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STABILISATION OF ENZYME STRUCTURES BY INHIBITORS

A Nuclear Magnetic Resonance Study of the Effect of Phosphate on the Acid Unfolding of Ribonuclease A

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The acid denaturation of bovine pancreatic ribonuclease A in the presence of 0.2 M sodium dihydrogen phosphate has been studied by n.m.r. spectroscopy. Phenylalanine, tyrosine and methionine resonances serve as monitors of the unfolding process. It is shown that the inhibitor shifts the equilibrium towards the native structure at acid pH. Exchange broadening of the C-2 resonances of the active site histidines, 12 and 119, occurs in the presence of phosphate, suggesting an equilibrium between native and unfolded structures. Stabilisation of the partially unfolded protein is observed at pH 1.5, as evidenced by the lack of the histidine resonance due to random coil protein. A scheme of the equilibria relating the various states of the protein is proposed.

The substrate-induced stabilisation of enzyme structure is an established principle. The difference in stability between the conformations of free and ligand bound enzymes has been studied extensively (Citri, 1973). The vast information available about bovine pancreatic ribonuclease A (RNase A) (Richards & Wyckoff, 1971) makes it a suitable model for the investigation of ligand-induced stabilisation of enzyme structure. It is known that phosphate stabilises RNase A against 8 M urea (Sela *et al.*, 1957; Nelson *et al.*, 1962; Bernard, 1968) and thermal denaturation at neutral (Hermans & Scheraga, 1961) and acidic pH (Ginsburg & Carroll, 1965). RNase A undergoes a conformational transition under acidic conditions. This process is accompanied by the exposure of a buried tyrosine residue (Tyr 92) (Bigelow, 1964). Recent n.m.r. studies have illustrated in depth the structural changes involved in the acid unfolding of the enzyme (Benz & Roberts, 1975). In addition to a Tyr residue a number of aliphatic amino acids e.g. Met, Ala, Val, Leu and Ileu, a Phe residue and His 119, His 12 and/or His 105 are perturbed by acid unfolding. N.M.R. spectroscopy affords, in principle, detailed information about the conformational states of proteins. In this paper, we describe a study of the acid denaturation of RNase A in the presence of phosphate with a view towards understanding the details of the stabilisation of native enzyme structure by inhibitors.

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MATERIAL AND METHODS

RNase A (type IA, Sigma Chemical Co. Lot 72C-1440) was purified according to the procedure of Taborsky (1959). Sodium dihydrogen phosphate (analar grade) was deuterated by evaporating D_2O solutions twice under *vacuo*.

Proton magnetic resonance spectra were recorded on a Varian HA-100 spectrometer equipped with a C-1024 time averaging computer. The probe temperature was 32°C. The residual HDO resonance was used for field frequency locking. Protein solutions (7.1 mM) were prepared in 0.2 M $NaCl/D_2O$. The pH was adjusted using DCl or NaOD and measured with a Toshniwal (India) pH meter, Type CL44. The reported values are direct meter readings uncorrected for the deuterium isotope effect. Sweep widths of 500 Hz with a scan rate of 5 Hz/sec were used without significant loss of resolution. Most spectra were obtained after 20–50 scans. Chemical shifts are expressed as δ p.p.m. downfield from 2,2′-dimethyl silapentane-5′-sulfonate (DSS).

RESULTS

It is known that inhibitors bind most strongly to RNase A at pH 5.5 (Hammes & Schimmel, 1965). However, addition of 0.2M phosphate to RNase A is largely without effect on the aliphatic and aromatic proton resonances of the protein (Figs. 1–6) at pH 5.5. Changes in the chemical shifts of the histidine peaks arise due to altered pKa values of His-12 and -119, the active site residues that bind phosphate (Meadows *et al.*, 1969). RNase A unfolds partially at acidic pH (Benz & Roberts, 1975). The effects of 0.2M phosphate on the n.m.r. spectra of RNase A at low pH are described below.

