Protonated polynucleotide structures. 18. Interaction of oligocytidylates with poly(G). B.L. Haas and W. Guschlbauer

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ABSTRACT The faculty for and degree of oligo(C)-poly(G) interaction is described as an essentially chain length-sensitive phenomenon. At neutral pH under suitable experimental conditions, oligocytidylates of chain length greater than four associate with poly(G) to form double-stranded structures, as does poly(C). The extent of complex formation increases with degree of polymerization. The complex at acid pH is shown to be triple-stranded, of stoichiometry 2C/1G. The observation of a 2G/1C artifact is discussed.

INTRODUCTION

Pochon and Michelson\(^2\) and Thiele and Guschlbauer\(^3\) demonstrated that poly(C) interacts with poly(G) to form multiple-stranded structures. The neutral complex is of stoichiometry 1C/1G; after titration to acid pH, a triple-stranded 2C/1G complex is evident. These complexes have characteristic CD spectra. The interaction spectra differ from the sum of the component spectra both in height and in peak position.

A number of conditions were determined by Thiele and Guschlbauer\(^3\) to be necessary for complex formation. 1) No interaction at neutral pH was observed unless the solution was heated briefly. 2) The influence of ionic strength was found to be more critical than for other double-stranded structures\(^4\) and was observed to be inverse to that favorable for poly(C)-poly(C)\(^\ast\) association. At low [Na\(^{\ast}\)] concentration no complexing occurred at pH 7.0; \(T_m\) values determined for acid complexes indicated that stability of triplexes also increases with increasing ionic strength. 3) Incomplete complex formation for solutions at low ionic strength was evidenced. Titration of solutions lower than 0.6 M in [Na\(^{\ast}\)] revealed the presence of a transition around the pK of poly(C), in addition to that indicative of triple-stranded complex formation. The amplitude of this step increased with decreasing ionic strength, suggesting that...
more and more poly(C) was free in solution as ionic strength diminished. More poly(C) was RNase-sensitive the lower the ionic strength. In view of the influence of chain length on oligocytidylic structure and self-association properties, it might be expected that degree of polymerization would also delimit oligonucleotide-polynucleotide complex formation. The following study demonstrated that, while the same types of complexes formed by poly(C) with poly(G) are formed between oligo(C) and poly(G), and that, while all of the factors influencing interaction between the polymers likewise affect that between poly(G) and the oligomers, this phenomenon is noticeably chain length-dependent.

MATERIAL AND METHODS

Oligocytidylicates were prepared by the alkaline hydrolysis of poly(C) (Miles Laboratories, Elkhart, Indiana) and subsequent column chromatography on DEAE cellulose.

Circular dichroism (CD) and UV spectra were recorded on a Jouan Dichrographe III and a Cary 15 spectrophotometer, respectively. A Tacussel Isis 4000 pH meter was employed for pH adjustment. At neutral pH the 1:1 mixtures were $1.0 \times 10^{-4}$ M in total nucleotide; the 2:1 acid mixtures were either $1.0 \times 10^{-4}$ M oligo(C), $0.5 \times 10^{-4}$ M poly(G) or $0.8 \times 10^{-4}$ M oligo(C), $0.4 \times 10^{-4}$ M poly(G).

OLIGO(C)-POLY(G) INTERACTION

1. The neutral complex. The pentamer and longer oligo(C) can interact with poly(G) at neutral pH, as evidenced by the similarity in shape of the CD spectra and those obtained for poly(C)-poly(G) (Fig. 1). The CD difference spectra (i.e., spectral sum of components - experimental spectrum) have the same shape as that for poly(C)-poly(G) under similar conditions (Fig. 2a); however, the amplitude of the difference spectrum increases as the size of the oligomer concerned does (Fig. 2a and b).

Evidently, molecular size is a factor in complex formation. Under no conditions can the tetramer be seen to interact with poly(G); larger oligomers do so to varying extent, depending on chain length; very long oligonucleotides ($N > 25$) act essentially like the polymer (Fig. 2a).

In order to determine to what extent a given oligomer is capable of interacting with poly(G), the spectra for solutions constant in
Figure 1: Circular dichroism (CD) spectra at neutral pH for the interaction between poly(G) and poly(C) (a), (Cp)_{14} (b) and (Cp)_{9} (c). ——— sum of components, —— experimental spectrum of the complexes.

