CD studies on ribonuclease A - oligonucleotides interactions

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ABSTRACT

The interaction of ApU, Aps\textsuperscript{4}U, Aps\textsuperscript{4}Up, ApAps\textsuperscript{4}Up and Gps\textsuperscript{4}U with RNase A was studied by CD difference spectroscopy. The use of 4-thiouridine (s\textsuperscript{4}U) containing oligonucleotides enables to distinguish between the interaction of the different components of the ligand with the enzyme. The mode of binding of the oligonucleotides to the enzyme is described. From this mode of binding it is explained why Aps\textsuperscript{4}U, for example, inhibits RNase A, while s\textsuperscript{4}UpA serves as a substrate.

INTRODUCTION

The interactions between various mononucleotides with RNase A have been studied in many ways, including gel filtration, kinetic studies, NMR and others (1). Circular dichroism (CD) difference spectrum in the far UV region has also been used for this purpose (2). As the absorption bands of RNase A and the common bases overlap, it is difficult to distinguish between the contribution of the enzyme and that of the nucleotide to the CD spectrum of their complex.

4-Thiouridine (s\textsuperscript{4}U) is an analog of uridine. The binding constant of s\textsuperscript{4}U 2'(3')p to RNase A is equal to that of U 2'(3')p (3), and oligonucleotides containing s\textsuperscript{4}U are split by RNase A. The use of s\textsuperscript{4}U 2'(3')p as a model of U 2'(3')p is advantageous because s\textsuperscript{4}U has an absorbance and circular dichroism which is red shifted 50 nm from the absorbance and circular dichroism of the common bases. It is possible therefore to distinguish between the contribution of this nucleotide to the spectrum and that of the enzyme. Moreover, the interaction between the transitions of the s\textsuperscript{4}U and the common bases in the oligonucleotide chain are minimal (4) and one can assume that the near UV region of the spectrum (\(\lambda > 300\) nm) is contributed by s\textsuperscript{4}U and the far UV region (\(\lambda < 300\) nm) by the enzyme and the common bases.

Samejima et al. (5) used s\textsuperscript{4}U 2'(3')p in CD studies on the interaction between RNase A and mononucleotides. They found that upon binding of s\textsuperscript{4}U 2'(3')p to RNase A the positive Cotton effect at 330 nm decreases. This change was explained as inversion of the Cotton effect of s\textsuperscript{4}U because the s\textsuperscript{4}U is restricted
by the enzyme to a new conformation.

Recently it was found in our laboratory that some uridine containing oligonucleotides inhibit RNase A (6). Among these inhibitors are ApU, ApUp and ApApUp, all of which can be classified as sequence isomers of UpA, a good substrate of RNase A. The lack of catalytic activity of RNase A on these isomers reflects a mode of binding to the enzyme different from that of a substrate.

In the present paper the changes in the CD spectra of the thiouridine containing oligonucleotides upon binding to RNase A are represented. The results are interpreted in terms of their mode of binding to the enzyme.

**MATERIALS AND METHODS**

s^4_U containing oligonucleotides were prepared and purified as described elsewhere (7). RNase A (Sigma x 5 crystallized, 60 Kunitz Units per milligram) was dissolved on the day of the experiment. Concentrations of RNase A and oligonucleotides were determined spectrophotometrically. ε_{278} (RNase A) = 9700 (8); ε_{331} (Ap_s^4_U) = 19,600; ε_{331} (A 2'-5' s^4_U) = 19,100; ε_{331} (s^4_U 2'-5' A) = 18,200 (7); ε_{258} (ApU) = 23,100 (9). It is assumed that Ap_s^4_U and ApAp_s^4_Up have the same ε_{331} as Ap_s^4_U. CD spectra in the region of 240-400 nm were measured on a Cary 60 spectropolarimeter equipped with a CD attachment 6001, using a pen period of 3 sec and a full range sensitivity of 40 millidegrees. Samples were heated at 60°C for 5 min, and measurements were taken at 26°C. In all measurements the light path was 1 mm. Samples were dissolved in 0.2 M imidazole-HCl buffer, pH 7.0. Difference spectra were calculated for every 2.5 nm. The dissociation constant of an oligonucleotide-RNase complex was determined according to Dahlquist (10), except that the nucleotide (N) was titrated with excess of enzyme (E). CD differences at 330 nm were measured for the different amounts of enzyme added. From the saturation curves obtained, the complex concentrations[EN] were calculated. Kd was determined from the plot of log [EN] versus log [E] according to the following linear equation:

