Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2451–2454, March 1995
Chemistry

Gas-phase folding and unfolding of cytochrome *c* cations

(electrospray ionization/Fourier-transform mass spectrometry/hydrogen/deuterium exchange/protein conformation)

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Contributed by Fred W. McLafferty, December 1, 1994

ABSTRACT **Water is thought to play a dominant role in protein folding, yet gaseous multiply protonated proteins from which the water has been completely removed show hydrogen/deuterium (H/D) exchange behavior similar to that used to identify conformations in solution. Indicative of the gas-phase accessibility to D_2O, multiply-charged (6+ to 17+) cytochrome *c* cations exchange at six (or more) distinct levels of 64 to 173 out of 198 exchangeable H atoms, with the 132 H level found at charge values 8+ to 17+. Infrared laser heating and fast collisions can apparently induce ions to unfold to exchange at a higher distinct level, while charge-stripping ions to lower charge values yields apparent folding as well as unfolding.**

Water is thought to be a key factor in the spontaneous folding of a protein into its bioactive conformation (1–5); "water interactions are of the essence in the function of real-life protein molecules" (6). However, a preliminary report (7) of the research described here gave hydrogen/deuterium (H/D) exchange evidence of at least three conformations of water-free cytochrome *c* cations in the gas phase. These multiply protonated species were formed by electrospray ionization, introduced into the high-vacuum region of the Fourier-transform mass spectrometer, and allowed to exchange with D_2O at 10^{-7} Torr (1 Torr = 133.3 Pa). In solution, the degree and sites of H/D exchange are well-established indicators of steric inaccessibility (8) and, thus, of protein conformation. To measure H/D exchange rates, these solution studies have used nuclear magnetic resonance (3, 9, 10), neutron diffraction (11), and mass spectrometry (MS), with MS employed to measure solution-phase H/D exchange rates (12–16), transient intermediates (17), and gaseous noncovalent complexes (18–21).

For multiprotonated cytochrome *c* in the gas phase (7), H/D exchange exhibited pseudo-first-order kinetics for 7+ to 15+ ions. Three distinct exchange levels were dominant, with some charge values exhibiting two of three levels, but the level of highest exchange occurred at an intermediate charge value. Although in solution nonionized cytochrome *c* mimics this behavior, with the native structure first denatured and then reorganized into the molten globule state with increasing H^+ concentration (4, 5, 22), the importance of water for *in vivo* folding appears well established: "the likelihood of a protein refolding in the gas phase is exceedingly small" (23). We report here more definitive high-resolution MS data showing that multiply protonated equine cytochrome *c* ions undergo H/D exchange with D_2O at six distinct levels, with conversion between these levels (presumably unfolding and folding) effected by infrared laser heating and high-velocity collisions or by reducing the number of charges on the ion.

MATERIALS AND METHODS

Solutions of 20 μM equine cytochrome *c* (Sigma) were electrosprayed [6+ to 9+ ions in pure aqueous solution and 10+ to 17+ ions in methanol/water/acetic acid, 76:22:2 (vol/vol)], and the resulting ions were transported by three rf-only quadrupoles through five stages of differential pumping to the trapped-ion cell in a 6.1-Tesla magnetic field (24). All-even or all-odd ionic charge states were isolated by stored waveform inverse Fourier transform (SWIFT) (25) and were allowed to react with D_2O at 1.1×10^{-7} Torr for 30 min (exchange was >98% complete) (7); the ion cell was evacuated for 10 min ($\sim 2 \times 10^{-8}$ Torr), and spectra were measured. IR irradiation of the ions used a 10.5- to 10.7-μm Synrad (Bothell, WA) continuous wave CO_2 laser (200 W/cm^2) through a ZnSe window. Ion-charge stripping used butylamine (2–20 s at $\approx 10^{-7}$ Torr), with the desired new charge state isolated by SWIFT (25). Improvements (24) in instrumentation over that in ref. 7 include a doubled magnetic field (higher resolution, mass accuracy, and ion-trapping efficiency), SWIFT isolation of charge values, lower (10^{-9} vs. 10^{-8} Torr) H_2O background, and improved electrospray conditions (26).