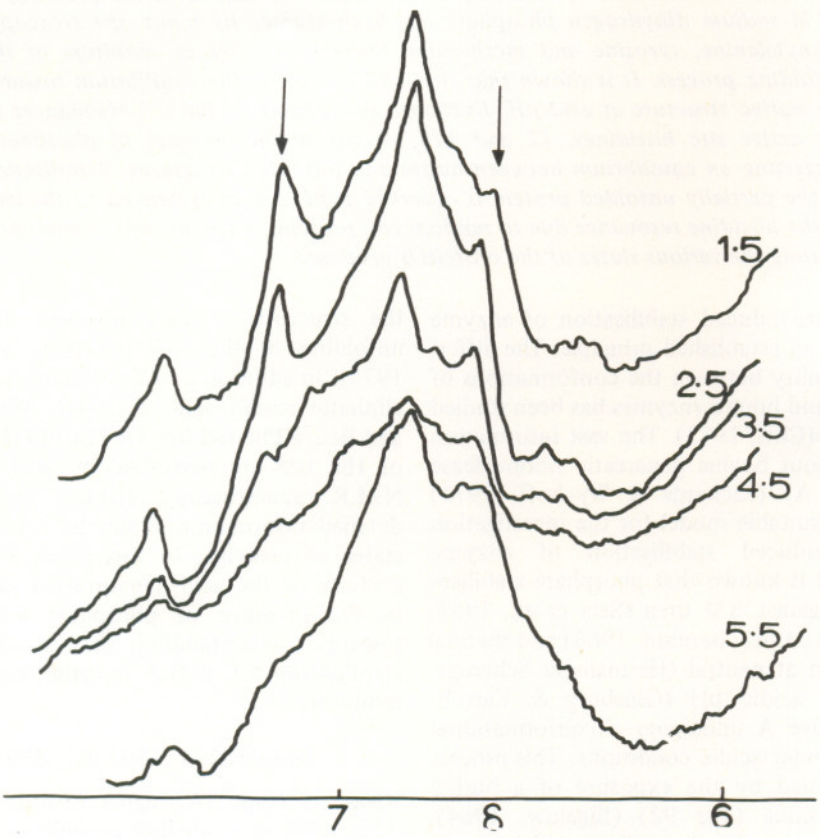


FIGURE 1

The aromatic region of the 100 MHz proton n.m.r. spectrum of RNase A (7.1 mM) in 0.2 M $NaCl/D_2O$ as a function of pH. Arrows indicate Phe and Tyr peaks that sharpen on unfolding.

Aromatic resonances. The aromatic proton resonances of RNase A with and without phosphate are shown in Figs. 1 and 2. Acid unfolding of RNase A is accompanied by the appearance of a sharp resonance at 7.13 δ, assigned to a phenylalanine residue (Benz & Roberts, 1975). A shoulder at high field due to a tyrosine residue is also observed at low pH. For the sake of clarity these peaks are indicated with arrows in the Figures. At pH 1.5 the aromatic resonances do not differ markedly in the presence of phosphate though the low field peak is less intense. This effect is seen better at pH 2.5, where the peak is clearly reduced in the presence of phosphate. Pronounced differences at the upfield edge of the aromatic envelope with and without phosphate are noticeable at pH 3.5. Phosphate causes the Tyr resonance to broaden. These effects are likely to result from a lower degree of acid unfolding in the enzyme-inhibitor complex. Raising the pH to 4.5 results in almost similar spectra from the enzyme and the inhibitor-enzyme complex.

Histidine $C_{(2)}$-H resonances. The effect of phosphate on the acid unfolding process is clearly illustrated by the His $C_{(2)}$-H peaks (Figs. 3 and 4). Three resolves resonances are observed at pH 4.5 in RNase A while only two peaks are observed in the presence of phosphate. The original assignment of the histidine resonances to specific residues has been recently revised (Markley, 1975; Patel *et al.*, 1975). We shall follow the revised assignment (indicated in Figs. 3 and 4). At pH 3.5