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-\log \frac{[EN]}{[N]} = \log [E] - \log K_d.
\]

**RESULTS AND DISCUSSION**

ApU binds strongly to RNase A with a Ki of 3 mM (6). The CD spectra of ApU, RNase A, and their complex is shown in Figure 1. The CD spectrum of ApU has two main Cotton effects, a positive one at 270 nm and a negative one at 250 nm (Fig. 1A). In the same region RNase A shows a broad and strong negative Cotton effect with a trough at 278 nm and a small peak at 240 nm. The spectrum of the
Figure 1. Circular dichroism of the complex of RNase A (0.45 mM) with ApU (0.50 mM). A. Spectrum of ApU (-- --) and RNase A (-----). B. Spectrum of the complex (-- --) and the calculated sum of the components (-----). C. Difference spectrum. For experimental details see Methods.

ApU-RNase A complex is shown in Fig. 1B. For comparison, the calculated sum of the components' spectra is also plotted. It is clear that there is a definite difference spectrum (Fig. 1c) caused by the interaction of RNase A with ApU. The Cotton effect around 270 nm decreases significantly, whereas there is a negligible change in the negative Cotton effect at 250 nm, and a small negative change at 240 nm.

As ApU has a Cotton effect at 270 nm it may be assumed that the change at this wavelength upon binding to the enzyme is caused by a change in the conformation of the oligonucleotide only. Previous reports concerning the interaction between mononucleotides and RNase A have reached the same conclusion (11).

Nevertheless the small negative change at 240 nm might be due to changes in the environment of tyrosine residues of the enzyme (12).

From the above CD difference spectrum (Fig. 1C) it is difficult to decide which base (A or U) interacts with the enzyme and causes the change of the spectrum. Therefore the use of 4-thiouridine instead of uridine moiety in the oligonucleotide was introduced.

Aps4U (Fig. 2A) has a positive Cotton effect at 330 nm and a negative ellipticity centered at 248 nm. The binding of Aps4U to RNase A causes a CD
Figure 2. Circular dichroism of the complex of RNase A (0.15 mM) with Aps U (0.50 mM). A. Spectrum of Aps U (- - -) and RNase A (---). B. Spectrum of the complex (---) and calculated sum of the components (---). C. Difference spectrum. For experimental details see Methods.

difference mainly around 330 nm where a large decrease in the Cotton effect is clearly observed (Fig. 2 B and C). A very small positive change appears at 250 nm and is followed by a negative change at shorter wavelengths. The decrease in the ellipticity of sU is probably the result of partial inversion of the Cotton effect. It is believed that this inversion is caused by a change from the anti- to the syn-conformation (1). The small difference found at about 250 nm may be explained by a small change in the asymmetry of the adenosine.

The CD difference at 330 nm was used to determine the dissociation constant of the Aps U - RNase A complex. Aps U was titrated by increasing amounts of RNase A (Fig. 3A) and from the respective CD amplitudes the dissociation constant was calculated according to Dahlquist (10) (Fig. 3B). The calculated dissociation constant is 0.44 mM and is of the same order of magnitude as that of ApU (6).

