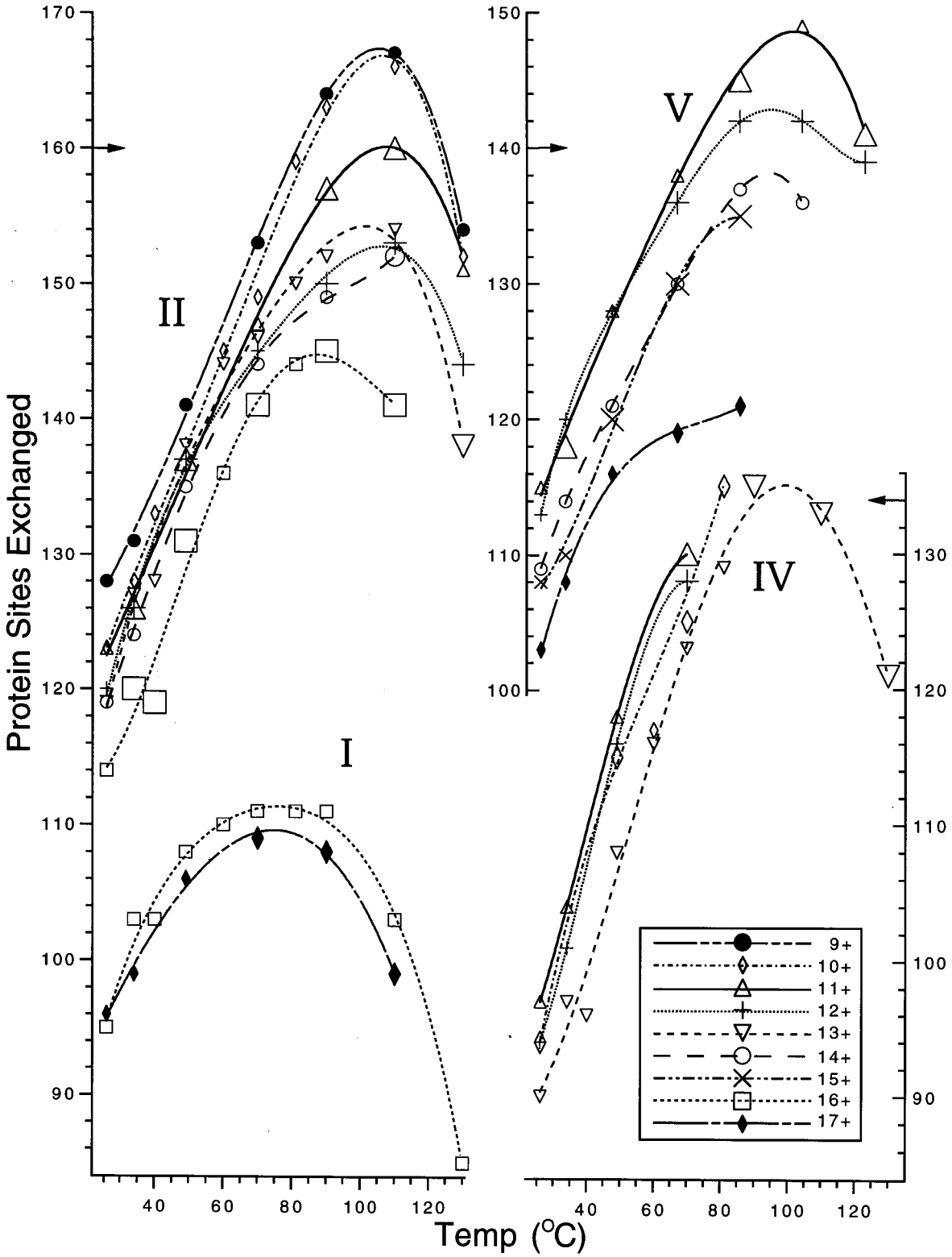

Figure 5. Number of protein H atoms of ESI cytochrome *c* ions exchanged by D_2O (30 min, 10^{-7} Torr) as a function of temperature and conformer state (I, II, IV, V). Large symbols, less accurate values; overlapping states made some assignments ambiguous (e.g., 17+). Reaction rates at 130 °C are half of those at 25 °C, so that the 130 °C values represent 90−95% completion vs ~98% at 25 °C.

