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2 J.P. Johnston, Biochem. Pharmac. *17*, 1285 (1968).
3 H.-Y.T. Yang and N.H. Neff, J. Pharmac. exp. Ther. *187*, 365 (1973).
4 H.-Y.T. Yang and N.H. Neff, J. Pharmac. exp. Ther. *189*, 733 (1974).
5 R.J. Baldessarini and J.E. Fischer, Biochem. Pharmac. *27*, 621 (1978).
6 J.J. Pisano, J.A. Oates, Jr., A. Karmen, A. Sjoerdsma and S. Udenfriend, J. biol. Chem. *236*, 898 (1961).
7 Y. Kakimoto and M.D. Armstrong, J. biol. Chem. *237*, 208 (1962).
8 A.A. Boulton and P.H. Wu, Can. J. Biochem. *50*, 261 (1972).
9 O. Suzuki, M. Oya, Y. Katsumata and M. Asano, Experientia *35*, 167 (1979).
10 G.G. Guilbault, P.J. Brignac, Jr, and M. Juneau, Analyt. Chem. *40*, 1256 (1968).
11 S.H. Snyder and E.D. Hendley, J. Pharmac. exp. Ther. *163*, 386 (1968).
12 J. Knoll and K. Magyar, in: Advances in Biochemical Psychopharmacology, vol. 5, p. 393- Ed. E. Costa and M. Sandler. Raven Press, New York 1972.
13 O. Suzuki, Y. Katsumata, M. Oya and T. Matsumoto, Biochem. Pharmac. *28*, 2237 (1979).
14 M.D. Houslay and K.F. Tipton, Biochem. J. *139*, 645 (1974).

Conformation of ribonuclease S-protein

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Summary. Ribonuclease S-protein exhibits a pH-dependent conformational transition between folded and unfolded states, and some unfolded S-protein persists up to pH 8. The histidine C2 proton resonance of the unfolded species was erroneously assigned by Bradbury et al.[1] to histidine residue 119 of the folded species.

Bradbury et al.[1] have attempted to explain our earlier observations[2] on the histidine residues of ribonuclease S-protein by invoking an 'irreversible denaturation' of S-protein, although they cited no evidence to support this assertion. Also, the peak which we had assigned to unfolded (or denatured) S-protein they assigned to histidine residue 119 of the folded S-protein. A test of which assignment is correct can be made on the basis of the behavior of this resonance under different experimental conditions.

In figure 1 are shown spectra of samples of S-protein at different pH values. It is clear that the peak labelled with the arrow increases in relative area as the pH is lowered, until at pH < 2 it corresponds to the equivalent histidine residues of unfolded S-protein. In order to confirm that this peak corresponded to unfolded or denatured S-protein at higher pH values, where significant amounts of folded S-protein are present, we heated a sample and added guanidine hydrochloride (^2H_2O-lyophilized) to another (figure 2). In both cases this peak clearly increased in area at the expense of other resonances, as expected for an unfolding process[5]. Consequently this must be the resonance (H-u) of the equivalent histidines of unfolded S-protein.

It might be argued that this resonance could also correspond to His 119 of the folded species if it happens to coincide under normal pH and temperature conditions with that of the unfolded material; this would imply that His 119 is in an essentially denatured environment in the globular S-protein. This seems unlikely since at pH values > 6 we observed 3 other resonances (figure 1), corresponding to the 3 histidine residues present in S-protein. 2 of these are sufficiently close (about 0.1 ppm) that they could not be resolved at the lower observing frequency (100 MHz) used by Bradbury et al.[1].

2 of these resonances were readily assigned[2] to His residues 48 and 105 on the basis of their near identity with resonances in spectra of ribonuclease A and S[6]. The third resonance, that of His 119, was the only resonance affected by the addition of Pi to the solution[2]. In fact, the most downfield peak in the spectra of S-protein at pH < 6 is always larger than that assigned to His 48 (figure 1), and consequently must correspond to 2 histidine residues.

The other peak, which varies significantly in area, we now confirm our assignment to unfolded material (H-u), since clearly the amount of this material depends upon the conditions. This resonance is a significant feature of spectra at all pH-values shown by Bradbury et al.[1], and we must conclude that there is some unfolded material present in all their S-protein samples. The value of '30%' estimated by Bradbury et al. for the amount of denatured material was derived from the relative area of this resonance found by us at pH 4.6, since we could not satisfactorily fit spectra quantitatively above this value[2]. In fact, a comparison of our spectra of S-protein with that of Bradbury et al.[1] at pH 4.5 (figure 9 of the cited paper) shows a remarkable consistency.

