$^{31}$P and $^1$H NMR Studies of the Effect of the Counteracting Osmolyte Trimethylamine-N-oxide on Interactions of Urea with Ribonuclease A

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Running Title: NMR Studies of TMAO, Urea and RNase A
SUMMARY

$^{31}$P NMR spectroscopy has been used to show that the activity of RNase A, which is lowered in the presence of urea, can be recovered with TMAO. A 1:1 ratio of TMAO:urea was sufficient to recover the enzyme activity. $^1$H NOESY NMR studies with RNase A have shown that even at relatively low effective concentrations of TMAO, some modification of the 3-dimensional structure of the biomolecule is apparent.
INTRODUCTION

Many organisms are known to accumulate small organic molecules (osmolytes) in their tissues in response to external osmotic stresses such as dehydration, freezing or high salinity. For example, the tissues of cartilaginous fishes characteristically contain high levels of urea, as part of a strategem for increasing blood osmolality in seawater. (1,2) It is generally known that urea can denature biological macromolecules, (3,4) however this effect can be offset by maintaining a cellular environment rich in ‘counteracting osmolytes’, particularly trimethylamine-N-oxide (TMAO) and glycine betaine. (3-5) There is much interest (4,6) in understanding the way in which counteracting osmolytes such as TMAO might offset the effects of urea on the function and/or conformation of enzymes. The present work seeks to address this issue by examining the (in vitro) effects of urea and TMAO on both the activity and 3-dimensional structure of a model protein, based on NMR spectroscopy. Bovine pancreatic ribonuclease A (RNase A) was selected as the model in this study, as it is a well-characterised protein (7,8) whose NMR spectrum has been fully analysed. (9) In addition, RNase A has previously been used in studies of counteraction of urea destabilisation by methylamines. (3) Although RNase A is not derived from tissues which normally experience high urea concentrations, the ‘counteracting osmolyte’ hypothesis is proposed (4,5) to be a general one, extending to proteins which are not subjected to high concentrations of urea in vivo.

EXPERIMENTAL PROCEDURES

Ribonuclease A (type XIIA), polyuridylic acid, trimethylamine-N-oxide (Sigma Chemical Company) and urea (BDH) were used without further purification.
$^{31}$P NMR Spectroscopy - $^{31}$P NMR assays were carried out at 25ºC in either a 0.1 M imidazole-HCl buffer (pH 6.98) or a 50 mM potassium hydrogen phthalate buffer (pH 3.98) containing NaCl (0.2 M), poly(U) (6 mM in P-O$^5$ bonds), urea (0-2 M), TMAO (0-1 M) and RNase (0.1 µM for pH 6.98, 2 µM for pH 3.98). Each reaction was monitored for 11-12 hours. Data were collected on a Varian Unity Inova spectrometer, operating at 202.299 MHz, using the following parameters: pulse width, 90°; acquisition time, 1.594 s; relaxation delay, 0.5 s; number of transients, 256; sweep width, 14215 Hz. $^{31}$P NMR spectra were referenced ($\delta = 0$ ppm) to external H$_3$PO$_4$ (0.3 M). Relative integrated intensities of the polyU peak were measured as a function of time and these data were fitted ($R > 98\%$) to an exponential function using Excel®. The lifetimes, listed in Table 1, were determined when the peak was 50% of the original intensity.

$^1$H NOESY Spectra - Samples of RNase A for $^1$H NMR NOESY spectra were prepared in 95% H$_2$O/5% D$_2$O (1.25 mM) and adjusted to pH 4 by addition of small amounts of HCl. Sodium 3-trimethylsilyl-2,2,3,3-$d_4$-propionate (TSP) (1 mM) was added as the internal reference ($\delta = 0$ ppm). Spectra were acquired on the Varian Unity Inova spectrometer, operating at 499.7393 MHz at 35ºC, in the phase-sensitive mode, with the carrier frequency at the middle of the spectrum. A mixing time of 0.15 s was used, and spectra were collected with 16 transients and 128 increments. Water suppression was achieved using the WETNOESY (10) pulse sequence. NOESY spectra were interpreted with the aid of the search program BBReader, (11) which assigned NOESY cross-peaks to the protons of particular amino acid residues. The reference structure data file (2aas.pdb) was obtained from the Protein Data Bank (12) and hydrogens were added using the MOLMOL program.(13) The reference NMR data file (bmr443.str, accession # 443) was obtained from the BioMagResBank. (14) Major peak deletions are listed in Table 2 and, in addition, the
following changes were noted: 15 mM TMAO - Glu49 HA-Ser50 H, Gln28 HA-Gln28 H, Val63 HG1-Val63 HA. 30 mM TMAO - Glu49 HA-Ser50 H, Gln28 H-Met30 HB2/HB3, Val57 HB-Val57 HA, (Thr45 HB-Thr45 H). 45 mM TMAO - Gln28 H-Met30 HB2/HB3, Gln28 HA-Gln28 H, (Thr45 HB-Thr45 H), (Tyr97 HA-Lys98 H), (Val47 HB-Thr82 H). Data in parentheses have confidence levels < 85%.

