"Denaturation" and Refolding of Cytochrome *c in Vacuo*

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Ion mobility measurements have been used to examine the conformations of the lower charge states of naked bovine cytochrome *c* ions in the gas phase. Conformations with mobilities close to those estimated for the native structure were observed for the +3 to +5 charge states, and a sharp unfolding transition, analogous to acid denaturation, occurs between the +5 and +7 charge states. Folded conformations were observed for the low charge states even when they were produced from unfolded high charge states. Thus cytochrome *c* refolds in the gas phase. Partial refolding occurs spontaneously, but there is an activation barrier associated with the final steps. These studies provide the first direct experimental evidence that unsolvated, gas-phase proteins can refold to conformations as compact as the native solution-phase structure.

Since the development of methods to put large biological molecules in the gas phase,[1] a variety of techniques have been used in an effort to examine their structures in this environment.[2−10] The ion mobility measurements employed here can resolve different conformations and provide an accurate measure of their average collision cross sections.[11] Information about the conformations present can then be deduced from the cross sections.[12] The experimental apparatus employed in the studies described here consists of an electrospray ion source and an injected ion drift tube.[13−15] Solutions of $\sim 5 \times 10^{-4}$ M bovine cytochrome *c* (Sigma Chemical Co.) in a 75:25 mixture of water and acetonitrile acidified with 0−4% acetic acid were electrosprayed in air. The ions enter the apparatus through a small aperture. They are carried through a heated desolvation region into the source vacuum chamber and focussed into a quadrupole mass spectrometer, where a particular charge state is selected.

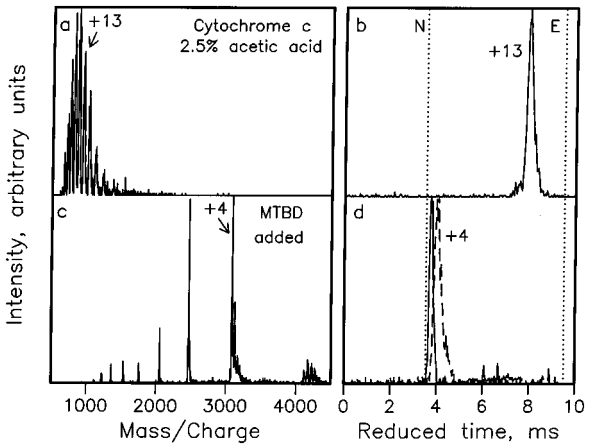

Figure 1. Electrospray ionization mass spectra measured for a cytochrome *c* solution acidified with 2.5% acetic acid: (a) without base added to the desolvation region; and (c) with MTBD added to the desolvation region. Part b shows the drift time distribution measured for the +13 charge state without the base added (injection energy 1300 eV). Part d shows the drift time distributions recorded for the +4 charge state with MTBD added to the desolvation region and with injection energies of 200 (dashed line) and 1400 eV (solid line). The dotted lines labeled N and E show the estimated drift times for the native conformation and the extended conformation described in the text, respectively.

The ions are then focussed into a low-energy ion beam and injected into the drift tube. The drift tube was operated with a helium buffer gas pressure of 2−5 Torr and with an electric field of 13.16 V/cm. Ions that exit the drift tube are focussed into a second quadrupole mass spectrometer. At the end of this quadrupole they are detected by an off-axis collision dynode and dual microchannel plates. Drift time distributions are obtained by injecting 10−50 μs pulses of ions into the drift tube and recording the arrival time distribution at the detector using a multichannel scaler.

Electrospray ionization of proteins results in a distribution of charge states, where the charge results mainly from protonation. Figure 1a shows the mass spectrum measured for cytochrome *c* ions generated by electrospraying a solution acidified with 2.5% acetic acid. Under these conditions cytochrome *c* is denatured in solution, which results in high charge states in the mass spectrum.[16] Figure 1b shows the drift time distribution recorded for the +13 charge state. The drift time distributions presented here are plotted against a reduced time scale given by the measured drift time multiplied by the charge. This accounts for the systematic decrease in the drift times with charge, and makes comparison between different charge states easier. There is a single, relatively narrow peak in the drift time distribution for the +13 charge state at a reduced time of around 8 ms. The measured drift times can be compared to drift times calculated for assumed geometries (see ref 14 for details). Estimated drift times for the native (labeled N) and an extended form of cytochrome *c* obtained by setting nearly all Φ and Ψ angles to 180° (labeled E) are shown by dotted lines in Figure 1. The estimated drift times are believed to be reliable to ±20%. For the +13 charge state, the peak is close to the drift time estimated for the extended form of cytochrome *c*. As discussed elsewhere,[14] an unfolded geometry appears to be the preferred gas phase conformation for all charge states above +6.