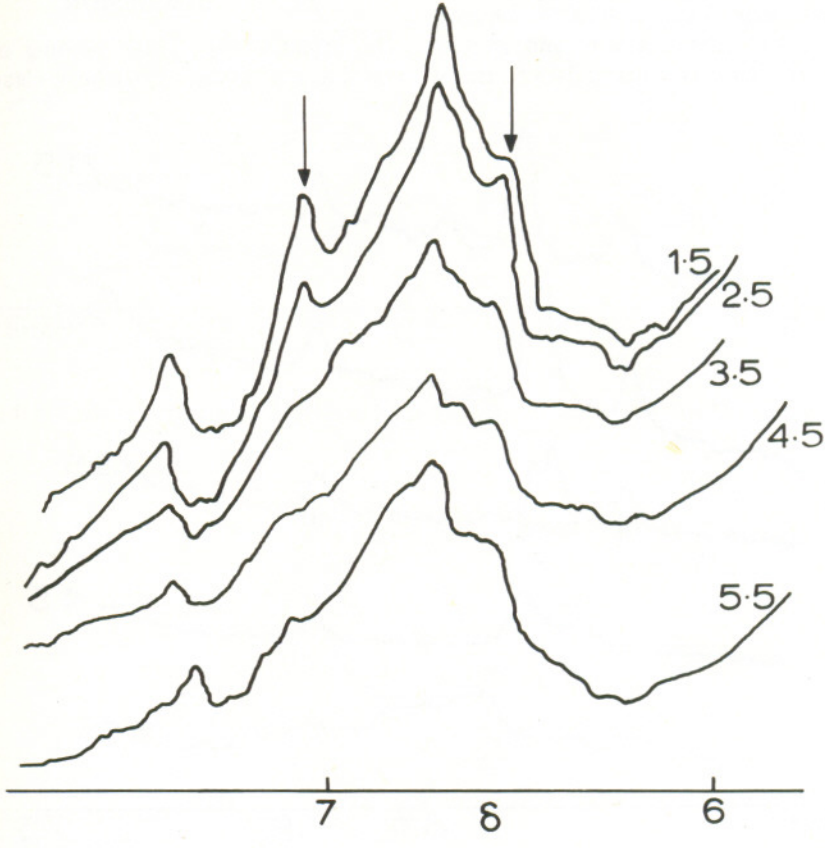


FIGURE 2.

The aromatic region of the n.m.r. spectrum of RNase A (7.1 mM) in 0.2 M $NaCl/D_2O$ + 0.2 M NaD_2PO_4 as a function of pH. Arrows indicate Phe and Tyr peaks that are monitors of unfolding.

and 2.5, RNase A – phosphate complexes yield only two $C_{(2)}$-H resonances corresponding to an intensity of two protons. However, the spectra of the free enzyme clearly shows four resonances. In the presence of inhibitor the peaks due to His-12 and -119 broaden and are difficult to detect. However, these resonances reappear at pH 1.5, leading to a spectrum closely resembling that of the free enzyme. It is of interest that the small peak at ~8.5 δ, in RNase A at pH 1.5, believed to be due to random coil protein (Benz & Roberts, 1975; Westmoreland & Matthews, 1973), is absent in the presence of phosphate.

Aliphatic resonances. The upfield region (0–3 δ) of the RNase A n.m.r. spectrum is complex and poorly resolved at 100 MHz. The information obtained from this region of the spectrum is rather limited. Figs. 5 and 6 show the aliphatic proton spectra of RNase A with and without phosphate over the pH range 1.5–5.5. Below pH 2.5, the RNase A spectrum is considerably altered suggesting acid unfolding of the molecule (Benz & Roberts, 1975). However, a sharp resonance at 2.07 δ appears even at pH 3.5. This may be tentatively assigned to a methionine residue (Sadler *et al.*, 1974; King & Bradbury, 1972). In the presence of phosphate this resonance appears broader and less intense, again suggesting stabilisation of the native enzyme structure. The rest of the upfield envelope appears qualitatively similar in the presence and absence of phosphate over the entire pH range studied. It is notable that while the aromatic resonances (Figs. 1, 2) are perturbed even at pH 4.5, the methyl and methylene peaks are affected only below pH 2.5.

DISCUSSION

The effect of phosphate binding on the acid unfolding of RNase A has been clearly demon-