With 30 min D_2O exchange, the major State II conformer 13+ ion (135 D exchange with no adducts) gave an additional +4D, +6D, +~9D, and +~12D for the +1, +2, +3, and +4 H_3PO_4 adducts, respectively. This is consistent with retention of State II and no protection of protein ion sites, with the additional D exchange due to the hydrogen atoms of the H_3PO_4 adducts. However, the low abundance State IV ions appear to have an additional +10D, +21D, and +>25D for the +1, +2, and +3 H_3PO_4 adducts; possibly these represent a mixture of State IV with States I and/or V. Recently, McLuckey has described similar hydrogen iodide adducts, reporting persuasive evidence that these are bonded to nonprotonated basic amino acids.[31]

(31) McLuckey, S. A.; Stephenson, J. L., Jr. *Anal. Chem.* **1997**, *69*, 281−285. McLuckey, S. A.; Stephenson, J. L., Jr. *J. Am. Chem. Soc.* **1997**, *119*, 1688−1696.

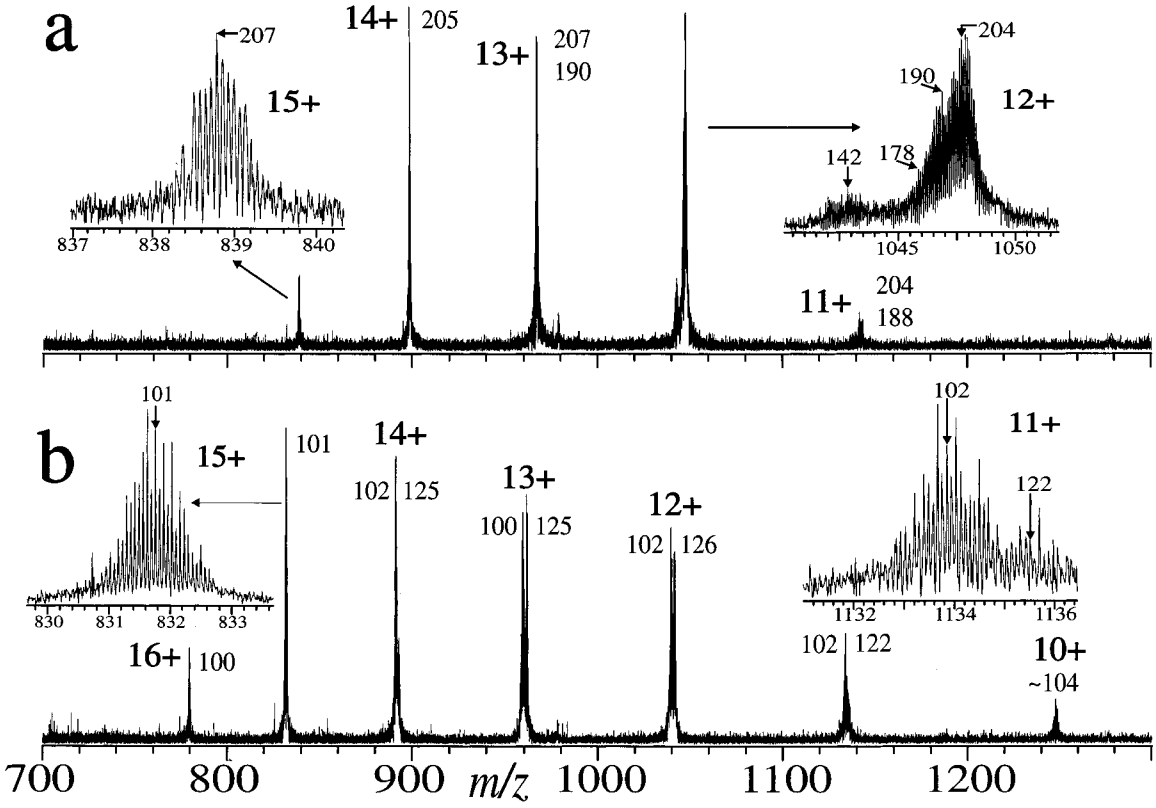

Figure 6. ESI/FTMS partial spectra from quadrupolar axialization in the presence of (a) D_2O for 30 min (upper right, 10 min) and (b) N_2 for 10 min (30 min gave nearly the same values), pump out to 10^{-9} Torr, and D_2O (10^{-7} Torr, no QA) for 30 min. Insets: *m*/*z* scale expansions.

