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Chemistry

Coexisting stable conformations of gaseous protein ions

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

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ABSTRACT **For further insight into the role of solvent in protein conformer stabilization, the structural and dynamic properties of protein ions *in vacuo* have been probed by hydrogen–deuterium exchange in a Fourier-transform mass spectrometer. Multiply charged ions generated by electrospray ionization of five proteins show exchange reactions with ^2H_2O at 10^{-7} torr (1 torr = 133.3 Pa) exhibiting pseudo-first-order kinetics. Gas-phase compactness of the S–S cross-linked RNase A relative to denatured S-derivatized RNase A is indicated by exchange of 35 and 135 hydrogen atoms, respectively. For pure cytochrome *c* ions, the existence of at least three distinct gaseous conformers is indicated by the substantially different values—52, 113, and 74—of reactive H atoms; the observation of these same values for ions of a number—2, 7, and 5, respectively—of different charge states indicates conformational insensitivity to coulombic forces. For each of these conformers, the compactness *in vacuo* indicated by these values corresponds directly to that of a known conformer structure in the solution from which the conformer ions are produced by electrospray. S-derivatized RNase A ions also exist as at least two gaseous conformers exchanging 50–140 H atoms. Gaseous conformer ions are isomerically stable for hours; removal of solvent greatly increases conformational rigidity. More specific ion–molecule reactions could provide further details of conformer structures.**

The relationship between the dynamic structure of proteins in solution and their biological activity has been of long-standing research interest. Protein folding is probably the least well understood step in the sequence of transformations relating genetic information with its expression by protein function (1). Dramatic new ionization methods for mass spectrometry (MS) have made possible the formation of protein ions in the gas phase to measure molecular weight and primary sequence information (2–4), even on fmol samples (5, 6). Recent studies indicate that protein conformations in solution can affect the resulting charge distribution of the gaseous multiply charged ions formed by electrospray ionization (ESI) (7–9) and that even noncovalent complexes can survive ESI to form gaseous multiply charged ions (10–15).

Critical information concerning solvent effects on the conformation and dynamic properties of proteins has come from NMR (16) and from isotope-exchange experiments with ^2H_2O (17), including those before and during ESI/MS (18, 19). With an activation energy of 17–20 kcal/mol (1 cal = 4.184 J) (17), the H/^{2}H exchange rate depends on the pH (17), electrostatic effects (20), proximity of the solvent-accessible surface (21), and conformational flexibility with hydrogen bond cleavage and formation during local unfolding and folding (22). Studies of gaseous proteins should help delineate the role of solvent in stabilizing protein conformations, but such previous studies have been mainly theoretical (23) because of the lack of experimental approaches. We report here that conformations of gaseous multiply charged protein ions can be characterized by their H/^{2}H exchange kinetics, providing definitive evidence of distinct stable conformations that can coexist in the gas phase.

MATERIALS AND METHODS

Protein solutions were electrosprayed and the resulting ions were transferred and accumulated for 5 s (24) in the analyzer cell of a Fourier-transform ion-cyclotron-resonance mass spectrometer with a 6.2-T magnet. Designated preliminary experiments were run on a similar instrument with a 2.8-T magnet as described (5, 6). The ions were allowed to react for different time periods (separate runs) with ^2H_2O before excitation and detection.

Purified RNase A was a gift from H. A. Scheraga (Cornell University). RNase A S-alkylated with 4-vinylpyridine (VP-RNase) was obtained from RNase A by reduction with dithiothreitol in 4 M guanidinium isothiocyanate (0.1 M Tris·HCl, pH 8); thiol blocking by alkylation with 4-vinylpyridine; followed by quenching, dialysis, and lyophilization. Other protein samples were obtained from Sigma.

RESULTS AND DISCUSSION

Gaseous multiply charged ions were formed from equine cytochrome *c* (12.3 kDa) by ESI (2, 4–6). These were assumed to be pure (e.g., no noncovalently bound water molecules), as they exhibit the expected isotopic peaks (Fig. 1 *Inset*) and molecular mass values (5, 6). These ions were allowed to react with ^2H_2O (10^{-7} torr; 1 torr = 133.3 Pa) for increasing time periods (Fig. 1), with the increasing mass reflecting the number of ^{2}H atoms exchanged for H atoms versus time. A plot of these values (Fig. 2*A*) is consistent (exponential regression coefficients, ≈0.995) with pseudo-first-order exchange kinetics for each charge state (Fig. 2*B*). The left intercept also indicates the maximum number of hydrogens exchangeable by this process. Data observable at >1000 s and at higher pressure indicate that other H/^{2}H exchange processes must be slower by at least a factor of 20. The number of exchangeable hydrogens is nearly independent of charge state, although higher values have been achieved with other electrospray conditions (other than capillary temperature and voltage offset). However, electrospraying cytochrome *c* from $CH_3O^2H/^2H_3O^+$ solution indicates exchanges of 187 hydrogens (subtracting the $^2H^+$ species added in ionization); in this molecule, there are 198 hydrogens bound to heteroatoms, the type of hydrogen expected to undergo H/^{2}H exchange (17).