Figure 2: CD difference spectra at neutral pH for the interaction between poly(G) and poly(C) or with oligocytidylylates (chain length indicated). Top: at 1 M [Na^+] , bottom: at 0.5 M [Na^+]
Figure 3: Titration of poly(G) with (Cp)$_{14}$ at pH 7.0 in 0.5 M \( [\text{Na}^+] \), heated after each addition of oligonucleotide. The numbers on the circular dichroism (CD) spectra indicate the ratio of the amounts of (Cp)$_{14}$ relative to that of poly(G). Insert: Change of CD at 280 nm as a function of the fraction of (Cp)$_{14}$. •: spectra measured immediately after heating, o: spectra measured after one night at 4°.

poly(G) concentration, but varying in (Cp)$_{14}$ concentration, were examined (Fig. 3). The CD spectra show three isosbestic points (at 293, 260 and 237 nm) up to an approximately equimolar oligo(C)-poly(G) mixture. Above this (Cp)$_{14}$ concentration the shape of the spectra change and the band at 272 nm, characteristic of free oligo(C), increases proportionately with oligomer concentration. A plot of the height of the CD signal at 280 nm versus the percentage of oligomer relative to polymer yields a non-linear plot (Fig. 3, Insert). The stoichiometry of complexes immediately after mixing and heating is only 0.8C/1G.
However, the same treatment of the data for these solutions after 24 hours of refrigeration indicates that this percentage augments to 90%. This same spectral amelioration is the case for other oligomers; furthermore, the conditions under which the solutions are treated can affect the efficiency of complex formation.

For example, for one series of studies (1 M [Na⁺]), complexes were observed only upon heating, as had been found to be the case for poly(C)-poly(G) interaction. But for 0.5 M [Na⁺], the spectra recorded before heating of the solution did not correspond to that of the components' sum. Complexing had already begun and heating improved the interaction (Fig. 4).

While heating seems to be necessary not only for poly(C)-poly(G), but also for oligo(C)-poly(G) interaction, oligomer-polymer complex formation is more complicated. Mixing curves (Fig. 5) determined from

![Graph showing CD difference spectra for the interaction between poly(G) and (Cp)₈ at neutral pH in 0.5 M Na⁺.](image)

*Figure 4: CD difference spectra for the interaction between poly(G) and (Cp)₈ at neutral pH in 0.5 M Na⁺. *- - - mixture without heating, --- mixture after heating.*
the spectra of (Cp)_g-poly(G) mixtures in view of verifying the one-to-one nature of the neutral complex indicated that, at pH 7.0, without heating a triple-stranded 2G/1C structure appeared to be predominant in solution (Fig. 5a); the heated solution contained both this and a 1G/1G complex (Fig. 5b); the same solution acidified and re-neutralized contained only the 1G/1G double-stranded complex. These facts are consistent with the finding of Lipsett6 that oligo(G)-poly(C) interaction consisted of the same 2G/1C triple-stranded structure at neutral pH. For the two polymers the only complex observed was the double-stranded poly(G)-poly(C) complex.2,3

It is necessary, then, to take into consideration these complications when comparing relative capacities of a series of oligomers for association with poly(G). Figure 5 demonstrates that, indeed, for a given oligomer complex formation is more or less complete, depending on the experimental conditions. However, for the series of oligocytidylates under identical conditions, it is evident that the chain length effect is a consistent factor in association capacity.

The (Cp)_N-poly(G) interaction at neutral pH is, like that of poly(C) with poly(G), influenced by ionic strength. The difference spectra for mixtures of (Cp)_g with poly(G) at different [Na^+ ] concentrations (Fig. 7a) indicate that complex formation is improved with

![Figure 5: Continuous variation experiments (mixing curves) for poly(G)-(Cp)_g in 0.5 M [Na^+ ], a) mixtures without heating at pH 6.5 , b) mixtures after heating at pH 6.5 , c) mixtures heated, titrated to pH 3.5 and returned to pH 6.5. • : 250 nm, ○ : 285 nm.](image)
Figure 6: Effect of chain length of oligocytidylylates on their interaction with poly(G) at neutral pH, 0.5 M [Na⁺]. CP difference signals as a function of chain length. •: without heating, ▲: mixtures heated, ○: mixtures heated, acidified and reneutralised (as in figure 5 c).

