Condensation of nucleic acids by intercalating aromatic cations

[light scattering/acridine orange/Novatrace/Mitoxantrone/1,4-dihydroxy-5,8-bis[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione (DHAQ)]

JAN KAPUSCINSKI AND ZBIGNIEW DARZYNKIEWICZ

Sloan-Kettering Institute for Cancer Research, D. Walker Laboratory, 145 Boston Post Road, Rye, NY 10580

Communicated by Zacharias Dische, August 9, 1984

ABSTRACT Certain intercalating aromatic cations, such as the fluorochrome acridine orange or the antitumor drug Mitoxantrone, induce condensation of nucleic acids in solutions. The appearance of the condensed form during titration of nucleic acids with these intercalating ligands can be quantitatively monitored by light scatter measurements. The resulting highly reproducible light scatter transition curves are typical of the cooperative processes, and the transitions occur at different critical concentrations of the ligands depending upon both the ligand itself and the primary structure (base and sugar composition) and the secondary structure (single- or double-stranded) of the nucleic acids. The mechanism of condensation of nucleic acids by intercalating cationic ligands is discussed in light of the model of interactions occurring between certain intercalators and single-stranded nucleic acids and compared with the condensation induced by polyvalent "simple" cations such as Co2+ or spermine**. The described phenomenon can have an application in analytical and preparative biochemistry for characterization of the primary and secondary structure of nucleic acids and for separation of the compounds. The possibility that the condensation plays a role in mutagenic and pharmacological effects of aromatic cations is considered.

Interactions between aromatic intercalating cations and nucleic acids at high binding density and high ligand concentration often result in precipitation of the product. Although this phenomenon was observed before (reviewed in ref. 1), conditions of the precipitate formation were not studied in detail nor were attempts made to characterize the molecular structure of the product. The precipitation was considered a hindrance in the titration experiments and the titrations were usually terminated at that point.

During the past several years we have studied interactions between nucleic acids and such ligands as 3,6-bis(dimethylamino)acridine (acridine orange, AO) or the antitumor drug 1,4-dihydroxy-5,8-bis[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione (DHAQ)* under conditions in which the products were insoluble in aqueous media and underwent precipitation (1-6).

In the course of those experiments we observed that the stage preceding precipitation—namely, the condensation (collapse) of the polymer—can be conveniently and accurately monitored by light scatter measurements (1, 3, 5). These experiments were extended to different types of nucleic acids and different ligands. The data revealed that the light scatter increase was in proportion to the quantity of nucleic acids and that the condensation occurred at different concentrations of the ligands, depending on the primary and secondary structure of the nucleic acid. This phenomenon is of interest because (i) it may be used to analyze the structure of nucleic acids and (ii) many of the intercalating ligands are antitumor drugs, mutagens, or both and the condensation may play a role in those effects. From the large library of the transition curves obtained for various types of nucleic acids and different ligands (antitumor drugs and fluorescent probes of nucleic acids), several representative experiments were chosen for this communication to describe the phenomenon and illustrate its potential for analytical purposes. More detailed studies will be the subject of a separate publication.

MATERIALS AND METHODS

Nucleic Acids. Phage MS2 RNA and Escherichia coli rRNA (16S and 23S) were obtained from Miles Laboratories, øX174 double-stranded (ds) circular replicative form (RF) DNA and øX174 single-stranded (ss) circular DNA were from Bethesda Research Laboratories, synthetic polynucleotides were from P-L Biochemicals, and calf thymus DNA was from Sigma. Preparation of the stock solutions was described before (4). Denatured DNA was prepared by heating sonicated DNA (3) for 10 min in 0.1 M NaCl/5 mM Hepes, pH 7.0, followed by rapid cooling to 0°C. Concentration of nucleic acids in stock solutions was determined by UV light absorption; the double-strandedness of homopolymer pairs and alternating copolymers was confirmed by their hyperchromicity (>30%) and their typical thermal denaturation curves (the same buffer, 1°C/min). The concentrations of nucleic acid are expressed as moles of nucleotides per liter.

Ligands. Cobaltic hexammine chloride was obtained from Eastman Kodak, DHAQ (Novatrace-HCl) from American Cyanamid (Pearl River, NY) was kindly provided by Z. A. Arlin, spermine-4HCl was from Sigma, and chromatographically purified AO was from Polysciences (Warrington, PA). Concentrations of Co2+, DHAQ, and AO stock solution were determined colorimetrically (2, 11, 27).