Oligonucleotides as ApUp and ApApUp have a uridine 3' monophosphate end group and therefore resemble a RNase A substrate more than ApU which lacks this end group. When the inhibition constant of ApUp was compared to that of ApU, it was found that the Ki of ApUp was lower than that of ApU (6). Further addition of an Ap at the 5' OH end of ApUp (ApApUp) slightly raised the Ki. The dissociation constants of the thio analogs (determined as described for Aps U)
Figure 3. A. Titration of Aps U (0.5 mM) with RNase A. B. Dahlquist plot for the determination of the dissociation constant of the complex. For details see Methods.

are 0.027 and 0.035 mM for Aps Up and ApAps Up respectively. These constants show the same trend as the Ki mentioned above, i.e., Aps Up < ApAps Up < Aps U. These differences cannot be interpreted as a result of additional charges, because the experiments were carried out at high salt concentrations. In order to elucidate whether these differences reflect a different kind of interaction, the CD spectra of Aps Up and ApAps Up were recorded in the presence and in the absence of RNase A. The CD spectrum of Aps Up (Fig. 4A) is very similar to that of Aps U (Fig. 2A). However, the difference spectrum of the Aps Up complex with RNase A (Fig. 4C) seems to differ from that of ApAps U complex (Fig. 2C). In the Aps Up complex the amplitude of the difference at 330 nm is much higher, and at 250 nm there is a significant positive change (Fig. 4C). The molar ellipticity differences at 330 nm of the above complexes were determined from the ellipticity differences at RNase A saturation. Since the values obtained ([θ] = 33000, 41000, 44000 for Aps U, Aps Up, and ApAps Up respectively) are similar, the higher amplitude of the CD difference in the Aps Up - RNase A complex (Fig. 4C), as compared to the Aps U - RNase A complex, reflects a higher percentage of complex in the mixture.

The CD spectrum of ApAps Up and the difference spectrum of the ApAps Up - RNase A complex in the near UV region (Fig. 5) is similar to the CD spectra and CD difference spectra of Aps U and Aps Up (Fig. 2 and Fig. 4). The far UV region is different, of course, because the two interacting adenosine residues contribute a split Cotton effect to the spectrum. The amplitude of this Cotton effect is reduced upon interaction with the enzyme, probably as a result of a change in the relative positions of the adenosine residues.

All the above mentioned s U containing oligonucleotides have positive
Figure 4. Circular dichroism of the complex of RNase A (0.46 mM) with Ap^4Up (0.50 mM). A. Spectrum of Ap^4Up (---) and RNase A (-----). B. Spectrum of the complex (---) and the calculated sum of the components (-----). C. Difference spectrum. For experimental details see Methods.

Figure 5. Circular dichroism of the complex RNase A (0.46 mM) with ApAps Up (0.54 mM). A. Spectrum of ApAps Up (---) and RNase A (-----). B. Spectrum of the complex (---) and the calculated sum of the components (-----). C. Difference spectrum. For experimental details see Methods.
Cotton effects in the region of 330 nm. Upon binding of these compounds to RNase A this Cotton effect is inverted, because the $^4\text{U}$ is restricted by the enzyme to a new conformation which has a negative Cotton effect.

It is suggested that $^4\text{U}$ residues in oligonucleotides (both substrates and inhibitors) bind to the pyrimidine site. Similar results were obtained with $^4\text{U} 2'(3')p$ - RNase A complex (5).

Pyrimidine nucleotides with the phosphate linked to their 2' OH (C 2'p and U 2'p) bind strongly to the pyrimidine site of RNase A and inhibit its activity (13). On the other hand, dinucleoside-monophosphates with the unnatural 2'-5' phosphodiester linkage like U 2'-5'A and A 2'-5'U are neither substrates nor inhibitors to RNase A (6). In addition, RNase A has no effect on the CD spectrum of their thio analogs ($^4\text{U} 2'-5'A$ and A 2'-5' $^4\text{U}$). It was shown that the conformation of the 2'-5' isomers is different from that of the respective 3'-5' isomers and that the 2'-5' isomers are more compact and rigid than that of the 3'-5' isomers (14). It can therefore be concluded that the above mentioned 2'-5' isomers do not bind to RNase A probably because the U or $^4\text{U}$ residues do not reach the pyrimidine site of the enzyme, due to the rigidity of the molecule.

