Controlling nucleic acid secondary structure by intercalation: effects of DNA strand length on coralyne-driven duplex disproportionation

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ABSTRACT

Small molecules that intercalate in DNA and RNA are powerful agents for controlling nucleic acid structural transitions. We recently demonstrated that coralyne, a small crescent-shaped molecule, can cause the complete and irreversible disproportionation of duplex poly(dA)·poly(dT) into triplex poly(dA)·poly(dT)·poly(dT) and a poly(dA) self-structure. Both DNA secondary structures that result from duplex disproportionation are stabilized by coralyne intercalation. In the present study, we show that the kinetics and thermodynamics of coralyne-driven duplex disproportionation strongly depend on oligonucleotide length. For example, disproportionation of duplex (dA)₁₆·(dT)₁₆ by coralyne reverts over the course of hours if the sample is maintained at 4°C. Coralyne-disproportionated (dA)₃₂·(dT)₃₂, on the other hand, only partially reverts to the duplex state over the course of days at the same temperature. Furthermore, the equilibrium state of a (dA)₁₆·(dT)₁₆ sample in the presence of coralyne at room temperature contains three different secondary structures [i.e. duplex, triplex and the (dA)₁₆ self-structure]. Even the well-studied process of triplex stabilization by coralyne binding is found to be a length-dependent phenomenon and more complicated than previously appreciated. Together these observations indicate that at least one secondary structure in our nucleic acid system [i.e. duplex, triplex or (dA)ₙ self-structure] binds coralyne in a length-dependent manner.

INTRODUCTION

For many years, small molecules that bind duplex DNA were primarily studied with the goal of understanding their activity in either the cause or treatment of cancer (1–5). Within the last decade, the proposed applications for small molecule–DNA interactions have increased substantially. For example, small molecules have now been developed that bind sequence specifically in the minor groove of duplex DNA and function as artificial gene regulation elements (6,7). In addition to duplex DNA, a number of laboratories have also worked towards the development of small molecules that specifically bind and stabilize triplex and G-quadruplex DNA (8–10). The use of such molecules in vivo to facilitate the formation of non-duplex DNA structures has principally been pursued as a possible route to antigenic therapy. The recent demonstration that a cationic porphyrin derivative can suppress gene expression by promoting the formation of a G-quadruplex in living cells clearly illustrates the potential for this approach in the development of new therapeutics (10).

Our laboratory is also interested in the use of small molecules as a general means to drive nucleic acid assembly and structural transitions. Part of our motivation in this pursuit comes from our recent proposal that small molecule intercalation may have played a central role in early life (i.e. the RNA world) by facilitating nucleic acid assembly and replication (11). In the same way, small molecule intercalation may provide a route to protein-free template-directed synthesis of nucleic acids (11). Towards this end, we have initiated studies of how small molecule binding to nucleic acids by intercalation can drive the assembly of multi-stranded DNA and RNA structures.

Coralyne (Scheme 1) is a small crescent-shaped molecule that is among a group of molecules known to preferentially intercalate DNA triplexes over duplexes and to increase the thermal stability of triplex DNA (12–16). Previously, we demonstrated that coralyne can also drive the complete and irreversible disproportionation of duplex poly(dA)·poly(dT) (17), i.e. coralyne causes the repartitioning of duplex poly(dA)·poly(dT) into coralyne-intercalated triplex poly(dA)·poly(dT)·poly(dT) and poly(dA) (Scheme 1). We also discovered that poly(dA) forms a self-structure in the presence of coralyne that is stable up to at least 47°C. Data from several experimental techniques indicate that this poly(dA) self-structure is a duplex with A·A base pairs that is intercalated up to a level of one coralyne molecule per 2 bp (17). This serendipitous discovery provides an excellent illustration of how small molecule intercalation can be used to drive nucleic acid assembly, because poly(dA) does not...
form a stable multi-stranded self-structure at neutral pH in the absence of coralyne.