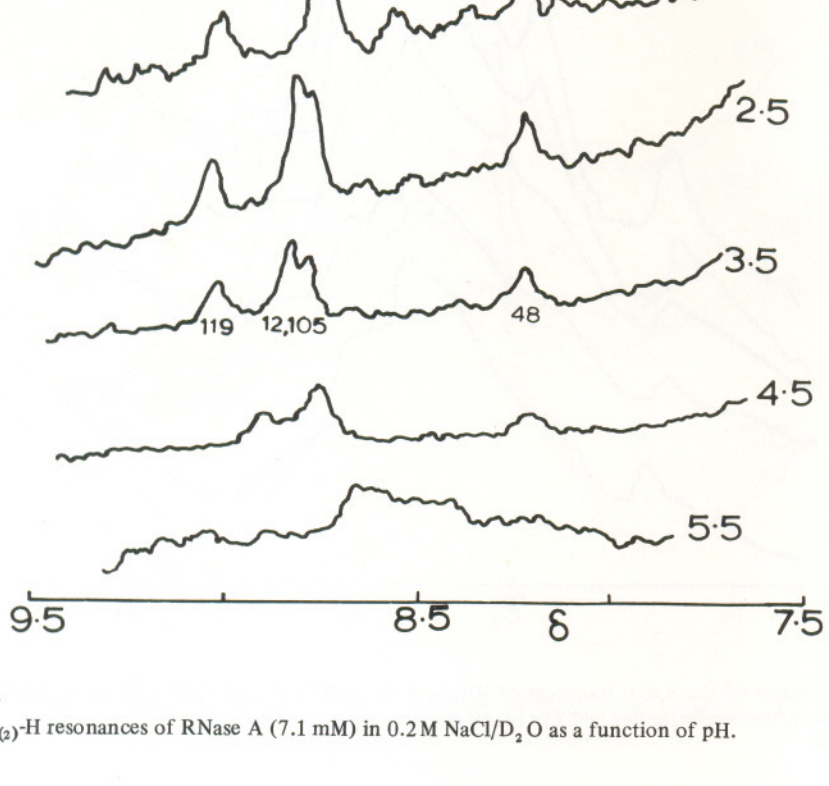


FIGURE 3.
Histidine $C_{(2)}$-H resonances of RNase A (7.1 mM) in 0.2 M NaCl/D_2O as a function of pH.

strated by the following observations:

(a) The differential behaviour of the His $C_{(2)}$-H resonances in the presence and absence of phosphate between pH 4.5 and 2.5.

(b) The sluggish appearance of the resonance at 7.13 δ due to a Phe residue in partially unfolded RNase A. Peaks tentatively assigned to a Tyr residue of unfolded protein are also less evident in the presence of phosphate.

(c) The partial disappearance or broadening of a Met S-CH_3 resonance in the phosphate RNase A complex.

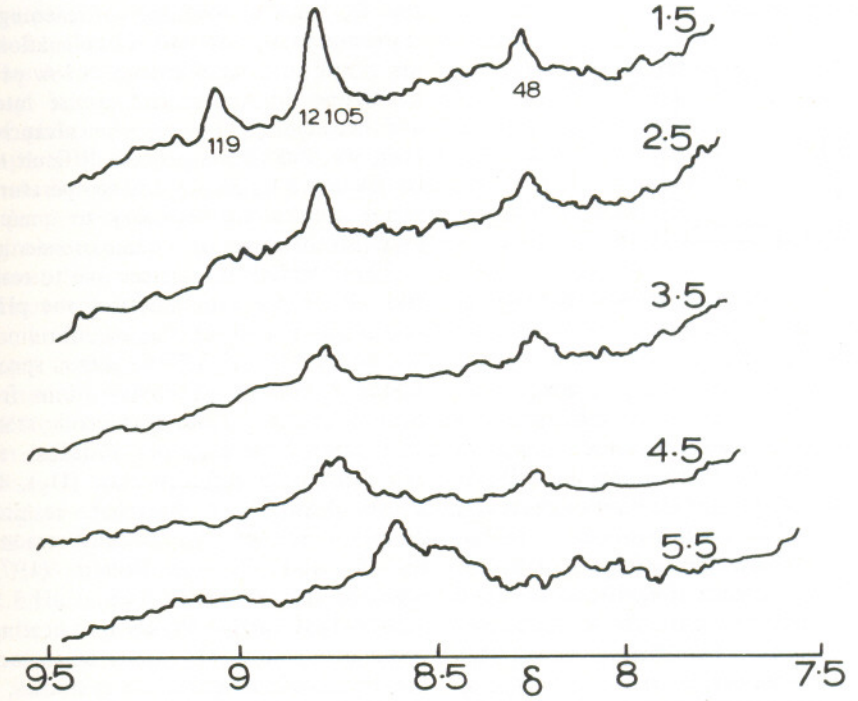


FIGURE 4.

Histidine $C_{(2)}$-H resonances of RNase A (7.1 mM) in 0.2 m NaCl/D_2O + 0.2 M NaD_2PO_4 as a function of pH.