Bradbury et al.[1] concluded that His 48 in S-protein 'titrates normally'. This is not borne out by their results, as well as ours, since a) the resonance of His 48 is broadened, especially at pH > 6, so that it was sometimes not possible to follow it, and b) its titration curve is shifted upfield relative to that of a normal histidine residue[1,2]. In fact, except for the absence of the low pH inflection, this curve is remarkably similar to that of His 48 in ribonuclease A or S[7], as we previously pointed out[2]. Consequently there is no basis for the statement of Bradbury et al.[1] that His 48 is 'accessible to solvent', and they quote no evidence for this other than their incorrect assertion that its titration curve is 'normal'. Thus, there is no apparent basis for the distinction between their conclusion, that 'the relative orientation of the C2 proton of His 48 and the ring of Tyr 25 remain about the same as in RNase-A'[1], and the conclusion they attribute to us of a 'highly structured environment of His-48'.

We have carefully referred to the material giving rise to the extra titrating C2 peak in NMR-spectra of S-protein as unfolded rather than denatured[2] since the term 'native' hardly applies to S-protein. However, the likelihood that this material is 'irreversibly denatured', as Bradbury et al.[1] assert can be discounted for the following reasons: a) spectra of S-protein were reversible as a function of pH (figure 1); b) the resonance which Bradbury et al.[1] assigned to His 119 in fact arises from unfolded material, and would indicate that they had as much 'denatured' S-protein in their sample as we had in our samples; c) if there was as