RESULTS

$^{31}$P NMR

The proposed mechanism for the general reaction of RNase A with RNA (Scheme 1) (8)

![Scheme 1 about here](http://www.jbc.org/)

involves enzyme-catalysed formation of cyclic phosphodiester intermediates, which are transformed ultimately to a monomeric phosphate species. The key amino acid residues involved in the process are His12 and His119. It has been shown that the RNase A-catalysed hydrolysis of polyuridylic acid (poly(U)) can be monitored by $^{31}$P NMR spectroscopy. (15,16) The present work uses $^{31}$P NMR as an assay method for determining the effects of osmolytes on the first (transphorylation) step of the reaction, by examining the rate of removal of poly(U) from the reaction mixture. $^{31}$P NMR spectra collected at different time intervals during the reaction show 4 groups of $^{31}$P resonances, corresponding to the presence of: polyuridylic acid (poly(U)), uridine 2',3'-cyclic phosphate (U>p), poly(U)-3'-(uridyl-2',3'-cyclic phosphate) (poly(U)U>p) and uridine 3'-phosphate (Up). (15) Representative $^{31}$P spectra, collected at time intervals during the RNase A-catalysed hydrolysis of poly(U), are displayed in Figure 1. The resonances correlate well with published data (15) and are

![Figure 1 about here](http://www.jbc.org/)
assigned as shown. Before addition of RNase A the peak assigned to poly(U) is observed as a sharp singlet, however in spectra collected after addition of the enzyme, a family of peaks is observed in this region, reflecting the increasing size heterogeneity of the poly(U) chain. Similarly, after initiation of the reaction a group of peaks is seen in the spectral region assigned to poly(U)U>p.

Figure 2 shows a set of four spectra corresponding to separate reaction mixtures containing either urea, urea + TMAO or no osmolytes. A comparison of these spectra with those in Figure 1 shows clearly that the intermediate species formed during the RNase A-catalysed reaction are also produced in the presence of TMAO and urea. Thus, it seems reasonable to assume that the general mechanism of the reaction is the same in each case. The spectra in Figure 2 were obtained with comparable initial concentrations of poly(U) and RNase A, at a convenient time (21 minutes) after reaction initiation. In the unmodified system (Figure 2A), resonances are observed for poly(U) and the cyclic phosphate intermediates poly(U)U>p and U>p. However, in the presence of 1 M urea (Figure 2B), only a relatively small amount of U>p is observed at the same time. Since U>p is the second identifiable species formed during the course of the reaction, the hydrolysis in this case is proceeding more slowly than in the unmodified situation. Spectra obtained from reaction mixtures containing both TMAO and urea (Figures 2C and 2D) are similar to the original (Figure 2A), which demonstrates that while the activity of RNase is modified in the presence of urea, this effect is counteracted by TMAO.

In order to examine the effects of different osmolyte concentrations and ratios on the reactivity of RNase A, a series of $^{31}$P NMR experiments was conducted with varying
concentrations of urea and TMAO. To facilitate comparisons, a ‘lifetime’ of poly(U) was used, representing the time required for the relative integral of the combined poly(U) resonances to reduce by half. The results shown (Table 1) are the means of either 2 or 3 experiments. From Table 1 it is evident that as the concentration of urea is increased, the lifetime of poly(U) also increases (entry 1 vs. entries 2-5). However, at constant concentration of urea (entries 6-8 and 9-11), the lifetime decreases with increasing TMAO concentration, such that for 1:1 urea:TMAO, the original (entry 1) reactivity is recovered. This result is observed for both of the urea concentrations (700 mM and 1 M) examined. Interestingly, in the presence of TMAO alone (entry 12), the lifetime is decreased relative to the osmolyte-free case, which demonstrates that, at least under these reaction conditions, TMAO itself does affect RNase A activity.

The $^{31}$P NMR results presented here do not show a correlation between urea concentration and the rate of the last step, viz. $U>p \rightarrow Up$, in the reaction sequence. It has been proposed (15) that after the first (transphorylation) step, the substrate is released from the enzyme active site before further reaction. This release takes place even though the two histidine residues (His12 and His119) are appropriately protonated and in position for the next step in the reaction. As the final step is not dependent upon $U>p$ being associated with the enzyme, it follows that the rate of production of Up should not be influenced by enzyme activity, provided appropriate concentrations of intermediates are maintained.