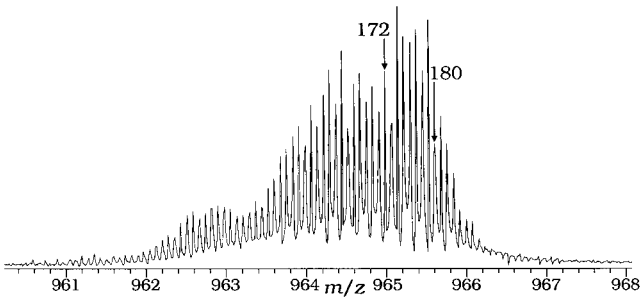

Figure 7. ESI/FTMS spectrum of SWIFT-selected 13+ ions that had exchanged 132 ± 10 D atoms, been subjected to SORI dissociation, and the remaining 13+ ions exposed again to D_2O (30 min). Arrows indicate the total number of added D atoms.

D Atom Localization by MS/MS. To specify the sites of H/D exchange, data were sought on the mass increase this causes in fragments of ionized cytochrome *c*.[17,32] State II 13+ ions that had exchanged an average of 132 D atoms (Figure 4, column I) were selected[21] and partially dissociated by SORI[23] collisional activation. The remaining undissociated 13+ ions were again exposed to D_2O (10^{-7} Torr, 30 min), resulting in the Figure 7 spectrum indicating extensive H/D scrambling by SORI; qualitatively similar spectra resulted from QA and IR laser activation of other charge values and conformer states.[33] If activation had scrambled the 132 D atoms uniformly over the 211 exchangeable H atoms (198 in solution + $13H^+$), each of these would contain 132/211 = 0.626 D atoms. Nondeuterated 13+ ions after SORI activation and subsequent D_2O exchange yield a ~2:3 ratio (spectrum not shown) of conformers containing ~108 and ~128 D atoms (similar to the QA values, Figure 6b). The remaining 103 and 83 exchangeable sites would now each contain 0.626 D atoms, for a total of 64 and 52 additional, predicting isotopic distributions centered at 108 + 64 = 172 D and 128 + 52 = 180 D for complete scrambling of the 132 D atoms. Thus (Figure 6), SORI and IR, as well as QA (Figure 5a), cause extensive but not complete H/D scrambling, consistent with unfolding of the conformers before dissociation.[33]

However, ions that SORI dissociate have much less time for scrambling than undissociated ions. Thus any H/D ratios of fragment ions that differ significantly from a statistical distribution should provide structural information on D atom locations. Deuterated molecular ions, selected[21] to represent a single charge value and conformational state, were dissociated;[23] in one case the 17+ ions were IR irradiated first to prepare State II ions. These MS/MS spectra give regional data (Figure 8) for the number of exchanged vs exchangeable hydrogens both for the protein H atoms and for just the amide H atoms (above, italics), assuming that the side chain sites exchange first; in cytochrome *c* the total amide and side chain sites are 100 and 98, respectively. The D atom content of a fragment ion was independent of its charge state within experimental error, although this can vary greatly for D_2O exchange *after* fragmentation (e.g., ~30% variation for 3+ vs 4+ of b_{25} and y_{29} from M^{13+}).

Secondary Conformer Structures. Despite the H/D scrambling, all of the spectra are consistent with relative protection for residues 22−25, 48−53, 65−75, and 91−93. This is shown most clearly by the 9+ and 13+ ions of highest D atom content whose Figure 8 values for much of these regions are still consistent with a negligible exchange of amide H atoms. If H/D exchange has only occurred at the side chain H atoms (none

(32) Wu, Q.; van Orden, S.; Cheng, X.; Bakhtiar, R.; Smith, R. D. *Anal. Chem.* **1995**, *67*, 2498−2509.

(33) Electron capture dissociation[28] of cytochrome *c* ions appears to cause far less H/D scrambling and more extensive dissociation; its use is under further investigation.