Surprisingly, the spectra of exchanged charge states 12+–14+ show (Fig. 1) peak splitting, indicating that two different

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Abbreviations: ESI, electrospray ionization; VP-RNase, S-alkylated RNase A; PA, proton affinity.

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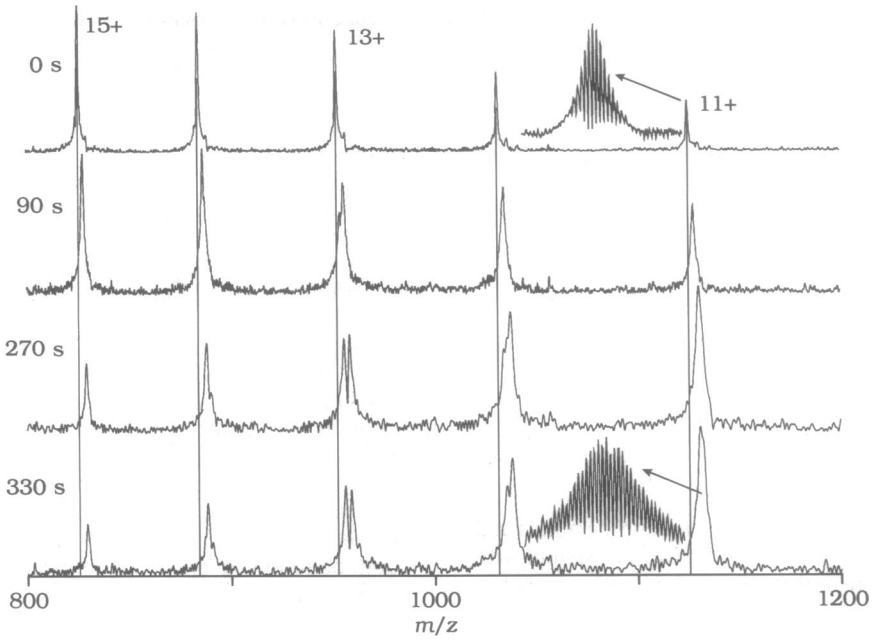

FIG. 1. Mass spectra of electrosprayed equine cytochrome *c* (20 μM in 76% CH_3OH/2% CH_3COOH/22% H_2O), allowing the ions to react for different times (separate runs) with ^2H_2O at 1.1×10^{-7} torr before excitation and detection. (*Insets*) Isotopic peaks (30,000 resolving power) measured in separate experiments.

ionic forms of the same molecular mass have distinctively different numbers of reactive hydrogens. These values are also observed for ions of other charges, with 74 ± 3 for the 12+–16+ charge states and 113 ± 1 for the 8+–14+ charge states (Fig. 3). Electrospraying cytochrome *c* from neutral solution yields 6+–9+ charge states, of which the 6+ and 7+ states show 53 ± 2 reactive H atoms; the 8+ charge state again shows peak splitting corresponding to 82 and 113 exchangeable hydrogens.

Extensive studies in solution have established the existence of four conformers of cytochrome *c* (25). Solution conformer III, neutral pH, is the native state, consistent with the gaseous 6+, 7+ charge state exhibiting the lowest number of exchangeable hydrogens (Fig. 3). In solution, state IIIa of somewhat tighter conformation is observed (25); it is conceivable that the 8+ state exchanging 82 H atoms represents a separate conformer related to state III or IIIa. With increased solution acidity, denaturation forms the completely unfolded state II, and finally forms state I that has a higher α-helicity; such more-compact denatured conformers are referred to as A states (26). The relative compactness of solution conformers I, II, and III corresponds well with the observed exchangeable H atom values of 74, 113, and 53, respectively (Fig. 3), for the gaseous conformers; although the more highly charged ions with their higher coulombic energy (27) would be expected to be more open, consistent with the increase from 53 to 113 exchangeable hydrogens, the anomalous decrease from 113 to 74 with even higher charge reflects the increased compactness of state I vs. state II. Furthermore, the gaseous conformers exchanging 53 and 74 H atoms do not isomerize appreciably in hours to the conformer exchanging 113 H atoms, as rate data at 10^{-6} torr (^2H_2O) still fit a single exponential.