A set of $^{31}$P NMR assays was also performed at pH 3.98 in order to allow comparison with the results of structural studies (vide infra). In concert with the data obtained at pH 6.98, it is found that the lifetime for poly(U) concentration is increased in the presence of urea,
compared with the unmodified case, however TMAO shows no counteracting effect. This observation may reflect that TMAO is mostly protonated at pH 4 (see Discussion).

NOESY Studies

Considerations of the mode of action of counteracting osmolytes on proteins have focused on whether there is a direct protein-osmolyte interaction (4,6) or an indirect, solvent mediated (‘water-structuring’) effect. (17) The present work uses 2-dimensional $^1$H NOESY NMR studies in order to probe aspects of the action of TMAO on RNase A.

$^1$H NMR has previously been used to determine the solution structure of RNase A (9) and the data are available on the Protein Data Bank (12) and BioMagResBank. (14) Hence, this protein is a convenient model for NOESY studies. The structural characterisation of RNase A relies heavily on NOESY spectra, and the search program BBReader, (11,18) used in the present study, has been developed to search such data.

The $^1$H NOESY NMR spectrum of RNase A was found to match that described previously, (9) obtained under the same experimental conditions. NOESY spectra of RNase A were then collected in the presence of varying concentrations of osmolytes, and compared to the osmolyte-free spectra. Cross-peaks which were removed (‘deleted’) from the baseline spectrum by the addition of the osmolyte indicated the particular hydrogen-hydrogen internuclear distance had increased to over 6Å. The deletions were assigned to specific amino acid residues using BBReader and therefore it was possible to determine which regions of the RNase A molecule were affected. Similar experiments have been reported with a hen egg white lysozyme/sorbitol system. (19) Spectra obtained with the relatively high concentrations of urea used in the $^{31}$P NMR assays were inconclusive due to difficulties in
obtaining useful data. NOESY spectra of mixtures of RNase A and lower concentrations of urea did not show any discernable effects. The study therefore focussed on an examination of the influence of TMAO alone, and Table 2 summarizes the persistent and reproducible deletions from the NOESY spectra over the range of TMAO concentrations examined and the related internuclear distances in the unmodified enzyme. The first two TMAO-induced spectral deletions (Table 2) correspond to sequential contacts but, notably, the latter two indicate substantial increases (>2 Å) in the natural hydrogen-hydrogen internuclear distances between two β-sheets and also between a helix and a sheet in RNase A.

[Table 2 about here]

Depending on the TMAO concentration, additional deletions were observed (cf experimental section). Most of the data were assigned with a confidence level of greater than 85% as determined from percentage ‘scores’ calculated by the BBReader program, on the basis both of the closeness of the chemical shift values to those in the published data, and of the distance between the two protons. These additional deletions were all short range intraresidue, sequential or i,i+2 contacts and as such do not provide additional structural information.

Two histidine residues (His12 and His119) have been identified in the active site of RNase A (8) and are therefore of interest for catalytic activity. None of the deletions noted involves either of these histidine residues. Furthermore, the NOESY spectrum of RNase A shows a cross-peak which can be confidently (94%) assigned to a correlation between His119 H and Phe120 HA protons. This peak is not altered by the addition of TMAO, suggesting that the osmolyte does not have a direct interaction at this active site area. However there is some indication that at least one of the subsite regions (viz Thr45, located in the B1 subsite, (8)) is modified by the presence of TMAO.
DISCUSSION

The effects of TMAO and other osmolytes on some of the physical properties of RNase A have been well documented. (3,5) For example, Burg and Peters have shown that urea lowers the midpoint of thermal transition (T_m) of RNase A, while glycerophosphocholine (GPC) and TMAO counteract this effect. (5) Yancey and Somero also noted that TMAO and β-alanine increase T_m and that this structure stabilization occurs even at relatively low concentrations (ca. 0.1 M). (3) The previous results are in concert with the findings reported here. However, the NMR data provide information at a more detailed molecular level, and show that the reactivity of the enzyme is decreased in the presence of urea, but is clearly recovered in urea/TMAO mixtures. Furthermore, it has long been postulated that the ratio of TMAO:urea is significant, and that an approximate 1:2 molar ratio often gives optimal counteraction. (3,20,21) Under the experimental conditions used in this work a 1:1 ratio is found to be sufficient to recover the activity of RNase A.

