# Detection of a Monomeric Intermediate Associated with Dimerization of Protein Hu by Mass Spectrometry

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Protein HU belongs to the class of type II DNA-binding proteins[1−3] and exists in all prokaryotes. The high sequence homology of HU among the various species suggests that the native topology in each case is very similar to the intertwined dimeric structure determined for protein HU from *Bacillus stearothermophilus* (HBst).[4,5] In contrast to the structures, however, the stabilities of different HU variants vary considerably.[6] Based on far-UV circular dichroism and differential scanning calorimetric data it has been reported that protein HU from *Bacillus subtilis* (HBsu) is unfolded in aqueous solution, but undergoes a folding transition to the native dimer on addition of salt.[7] Here we show that, by manipulating the ionic strength, distinct conformational changes associated with the folding and dimerization of HBsu can be observed by using nanoflow electrospray ionization mass spectrometry (ESI-MS).

ESI mass spectra of HBsu (Figure 1) were obtained from aqueous solutions containing different concentrations of ammonium acetate ($NH_4Ac$). ESI-MS[8] utilizes a soft-ionization technique capable of producing, from protein molecules in solution, multiply charged gas-phase ions in which noncovalent features of the protein are maintained.[9] The charge on each positive ion is determined primarily by the number of labile protons attached to the basic sites on the solvent-exposed protein surface, which depends on both instrument parameters and solvent conditions.[10,11] In particular, a decrease in the number of positive charges is predicted when the conformation of a protein molecule becomes more compact.

The mass spectrum of HBsu[12] obtained from a solution without salt (Figure 1A) exhibits a broad distribution of charge states, ranging from 6+ to 16+ with 13+ being the most intense. The mass derived from the sequence of these peaks, 9882(±1) Da, is consistent with the predicted mass, 9884 Da, of the intact monomer of HBsu.[13] By contrast, the mass spectrum obtained from a solution with a high ionic strength (Figure 1C) exhibits a single charge-state distribution with a marked shift to higher mass/charge values relative to the charge-state distribution shown in Figure 1A.[14] The charge-state distribution in Figure 1C comprises only two significant charge states. The mass derived from these peaks is 19764(±1) Da, i.e., the mass of the noncovalent dimer.[15] Figure 1B shows a mass spectrum of HBsu obtained from a

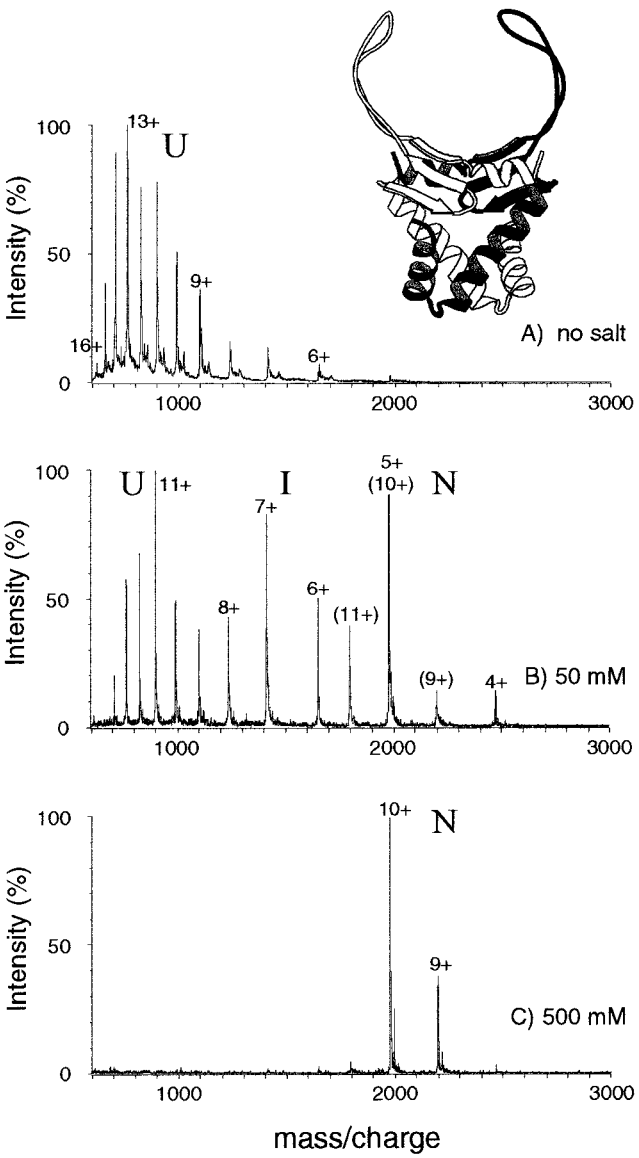


**Figure 1.** ESI mass spectra of HBsu at different $NH_4Ac$ concentrations.[29] The aqueous protein solutions (A) without salt and (B) containing 50 mM and (C) 500 mM $NH_4Ac$ were adjusted to pH 5.0 using formic acid. Labels U, I, and N indicate the charge-state distributions corresponding to the unfolded protein, a monomeric folding intermediate, and the native dimer, respectively. The labels on the peaks, *n*+, indicate the number of excess positive charges, *n*, on the protein ion. Labels in parentheses in the middle panel correspond to charge states of N. Inset: Solution structure of HBst[4] (PDB entry 1HUE) drawn with the program MOLSCRIPT.[30] The light and dark ribbons emphasize the fold of the two monomers in the dimer.[31]

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(12) HBsu was cloned, overexpressed, and purified as described previously.[32]
(13) The mass was calculated by using the program MASSLYNX 2.3 (Micromass UK).