In order to generate the lower charge states, MTBD (1,3,4,6,7,8-hexahydro-1-methyl-2*H*-pyrimido[1,2-*a*]pyrimidine, Aldrich Chemical Company), a strong base, was introduced

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into the desolvation region through a heated inlet line and leak valve. Figure 1c shows the mass spectrum recorded after the addition of MTBD. Proton stripping reactions have caused the mass spectrum to shift to substantially lower charge states. The gas-phase basicity of MTBD is large enough to generate the +3 charge state.[3] The dominant peaks in the mass spectrum shown in Figure 1c are due to the +4 and +5 charge states at *m*/*z* ~3100 and ~2400, respectively. The small peaks at *m*/*z* ~4100 are due to the +3 charge state. At higher base concentrations the +3 charge state dominates. There are several peaks present for the +3 and +4 charge states which result from the addition of MTBD molecules.[3] The dashed line in Figure 1d shows the drift time distribution recorded for the +4 charge state. There is a single relatively sharp peak close to the drift time expected for the native conformation. Under the conditions employed, cytochrome *c* is denatured in solution, and the drift time distributions for the high charge states show extended conformations, so cytochrome *c* must refold in the gas phase in less than the 50−100 ms residence time in the desolvation region. As the ions enter the drift tube they are heated and then cooled by collisions with the buffer gas.[17] At high injection energies this may result in conformational changes. The solid line in Figure 1d shows the distribution measured for the +4 charge state with a high injection energy. The +4 charge state folds more tightly after being collisionally heated. Similar results have been obtained for the +3 and +5 charge states. These results indicate that after proton stripping, partial refolding occurs spontaneously, but there appears to be a barrier associated with the final steps. This implies that during the spontaneous refolding process the protein becomes trapped in a metastable conformation.

The proton stripping experiments described above are conceptually similar to experiments recently reported by McLafferty and Williams and their collaborators.[2,5] Gross et al.[2] reported indirect evidence, from gas-phase basicity measurements, for the folding of lysozyme following charge stripping. Wood et al.[5] have employed H/D exchange to examine the conformations generated by proton stripping to the +7 charge state of cytochrome *c* and found a substantial reduction in the number of exchangeable hydrogens. In contrast, we find that it is possible to generate the +7 charge state in a conformation almost as compact as the native conformation by electrospraying an unacidified solution.[14] However, the +7 charge state generated by charge stripping from higher charge states *does not refold to this conformation*. Furthermore, the compact conformation generated from an unacidified solution unfolds when collisionally heated, indicating that the extended conformation is preferred in the gas phase for this charge state.

Figure 2 shows drift time distributions recorded for the +3 to +9 charge states at high injection energies, where the drift time distribution is independent of the means of production and the features present are believed to reflect the most stable gas phase conformations. The dashed line shows the drift time expected for the native conformation. For the +3 to +5 charge states a single relatively narrow peak is observed with a drift time close to that expected for the native conformation. The peak moves slightly to the right as the charge increases. This may reflect a slight expansion of the protein due to Coulomb repulsion. For the +6 charge state a dramatic change occurs. The peak with the drift time close to that expected for the native conformation has disappeared, and it is replaced by two broader peaks at significantly longer times. With the addition of another charge to give the +7 charge state, the peak at shorter time in the distribution for the +6 charge state disappears, and it is replaced by a relatively narrow peak at even longer time. For the higher charge states the peak at shorter times gradually disappears.

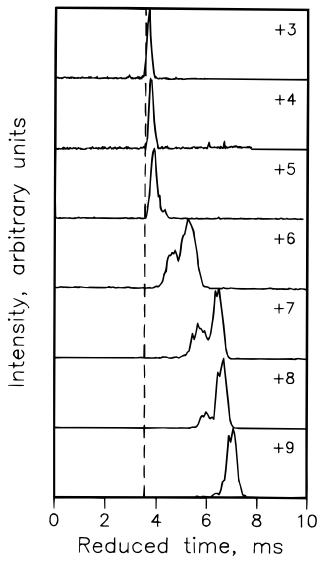

Figure 2. Drift time distributions recorded for +3 to +9 charge states of cytochrome *c* at high (1000−1800 eV) injection energy. The drift time distributions are plotted against a reduced drift time scale obtained by multiplying the measured drift times by the charge. The dashed line shows the drift time estimated for the native conformation.

A remarkable feature of the results shown in Figure 2 is the sudden change in the structure between the +5 and +7 charge states where the protein abruptly unfolds to an extended conformation. It seems likely that this sharp unfolding transition results from Coulomb repulsion. For the higher charge states, Coulomb repulsion presumably overcomes the intramolecular interactions holding the protein in a folded conformation. In many respects the results shown in Figure 2 are analogous to acid denaturation in solution. In solution, the average charge on cytochrome *c* at *p*H 7 is ~+5 for the reduced form and ~+6 for the oxidized form.[18] As the pH is lowered, the charge increases to ~+11 and ~+12 at pH 4. Below pH 4 cytochrome *c* denatures.[19] Native cytochrome *c* can tolerate these high charge states in solution because it is in a liquid with a high dielectric constant. In the gas phase, folded conformations are only stable for charge states below +6.

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