As further evidence of the relationship between the degree of folding and the number of exchangeable hydrogens, disulfide cross-linked RNase A (13.7 kDa) and its derivative

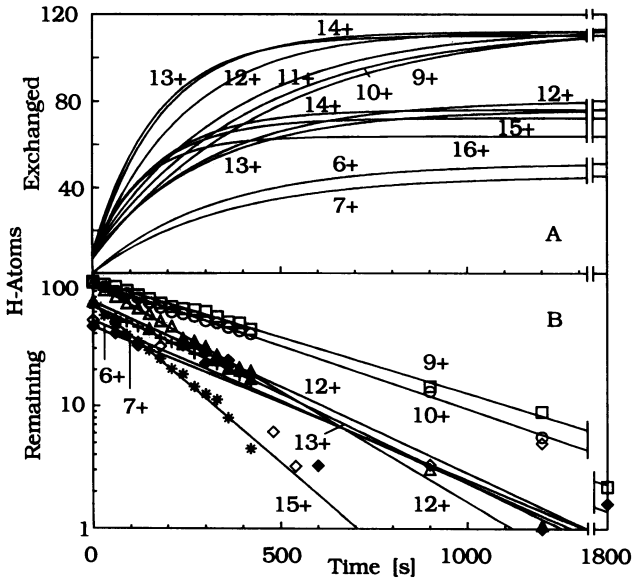

FIG. 2. (*A*) Exponential fit of mass increase in Da (exchanged H atoms) vs. time for different cytochrome *c* charge states. (*B*) Logarithmic plot of the data in *A*; maximum number of reactive hydrogens corresponds to the remaining reactive H atoms at time 0.

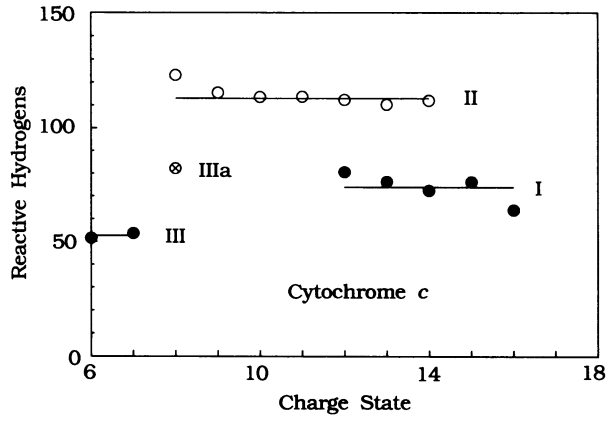

FIG. 3. Number of reactive H atoms observed for possible conformers of cytochrome *c*.

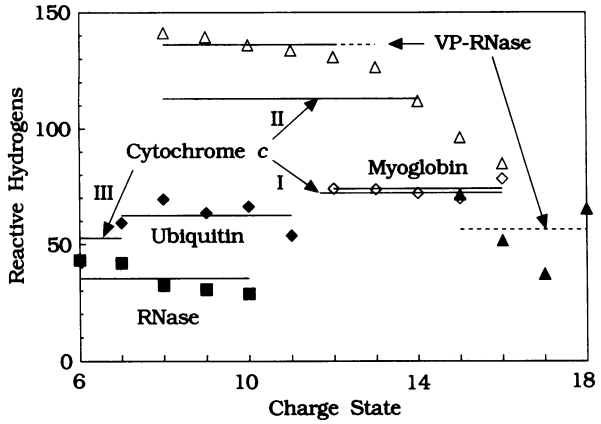

FIG. 4. Number of reactive H atoms observed for the indicated protein conformers.

VP-RNase formed by cleavage of its four disulfide bonds and alkylation with 4-vinylpyridine were studied. Consistent with its covalent-bond stabilization, the compact RNase A exchanges only 32 ± 2 H atoms (Fig. 4), far less than the values for the denatured VP-RNase. For this, again the intermediate charge states react in what appear to be different conformations, with the lower charge states exchanging 135 ± 2 H atoms and the higher charge states exchanging ≈60 H atoms. This decrease in available sites at higher charge, as observed above for cytochrome *c*, again could be indicative of A state (26) formation; this could be enhanced by the high concentration of organic solvent used in electrospray (28). The 6+–10+ RNase A data actually fit better a double exponential plot, representing a fast reaction exchanging 25 ± 1 H atoms and a much slower reaction that could possibly represent interconversion of conformers.