The effect of the substitution of the adenosine in ApU by guanosine was also studied. GpU inhibits RNase A with a slightly higher Ki than ApU (6.1 mM) (6). The CD difference spectrum of its analog, Gps$^4\text{U}$ is shown in Fig. 6. The spectrum of Gps$^4\text{U}$ (Fig. 6A) has a positive Cotton effect contributed by the $^4\text{U}$, similar to that of Aps$^4\text{U}$ (Fig. 2A). However, the rest of the spectrum, a positive ellipticity at 263 nm and a negative ellipticity at 287 nm, differs from the spectrum of Aps$^4\text{U}$ and is mainly attributed to the guanosine moiety. The difference spectrum (Fig. 6C) shows that the Cotton effect of $^4\text{U}$ in Gps$^4\text{U}$ decreases significantly in the complex with RNase A, but there is only a negligible difference in the region contributed by guanosine. From these results it is concluded that $^4\text{U}$ in Gps$^4\text{U}$ is bound to RNase A, and its conformation is changed upon interacting with the enzyme. However, the lack of a significant change in the optical activity of A and G in these complexes does not imply a lack of interaction between these bases and the enzyme (12).

In order to clarify the contribution of the purines to the binding of the above mentioned compounds to RNase A, the following experiment was carried out. Two competing nucleotides were added to an Aps$^4\text{U}$ - RNase A complex: U 2'(3')p which binds to the pyrimidine site of the enzyme, and A 5'p which binds to the purine site of the enzyme. The phosphates of these two mononucleotides bind to the same site ($P_1$) (13). The relative amount of
bound Aps\textsuperscript{4}U in the presence of increasing concentrations of the added nucleotides is shown in Fig. 7. The addition of 1.1 mM of U 2'(3')p reduced to half the amount of bound Aps\textsuperscript{4}U, whereas the effect due to 15 mM of A 5'p is small and within the experimental error. It is justified to compare these concentrations as they are proportional to the dissociation constants of the respective complexes (15). The fact that U 2'(3')p displaces Aps\textsuperscript{4}U from the complex proves that the interaction of the pyrimidine in the oligonucleotide with the pyrimidine site is essential for its binding to RNase A. On the other hand, the fact that A 5'p cannot displace Aps\textsuperscript{4}U from the complex suggests that their binding sites do not overlap, i.e., the purine of Pups\textsuperscript{4}U is not bound to the purine site of RNase A and its phosphate does not bind to the P\textsubscript{i} site. This is in contrast to the case of UpA (as substrate) in which the purine interacts with the purine site of the enzyme (13).

Sawada and Irie (16) found that pUp binds to RNase A stronger than U3'p and therefore an additional site for the 5' phosphate was implied. We suggest that the phosphate of Aps\textsuperscript{4}U is located at this additional site. From the similarity between the complexes of RNase A with Aps\textsuperscript{4}U and Aps\textsuperscript{4}Up (Figs. 2, 4), it can be concluded that their conformation in the binding site is the
Figure 7. The effect of competing nucleotides on the Aps^4U - RNase A complex. A mixture of RNase A (0.77 mM) and Aps^4U (0.46 mM) is titrated with U 2'(3')p (----) and A 5'p (---). The CD amplitude at 330 nm was measured and the relative amount of complex calculated.

The lower dissociation constant of Aps^4Up is attributed to the binding of the additional 3' phosphate to the P1 site.

Previous studies concerning the interaction of RNase A with nucleotides dealt mainly with mononucleotides. The analysis of the interaction with oligonucleotides is complex due to the similar optical properties of the various bases. The use of 4-thiouridine containing oligonucleotides enabled us to distinguish between the interaction of the different components of the oligonucleotide.

From the results presented in this communication it is clear that the Aps^4U binds to RNase A through its s^4U5'p residue which binds to the pyrimidine site of the enzyme. Upon binding of the Aps^4U to RNase A the s^4U residue adopts the conformation of s^4U 2'(3')p or s^4UpA bound to the enzyme. However, in the case of Aps^4U the adenosine residue is not bound to the purine site of the enzyme and the phosphodiester bond is at a phosphate site distinct from the catalytic site (P1). This mode of binding explains why Aps^4U is an inhibitor to RNase A while s^4UpA is a substrate. This applies also to the other inhibitors described in this paper.

REFERENCES