Buffers and Solutions. All buffers contained 5 mM Hepes, pH 7.0, with addition of NaCl (0.01 or 0.15 M for the experiment with Co2+ and spermine** or AO and DHAQ, respectively) and 1 mM EDTA (optionally as described in the text). The buffers were filtered through 0.45-μm-pore Millex Millipore filters. In the experiment with AO and DHAQ, the final solution contained 0.1% Triton X-100, a nonionic detergent added to protect the colloid against rapid agglomeration.

Light Scattering Measurement. Aliquots containing 2 ml of nucleic acid (0.2-10 μM) in the buffer were placed in a quartz cuvette in the thermostatic holder (25 ± 0.1°C) of an SLM 4800 spectrofluorimeter (SLM Instruments, Urbana, IL). Light scattering was measured at 90° to the incident light. Both monochromators were set to the same wavelength (350 nm in the experiments with AO, spermine, and DHAQ and 390 nm when Co2+ was used); the bandwidths were 10 nm.

Abbreviations: AO, acridine orange; DHAQ, 1,4-dihydroxy-5,8-bis[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione; ds, double-stranded; ss, single-stranded.

*The abbreviation most common in the literature. Two trade names (Novatrace and Mitoxantrone) and National Service Center numbers (NSC-279836 and NSC-301739) are also often used.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.
were 4 nm. The sample and the blank (buffer only) were treated with small volumes (5-10 μl) of the ligand (concentration L) stock solution (0.1-10 μM); after the addition, the contents of the cuvettes were gently mixed and incubated for 10 min [the time necessary to obtain a stable reading (6)], and the light scattering was measured.

The results were corrected for the effect produced by the ligand alone (blank) and expressed as I/I₀, in which I₀ and I are the intensities of the light scattered by the sample before and after addition of the ligand, respectively. The data were then processed by computer (HP 9826) using a "cubic spline" interpolating program and smoothed, and the first derivative was taken and drawn by digital plotter (HP 7225A).

RESULTS

Titration of nucleic acids with the aromatic cations resulted in an increase in light scatter (Fig. 1). The light scatter transition curves were typical of the cooperative process. The increase in intensity of light scatter remained stoichiometric in relation to the quantity of the polymer; the integrated area (S) under the derivative curves [d(I/I₀)/dL] correlated with the concentration of nucleic acids in the solution. Thus, when the appropriate calibration curves were made (e.g., such as in Fig. 1C for DHAQ) it was possible to estimate the concentration of nucleic acids in solution on the basis of the increase in light scatter during the titration.

The light scatter curves representing condensation of various types of nucleic acids induced by AO are shown in Fig. 2. In experiments with synthetic homopolymers (Fig. 2A), the concentration of all polymers was similar, yet the nucleic acids condensed at distinctly different concentrations of ligand, depending on their primary structure.

In general, the condensation of homoribopolymers occurred at lower ligand concentrations than the condensation of their deoxyribo analogs [e.g., poly(rA) vs. poly(dA) or poly(rU) vs. poly(dU)]. A similar relationship was observed for other ribo vs. deoxyribo analog pairs as well as in the case of titration with DHAQ (not shown).

Natural RNAs (rRNA, MS2 RNA) underwent condensation at lower AO concentrations than either ss or ds DNA (Fig. 2B). The latter, in turn, condensed at higher AO concentrations than ss DNA. The integrated area under the derivative titration curve of ds DNA (Fig. 2B) was approximately half that of ss DNA measured at equimolar concentration. This phenomenon has been confirmed in repeated experiments with different types of ss and ds DNA (not shown). Thus, when the denatured DNA was titrated, the increase in light scatter intensity measured as S was approximately twice that of native DNA (per mole of nucleotide).