higher ionic strength. A plot of the heights of the difference spectra versus ionic strength (Fig. 7b) shows that, below 0.01 M [Na⁺], very little association occurs, even after heating of the solution. Use of ionic strengths greater than 0.5 M does not significantly affect the extent of complexing. However, as was found for poly(C)·poly(G), complexes formed at high ionic strength and then dialysed to lower ionic strength did not dissociate.

2. The acid complex. Acidification of the oligo(Cp)ₙ·poly(G) solutions results in a characteristic change in CD spectra (Fig. 8); the spectra for these interactions are qualitatively the same as that for poly(C)·poly(C')·poly(G). The mixing curves determined for (Cp)ₙ·
poly(G) interaction at acid pH clearly indicate that, while at pH 4.0 (Fig. 9a) the solutions are a complex mixture of 1C/1G, 2G/1C and 2C/1G structures, at pH 3.2 the oligo(C)-oligo(C*)-poly(G) complex predominates (Fig. 9b). Again, no interaction was observed for (Cp)₄.

The titration curves for oligo(C)-poly(G) interaction in 1 M [Na⁺] (Fig. 10) are interesting in character. Whereas poly(C)-poly(G) and (Cp)₂₅-poly(G) mixtures furnish curves with a single transition corresponding to the formation of the acid complex (step b in the figure), mixtures of poly(G) with smaller oligonucleotides undergo

![Graph showing titration curves for oligo(C)-poly(G) interaction.](image)

**Figure 7**: Effect of ionic strength on the interaction between poly(G) and (Cp)₉ at pH 7. Top: CD difference spectra in ---0.005 M [Na⁺], 0.01 M [Na⁺], -0.1 M [Na⁺] and -0.25 M [Na⁺]. Bottom: CD difference signal at 280 nm. Upper line (symbols as in top panel): after heating, lower line (Δ): without heating.
Figure 8: CD spectra at acid pH and high ionic strength for the interaction between poly(G) and poly(C) or oligocytidylates (chain length indicated).

Figure 9: Continuous variation experiments (mixing curves) for poly(G) and (Cp)_g at pH 4.0 (a) and 3.2 (b) in 0.5 M [Na^+] . CD at 250 (\bullet) and 285 nm (\Delta).
two distinct transitions. This fact is reminiscent of the curves described for poly(C)-poly(G) association at low ionic strength.\(^3\) The occurrence of a step in the titration around the pK of poly(C), which step increased in importance with decreasing ionic strength, was shown to reflect poly(C) free in solution due to incomplete complex formation at pH 7.0.

The fact that, even at high ionic strength, this step (step a in the figure) is visible for oligomers smaller than \((\text{Cp})_{25}\), and that the relative size of the step increases with decreasing chain length at constant ionic strength, confirms the importance of degree of polymerization for neutral complex formation. The pK of this transition varies with chain length and with ionic strength and is comparable to the pK's for self-association of free oligomer material.\(^7\)

**Figure 10:** Titration of mixtures of poly(G) and \((\text{Cp})_n\) (n = chain length indicated in the figure) in a 1:2 ratio. The solutions were prepared in 1 M [Na\(^+\)] and heated at neutral pH before titration.
The second step in the titration (step b) corresponds to the formation of the acid complex. The $T_m$'s for these acid complexes, even in 0.15 M [Na$^+$], are very high. Still they demonstrated that stability increases with chain length. The $T_m$ of the acid triplex for (Cp)$_7$ in 0.15 M salt was 66°C; that for (Cp)$_8$ was 75°C; the $T_m$ for the nonamer triplex was 80°C. For higher chain lengths the end of the melting curve could not be observed. At higher ionic strengths $T_m$ measurements become impossible ($T_m > 100^\circ$C).