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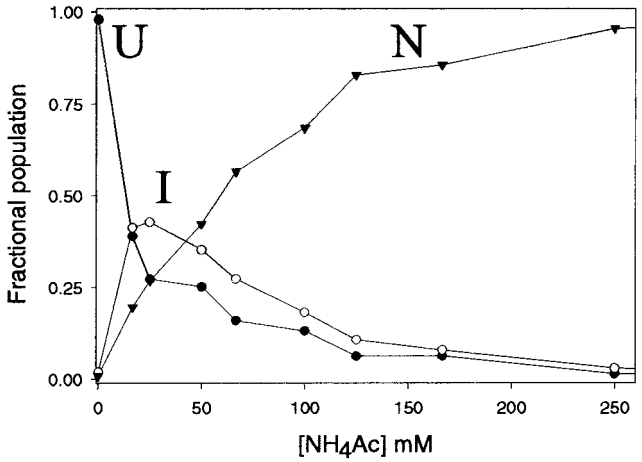


**Figure 2.** Fractional populations of the conformational states U, I, and N of HBsu as a function of the $NH_4Ac$ concentration. The fractions of U (closed circles), I (open circles), and N (closed triangles) from each ESI mass spectrum were obtained by fitting the intensities of all charge states to a sum of three Gaussian functions.

solution containing 50 mM $NH_4Ac$. Remarkably, this spectrum exhibits three distinct charge-state distributions. Those corresponding to the unfolded monomer and the native dimer are easily identified by comparison with parts A and C of Figure 1, respectively. The third charge-state distribution lies between them, ranging from 4+ to 10+ with the most intense peak of 7+, and corresponds to the mass of the monomer.

We suggest that the wide range of charge states of the unfolded protein (U) reflects the conformational heterogeneity associated with a highly disordered protein,[16] and that the narrower charge-state distribution at lower charge of the second monomer (I), observed at intermediate salt concentrations, indicates that this monomer is partially folded. The folded molecule in the native dimer (N) adopts a well-defined, compact conformation, as indicated by the very narrow width of the corresponding charge-state distribution and a further shift toward a lower center of charge of 5+ (10+ for the dimer).[17] The fractional intensities of the signals of the three conformational states U, I, and N as a function of $NH_4Ac$ are presented in Figure 2. The partially folded monomer, I, is clearly present at $NH_4Ac$ concentrations in the range of 10 to 100 mM with a maximum population of approximately 40(±5)% at about 30 mM $NH_4Ac$. The presence of both U and N at these salt concentrations indicates that the protein interconverts among the three conformational states.

Hydrogen exchange measurements[18−21] were carried out at three salt concentrations.[22] At 500 mM $NH_4Ac$, 41(±3) sites per monomeric subunit of HBsu remained unexchanged, a number in good agreement with NMR data obtained for the HBst dimer[23] which revealed 45 protected amide protons per subunit. However, at 5 and 50 mM $NH_4Ac$ exchange of all labile protons with deuterium occurred after approximately 1 and 2 h, respectively, the difference in exchange rate being attributable to the decrease in the fraction of unfolded protein at higher salt concentrations. The substantial difference in hydrogen exchange protection between HBsu at low and high salt concentrations is a consequence of the interconversion of the various conformational states of the protein which occurs in solution.[21,24]

These results suggest that the distribution of charge states detected in a protein ESI mass spectrum reflects the heterogeneity of conformational states in protein folding. A decrease of both the actual charge and the width of distinct charge-state distributions can be interpreted in terms of the funnel-like nature of an energy landscape which directs the ensemble of protein molecules toward more compact structures, and ultimately to the native state.[25,26] In the specific case of HBsu, ESI-MS allows us to detect this phenomenon, but also to put forward a folding mechanism for the protein. The question of how an intertwined dimeric structure can form during folding is a particularly intriguing one. For the Arc repressor, structurally related to HU, a three-state folding mechanism has been proposed, but controversy exists as to whether the intermediate state is a monomer[27] or a loosely folded dimer.[28] For protein HU we show unambiguously using ESI-MS the presence of a partially structured monomeric intermediate. We suggest that the folding process initially results in such species which are capable of recognizing other monomers with a similar fold, and which subsequently assemble to the native dimeric structure.

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(14) A preliminary search of salt bridges in HBst suggests that the charge difference between the predominant charge states in parts A and C of Figure 1 can be rationalized by the formation of nine intramonomeric salt bridges per monomer in the HBsu dimer which are likely to be absent in the unfolded protein.

(15) The charge states corresponding to the dimer are well resolved and not significantly broader than the charge states of the monomeric form, which may suggest that water molecules are not involved in the maintenance of this dimeric protein structure. A similar observation has been made for transthyretin.[33]

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