RESULTS AND DISCUSSION

Equine cytochrome *c* in solution exchanges 144 of the 198 hydrogens bound to heteroatoms or to the imidazole C-2 of histidine (9, 10) in 20 min when in neutral solution; 154 exchanges are observed under acidic conditions (22) where equine cytochrome *c* exists as a compact A-state. Up to 190 and 193 exchanges are observed in reduced and oxidized forms, respectively, after 1 month in neutral solution (10). For electrosprayed equine cytochrome *c* ions, charge values from 7+ to 17+ (Fig. 1*A*) undergo H/D exchange at four (or more) discrete exchange levels (Fig. 1*B*) of 113 (state I), 133 (state II), 100 (state IV), and 120 (state V); from Fig. 2 it is possible that states IV and V each represent pairs of levels. In many cases, ions of one charge value evidence multiple exchange envelopes (Fig. 1*B*), indicating that ions of the same primary structure with the same number of protons can differ in their number of accessible Hs. The mass difference of two such exchange envelopes could not be due to a noncovalently bound molecule such as D_2O because this mass difference increases with increasing extent of H/D exchange (7). Profiles of H/D exchange level with time exhibit pseudo-first-order kinetics for each charge state (7); rate constants, $k_{H/D}$, for some of these are given in Table 1. H/D exchange reactions with deuteriated methanol (CH_3OD) gave similar exchange levels with 2–3 times higher $k_{H/D}$, while that for reaction with CH_3COOD was 2–3 orders of magnitude higher. As proposed earlier (7), these exchange levels could correlate with different conformational intermediates for these gas-phase protein ions.

Consistent with conformational stability over a substantial pH range in solution (1–6, 8–11), the highest of these four exchange levels (133 ± 5, state II) is observed over the 8+ to 16+ charge values (overlapping with the 124 level, state V′, *vide infra*), a doubling of electrostatic charge. Inconsistent with

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Abbreviation: H/D, hydrogen/deuterium.

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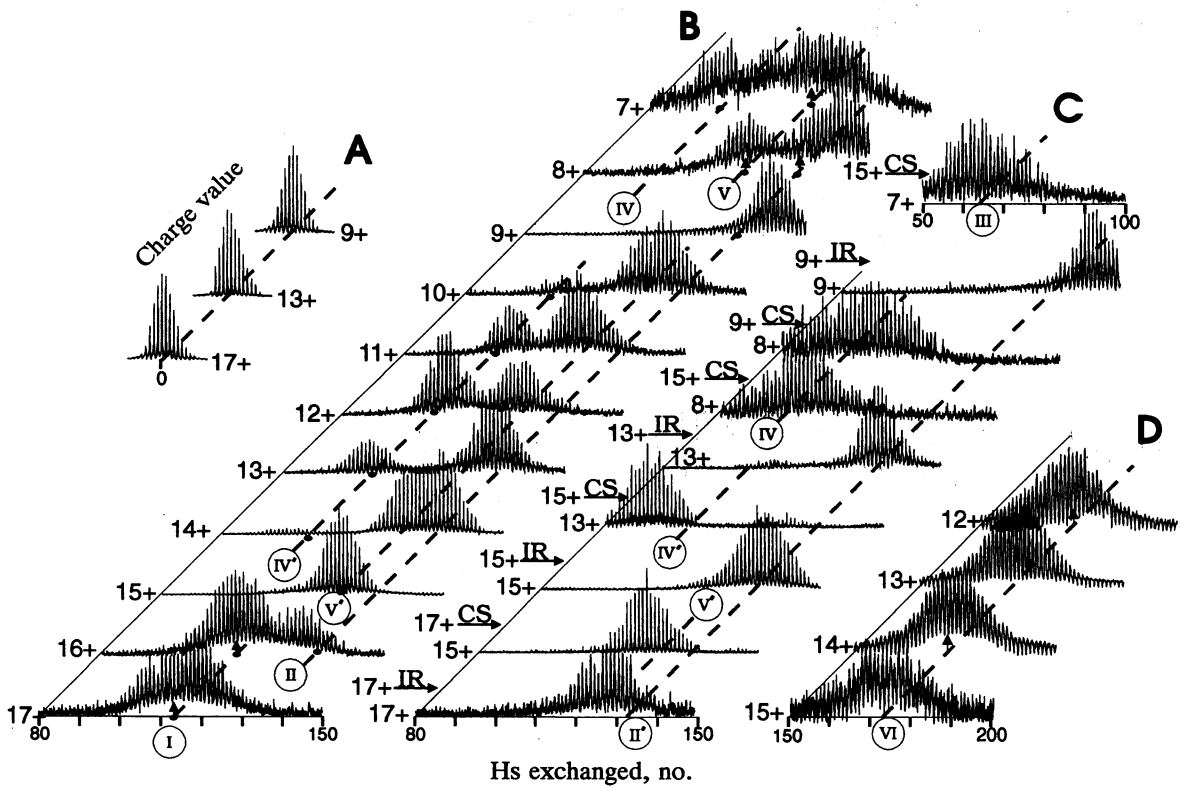

FIG. 1. Isotopic peak clusters of electrosprayed equine cytochrome *c*. (*A*) Typical precursor $(M + nH)^{n+}$ ions (most abundant isotopic peak contains seven ^{13}C atoms and ^{56}Fe). (*B*) After gaseous D_2O exchange. (*C*) After IR irradiation or charge-stripping (CS). (*D*) After quadrupolar axialization collisions.

this, the 16+ ions contain eight (presumably exchangeable) more protons than the 8+ ions; these and the earlier data (7) show no effect of these on the H/D exchange value, which is under further investigation. The 16+ ions show (Fig. 1*B*) an additional exchange envelope that is also in the 17+ for 113 ± 3 Hs exchanged (state I), corresponding to a more ordered structure. Possibly analogous, cytochrome *c* in solution forms a more compact A-state from the denatured state with decreasing pH (4, 5).

