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Formation of a CH−π Contact in the Core of Native Barstar during Folding

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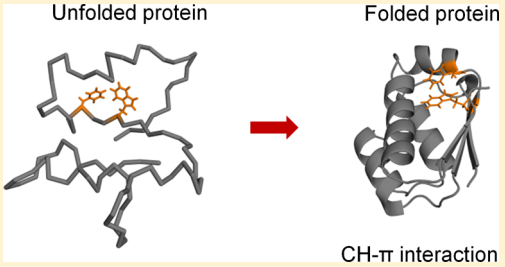
Supporting Information

ABSTRACT: An important part of the protein folding process is the consolidation of the protein core through the formation of specific, directional contacts after the initial hydrophobic collapse. Here, we simultaneously monitor formation of core contacts and assembly of secondary structure through salt-induced folding by using resonance Raman spectroscopy. Unfolded barstar at pH 12 was refolded by gradual addition of sodium sulfate salt. Altered spectral characteristics of the Trp53 residue suggest that the core of the protein attains a CH−π interaction at a low concentration of the salt, with an increase in the packing density. Further increase in salt concentration produces a reduction in the solvent accessibility of the core. These data provide evidence that the core of the protein becomes rigid upon the addition of 0.6 M sodium sulfate. This is the first time that the formation of a CH−π interaction has been directly monitored during the folding of a protein.



INTRODUCTION

The understanding of the role of specific interactions during the folding of a protein from a random chain unfolded conformation to the functionally relevant tertiary structure is a major problem in biology. Hydrophobic, hydrogen bonding, packing, and other interactions have been found to play important roles during protein folding.[1,2] Nothing, however, is known about the formation of a specific CH−π interaction during a protein folding reaction.[3,4] CH−π interactions have a high probability of occurrence inside a protein,[5] as suggested by survey studies of the structures of several proteins, and it has been suggested that they are important in determining the stability and function of proteins.[5−12] To monitor the formation of CH−π interactions during folding has been a challenge. Not only is a suitable probe and a sensitive experimental technique which can directly measure these interactions required, but a suitable protein model in which the CH−π interaction can be detected experimentally is also needed.

The protein barstar, an inhibitor of the ribonuclease barnase, has been systematically used during the past 20 years as a model protein to study protein folding and unfolding reactions.[13−27] Barstar contains three tryptophans (Trp38, Trp44, and Trp53) residues, with Trp53 present inside the core of the protein, completely sequestered away from the solvent. The crystal structure of barstar also shows that the side chain of Trp53 is inside a hydrophobic environment surrounded by several aliphatic residues.[28] Its position in the protein has made Trp53 a useful probe to get insights into the structural changes inside the core as well as on the changes in the solvent accessibility of the core during the folding and unfolding reactions of barstar.

In a previous study from our group, UVRR spectroscopy was used to characterize the local environment of Trp53 inside barstar. In that study, it was shown that the Raman bands of Trp53 provide experimental evidence of the CH−π interaction as well as steric interactions with Phe56 and Ile5, respectively. The fact that UVRR spectroscopy is sensitive to the CH−π and steric interactions between Trp53 and neighboring amino acid residues suggests that this technique can be used to directly monitor the formation of specific interactions during structural changes of the protein. It has been difficult to directly monitor the development of a specific interaction during the folding of any protein, without resorting to chemical labeling and mutagenesis, and both these procedures can potentially perturb the interaction being studied. On the other hand, UVRR spectroscopy offers itself as a nonperturbative probe to monitor the formation of specific interactions.

The ability of the resonance Raman spectroscopy to monitor the folding and unfolding reactions of proteins is well-known.[29−40] This, together with the sensitivity of the UVRR spectrum of Trp to capture information about relevant interactions in proteins, makes UVRR spectroscopy an excellent methodology to study folding using barstar as a model.[41−49] In this study, the salt-induced equilibrium refolding reaction of a single Trp (Trp53) containing mutant form of barstar was monitored using UVRR spectroscopy. Excitation with a wavelength at the red edge of the Bb absorption band of Trp allows the changes in the Raman bands of Trp53 to be monitored.

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As demonstrated by previous studies, the completely unfolded form of barstar in a solution at pH 12 can be progressively refolded by adding an increasing concentration of sodium sulfate (Na_2SO_4).[19,50] At this pH value and in the absence of salt, barstar adopts a random coil conformation (D form) similar to that of the unfolded protein in high concentrations of denaturants such as urea or guanidine hydrochloride.[18] The equilibrium study of the refolding of barstar demonstrated that at low concentrations of Na_2SO_4 (0.05−0.1 M) the protein chain collapses in to a premolten globule form (P form) with 20−30% more volume than the native state.[19,50] With an increase in salt concentration, the protein transforms gradually to a dry molten globule (B form) with a consolidated core similar to that of the native protein. Information about changes in solvent accessibility as well as about the formation of secondary structure was also obtained.