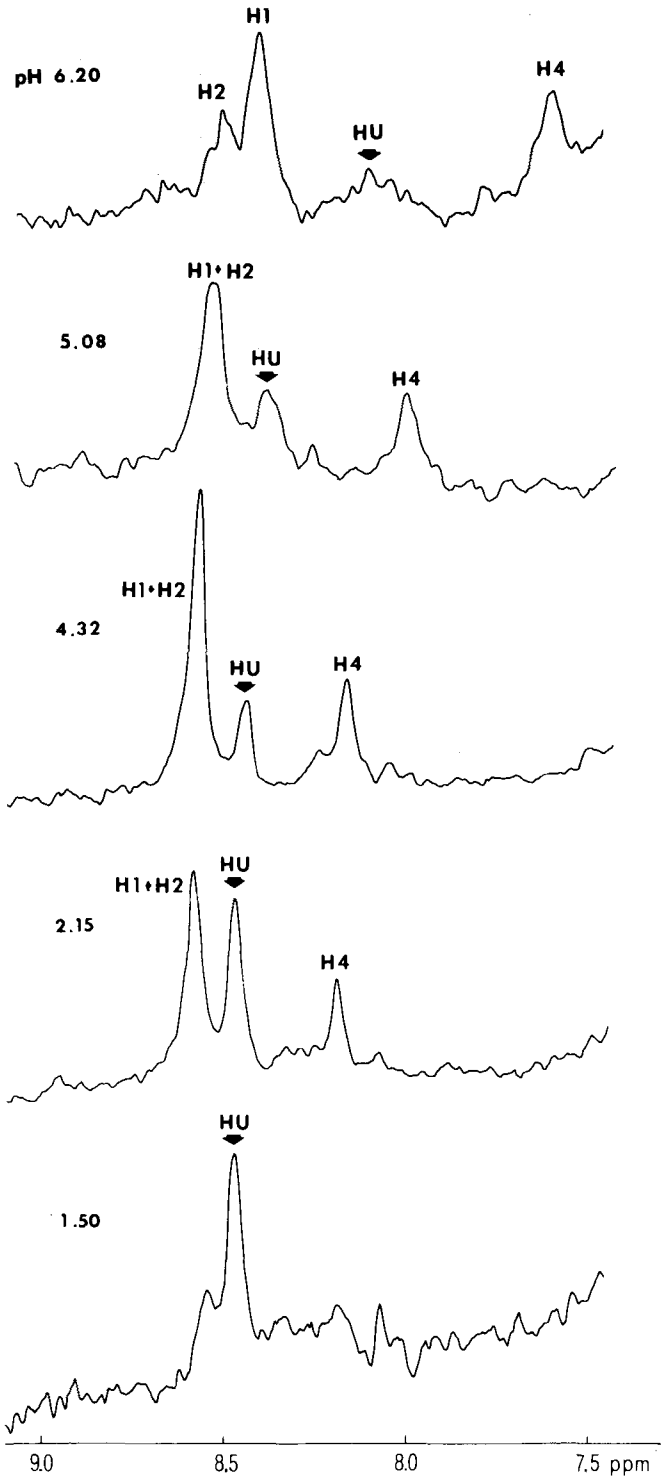

Fig. 1. NMR-spectra at 220 MHz of ribonuclease S-protein (10 mg/ml) in ^2H_2O (0.1 M NaCl). Spectra at pH-values 6.20, 5.08 and 1.50 were recorded on a sample derived from active ribonuclease S[3] following column chromatography in 50% aqueous acetic acid[4]. Spectra at pH values 4.32 and 2.15 were from a sample obtained from Sigma Chemical Co. All samples gave essentially equivalent spectra at the same pH-values (see also Shindo and Cohen[2]). The peak denoted by the arrow increased in area as pH is lowered and is assigned to the equivalent histidines of unfolded S-protein (H-u). The 2 peaks to low field (8.5 and 8.4 ppm) in the spectrum at pH 6.2 are due to His residues 119 (H-2) and 105 (H-1) of folded S-protein, respectively. The resonance (H4) of His 48 is upfield, for example at 8.0 ppm at pH 5.08.

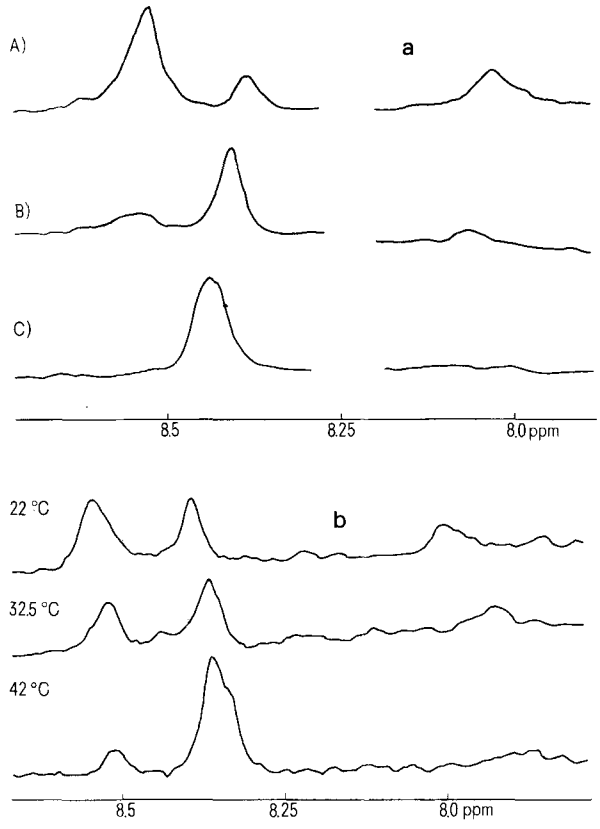

Fig. 2. *a* NMR-spectra at 220 MHz of S-protein at pH 5.15. *A* Sample (Sigma) from figure 1 after reducing pH to 1.5 for 2 h, increasing to pH 6, re-lyophilizing and re-adjusting to pH 5.15. *B* Same sample with about 0.7 mole ratio of guanidine hydrochloride added. *C* Containing about 1.5 mole ratio of guanidine hydrochloride. The peak which increases in area corresponds to the equivalent histidine residues of unfolded S-protein (H-u).

b NMR-spectra at 220 MHz of -S-protein (Sigma) at pH 5.0 at 3 temperatures. The peak which increases in area with temperature corresponds to the equivalent histidine residues of unfolded S-protein (H-u).

much as 30% 'irreversibly denatured' material present in S-protein then large differences in enzymatic activity between samples of ribonuclease A (or S) and S' would have been found previously, whereas this is not the case[8,9]. Consequently, we conclude that Bradbury et al's[1] assertion that significant amounts of 'irreversibly denatured' material is present in S-protein samples prepared by the standard methods is unfounded.

1 J.H. Bradbury, M.W. Crompton and J.S. Teh, Eur. J. Biochem. *81*, 411 (1977).
2 H. Shindo and J.S. Cohen, J. biol. Chem. *251*, 2648 (1976).
3 E.M. Crook, A.P. Mathias and B.R. Rabin, Biochem. J. *74*, 234 (1960).
4 M.S. Doscher, Meth. Enzymol. *2*, 640 (1967).
5 H. Epstein, A.N. Schechter and J.S. Cohen, Proc. nat. Acad. Sci. USA *68*, 2042 (1971).
6 H. Shindo, M.B. Hayes and J.S. Cohen, J. biol. Chem. *251*, 2644 (1976).
7 H. Shindo and J.S. Cohen, J. biol. Chem. *250*, 8874 (1976).
8 T. Takahashi, M. Irie and T. Ukita, J. Biochem., Tokyo *65*, 55 (1969).
9 T.Y. Tsong, R.P. Hearn, D.P. Wrathall and J.M. Sturtevant, Biochemistry *9*, 2666 (1970).