In the present work, we report how DNA strand length can affect the ability of coralyne binding to control DNA secondary structure. Coralyne is shown to cause the complete disproportionation of duplex (dA)$_{16}$-(dT)$_{16}$ at 36°C, as we previously demonstrated for duplex poly(dA)-poly(dT). However, our studies of duplex (dA)$_{16}$-(dT)$_{16}$ have revealed that duplex disproportionation by coralyne is not strictly irreversible. Over the course of hours at 4°C, a disproportioned (dA)$_{16}$-(dT)$_{16}$ sample reverts back to the duplex state from the coralyne-intercalated triplex and (dA)$_{16}$ self-structure. Furthermore, at room temperature a disproportioned duplex (dA)$_{16}$-(dT)$_{16}$ sample requires days to reach equilibrium, and the equilibrium state contains a mixture of duplex, triplex and (dA)$_{16}$ self-structure. Coralyne-disproportioned samples of duplex (dA)$_{32}$-(dT)$_{32}$ and duplex poly(dA)-poly(dT) were found to require several weeks to reach structural equilibrium. We also show that even the kinetics of triplex stabilization by coralyne depend on oligonucleotide length. The results reported here illustrate the potential for small molecule binding to be used in concert with temperature to drive nucleic acid structural transitions.

**RESULTS AND DISCUSSION**

Complete triplex formation and coralyne intercalation by (dA)$_{16}$-(dT)$_{16}$ is inhibited by a kinetic trap at 4°C

The pyrimidine triplex (dA)$_{16}$-(dT)$_{16}$-(dT)$_{16}$ is not stable under the solution conditions of our study, even at 4°C. This is illustrated by the fact that a sample with a 1:2 molar ratio of (dA)$_{16}$ and (dT)$_{16}$ exhibits a CD spectrum that is virtually identical to that of the (dA)$_{16}$-(dT)$_{16}$ duplex in a sample containing a 1:1 molar ratio of (dA)$_{16}$ and (dT)$_{16}$ (Fig. 1A). Additionally, the CD melting profiles of these two samples exhibit the same single melting transition (i.e. $T_m$) at 37°C (Fig. 2A). Thus, the only secondary structure present in the sample with a 1:2 molar ratio of (dA)$_{16}$ and (dT)$_{16}$ is the duplex (dA)$_{16}$-(dT)$_{16}$, which coexists with an equal molar equivalent of single-stranded (dT)$_{16}$. We will refer to a sample in this state as (dA)$_{16}$-(dT)$_{16}$ + (dT)$_{16}$, as opposed to (dA)$_{16}$-(dT)$_{16}$-(dT)$_{16}$, to emphasize that in this sample the triplex secondary structure is either absent or in equilibrium with an appreciable amount of duplex and single-stranded (dT)$_{16}$. The absence of triplex secondary structure in the (dA)$_{16}$-(dT)$_{16}$ + (dT)$_{16}$ sample is somewhat particular to the conditions of our study, as the addition of divalent cations can facilitate triplex formation by oligonucleotides of this length and sequence at 4°C (18). However, in order to be consistent with our past studies (17), and given our specific aim to explore nucleic acid stabilization by intercalation, we have used solution conditions in which the triplex...
(dA)16´(dT)16´(dT)16 is unstable in the absence of coralyne intercalation. Coralyne has previously been shown to intercalate triplex DNA and thereby enhance the thermal stability of the triplex secondary structure (19). The addition of coralyne to a (dA)16´(dT)16 + (dT)16 sample at 4°C results in an appreciable change in the sample CD spectrum (Fig. 1B). However, complete triplex formation and coralyne intercalation does not appear to occur upon coralyne addition, as the coralyne-intercalated triplex produces small positive CD bands at ~340 and ~440 nm (17). Local maxima are observed at these wavelengths, but appear to ride atop a broad negative band that has a minimum at 350 nm (Fig. 1B, inset). The persistence of duplex secondary structure in this sample is supported by the observation of similar (but more intense) negative CD bands at ~350 nm for a sample of duplex (dA)16´(dT)16 with coralyne at the same temperature (vide infra). This indicates that coralyne does not cause the complete and spontaneous formation of a triplex in this sample when it is maintained at 4°C, which is surprising given the fact that coralyne is well known to greatly increase the melting temperature of A-T-T triplexes (12).