EXPERIMENTAL METHODS

In this study, UVRR spectroscopy was used to monitor the salt-induced equilibrium refolding reaction of barstar. The UVRR bands of Trp53 were used as markers for the formation of the CH−π and steric interactions and of changes in the packing density in the core of the protein, as well as in solvent accessibility, during the refolding reaction.

To check the effect of the high pH on the Raman bands of tryptophan and tyrosine, the spectra of the analogues *N*-acetyltryptophanoamide (NATA) and *N*-acetyltyrosinamide (NAYA) in buffer solutions at pH 7 and pH 12 were recorded. Figure S1 shows a comparison of the spectra of NATA at both pH values. Spectral characteristics of NATA are unaffected by pH. However, in the case of NAYA, there are appreciable differences between the two spectra due to deprotonation at higher pH (Figure S2). Thus, it is expected that at high pH the protein spectrum will contain contributions from deprotonated Tyr. The similarity of the spectra of NATA at different pH values suggests that the Raman bands of Trp53 can be used to monitor the changes in the core of barstar during refolding at high pH and at the same time allow a direct comparison with the native state at pH 7 without any major contribution arising from the difference in pH.

Spectra of the W38FW44FC82A variant of barstar at pH 12 in the presence of different concentrations of Na_2SO_4 are shown in Figure 1. The spectrum of the unfolded state in the absence of Na_2SO_4 (D form) is shown at the bottom, and that of the native state at pH 7 is shown at the top. Spectra of the protein in the absence and in the presence of 0.1 M Na_2SO_4 at pH 12 do not show significant spectral differences (Figure S3). Previous studies of the refolding reaction of barstar have found that at 0.1 M Na_2SO_4 the protein collapses to a premolten globule (P form), which has 20−30% more volume than the native state, and has a solvated core. In the same study it was also determined that the P form lacks any specific packing at the core.[19,50] In agreement with that observation, here it is seen that at this salt concentration the W18 and Fermi doublet bands of Trp53 do not show native-like characteristics. Because it was not possible to use the internal standard for recording the spectrum of the D form, it is not possible from our study to determine the difference in the solvent accessibility of the side chain of Trp53 between the D and P forms.

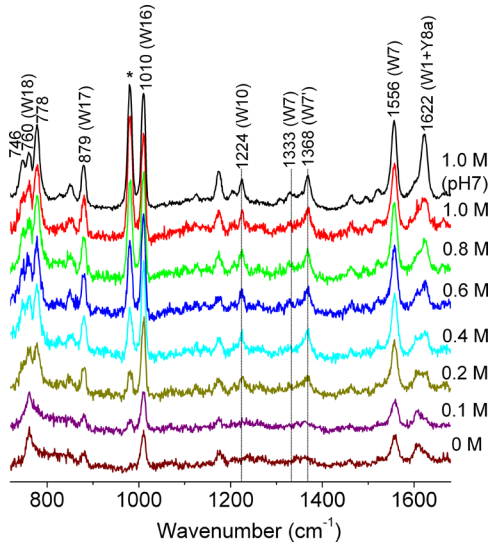

Figure 1. Spectra of W38FW44FC82A variant of barstar at pH 12 recorded at indicated concentrations of Na_2SO_4. Asterisk (∗) marks the 981 cm^{-1} band of Na_2SO_4. The spectrum at the bottom represents the unfolded state in the absence of salt, and the one at the top is the spectrum of the native state at pH 7. Spectra were normalized to the intensity of the 981 cm^{-1} band of Na_2SO_4 at 1 M.

RESULTS AND DISCUSSION

An increase in the concentration of the salt to 0.2 M produces large changes in the positions and intensities of the Raman bands of Trp53 (Figure 1). The relative intensity of the W18 (759 cm^{-1}) band decreases with respect to the other bands (i.e., W16 and W3) in the spectrum. Two new bands at 746 and 778 cm^{-1} emerge, with the latter becoming even more intense than the W18 band. Because of the low signal-to-noise ratio in the spectra at 0.1 M Na_2SO_4, it was difficult to deconvolute the W10 and Fermi doublet bands accurately to make an estimation of the actual spectral changes. However, it can be qualitatively seen that there is an increase in the intensity of the second component (band) of the Fermi doublet with respect to the first one. The intensity ratio $R = I(\sim 1360\ cm^{-1})/I(\sim 1340\ cm^{-1})$ has been previously correlated with the hydrophobicity of the local environment of Trp.[45,48,49] An increase in the ratio for Trp53 indicates that its local environment has become more hydrophobic at 0.2 M than at 0.1 M Na_2SO_4. It seems that in 0.2 M Na_2SO_4 the side chains of several aliphatic amino acids come closer to the side chain of Trp53.[51]