The data in Fig. 2 indicated that it was possible to identify the type of the polymer on the basis of the concentration of ligand at which the condensation occurred. We propose to use the "critical free ligand concentration" estimate, Cᵣ, which can be obtained from the light scatter titration experiment, as a characteristic feature of a particular type of nucleic acid

\[ Cᵣ = Cᵢ - Cᵢ \]
Table 1. Critical free ligand concentrations of AO and DHAQ

<table>
<thead>
<tr>
<th>Polymer</th>
<th>AO, μM</th>
<th>DHAQ, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ss Poly(dC)</td>
<td>3.4 (b)</td>
<td>0.8</td>
</tr>
<tr>
<td>Poly(dA)</td>
<td>13.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Poly(dT)</td>
<td>17.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Poly(dU)</td>
<td>23.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Poly(dA)poly(dU)</td>
<td>23.7</td>
<td>1.5</td>
</tr>
</tbody>
</table>

| ds Poly(dA)poly(dU)      | 22.6 (b)| 2.6      |
| Poly(dA-dU)poly(dA-dU)   | 24.2   | 23.0     |
| Poly(dA)poly(dT)         | 29.3 (b)| 3.2      |
| Poly(dA-dT)poly(dA-dT)   | 35.0   | 26.5     |
| Poly(dT)poly(dC)         | 14.7 (b)| 2.7 (b)  |
| Poly(dT)poly(d-dC)       | 42.2   | 39.5     |
| Poly(dA-dC)poly(d-dC)    | 50.5   | 40.9     |

Natural
- Native calf thymus DNA: 51.5
- Denatured calf thymus DNA: 49.8
- ss dX174 DNA: 27.6
- Denatured calf thymus DNA: 24.6
- ss dX174 DNA: 13.8
- rRNA (16S + 23S): 7.4

Polymers (5 ± 1 μM) were titrated at 25 ± 0.1°C in buffer containing 0.15 M NaCl, 5 mM Hepes, 1 mM EDTA, and 0.1% Triton X-100 at pH 7.0. The critical transition midpoint (Cm) is the concentration of the ligand at which the condensation of the polymer (Cp) is in the range of 0.5 ≤ Cp < 1.0 (6).

In numerous experiments we observed that Cm is independent of the concentration of nucleic acids—e.g., for AO Cm = 48.1 ± 3.0 μM, six measurements within the calf thymus DNA concentration range 5–70 μM (6) and, therefore, can be a useful parameter for polymer characterization. Table 1 lists Cm values for several ss and ds DNAs titrated with AO or DHAQ. In experiments with ss DNA, the polymer condensed at distinctly different concentrations of the ligands, depending on their primary structure, regardless of whether AO or DHAQ was used.

The Cm values for ss deoxiribo polymers were lower than for their ds forms. This was true also for the ribo polymers (not shown) and for natural nucleic acids (Fig. 2B). The difference between ds and ss forms was of such degree that titration of the ss forms of viral DNA with AO (Fig. 2C) yielded two completely separated transition curves. Similar results were obtained when mixtures of thermally denatured and native calf thymus DNA were titrated with AO or DHAQ (not shown).

An interesting selectivity was evident when deoxiribopolymer pairs were compared with their alternating (purine-pyrimidine) analogs (Table 1). All three alternating polymers were characterized by severalfold higher Cm values in comparison with the corresponding homopolymer pairs when reacting with DHAQ. A similar phenomenon was seen in the case of RNA polymers (not shown). Note that natural ds DNA, which represents a mixture of both structures, had intermediate Cm values (DHAQ, Table 1).

It is known that neutralization of the phosphate charge of the nucleic acid, which can be achieved by addition of "simple" cations with a valency of 3 or higher (e.g., inorganic cations), results in collapse of the polymer (7). It was of interest, therefore, to compare the condensation of nucleic acids induced by those simple cations with the condensation obtained by intercalating monovalent cations (Table 2). For the simple cations (Co, spermine), the Cm value increased in the order native DNA, denatured DNA, RNA. This order was reversed in the case of condensation induced by AO or DHAQ. Another difference was the sensitivity of the process to EDTA. Whereas 1 mM EDTA increased the Cm values for the simple cations severalfold, the increase was lower than 40% in the case of AO or DHAQ (Table 2).

**DISCUSSION**

The experiments presented above demonstrate that nucleic acids undergo condensation upon interaction with certain intercalating (8) cations. The process can be monitored by light scatter measurements. Two aspects of these findings, the possible mechanisms of the phenomenon and its practical application, require discussion.