Heating the (dA)16´(dT)16 + (dT)16 sample with added coralyne to 30°C causes the CD spectrum of this sample to adopt spectral features that are typical of a DNA triplex with intercalated coralyne, including the appearance of small positive CD bands at ~340 and ~440 nm (Fig. 1C, inset). The CD spectrum of the (dA)16´(dT)16 sample at 30°C without coralyne, on the other hand, remains similar to that of duplex (dA)16´(dT)16 (Fig. 1A and C). Thus, heating of the (dA)16´(dT)16 sample with coralyne to 30°C actually promotes the formation of triplex (dA)16´(dT)16´(dT)16. The CD heating profile for triplex (dA)16´(dT)16´(dT)16 with added coralyne reveals that the intercalated triplex melts at 46°C into single strands in a single transition ($T_m$) (Fig. 2B). The CD spectrum of triplex (dA)16´(dT)16´(dT)16 with coralyne at 4°C...
Several experimental observations indicate that upon heating, duplex (dA)\textsubscript{16}(dT)\textsubscript{16} in the presence of coralyne undergoes disproportionation into 0.50 molar equivalents of triplex (dA)\textsubscript{16}(dT)\textsubscript{16}(dT)\textsubscript{16} and 0.50 molar equivalents of (dG)\textsubscript{16}. These observations include: heating of the (dA)\textsubscript{16}(dT)\textsubscript{16} sample with added coralyne to 36°C dramatically reduces the magnitude of the duplex-specific coralyne CD bands at 300 and 350 nm (Fig. 4B); the shapes of the DNA bands between 200 and 260 nm change with heating to more closely resemble those of a DNA duplex (Fig. 4B); the CD spectrum of the (dA)\textsubscript{16}(dT)\textsubscript{16} sample with coralyne at 36°C shows small positive bands at ~340 and ~440 nm (Fig. 4B, inset), similar to CD bands observed for coralyne intercalated in the (dA)\textsubscript{16}(dT)\textsubscript{16}(dT)\textsubscript{16} sample with added coralyne (Fig. 1D, inset). Additionally, the melting profile of the (dA)\textsubscript{16}(dT)\textsubscript{16} sample with coralyne added exhibits a transition at 46°C (Fig. 2C), which is the same temperature at which the coralyne-intercalated duplex (dA)\textsubscript{16}(dT)\textsubscript{16}(dT)\textsubscript{16} melts (Fig. 2B). The magnitude of this transition in the CD melting profile of the duplex sample with added coralyne is half the transition in the triplex sample with added coralyne (Fig. 2B and C), which is also consistent with...
Thus, the (dA)16·(dT)16 sample with coralyne can be assigned to the state when the sample is returned to 4 °C. The process of duplex disproportionation by coralyne in the (dA)16·(dT)16 sample during the first sample heating is indicated by a broad transition that is centered at ~23 °C (Fig. 2C). During the second heating of the same sample, this broad transition is absent (Fig. 2C). This indicates that the intercalated triplex and (dA)16 of a coralyne-disproportioned (dA)16·(dT)16 sample do not immediately revert back to the duplex state when the sample is returned to 4 °C. This lack of reversion from the disproportionated state is also supported by the fact that the CD spectrum of the coralyne-disproportioned (dA)16·(dT)16 at 4 °C after heat cycling (from 4 to 75 °C and back to 4 °C) is radically different from the CD spectrum of the sample prior to heating (Fig. 4A and C). Furthermore, there is an excellent match between the CD spectrum of coralyne-disproportioned (dA)16·(dT)16 sample and a composite CD spectrum generated by the summation of a CD spectrum of coralyne-intercalated triplex (dA)16·(dT)16·(dT)16 and the CD spectrum of (dA)16 in the presence of coralyne (Fig. 4C).

Our previous investigations revealed that poly(dA) adopts a self-structure with A-A base pairs that is completely dependent on coralyne intercalation for stability (17). Here we show that much shorter homo(dA) strands also form the (dA)n self-structure in the presence of coralyne. In Figure 4D, the CD spectra are presented for (dA)16 at 4 °C in the presence and absence of coralyne. The addition of coralyne to the (dA)16 sample leads to the appearance of a significant CD band at ~340 nm that indicates coralyne binding. The CD spectrum between 220 and 270 nm of (dA)16 in the presence of coralyne also differs significantly from (dA)16 in the absence of coralyne, indicating a significant change in the secondary structure of (dA)16 upon coralyne binding. The CD melting profile for (dA)16 with coralyne shows a relatively broad melting transition centered at ~25 °C (Fig. 2D).