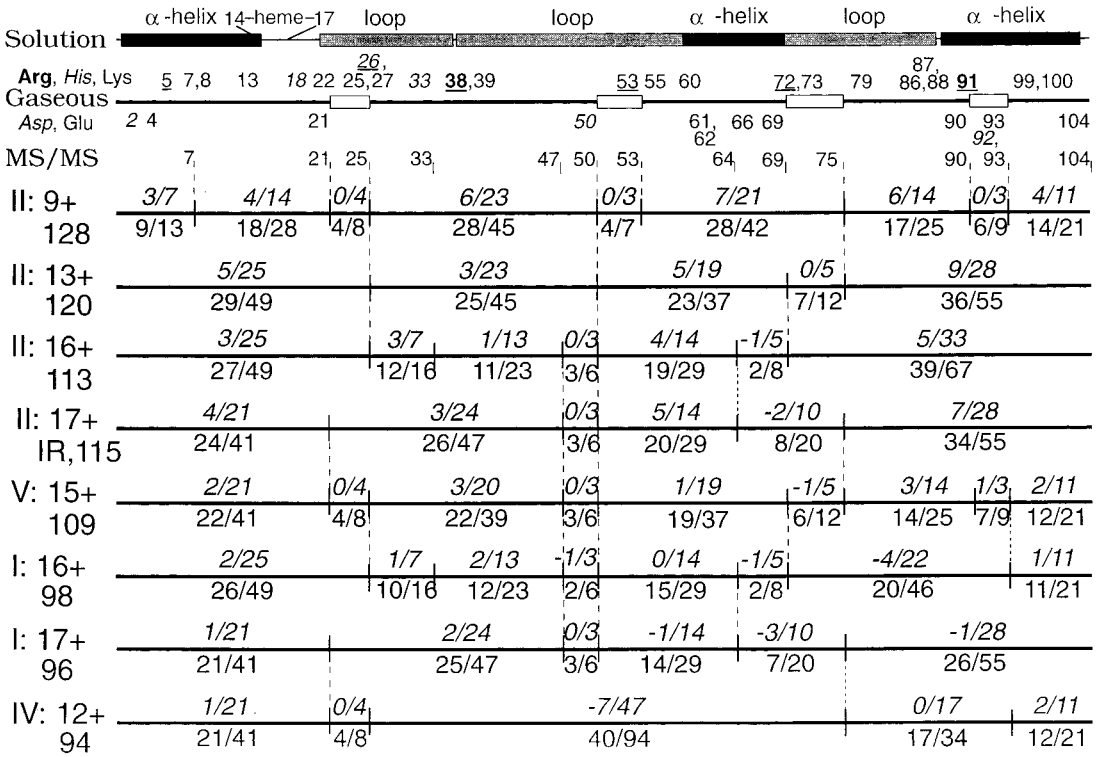

Figure 8. MS/MS dissociation of deuterated cytochrome *c* ions (left column: conformer state, charge value, protein D atom content): D atoms added (value below line) to fragment ions vs total exchangeable protein H atoms (above line in italics, amide H atoms exchanged, assuming that side chain sites exchange first). Top: Most stable regions for solution and gaseous ions, with locations of basic (6 most basic underlined) and acidic residues.

at amide H atoms), this is consistent with an α-helical structure in these four regions. In solution, zwitterionic stabilization of the α-helix is effected by 1,4 placement (less so by 1,3) of acidic (Asp, Glu) and basic (Lys, Arg, His) amino acids.[3] Singly charged gaseous ion salt bridges are further stabilized by incorporation of a second basic residue.[34] Such +, −, + triads exist in all four protected regions (Figure 8, top): Glu^{21}-$Lys^{22,25}$ (and $His^{18,26}$), Asp^{50}-Lys,[53,55] Glu^{69}-Lys,[72,73] and $Glu^{90,92}$(Asp^{93})-Lys^{86-88} (Arg^{91}). Thus these α-helical regions should include at least residues 21−25, 50−55, 69−73, and 87−93 (the *n* to *n* + 4 connectivity of the α-helix would presumably require an additional three N-terminal residues). Of little apparent effect is the more closely spaced pair $Glu^{61,62}$-Lys^{60}, as well as the pairs Glu^{4}(Asp^{2})-$Lys^{5,7,8}$ and Glu^{104}-$Lys^{99,100}$ that could be destabilized by terminal fraying.[3] Of the 24 basic amino acids of ionized cytochrome *c*, the six most basic, as determined by Williams[11] (underlined in Figure 8), are Arg^{38}, Arg^{91}, Lys^{5}, His^{26}, Lys^{53}, and Lys^{72}; one of these is present in each of the four postulated salt bridging regions.