The collapse (condensation) of DNA may be caused by addition of various cations to dilute solutions of the polymer (reviewed in refs. 7 and 9). The structure of the collapsed DNA is not well characterized. Intramolecular segment-segment interactions, normally precluded by the strong repulsive forces of the charged phosphates of the backbone, may become dominant in the compact state (10). In aqueous solutions, cations with a valence of 3 or higher are required to neutralize the charge and cause condensation. After charge neutralization the collapse of DNA becomes a spontaneous process (10). Condensation of ds DNA induced by Co, spermine, or spermine results in toroidal or doughnut-shaped forms, which retain the local B conformation (9, 11–15). The increase in light scatter parallels the transition due to the fact that there is an increase in form factor (transition

Table 2. Critical free ligand concentrations with AO, DHAQ, and simple cations

<table>
<thead>
<tr>
<th>Polymer</th>
<th>AO, μM</th>
<th>DHAQ, μM</th>
<th>Co, μM</th>
<th>Spermine, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Native DNA</td>
<td>43.9</td>
<td>48.1</td>
<td>16.0</td>
<td>16.3</td>
</tr>
<tr>
<td>Denatured DNA</td>
<td>27.2</td>
<td>27.5</td>
<td>7.8</td>
<td>10.5</td>
</tr>
<tr>
<td>MS2 RNA</td>
<td>11.7</td>
<td>11.7</td>
<td>4.8</td>
<td>5.4</td>
</tr>
<tr>
<td>rRNA (16S + 23S)</td>
<td>8.3</td>
<td>8.3</td>
<td>2.7</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Titrations of polymers (1–5 μM) were performed in buffers containing 5 mM Hepes, pH 7.0, and different NaCl and EDTA concentrations as indicated, respectively: a, 0.15 M and 1 mM; b, 0.15 M and 0; c, 10 mM and 1 mM; d, 10 M and 0. Triton X-100 was present at 0.1% in the buffers in experiments with AO and DHAQ; its presence has a minor effect (<30%) on the value of Cm (not shown).

Because of high valency of Co or spermine, the amount of the bound ligand (Cm) is relatively small as compared to Cm. Therefore, no correction for Cm in calculation of Cm has been made (Cm ~ Cm).
Biochemistry: Kapuscin and Darzynkiewicz

from a Gaussian chain to a sphere of the scatterers (12). The transition is cooperative and can be reversed by monovalent cations (9, 11).

There are several differences between the condensation induced by the simple multivalent (≥+3) cations and the condensation caused by the intercalating aromatic ligands, suggesting that the molecular mechanisms of the phenomenon are different. Thus, some intercalators (e.g., AO) are monovalent cations. The simple monovalent cations (e.g., inorganic cations) neither induce DNA condensation nor, as described presently, interfere with the condensation caused by the intercalating ligands. Also, the sensitivity to EDTA of the condensation induced by simple cations is much higher than the condensation induced by AO or DHAQ, and the relative reactivities of native DNA, denatured DNA, and RNA are reversed.

A model describing binding of the intercalating cations was recently proposed by us (1, 4, 5); it explains both the condensation of the product and specificity of the phenomenon in relation to the ligand or polymer structure. According to this model, the initial attachment of the ligand (nucleation) is via insertion of the planar aromatic ring between the adjacent bases of the ss polymer. The cooperative process follows the nucleation. Namely, from the nucleation point the binding progresses along the polymer molecule and “stacks” of the alternating sequence ligand–base are thus formed. Both electrostatic forces and forces involved in ligand–base stacking (London–Van der Waals) take part in this binding and a charge neutralization of the polymer leads to polymer condensation. Hydrophobic properties and other nonionic forces (10) may additionally contribute to polymer collapse. The above model differs from the traditional dye–dye stacking model (16) in which the stacks of the ligands are believed to be electrostatically attached to the phosphates, on the backbone side of the polymer (for further discussion see refs. 1 and 4). According to our model the affinity of ligand binding correlates with base composition because the base–ligand interactions take place during both the nucleation and cooperative processes. The structure of the sugar moiety (ribo vs. deoxyribo) may also play a role—e.g., due to effects on the dynamic properties of the polymer (10, 17).