Spectral characteristics shown by the W18, W10, and the Fermi doublet bands of Trp53 at pH 12 in the presence of 0.2 M of Na_2SO_4 are similar to those found in the spectrum of the native protein at pH 7 and are maintained at higher concentrations of the salt. We have previously described the characteristics of W18, W10, and W7 in the spectrum of the native state. These suggest a CH−π interaction between the side chain of Trp53 and the CH groups of Phe56 and a steric interaction with the methyl group of Ile5. Similarities in spectral characteristics of the protein at pH 12 in the presence of 0.2 M Na_2SO_4 provide evidence that part of the protein core has become tightly packed and that native-like interactions (CH−π, steric, and then packing interactions) are already present at this stage. This is a surprising result considering that previous time-resolved fluorescence decay measurements had suggested that the side chain of Trp53 is able to undergo local motion at this concentration of Na_2SO_4 and reaches maximum rigidity only at

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a concentration of ~0.6 M Na_2SO_4.[50] The Raman data in this study shows that the motion of the side chain of Trp53 in 0.2 M Na_2SO_4 is sufficiently restrained so that the CH groups of Phe56 and the methyl groups of Ile5 are able to perturb the electronic density of the indole ring in a manner similar to that in the native state.

More details on the equilibrium refolding reaction of barstar can be obtained by monitoring changes in intensities of specific bands with respect to salt concentration. Change in absolute differential cross sections of the W17 and W16 Raman bands is shown in Figure 2. Initially, there is an increase in the intensities of the bands upon increasing the concentration of Na_2SO_4 to 0.2 M. It is known that a decrease in the water accessibility of the indole ring produces an increase in the Raman cross section of Trp.[42] This intensity change suggests a decrease in the solvent accessibility of the side chain of Trp53 due to the increase in the packing density of the core. The increase in intensity also demonstrates that the P form still has a wet core. Further addition of salt up to a concentration of 0.4 M further increases the intensities of the bands. In the range of 0.4−1 M Na_2SO_4, the Raman intensities remain approximately constant. The intrinsic fluorescence spectrum of Trp53 suggested that during the transition from the unfolded form to the B form water is gradually excluded from the core. In the concentration range 0.6−1 M , the fluorescence intensity shows only subtle changes, in agreement with the changes in the intensities of the Raman bands of Trp53.

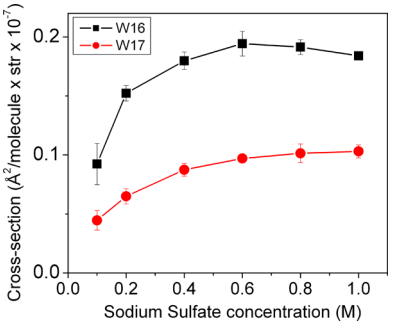

Figure 2. Intensity changes of the W16 and W17 UV resonance Raman bands of Trp53 during refolding as a function of Na_2SO_4 concentration.

Changes in the W18 region of Trp53 follow a more complex trend (Figure 1). There is a decrease in the intensity of the W18 band relative to the intensities of the other bands of the spectrum upon addition of 0.2 M Na_2SO_4. New bands at 746 and 778 cm^{-1} are observed which are resonance-enhanced due to the formation of the CH−π interactions.[51] At higher concentrations of Na_2SO_4 these two bands continue to gain intensity, likely because of the increase in packing density as well as in the strength of the CH−π interactions. At concentrations higher than 0.4 M, the relative intensity of the bands in the W18 region attains a value similar to that in the spectrum at 1.0 M Na_2SO_4. Figures 3a, 3b, and 3c show the difference spectra obtained by subtracting the spectra at 0.4, 0.6, and 0.8 M from the spectrum at 1 M Na_2SO_4, respectively. The comparison shows that there are only small differences at 0.4 M and imply that the interactions inside the core as well as solvent accessibility are similar for Na_2SO_4 concentrations in the range from 0.6 to 1.0 M.

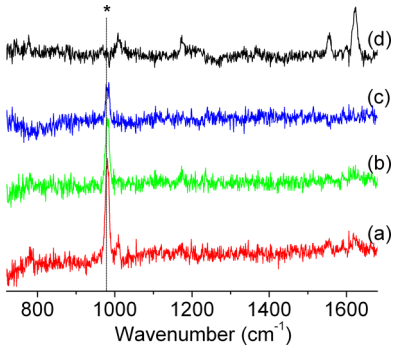

Figure 3. (a, b, c) Difference spectra obtained by subtracting the spectra of the refolded protein at pH 12 at 0.4, 0.6, and 0.8 M Na_2SO_4, respectively, from the spectrum at 1 M Na_2SO_4. (d) Difference spectrum obtained by subtracting the spectrum of the protein at pH 12 from that of the native protein at pH 7 containing 1 M Na_2SO_4. Asterisk (*) represents the 981 cm^{-1} band of Na_2SO_4.