The lowest level of normal H/D exchange (95 ± 3, state IV), correlating with a less accessible and more ordered structure, is observed for the 6+ (marginal signal/noise ratio), 7+, and 8+ (just discernable) charge values. For charge values 10+ to 14+, an H/D exchange level of 102 ± 5 (state IV′) is observed, close to that for state IV; these could also be the same state, with an insensitivity to charge value similar to that for state II. Both the 7+ and 8+ charge values exhibit an additional exchange envelope (116 ± 1) not present in 6+ or 9+, with a similar, although slightly higher, exchange level (124 ± 2) for the 10+ to 16+ charge values that coexists with state II; these could also be the same states and are designated V and V′, respectively.

In solution, heating induces thermal denaturation of cytochrome *c* (27); here, gas phase heating is effected with IR laser irradiation (28). For ions of charge values 7+ to 10+ and 13+ to 17+, irradiation for 80–100 ms results (Figs. 1*C* and 2) in

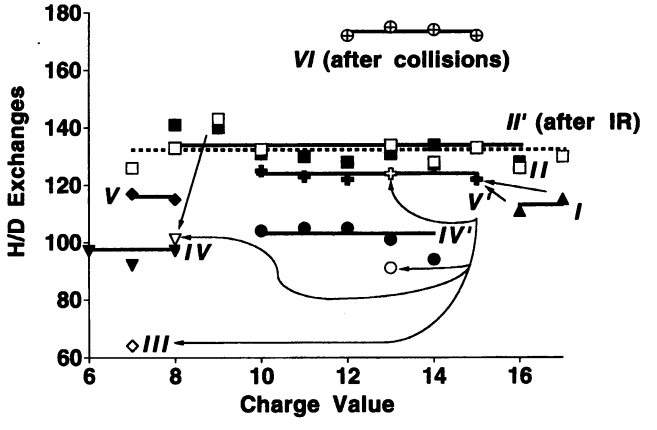

FIG. 2. H/D exchange levels (no. of H/D exchanges) vs. charge value. Solid symbols, states from electrosprayed ions; open symbols, states altered by irradiation, charge-stripping, or collisions.

Table 1. Rate constant, $k_{H/D}$, for H/D exchange reactions of the indicated conformers of multiply protonated cytochrome *c* ions allowed to react with D_2O at 1.1×10^{-7} Torr ($\times 10^{-13}$ cm^3 s^{-1} molecule^{-1})

	$k_{H/D} \times 10^{13}$, cm^3·s^{-1}·molecule^{-1}			
Ion	I	II	IV	V
17+	46			
16+	25	*		
15+				11
14+		22	11	*
13+		22	*	
12+		16	21	*
11+		12	18	*
10+		10	*	*
9+		9		
8+		6	*	7
7+			11	5

*Conformer is present but rate constants could not be ascertained because of low signal/noise ratio for some H/D exchange vs. time reactions or because of difficulty in assigning H/D exchange values for state V based on overlap with state II.

only one dominant level of H/D exchange (132 ± 5, state II′, open squares), matching the exchange level for state II (133 ± 5). These results are consistent with thermal unfolding of the more compact structure that stops when the intermediate state II is reached and with the conformational similarities of the more compact structures. The irradiated 13+ ions show a small amount of residual state IV′, indicating that IV′ → II′ is a one-step process; irradiation of the 10+ and 14+ ions gave similar results. Interestingly, 11+ and 12+ ions exhibit no change in H/D exchange behavior when irradiated for any period up to the onset of dissociation (160 ms and 150 ms, respectively). Although no decrease in exchange level is observed with a 30-min delay immediately after IR irradiation, evidence for refolding is being sought in longer time-scale experiments.

For the compact A ("molten globule") state of cytochrome *c* in solution (pH ≈ 1), decreasing the H^+ concentration induces denaturation, while a further decrease of the H^+ concentration (pH ≈ 7) yields the native conformation (4, 5). Here, an analogous gas-phase experiment utilizes a high proton-affinity agent to remove H^+ ions from multiply protonated proteins (29, 30). Reaction of the 17+ ions (state I, exchanging 115 H) with butylamine to form 15+ followed by H/D exchange shows (Fig. 1*C*) a narrowed and slightly increased exchange level of 120, matching the 15+ ions formed directly by electrospray. Similarly, charge-stripping 16+ (normally a 111/127 doublet) to 15+ results in only a single exchange level (123), consistent with formation of V′ from states I and II by unfolding and folding, respectively. Reaction of 15+ ions (state V′) with butylamine, isolation of the resulting 13+, and H/D exchange yield a doublet of exchange levels. If the less abundant 124 exchange level represents unchanged V′, this again indicates stepwise isomerization; the new 91 exchanging state represents a substantial folding of V′, possibly state IV′ (102 exchanges). These H/D exchange reactions also follow first-order kinetics.

