NMR STUDIES OF THE UNFOLDING OF RIBONUCLEASE BY GUANIDINE HYDROCHLORIDE. EVIDENCE FOR INTERMEDIATE STATES

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1. Introduction

Studies of the unfolding and refolding of proteins constitute a useful approach to an understanding of the relationships between primary sequence and three-dimensional structure. Information concerning the pathway of unfolding or refolding can only be obtained if states intermediate between the native and the random-coil structures can be detected, for example spectroscopically. Only rarely have such intermediates been detected by the methods (such as optical rotation, ultraviolet absorption) commonly used to study denaturation [1, 2]. In the case of ribonuclease, there is some evidence for the existence of intermediates in thermal unfolding [1, 3], notably from the elegant kinetic experiments of Tsong et al. [4–6], but Salahuddin and Tanford [7] concluded that the guanidine hydrochloride denaturation is a "two-state" process.

High-resolution NMR can provide information on the environment of individual residues in proteins during conformational transitions [8]. This makes NMR particularly valuable in the study of denaturation, as has been illustrated by experiments with lysozyme [9], staphylococcal nuclease [10, 11] and ribonuclease [12–14]. The studies of staphylococcal nuclease in particular gave strong evidence for the existence of intermediates in unfolding.

We report here a study of the behaviour of the C2 proton resonances of the four histidine residues of ribonuclease during unfolding by guanidine hydrochloride. We find evidence that at least two intermediate states exist, and since the histidine resonances have been assigned to individual residues in the sequences of ribonuclease [15] we can begin to characterise these states.

2. Materials and methods

Bovine pancreatic ribonuclease (Worthington RAF grade; phosphate free) was treated with D_2O (99.8%; Norsk Hydro-elektrisk) as previously described [16] to replace all exchangeable protons with deuterium. It was then dissolved in 0.2 M deuteroacetate in D_2O, pH (meter reading) 5.5, to a final conc. of 10^{-4} to 2×10^{-3} M. Guanidine hydrochloride (Sigma) was recrystallised from ethanol/benzene, then lyophilised repeatedly from D_2O; it was stored in a dessicator for up to one month. A series of accurately weighed amounts of the deuterated guanidine hydrochloride (GuDCl) were added to the protein solution (2–3 ml), and the GuDCl concentration was corrected for the small changes in volume which resulted. After each addition of guanidine the pH was readjusted to pH 5.5 when necessary.

^1H NMR spectra were obtained at 100 MHz using a Varian XL-100-15 spectrometer, with Fourier transform facilities controlled by a VDM 620i computer. The spectrometer was field-frequency locked to the deuterium in the solvent, and all solutions contained 10^{-3}M dioxane as internal reference. All chemical shifts are given with respect to this dioxane reference. The sample temperature was 33.0 ± 0.2°. Spectra are the result of accumulating 250 transients, with an acquisition time of either 1.00 or 4.00 sec; in the latter case the large water peak was continuously irradiated with a second r.f. field (this necessitated a slight modification to the instrument). Direct measurement of the spin-lattice relaxation times of these C2 protons in the presence of 1.5–3.5 M GuDCl showed that the relaxation times of both the "native" and "denatured" resonances (see below) were constant over this concentration range. Therefore changes in area of the res-

onances reflect only changes in the fraction of the molecules in a particular conformational state.

3. Results and discussion

The cooperative unfolding of ribonuclease as observed by optical rotation or ultraviolet absorption occurs between about 1.5 M and 3.5 M GuHCl under the conditions of pH and temperature used here [7]. In the present paper we shall be concerned only with this concentration range; the marked downfield shifts of the C2–H resonances of histidines 12 and 119 which occur at lower GuDCl concentrations and which, for histidine 119, are continuing in this concentration range will be described elsewhere (Benz and Roberts, in preparation).

Fig. 1 shows the C2–H resonances of the four histidine residues of ribonuclease at various concentrations of GuDCl. The unfolding transition is manifest in these spectra by a decrease in the area of the resonances corresponding to the different histidine residues in the folded protein (in the native protein these four resonances have equal area), together with an increase in the area of a single resonance at 481 Hz from dioxane (fig. 1) corresponding to the histidines in the unfolded protein. In the unfolded protein, the magnetic environment of all four histidine residues is the same, so the four C2 protons give rise to a single resonance line. Since separate and moderately sharp resonances are seen for the native and unfolded states, the exchange between these states must be slow on the NMR time-scale; in this case the rate of exchange must be less than about 70 sec^{-1}. Salahuddin and Tanford [7] find approx. 0.002 sec^{-1} for the overall denaturation at 2.88 M GuHCl. Furthermore, since only two resonances are seen for each histidine residue, each histidine seems to exist in only two major conformational states in this range of GuDCl concentration *.

However, if one examines in detail the dependence of the areas of the different C2–H resonances on

* This is an oversimplification, since the "folded" and "unfolded" peaks could each represent an average of several states in rapid exchange. There is fairly good evidence that this is not the case for histidines 12, 48 and 105, though the "folded" peak of histidine 119 probably does represent more than one state. The point is discussed in detail by Benz and Roberts (in preparation).