In order to base the NOESY analysis on the established data, it was necessary to obtain spectra under the same experimental conditions. In particular, measurements at pH 4 were required. It should be noted that at this pH, most of the TMAO (pK_a 5.13 (22)) is in the protonated conjugate acid form and the counteracting effect was not apparent (vide supra). Notwithstanding these limitations, the NOESY experiments indicate that, even at low effective concentrations, TMAO can cause detectable structural changes in some segments of the enzyme. This contrasts with the hen egg white lysozyme study, where a much larger concentration of the perturbing compound was required in order to induce any observable changes. (19) In that case, two new cross-peaks were observed in the NOESY spectrum, and some chemical shift changes were also noted.
As noted above, there are at least two ways in which TMAO might influence enzyme structure and activity. These include a direct osmolyte-protein interaction or a solvent-mediated effect which induces a ‘global’ change in the environment around the protein. For example, it has been proposed that organic osmolytes can influence hydrogen bonding of solvent molecules over long distances. (23) This work has shown that TMAO alone at pH 4 does not induce changes in the RNase molecule at or near the active site and, at best, minor structural changes are introduced in other regions of the molecule. The osmolyte has a more dispersed, global effect on the protein, which is presumably effected via the solvent, rather than a specific interaction at particular site(s).

In summary, by using $^{31}$P NMR spectroscopy, it has been demonstrated unequivocally that the activity of RNase A, lowered in the presence of urea, can be recovered with TMAO in molar ratios of approximately 1:1. NOESY studies with RNase A have shown that even at relatively low effective concentrations of TMAO some, albeit small, changes in the 3-dimensional structure of the biomolecule can be detected. Minor changes in protein structure at locations outside the active site have been postulated previously to affect kinetic properties. (24)

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Figure 1: $^{31}$P NMR spectra showing the species present during the hydrolysis of poly(U) by RNase A (0.1 M imidazole·HCl buffer + 0.2 M NaCl, pH 6.98, poly(U) concentration 6 mM, RNase A concentration 0.1 µM). Data collected (A) 1 min, (B) 31 min, (C) 161 min and (D) 411 min after addition of RNase A.
Figure 2: $^{31}P$ NMR spectra of the hydrolysis of poly(U) by RNase A, in the presence of osmolytes (0.1 M imidazole·HCl buffer + 0.2 M NaCl, pH 6.98, poly(U) concentration 6 mM, RNase A concentration 0.1 µM). All spectra shown were collected 21 min after addition of RNase A. (A) no osmolytes present, (B) 1 M urea, (C) 1 M urea + 500 mM TMAO, (D) 1 M urea + 1 M TMAO.
Scheme 1: Proposed mechanism for RNase A-catalysed transphorylation/hydrolysis of RNA
(A = His119, B = His12) (8).
Table 1: Mean ‘lifetimes’ of poly(U) reaction with RNase A, in the presence and absence of osmolytes. Experimental conditions: 0.1 M imidazole-HCl buffer + 0.2 M NaCl, pH 6.98, RNase concentration 0.1 µM. 6 mM poly(U) implies a concentration of 6 mM in P-O5' bonds.

| Entry | Experimental Conditions                          | ‘Lifetime’ (min) |
|-------|------------------------------------------------|------------------|
| 1     | 6 mM poly(U)                                    | 42               |
| 2     | 6 mM poly(U) + 100 mM urea                      | 49               |
| 3     | 6 mM poly(U) + 700 mM urea                      | 61               |
| 4     | 6 mM poly(U) + 1 M urea                         | 70               |
| 5     | 6 mM poly(U) + 2 M urea                         | 113              |
| 6     | 6 mM poly(U) + 700 mM urea + 233 mM TMAO       | 52               |
| 7     | 6 mM poly(U) + 700 mM urea + 350 mM TMAO       | 52               |
| 8     | 6 mM poly(U) + 700 mM urea + 700 mM TMAO       | 41               |
| 9     | 6 mM poly(U) + 1 M urea + 333 mM TMAO          | 60               |
| 10    | 6 mM poly(U) + 1 M urea + 500 mM TMAO          | 56               |
| 11    | 6 mM poly(U) + 1 M urea + 1 M TMAO             | 42               |
| 12    | 6 mM poly(U) + 1 M TMAO                        | 31               |
Table 2: Cross-peak Deletions for (15 – 45 mM) TMAO-affected NOESY Spectra of RNase A and Related Hydrogen-Hydrogen Internuclear Distances for the Unmodified Enzyme. Assignments using BBReader. (14) Confidence levels (i.e. BBReader calculated ‘score’) > 85%. Nomenclature as in BioMagResBank. (25)

| Deletion                        | distance /Å |
|--------------------------------|-------------|
| His48 HA-Glu49 H               | 2.72        |
| Ile106 HG2-His105 HB3/HB2      | 5.29        |
| Ser80 HA-Lys104 H              | 4.03        |
| Phe8 HA-Phe120 H               | 4.16        |
Figure 1

[Diagram showing NMR spectra of Poly(U), U>p, and Up]
Figure 2
Scheme 1
$^{31}$P and $^1$H NMR studies of the effect of the counteracting osmolyte trimethylamine-N-oxide on interactions of urea with ribonuclease

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