This expected[2] enhanced stability for secondary structures in the gas phase is consistent with the spontaneous folding of denatured cytochrome *c* upon ESI transfer to a nonaqueous environment or after denaturation by QA collisional activation (Figure 6). The bonding of amide H atoms between turns of an α-helix should protect such H atoms (100 of the 198 exchangeable hydrogens) from D_2O exchange, while the exposed edges and turns of a *β*-sheet[9] should increase H-exchange to substantially above 50%. Thus a combination of these secondary structures could account for States I, II, IV, and V, as their 7+ ions of these conformers would have 50% to 65% of their exchangeable H atoms exposed. The State IV conformer could be mainly an α-helix; its 99 exchangeable H atoms for the 7+ ions (Figure 3) would be accounted for by the 98 side chain H atoms if its seven protons transfer to less basic sites fast enough that none protect adjacent exchangeable sites (and one proton would transfer too slowly in the 8+ State IV ions). Alternatively, α-helix formation could be incomplete (e.g., frayed ends)[3] balanced by protection provided by a larger fraction of the protein's protons or even the covalently bound heme. The additional protons on the State IV 12^+ ions (Figure 8) appear to protect sites on the central residues 26−75. This and the additional ∼9 sites protected in State VI probably involve tertiary structures (vide infra).

Figure 3 indicates that ∼12 of the sites protected in the State IV conformer are exposed in State I. Comparing the State IV 12+ MS/MS data (Figure 8) to those of State I 16+ and 17+ ions (which should have 8 or 10 more sites protected by the additional 4 or 5 protons distributed more randomly), the increased exposure of the State I conformer is largely between residues 26 and 47; there are no acidic residues in this region. Similarly the unfolding of State I → State V (15+, 109 D) exposes ∼9 amide H atoms of which the bulk appear to be between residues 76 and 86, a region that also contains no acidic residues and is equally exposed in the State II conformer. The majority of the further ∼9 D atom unfolding of State V → State II appears to occur near residues 54−64 for which Glu^{61}, Glu^{62}/Lys^{60} could provide some helical stabilization. Thus the regional unfoldings in each of these conformer changes could be due to either conversion to a classical *β*-sheet or more extensive denaturation. Besides these possible α-helix and *β*-sheet secondary structures, tertiary protection of the heme group (covalently bound at Cys^{14} and Cys^{17}) may occur; this protection appears to be less in State II, as the heme fragment ions (616.6 Da, Fe^{3+}) from State V (15+, 109 D) and State II (9+, 128 D) contain 0.8 and 1.8 D atoms, respectively.

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Tertiary Structures. State III ions that exchange only ~56 of the possible 198 H atoms (Figure 3) must surely have a tertiary structure, but these ions could not be dissociated by SORI.[33] The solution native conformation has three α-helical regions,[4,5] but these show little correspondence to the most stable four regions for the gaseous ions (top, Figure 8). Although in solution (both pH 7 and 1) the interacting N- and C-terminal helices are the last to be denatured,[5,6] these appear to be the most exposed in the gaseous State IV, as well as even more open in State II; the solution interaction between the terminal helices is stabilized by hydrophobic forces that should be nearly neutralized by removing the surrounding water. A variety of compact structures are conceivable for the State III ions; the assembly of α-helices into a polyhedron is common in solution.[35]

Conclusions

Of the structural possibilities for gaseous conformers examined by Wolynes,[2] an inside out protein with apolar residues outside is clearly untenable. These data instead support his favored explanation: "Without the alternative of hydrogen bonding to the solvent, the classical secondary structures should be even more thermodynamically stable"; α-helix stabilization is generally consistent with all of our experimental data. These data for the secondary gaseous structures I, II, IV, and V are consistent with mixtures of α-helices and *β*-sheets;[9] these are insensitive to electrostatic repulsion, with the α-helix especially stabilized by salt bridging and solvation of the protonated side chains to protect additional exchangeable H atoms. However, this stabilization produces α-helicity in substantially different regions of gaseous versus solution cytochrome *c*, indicating loss of the tertiary stabilization of the native solution structure by ESI removal from water; the nonaqueous environment could stabilize a polyhedron of α-helices.

Although loss of hydrophobic bonding destabilizes the terminal helices, the denatured form is no longer stable under any ionic charge conditions. Evolutionary optimization has resulted in a sequence yielding a single native conformer for a specific biological function *in vivo*; the same sequence without water yields a multiplicity of conformers of similar stabilities. Thus theoretical predictions of hydrogen bonding and van der Waals effects alone could be tested by predicting a sequence that would give only one gaseous conformer.

A new dissociation method that minimizes H/D scrambling[33] and parallel experiments with other cytochrome *c* isozymes should provide more details. Chiral recognition in the deprotonation of cytochrome *c* ions[36] could yield further conformational characterization. Finally, because the intramolecular complex structures are changed so dramatically by removing water to yield multiple structures of very similar stabilities, inferring the stability of a intermolecular noncovalent complex in one media from that found in another[12,15] should be done with caution.

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