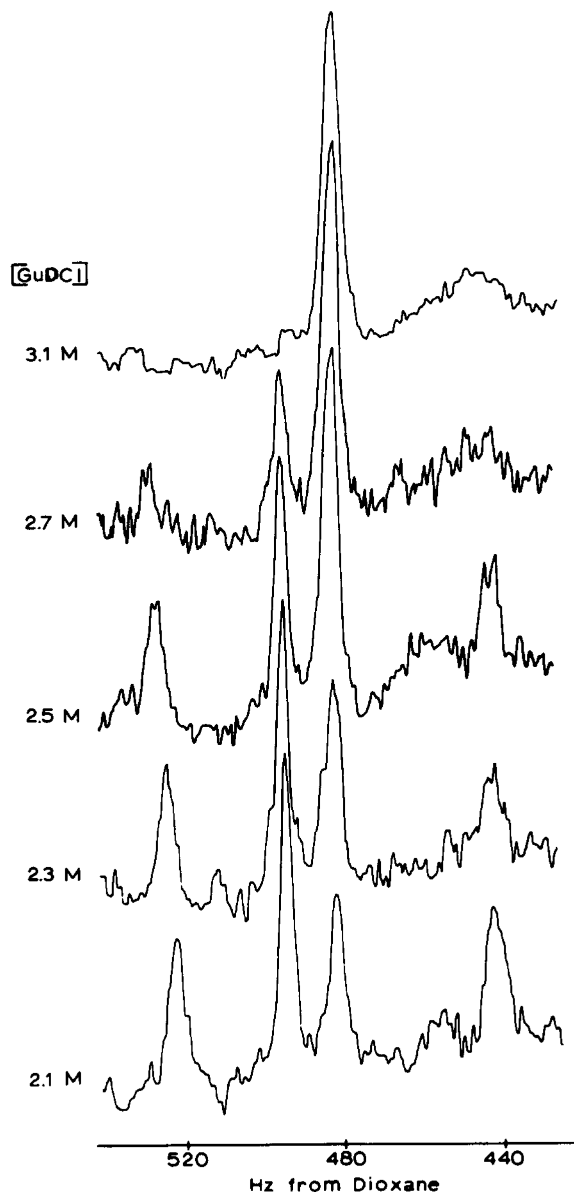

Fig. 1. Histidine C2–H resonances of bovine pancreatic ribonuclease at different concentrations of GuDCl. The assignments of the resonances are: 521 Hz, His 119; 493 Hz, His 105 and His 12; 481 Hz, "denatured" peak; 441 Hz, His 48.

GuDCl concentration, it is apparent that the conformational transition of all the histidines does not take place at the same GuDCl concentration. As the concentration of GuDCl is increased, the first change is seen in the resonance corresponding to the C2 protons

of residues 12 and 105; at 2.1 M GuDCl, the area of this peak has decreased significantly, and the denatured peak has appeared. Since the C4 proton of histidine 105 shows no change in area at this GuDCl concentration, the decrease in area of the overlapped C2–H resonance must be due to histidine 12. At slightly higher GuDCl concentrations (2.3–2.4 M) the resonances of histidines 119 and 48 decrease in area; within the accuracy of the present measurements * the areas of these two resonances show the same dependence on GuDCl concentration. At 2.8 M GuDCl, essentially the only "native" C2–H resonance remaining is that of histidine 105, and finally this also decreases, until at 3.1 M GuDCl only the peak corresponding to the unfolded protein is seen. No further changes were seen up to 5 M GuDCl, the highest concentration used, except that the broad resonance at 442 Hz disappears *.

In a recent NMR study of the unfolding of ribonuclease by various means, Bradbury and King [13] did not observe any differential effects of GuDCl such as those reported here. The experiments were carried out at 60 MHz, which may have made it more difficult to detect the relatively small differences in behaviour of the different histidines. More importantly, however, Bradbury and King [13] showed that their commercial ribonuclease preparation contained five sulphate ions per mole of ribonuclease; sulphate is known to have substantial effects on the unfolding of ribonuclease [17].

In order to interpret the differential behaviour of the C2–H resonances shown in fig. 1 it is important to note that, although the spectra shown were obtained at a ribonuclease concentration of 2 mM, some 10-fold greater than that usually used for optical studies of denaturation, no concentration dependence of the phenomena reported here was observed. Furthermore, the changes in the NMR spectrum were completely reversible, with no indication of hysteresis (it has not always been possible to demonstrate reversibility in earlier NMR studies carried out at the higher protein concentrations required by the continuous-wave time-averaging technique). Thus the differential effects of GuDCl on the four histidine residues cannot be attributed to any protein–protein interactions. In addition, all the area changes reported here occur within a GuDCl concentration range corresponding to the cooperative unfolding transition observed by other techniques, and the final product is clearly, by NMR criteria [8, 18] a random coil (though the disulphide bonds remain intact). It appears, therefore, that the phenomena we have observed are indeed giving information on the pathway of unfolding of ribonuclease. On the basis of the changes in the histidine C2–H resonances, we can define two intermediate states: one in which histidine 12 is in a solvent environment, while the other three histidines are still in a folded structure, and a second in which only histidine 105 remains in a non-solvent environment. Since there is no a priori correlation between the magnitude of a chemical shift change and the magnitude of the conformational change producing it, we cannot characterise these intermediates more precisely than this at the present time. The conflict between these results and the optical spectroscopic studies of Salahuddin and Tanford [7] is probably only apparent; it is likely that the NMR results reflect only small deviations from "two-state" behaviour. However, these small deviations are of considerable importance since they open the way to a description of the pathway of unfolding. Clearly much more information is required before a detailed description of this pathway is possible, but since histidine 12 is the first to "unfold" in acid and urea denaturation (Benz and Roberts, in preparation) as well as in GuDCl, it is tempting to speculate that a common first stage of unfolding, involving the N-terminal (S-peptide) region of the molecule, may exist, and experiments to test this are in progress.

* The accuracy with which the area of the histidine 48 resonance can be determined is limited by the broad "peak" which appears under this histidine resonance at ca. 2.5 M GuDCl. The nature of the broad peak is currently under investigation.

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