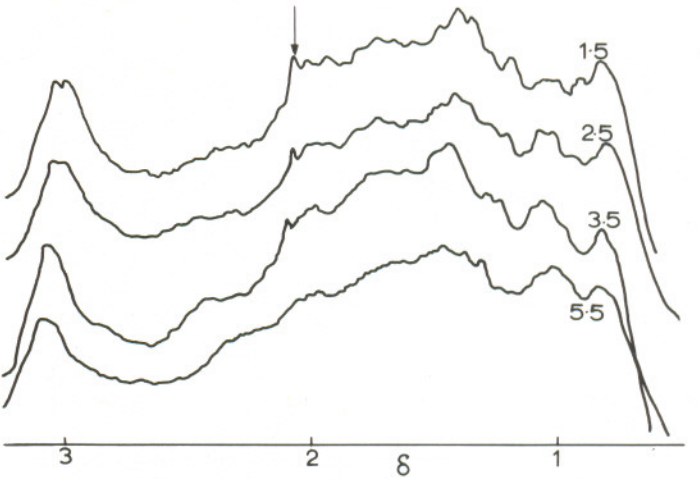
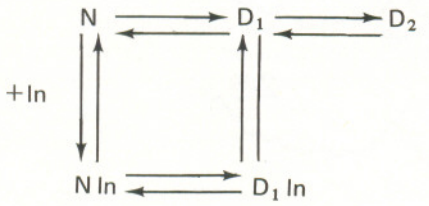


FIGURE 5.

Upfield region of the n.m.r. spectrum of RNase A (7.1 mM) in 0.2 M NaCl/D_2O as a function of pH. Arrow indicates the resonance that is tentatively assigned to a Met residue.

It should also be noted that the spectra of free enzyme and enzyme-phosphate mixtures differ even at pH 1.5, where ligand binding is likely to be minimal. Most significantly, the 'random coil' peak at ~8.5 δ is absent in the presence of phosphate. These results may be rationalised by considering the equilibrium scheme outlined below.



Phospate binds RNase A most effectively at pH 5.5 (Hammes & Schimmel, 1965). At the concentration (0.2 M) used in this study the protein would exist entirely in the inhibitor bound state (NIn). The n.m.r. spectra at pH 5.5 point to gross conformational similarities between NIn and the native enzyme (N). As the pH is lowered, phosphate binding decreases and the equilibrium shifts towards N. In the absence of phosphate, the unfolding of RNase A leads to the appearance of significant amounts of a partially unfolded state D_1 at acidic pH, as early as pH 4.5. The Phe, Tyr and Met resonances discussed earlier are characteristic of D_1. The histidine region of the n.m.r. spectrum is dramatically different in the pH range 4.5 to 2.5, in the presence of phosphate. Only two peaks due to His-48 and -105 are observed. The resonances due to His-12 and -119 broaden and disappear in this pH range. We suggest that binding of phosphate to the active site histidines results in a stabilisation of the structure NIn. At low pH the exchange of His-12 and -119 between their environments in NIn and D_1 leads to exchange broadening of their resonances. An alternative explanation, involving a conformational change at low pH leading to strong intramolecular dipolar interactions and broadening, may also be advanced. However, we find such a process difficult to visualise. Detailed frequency and temperature dependence studies are necessary to unequivocally establish the origin of the line broadening.

The histidine resonances due to residues 12 and 119 reappear on lowering the pH to 1.5, suggesting a shift of the equilibrium towards N and D_1. At pH 1.5 the n.m.r. spectrum of RNase A consists of contributions from two conformations – a random coil state (D_2) characterised by only one histidine resonance and a partially unfolded state (D_1), discussed above. Addition of phosphate results in the disappearance of the histidine resonance of the D_2 state. Benz & Roberts (1975) have observed this resonance even at pH 3.5. These authors have further shown that heating RNase A samples at low pH results in an increase of the D_2 histidine peak. In our studies, an optimistic view of the n.m.r. spectrum at pH 3.5 would indicate the presence of D_2. However, the poor signal-to-noise ratios under our experi-