The acid complex is, again, favored by high ionic strength, as judged from the heights of the difference spectra for (Cp)$_n$-poly(G) mixtures at different ionic strengths (Fig. 11). The pK of the transition varies with ionic strength in like fashion to the variation for poly(C)-poly(G) interaction, i.e., varying from 3.55 at 1 M [Na$^+$] to 4.1 for 0.01 M [Na$^+$] (Fig. 12, step b and insert). Note that the titration of free oligomer at higher pH (step a) is less important for high than for low ionic strength, which corroborates the conclusions on the extent of complex formation at neutral pH.

![Figure 11: Effect of ionic strength on the acid complex between poly(G) and (Cp)$_9$. CD difference spectra in --- 0.25 M [Na$^+$]; --- 0.1 M [Na$^+$] and --- 0.01 M [Na$^+$].](image)
DISCUSSION

The model for the double-stranded complex formation between poly(C) and poly(G) consists of Watson-Crick-type base pairing between guanine and cytosine residues:

![Diagram of base pairing](image)

It is quite logical to suppose that oligocytidylic species smaller than poly(C) could associate in the same fashion with poly(G) but that

![Figure 12: Effect of ionic strength on the titration of the complex poly(G)·(Cp)_g](image)

**Figure 12:** Effect of ionic strength on the titration of the complex poly(G)·(Cp)_g. Equimolar mixtures of polymer and oligomer were heated at neutral pH before titration. Insert: pK of titration step b as a function of ionic strength.
the faculty for an extent of interaction would be chain length-dependent.

Indeed, we find that, for our set of experimental conditions, 1) small oligonucleotides (N < 4) do not form complexes with poly(G); oligomers larger than this do so, and do so with greater facility as chain length increases. Large oligocytidylic acids (N > 25) interact with poly(G) to the same extent as does poly(C). This fact is in agreement with the polynucleotide-oligonucleotide studies of Michelson and Monny. These authors, while they did not enter into a quantitative treatment of complex formation, did note abrupt changes in the progression of Tm and pK values at the level of oligomers of chain length greater than 20 residues. These large oligonucleotides behaved like the polymer.

But interaction of oligo(C) of intermediate (25 < N > 4) chain length with poly(G) is complicated by the formation, at neutral pH, of a triplex of stoichiometry 2G/1C. Such structures have been reported previously for oligo(G)-poly(C)\(^5\) and poly(I)-poly(mC)\(^9\) interactions. In the present case the existence of the 2G/1C structure can be explained by the auto-association of poly(G) and the inability of small oligocytidylic acids to displace one of the G strands thus associated.

Poly(G) has a well-documented reputation for forming multiple-stranded structures.\(^{10,11}\) In the instance of poly(G)-poly(C) interaction, heating is sufficient to separate poly(G) strands and upset the equilibrium in favor of poly(G)-poly(G) formation.\(^3\) But for oligonucleotides of relatively short length heating does not suffice. While, upon reassociation, some, the amount depending on oligomer chain length, oligo(C)\(^{+}\)poly(G) is formed, a good part of the solution still consists of the 2G/1C triplex. (See Fig. 5.)

With titration, the 2C/1G complex appears. At pH 4.0 all three structures (1C/1G, 2G/1C, 2C/1G) are observed; however, at lower pH (3.2) the only structure present in solution seems to be the oligo(C)\(^{+}\)oligo(C)\(^{+}\)poly(G) complex. The fact that this 2 pyrimidine/1 purine complex is more stable than the inverse is in agreement with other findings on triple-stranded structures involving the G-C (or I-C) series, both for the polymers\(^5,12\) and for the case in which the purine element is a monomer.\(^13\) Furthermore, upon reneutralization of the oligomer-polymer solution, we no longer find the 2G/1C triplex. This again implies that the 2 pyrimidine/1 purine complex is more stable than the 2 purine/1 pyrimidine structure at acid pH and also suggests that the
latter is not a true stoichiometric complex but a mere artifact.

The formation of the acid complex is, again, sensitive to oligo-
nucleotide chain length. One would imagine that these three-stranded
oligo(C)-oligo(C')-poly(G) structures are arranged in the same manner
as poly(C)-poly(C')-poly(G), with one Watson-Crick and one Hoogsteen
pair. The same sort of binding scheme was found for poly(C)-guanosine
interaction.13

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