More extensive reaction of the 15+ charge state with butylamine to form 7+ dramatically halves the exchange level (64, state III, Fig. 1*C*), even lower than the lowest H/D exchange level for species from direct electrospray. In fact, the 64 value correlates far better with the previous value of 53 for 7+ from the different electrospray device and higher H_2O background (diluted D_2O) during H/D exchange (a 17% lower value) (7). The value of 64 represents only one-third of the 198 exchangeable hydrogens, a much more highly ordered structure; in solution, the most highly folded native state also exists at low H^+ concentration (neutral pH), while a somewhat more accessible native-like "tight" state forms with decreasing pH (4, 5), consistent with gaseous state IV. In fact, reaction of butylamine with either 15+ (V′) or 9+ (II) to form 8+ followed by H/D exchange yields spectra of identical (although broad) 101 exchange levels (Fig. 1*C*); this could be state IV, correlating with the 82 (18% lower, "state IIIa") value found previously (7) for the electrosprayed 8+ ions. The preference for "tighter" (fewer exchangeable Hs) states from gaseous charge exchange than from electrospray could result from more efficient formation of minimum energy states by using slow gas-phase folding rather than the more sudden (nonequilibrium) electrospray process (31).

Quadrupolar ion axialization using high excitation amplitudes effects high-velocity collisions (32); in this mode, an even higher exchange level (173 ± 2, state VI) was achieved for 12+ to 15+ ions (Fig. 1*D*). This value is much closer to the 198 value expected for complete denaturation, consistent with an even more accessible structure, and is under further investigation.

Of the (at least) six distinct cationic states identified in the gas phase representing 64 to 173 exchangeable hydrogens, I can be unfolded to V′; I, IV, IV′, V, and V′ to II; and at least II, IV′, and V′ to the nearly denatured VI. V′ can be folded to III, IV, or IV′, and II folded to IV. These observations are consistent with energy valleys in the folding process, or "conformational substates" as described by Frauenfelder and Wolynes (33). The fast H/D exchange solution-phase behavior for native equine cytochrome *c* (144 exchangeable Hs) (10) does not match any of the levels we have observed. Particularly striking is the difference observed between our least exchangeable species, state III (64), sprayed out of native solution conditions, and the solution-phase native state. Although this may be attributable to numerous factors, it is possible that the least accessible structures in solution and the gas phase are different, though thermodynamic arguments favor the native structure in-vacuo (34). Gas-phase hydrogens that are sterically hindered or involved in hydrogen bonding would not be expected to undergo rapid H/D exchange.

CONCLUSIONS

The gaseous multiply protonated ions of equine cytochrome *c* exist in at least six different states, as characterized by their accessible sites for H/D exchange. Levels for this of several of these reactive states can be altered to match those of other states by manipulating them with IR irradiation, high velocity collisions, or proton stripping; such changes appear to occur in discrete steps. The gas phase may thus provide a complementary environment for the study of solution phase transient folding intermediates (conformational substates), aided by the slow kinetics of conformational changes uncatalyzed by solvent.

Whether gas-phase H/D exchange mechanisms proposed for singly-charged amino acids (35, 36) apply for multiply protonated ions as well as H/D exchange of negative $(M - nH)^{n-}$ ions and other isozymes needs to be examined. The identity of states such as IV′ vs. IV, V′ vs. V, and I vs. IV could be examined by removing protons from a gaseous deuteriated state to convert one state to the other and then determining if further H/D exchangeable sites are exposed. Tandem MS (37) might be used to dissociate these gaseous deuteriated states and to assay H/D exchange in the subunits; subunit exchange is a far more sensitive test of conformer identity (3, 9, 10, 22). Such a comparison of the gas-phase states with each other and with solution-phase deuteriated states should provide a much more critical delineation of gas- vs. solution-phase folding. Perhaps another quotation could prove appropriate: "it is the removal of water that induces a protein to form a three-dimensional structure" (2).

We gratefully acknowledge helpful discussions with D. Suckau, H. A. Scheraga, B. T. Chait, P. A. Karplus, W. A. Eaton, J. P. Speir, M. W. Senko, N. L. Kelleher, and Z. Guan and the financial support of the National Institutes of Health and the Kodak Co. (for a fellowship to R.A.C.).

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