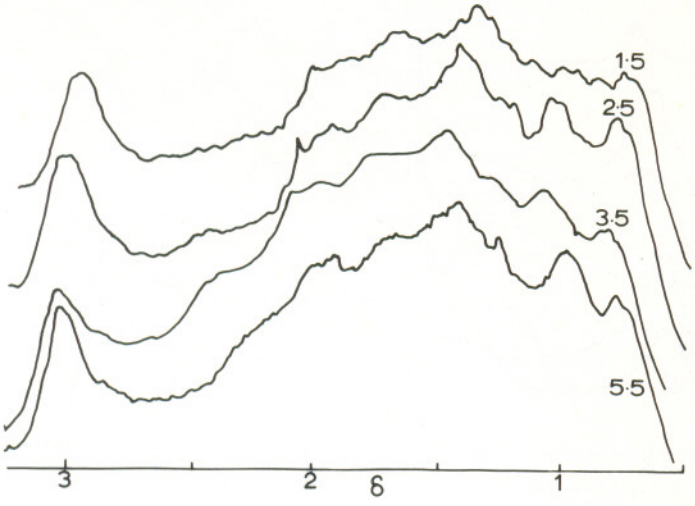


FIGURE 6.

Upfield region of the n.m.r. spectrum of RNase A (7.1 mM) in 0.2 M $NaCl/D_2O$ + 0.2 M NaD_2PO_4 as a function of pH.

mental conditions preclude further analysis. The absence of the D_2 peak in phosphate-RNase A mixtures suggests stabilisation of the D_1 state. We propose that an interaction of phosphate with D_1 shifts the equilibrium towards D_1 and hence D_1In. The lowest pKa of phosphate is 2.1. However interactions with the 'positive patch' of residues, (Lys-7, Arg-10, Arg-39, His-12, Lys-41, His-119) in the vicinity of the RNase A active site (Richards & Wyckoff 1971), may lower this value substantially. Binding of phosphate in the form $(H_2PO_4)^-$ may be important even at pH $\sim$ 1.5. It is likely that the state D_2 will be populated to a greater extent in the presence of phosphate, in a more denaturing environment. The equilibrium proposed between NIn and D_1In is purely speculative and is not supported by any data presented here. It is, however, an intriguing possibility, as the partial unfolding of a protein structure, without altering active site geometry, may leave inhibitor binding unaffected. In this context it should be noted that ϵ-41-dinitrophenyl-RNase A has the ability to bind 3′-CMP, though the native and modified proteins differ considerably in overall structure (Ettinger & Hirs, 1968; Allewell *et al.*, 1973).

Cohen *et al.* (1973) have observed acid inflections in the titration curves of His-12 and -119 between pH 3 and 5. Phosphate binding decreases the pKa of this inflection slightly. Interactions of these histidine residues with proximate carboxyl groups have been involved to explain these observations. It is likely that local conformational changes, as observed here, may partially contribute to the inflections. It must be stressed that even at pH 4.5 the local environments of Phe, Tyr and Met residues are altered. Donovan (1965) has observed small perturbations of Phe and Tyr residues between pH 5 and 2 by u.v. difference spectroscopy. A sequence of the unfolding of RNase on lowering the pH may be postulated, with the aid of information available in the literature (Benz & Roberts, 1975; Westmoreland & Matthews, 1973; King & Bradbury, 1972). In the initial stage a Phe residue, Tyr-92, and possibly Met-29 are exposed to the solvent. This is followed by change in the environment of His-12, His-119 and/or His-105. The third stage that sets in below pH 2.5 results in the perturbation of a number of residues like Ala, Val, Leu and Ileu as seen by changes in the methyl proton resonances.

0.2 M phosphate is routinely used in the purification of RNase A (Hirs *et al.*, 1953). We have shown elsewhere that a 'trapped' conformation of a RNase A derivative isolated after exposure to strongly acidic media reverts to a conformation indistinguishable from the native structure in the presence of phosphate (Das, 1975). Conformational changes in RNase A on binding the nucleotide inhibitors 2′ and 3′-CMP have been observed by H-D exchange studies (Nonnenmacher *et al.*, 1971), n.m.r. spectroscopy (Meadows *et al.*, 1969). However, the binding of phosphate at pH 5.5 does not lead to changes detectable by n.m.r. (Meadows *et al.*, 1969). It is indeed interesting that RNase A could be crystallised only in the presence of anions like phosphate, sulphate and arsenate, which are known to interact at the active site (Kartha *et al.*, 1967). The enzyme may exist in a number of closely related conformational states at pH 5.5. Addition of phosphate shifts the equilibria to a stable inhibitor bound form. Our results strongly support the view that phosphate tightens the structure in the vicinity of the active site and also retards unfolding at more remote regions, as evidenced by the behaviour of Met, Phe and Tyr residues.

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