The process of duplex disproportionation by coralyne in the (dA)16·(dT)16 sample during the first sample heating is indicated by a broad transition that is centered at ~23 °C (Fig. 2C). During the second heating of the same sample, this broad transition is absent (Fig. 2C). This indicates that the intercalated triplex and (dA)16 of a coralyne-disproportioned duplex sample do not immediately revert back to the duplex state when the sample is returned to 4 °C. This lack of reversion from the disproportionated state is also supported by the fact that the CD spectrum of the coralyne-disproportioned (dA)16·(dT)16 at 4 °C after heat cycling (from 4 to 75 and
Three distinct DNA secondary structures coexist in equilibrium at 22°C

Our observation that the coralyne-disproportioned state of (dA)16(dT)16 is thermodynamically favored at 30°C, whereas the duplex state is favored at 4°C, immediately suggested that for some range of temperature duplex (dA)16(dT)16 and coralyne-disproportioned (dA)16(dT)16 will coexist in equilibrium. To directly investigate this possibility, we studied the equilibrium state of a (dA)16(dT)16 sample at 22°C (room temperature). For this investigation, 0.25 molar equivalents of coralyne per base pair were added to a duplex (dA)16(dT)16 sample at 22°C and the CD spectrum was acquired immediately (Fig. 5B). The sample was then heated to 75°C and cooled back to 4°C to ensure complete disproportionation (and consistency with other experiments). The disproportionated sample was then moved to room temperature and CD spectra were collected at 22°C on a regular basis. The CD spectrum of the sample changed with time, as illustrated by a graph of the CD signal at 293 nm, until achieving equilibrium after ~5 days (Fig. 5B, inset). The CD spectrum of the sample at equilibrium is intermediate between the CD spectrum of the sample at 22°C before heating to disproportionation and the CD spectrum acquired at 22°C immediately after disproportionation. Based upon a least squares best fit of the equilibrium spectrum as a weighted sum of the spectra before and after disproportionation, it appears that the equilibrium state of the (dA)16(dT)16 sample with coralyne at 22°C is 40% duplex and 60% disproportioned duplex [i.e. triplex and (dA)16 self-structure] (Scheme 2).

Previous studies have shown that increasing the temperature of a (dA)16(dT)16 duplex sample with divalent cations or high monovalent cation concentration can cause duplex disproportionation in the absence of intercalation (21-23). However, without intercalation, duplex disproportionation is typically not complete before the sample reaches a temperature at which all secondary structures melt into single strands, and disproportionation can be readily reversed by a decrease in sample temperature (21-23). Our results indicate that coralyne intercalation is working synergistically with temperature to drive duplex disproportionation, for both intercalation and increased temperature (i.e. above ~30°C) appear to be necessary for our duplex DNA samples to achieve complete disproportionation.

The equilibrium point and kinetics of reversion from the coralyne-disproportioned state at 4°C depend upon DNA strand length

We previously reported that coralyne causes the irreversible disproportionation of duplex poly(dA)-poly(dT) based upon our observation that the spectrum of a coralyne-disproportioned poly(dA)-poly(dT) sample was stable at 4°C for >1 day...
We also observed that duplex poly(dA)-poly(dT) does not undergo disproportionation for a matter of months if coralyne is added at 4°C and the sample is continually maintained at 4°C. Combined, these observations lead us to conclude that duplex poly(dA)-poly(dT) does not spontaneously disproportionate when coralyne is added at 4°C because the duplex structure (with associated coralyne) acts as a kinetic barrier to disproportionation and that heating above 30°C is necessary to overcome this barrier. However, the results presented above with (dA)_{16}(dT)_{16} suggest a different assignment of which DNA secondary structures in the presence of coralyne at 4°C are thermodynamically favored and which are kinetic traps.

To examine the effect of DNA length on the propensity for a disproportioned sample to revert to the duplex state at 4°C, we repeated the same experiment described above for monitoring (dA)_{16}(dT)_{16} reversion, except with the 32mer duplex (dA)_{32}(dT)_{32}. The CD spectra of coralyne-disproportioned (dA)_{32}(dT)_{32} also changes over time at 4°C (Fig. 6A). However, there are two significant differences between 32mer and 16mer duplex reversion. First, coralyne-disproportioned (dA)_{32}(dT)_{32} requires several days to reach equilibrium at 4°C, rather than several hours, with an exponential decay time constant of ~7 days (Fig. 6A, inset). Secondly, the equilibrium state of the 32mer sample at 4°C is a partially disproportioned state, much like that reached by the 16mer sample at 22°C. Thus, doubling oligonucleotide length from 16 to 32 actually shifts the secondary structure equilibrium at 4°C towards the disproportionated state.