If the condensation of the polymer is a consequence of binding as proposed by our model (1, 3–5), a correlation between the binding affinity of a particular ligand (related to base or sugar composition) and polymer condensation would be expected. Experiments, therefore, have been done to compare the intrinsic (Kc) and cooperative (Kc') association constants of AO binding to various polymers, with critical free ligand concentration (C*) calculated from the light scatter transition curves. A close relationship between Kc or Kc' and the reciprocal of C* is evident (Fig. 3), which supports our interpretation of the condensation phenomenon. The deviation of the Kc value for poly(rI) may be the result of the tendency of this polymer to form poly-stranded structures (2, 18).

Results from Table 1 indicate that, among the polymers with the same sugar moiety, the ligand–base interactions play a dominant role over the secondary structure of ss nucleic acids (coil vs. helix conformation). If the latter predominated, the relative reactivities of different ligands to the same polymers would be similar, which is not the case.

Condensation of ds nucleic acids is similar in principle to that of ss nucleic acids except that partial denaturation must precede the condensation. Namely, the intercalating ligand may attach to the ss sections of the ds polymer (e.g., “loose” ends or “breathing” regions) in a manner similar to that discussed above. Due to cooperativity, the reaction will expand from the nucleation point, resulting in denaturation of the adjacent sections. The thermodynamics of such binding and biochemical evidence of DNA denaturation by the intercalators were recently presented (1, 3–5). The data in support of DNA denaturation by intercalating ligands were recently reviewed by us (6), and in this context some results of AO-induced condensation of DNA were described. Because of the denaturation step, which requires higher concentrations of the ligand, C* values of the ds forms are higher than those of the ss forms.

The light scatter increase during titration of ds nucleic acid is only one-half of that of ss nucleic acid (Fig. 2B). This difference, also observed with calf thymus DNA, may be due to incomplete strand separation during the ligand-induced denaturation step (5). Namely, because the intensity of the scattered light is proportional to the number of macromolecules (20), at the same concentration of the polymer there may be twice as many scatterers in a solution of ss or ther- mally denatured nucleic acid compared with its ds form.

In contrast to the intercalating ligands, the simple cations preferentially induce condensation of ds rather than ss nucleic acids. The higher reactivity of the ds polymers can be explained by their higher axial charge density (21, 22) and exclusively ionically polymer–ligand interaction.

Regardless of the exact nature of the interactions responsible for the condensation, the phenomenon has immediate practical application. Thus, the light scatter changes occurring as a result of ligand binding can be used to characterize the primary and secondary structure of nucleic acids in solutions. The values of C* (for a given ligand) are different and characteristic for different nucleic acids. The resolution of this analytical method is high, which allows one in certain situations to separate transitions in samples containing two mixed nucleic acids of different primary or secondary structure (e.g., Fig. 2C). The method is quantitative, simple, rapid, versatile, and nondestructive for nucleic acids; the latter can be fully recovered from the insoluble complexes (5). As little as 0.1 µg of nucleic acid can be analyzed in a single sample in a standard fluorimeter. It is possible to construct a dedicated instrument in which the titration and light scatter measurements, either in a static or a flow channel, can be made automatic. This approach can also be applied for preparative purposes. From mixture of nucleic acids in solutions it is possible to separate those that undergo condensa-

![Fig. 3. Relationship between intrinsic (Kc; ○) or cooperative (Kc; ○) association constants of AO (data from ref. 4) and the reciprocal of critical free AO concentration (C*) in reactions with the homobipolymers: U, poly(rU); C, poly(rC); A, poly(rA); I, poly(I). A deviation in the Kc value of poly(rI) may be due to the fact that this polymer has a tendency to form multistranded structures, especially at high salt concentrations (2, 18).](image-url)
tion at different $C_c$ values; they can be removed simply by either low-speed centrifugation or filtration (1, 5). The technique, therefore, may have wide application in analytical and preparative biochemistry.

The ligands inducing condensation of nucleic acids that we have studied exhibit antitumor and mutagenic properties (reviewed in refs. 23 and 24). The condensation effects can be observed at the pharmacological range of their concentrations (23, 25, 26). Thus, the possible role of this phenomenon in antitumor or mutagenic properties of the intercalating cations should be considered.

We are grateful to Dr. Myron R. Melamed for his constant support and encouragement, Dr. Frank Traganos for helpful discussion, and Mrs. Sally Carter and Miss Robin Nager for their assistance in the preparation of the manuscript. This work was supported by U.S. Public Health Service Grants CA 28704 and CA 23296.