The number of such exchangeable hydrogens was determined also for ubiquitin (8.5 kDa) and myoglobin (17 kDa); these data were measured on the 2.8-T (5, 6) instrument of lower resolving power, so the reactivities of different conformers would not necessarily have been observable. These values are less dependent on molecular mass (Fig. 4) than on conformation and, again, show little dependence on charge state. Equine, porcine, rabbit, and chicken cytochrome *c* show small but distinguishable differences in their number of exchangeable hydrogens.

Although the number of exchangeable H atoms is hardly affected by increasing the number of charges on the ion, this does increase the rate constants for H/^{2}H exchange (Fig. 5). This is by far the most dramatic for RNase A, increasing an order of magnitude from +6 to +10; thus, little of this is due

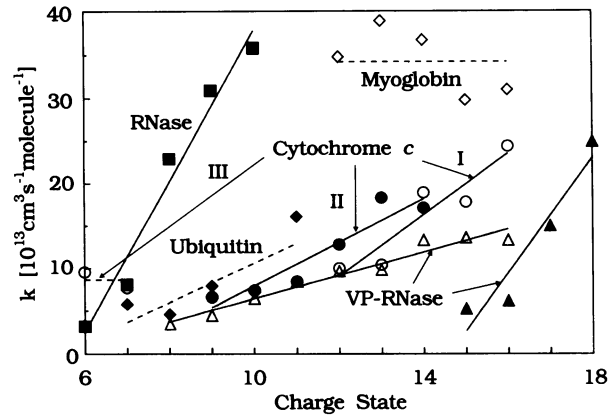

FIG. 5. Rate constant, *k*, for H/^{2}H exchange reactions of the charge states of the indicated conformers, derived as in Fig. 2.

to reactions at individual charge sites. Qualitatively, the extent of rate constant increase with increasing charge is dependent on conformer compactness (e.g., cytochrome *c*, VP-RNase); higher compactness should cause a much greater increase in charge density with an increasing number of charges.

Isotope exchange in gaseous ion–molecule reactions appears to take place as a multistep reaction involving proton transfers and rearrangements in a long-lived activated ion–molecule complex (29–31). This H/^{2}H exchange requires a low difference (<≈25 kcal/mol) between the proton affinity (PA) of the reacting molecule and that of water (31, 32). For amino acid side chains, aryl and O-linked H atoms have PA values that are 20–30 kcal/mol above that of water, while N-linked H atoms have PA values >40 kcal/mol higher (33); the latter sites should be more favorable for H/^{2}H exchange in solution. The higher exchange reactivity at higher ion charges and in more compact conformers could be due to a coulombic decrease in the effective PA values of reactive groups. In addition, initial H/^{2}H exchange at a site of low PA could be followed by its fast intramolecular H/^{2}H exchange (34) with a site of higher PA.

CONCLUSIONS

In solution, the H/^{2}H exchange of cytochrome *c* involves nearly all its labile hydrogen atoms, indicating a fast dynamic equilibrium between its conformers. In sharp contrast, its gaseous ions can exist in at least three different reactive forms that do not interconvert in hours. Thus, solvent stabilization of solution conformations is not of overriding importance; in fact, this indicates the opposite. Presumably, these conformers are stabilized by intramolecular noncovalent interactions; similar intermolecular interactions provide conformationally dependent stabilization for bimolecular complex ions in the gas phase (10–15). The apparent correlation observed here between the compactness of the well-characterized cytochrome *c* conformers I, II, and III in solution and the extent of H/^{2}H exchange of the corresponding ionic conformers indicates that the stabilizing interactions in solution and *in vacuo* are of a similar noncovalent nature, involving hydrogen and ionic bonding (35) and Van der Waals interactions (36). Water molecules in aqueous solution apparently screen such stabilizing interactions (35), making possible the dynamic conformational processes such as protein folding that are necessary for biological activity. To the extent that these stable gaseous ion conformers are characteristic of the conformations of their precursor proteins in solution, a variety of selective ion–molecule reactions can be envisioned that could provide valuable complementary structural information with unusual speed and sensitivity.

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