The reversion of a coralyne-disproportioned poly(dA)-poly(dT) sample at 4°C was investigated as well. In an experiment similar to those described above, 0.25 molar equivalents of coralyne were added to a poly(dA)-poly(dT) sample at 4°C. This sample was then heated to 95°C, which is above the melting temperature of the coralyne-intercalated poly(dA)-poly(dT) poly(dT) triplex (i.e. 87°C), and then cooled back to 4°C. The disproportionated sample was maintained and monitored by CD at 4°C for several months (Fig. 6B). CD spectra indicate that the secondary structures within a coralyne-disproportioned poly(dA)-poly(dT) sample at 4°C do change over time, however, the equilibrium state of this sample is more difficult to interpret than that of the 16mer or the 32mer samples. The approach to equilibrium by the polynucleotide sample is best fitted by a double exponential, with a fast time constant of 1.4 days and a slow time constant of 28 days (Fig. 6B, inset). The equilibrium state that is finally reached by this sample does not appear to be intermediate between that of the same sample before and after heating (Fig. 6B). Thus, changing DNA length from 32 to ~300 nt (i.e. poly(dA), poly(dT)) can change the secondary structures that are favored by a (dA)_{n}(dT)_{n} sample in the presence of coralyne at 4°C.

In aqueous solution, coralyne may form a hydration product across the C=N bond that would destroy planarity and potentially inhibit intercalation of DNA. Considering the long incubation times required for coralyne-disproportioned samples to reach equilibrium at 4°C (Figs 5 and 6), it was important to determine if coralyne degradation is at all responsible for reversion from the disproportionated state. To investigate this possibility, after 9 months of storage at 4°C from the time of coralyne addition, CD spectra were acquired for the same samples from which reversion time constants were measured. All three samples (i.e. 16mer, 32mer and polymer duplex samples) exhibited an appreciable reduction in coralyne absorption bands (25–40%) with respect to the original sample spectra, which could be indicative of coralyne degradation. Nevertheless, upon heat cycling to 75°C and back to 4°C, all three samples completely returned to the coralyne-disproportionated state. These observations confirm that the reversion time constants reported in this study are principally due to the kinetics of DNA secondary structure rearrangements, rather than coralyne degradation.
Our observation that the secondary structure equilibrium of a (dA)n-(dT)n sample with coralyne at 4°C is tilted towards the duplex state for shorter DNA strand lengths suggests that coralyne binds the 16mer duplex with a greater per base pair free energy than longer duplexes and/or coralyne binds the secondary structures in a disproportioned 16mer sample with a lesser per base pair free energy than in disproportionated samples with longer DNA strands. While either case would be somewhat surprising, Hopkins et al. [22] have shown that the average ΔH for dissociation of an A-T base pair is 3.0 kcal mol⁻¹ greater for poly(dA)-poly(dT) than for (dA)19-(dT)19. Furthermore, the average ΔH for dissociation of the A-T Hoogsteen base pair in a poly(dA)·poly(dT)·poly(dT) triplex is nearly twice that of a (dA)19-(dT)19-(dT)19 triplex (22). These previous studies indicate that the precise structures (either static or dynamic) of the duplexes and the triplexes of the present study likely depend upon DNA strand length. If coralyne binding is modulated by these length-dependent structures, this could be the origin of the length-dependent secondary structure equilibrium at 4°C. We propose that the length dependence of this equilibrium results primarily from a length-dependent binding of coralyne to (dA)n-(dT)n duplexes. Our basis for this proposal is the observation that CD spectra of (dA)n-(dT)n duplex samples containing coralyne change with strand length to a much greater extent than the spectra of coralyne-disproportionated samples at the same temperature (Figs 5 and 6). This could result from the exact mode/geometry of coralyne binding to a (dA)n-(dT)n duplex being a length-dependent phenomenon.

CONCLUSION

In conclusion, we have shown that nucleic acid binding by a small molecule, such as coralyne, is a powerful means to control DNA secondary structure. However, we have also shown that the kinetics and thermodynamics of DNA structure formation in the presence of small molecule intercalators can be complex. Even our rather minimalist system composed of (dA)n-(dT)n duplexes has revealed several aspects of DNA secondary structure formation that are likely to depend upon strand length. This length dependence may, in part, result from a length-dependent binding of coralyne to (dA)n-(dT)n duplexes. In any case, if we consider that the three DNA secondary structure states of this study (i.e. duplex, triplex, (dA)n self-structure) are likely to have different enthalpies and entropies of coralyne binding, it is then perhaps not so surprising that coralyne binding can produce a complex relationship between temperature and DNA secondary structure. One aspect of DNA–coralyne interactions that we have not explored in the present work is the possible effect of coralyne concentration on DNA secondary structure equilibrium. It is altogether possible that altering intercalator concentration could be a means to control secondary structure equilibrium at a particular temperature.

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