The time-resolved fluorescence anisotropy decay measurements carried out by Rami et al. showed that for concentrations of salt lower than 0.6 M two components of Trp fluorescence with different rotational correlation times can be observed.[50] The component with the shorter correlation time was associated with the local motion of the side chain of Trp53. It was shown that, for this component, the amplitudes (time decay) of the total anisotropy decay were 46% (0.63 ns), 27% (0.78 ns), and 8% (1.06 ns) for 0.1, 0.2, and 0.5 M Na_2SO_4, respectively. From 0.6 to 1 M Na_2SO_4, the component with the shorter decay is not present, suggesting that the side chain is rigid in the core. This observation demonstrated that in 0.2 M Na_2SO_4 the side chain of Trp53 still undergoes some local movement. The comparison of the relative intensity of the bands in the W18 region between the refolded protein in 0.2 and 1 M Na_2SO_4 also indicates that the interactions between the Trp53 side chain and the surrounding residues have not reach maximum strength in 0.2 M Na_2SO_4 which allows the possibility of Trp53 local movement at this salt concentration.

Figure 3d shows the difference spectrum obtained by subtracting the spectrum at pH 12 containing 1 M Na_2SO_4 (B form) from that of the native protein at pH 7 containing the same concentration of salt. The difference shows small variations in the bands in the W18 region as well as in the W10 and the Fermi doublet bands of Trp53. Major differences occur in the spectral region dominated by Tyr bands due to the fact that Tyr deprotonates to tyrosinate at pH 12, and this produces several spectral changes with respect to the Tyr bands at pH 7 (Figure S2). Similarity in the UVRR bands of Trp53 implies that in 1 M Na_2SO_4 at pH 12 the local region of the core around Trp53 is consolidated. This result is in agreement with a previous study of the refolding of barstar under similar conditions.[19] It was shown that by adding 1 M Na_2SO_4 to the completely unfolded protein at pH 12, the protein transforms from the P form to the B form in which the secondary and tertiary structure are 65% and 40% similar to that of the native state, respectively, and the compactness of the core of the protein is also developed. This comparison as well as the similarity between the spectra at pH 12 at 0.6 and 1 M Na_2SO_4, implies that during the refolding reaction the core is already consolidated at the former concentration. This result is in correspondence with that obtained by Rami et al. using time-resolved fluorescence anisotropy measurements.[50] They found that the decay parameters (rotational amplitude and correlation

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time) do not change in the range of Na_2SO_4 concentrations from 0.6 to 1 M.

CONCLUSION

From the analysis of the UVRR bands of Trp53, it is possible to construct a scheme with the different steps of the core formation during the refolding reaction of barstar. (1) Initially, in the absence of Na_2SO_4, the protein is in a random coil form with no appreciable structure. (2) At 0.1 M Na_2SO_4 the protein has a conformation with a wet and unstructured core. (3) An increase of Na_2SO_4 concentration to 0.2 M produces an increase in the hydrophobicity of the environment around Trp53, the formation of native-like structure (CH−π and steric interactions), and a simultaneous increase in packing density inside the core. At this stage, some water molecules have been expelled from the core. (4) From 0.2 to 0.6 M Na_2SO_4, the core becomes dry and structurally consolidated. (5) From 0.6 to 1.0 M Na_2SO_4, the solvent accessibility and the interactions between side chains inside the core of the refolded protein are similar to those in the native state.

CH−π interactions are known to occur inside many proteins. The side chain of Trp is involved usually as an acceptor in these interactions. The ability of UVRR spectroscopy to detect the CH−π interaction between the side chain of Trp and CH groups of other amino acids has been demonstrated recently.[51,52] Here, we have used this sensitivity to monitor the formation of the CH−π interaction inside the core of the protein barstar during an equilibrium refolding reaction. It was also possible to monitor an increase in the packing density of the core during the process. Based on the results, the steps involved in the core consolidation during the refolding reaction of barstar have been described. We found that at a low concentration of Na_2SO_4 (0.2 M) there is a formation of the CH−π and steric interactions inside the core, and there is also an increase in the hydrophobicity of the local environment around the side chain of Trp53. At a higher concentration of Na_2SO_4 (0.6−1 M), the Raman spectral characteristics of the refolded protein at pH 12 are similar to those of the native state. This is the first time that the formation of CH−π interactions has been detected directly during the folding of a protein. The results obtained here serve as a starting point to study other folding and unfolding reactions of barstar by using UVRR spectroscopy.

ASSOCIATED CONTENT

Supporting Information

Detailed materials and methods and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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