Fluorimetric detection of different structures induced by concentration changes of alkaline and alkaline-earth counterions on covalently closed DNA

L. MASOTTI,* M. L. BARCELLONA,† J. von BERGER,* and M. AVITABILE†

*Institute of Biological Chemistry, University of Parma, via Gramsci 14, 43100 Parma, Italy; and †Institute of Biological Chemistry, University of Catania, viale A. Doria 6, 95125 Catania, Italy

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Fluorimetric studies on the complex formed by the intercalation of 4′-6-diamidino-2-phenylindole·2HCl into ccDNA from Col E1 plasmid show that in the presence of increasing concentrations of alkaline and alkaline-earth counterions structural transitions occur and three dye-ccDNA complexes are formed, each stable within a defined salt-concentration range. The structural transitions detected do not depend on the nature or the electric charge of the counterions; only Ca²⁺ shows a quite different behaviour. Such observations suggest that Ca²⁺ possesses an ionic radius and charge, which combined make its interaction with the nucleic acid unique.

A large number of covalently closed double-stranded DNAs has been isolated from various sources; they display a great variability of secondary and tertiary structures that are altered by the changes in the environmental conditions (1-5).

The structural details regarding the overall geometry as well as the possible conformational changes occuring in solution for such macromolecules are of wide biological relevance and are the object of growing attention. In fact several basic questions regarding structural and functional properties of ccDNA still need clear answers: how is the genetic expression regulated? How influential is the primary structure in determining secondary and tertiary structural transitions? How and to what extent do counterions influence the secondary and tertiary structures in vivo? We have approached this last problem by studying the effects of counterions on ccDNA structure in vitro.

Circular dichroism studies (6) showed that KCl induces ccDNA to undergo conformational changes in the same salt-concentration range required for its transcriptional activity. It is known that the bicationic dye 4′-6-diamidino-2-phenylindole·2HCl (DAPI) forms a fluorescent complex with DNA, binding specifically to A+T base pairs (7-9). Thermodynamic studies (10), and ultraviolet absorption as well as steady-state and fluorescence quenching measurements (11) show that the dye intercalates into the nucleic acid. The use of DAPI in the presence of increasing concentrations of KCl showed that, in the
salt-concentration range assayed, three different dye-ccDNA complexes are formed, each stable within a defined concentration range (12).

This paper reports fluorescence studies of the effects of various concentrations of alkaline and alkaline-earth counterions on the structure of ccDNA.

**Materials and Methods**

DAPI (Serva) was checked for purity by means of thin-layer chromatography, dissolved in double-distilled water, and used at a concentration of 2.1 x 10^{-6} M, determined spectrophotometrically (ε_{262} = 18636 litre·mol^{-1}·cm^{-1}). ccDNA, from Col E1 plasmid (Sigma Chemical Co.), was also dissolved in double-distilled water and used as a stock solution at a concentration of 3.4 x 10^{-4} M, also determined spectrophotometrically (ε_{260} = 6600 litre·mol^{-1}·cm^{-1}).

Fluorimetric measurements were carried out using a Perkin-Elmer MPF 44A spectrofluorimeter, equipped with a DCSU2 accessory. The excitation and the emission wavelengths were set at 340 nm and 442 nm respectively. The samples, in cuvettes of 1 cm pathlength, were maintained at 25°C with continuous direct monitoring of the sample temperature by means of a thermocouple. In all the experiments, each sample was allowed to equilibrate for 5 min.

The effects of counterion concentrations on the DAPI-ccDNA complex were studied at dye-saturating conditions, adding to the solution the required volumes of a 4 M stock solution of the appropriate chloride. The maximum concentration of the BaCl_2 stock solution was only 1.2 M, due to the limited solubility of the salt. Fluorescence intensities were then corrected for dilution. In the upper concentration range, salts were added as appropriate weights of powder.

The measurements for the Scatchard plots were carried out by stepwise addition of 50-μl portions of the ccDNA stock solution. Measurements performed by the dilution titration technique (13) gave identical results within the experimental error.

**Results and Discussion**

Fig. 1 shows that both the emission and the excitation maxima increase according to the amount of ccDNA available for binding. Moreover, each excitation maximum was red-shifted as a consequence of the dye-ccDNA complex formation, where DAPI intercalates into nucleic acid.

The fluorescence intensity pattern obtained at different alkaline-ion concentrations is shown in Fig. 2a. For all the ions assayed it is evident that the curve was virtually the same and steps can be observed in three salt-concentration ranges: below 5.3 x 10^{-2} M, from 2.2 x 10^{-1} M to 1.2 M, and above 2.9 M. Among the alkaline-earth cations assayed, Mg^{2+} and Ba^{2+} exhibited the same behaviour as the alkaline ions (Fig. 2b). However, when Ca^{2+} interacted with the DAPI-ccDNA complex, three steps were also observed, but they all occurred at lower salt concentrations: below 1 x 10^{-2} M CaCl_2, from
2.8 x 10⁻¹ M to 5.8 x 10⁻¹ M, and above 9.8 x 10⁻¹ M. In Figs. 3a and 3b, more detailed studies on the dependence of fluorescence intensity on cation concentrations are reported. The shape of the curves seems to indicate minor rearrangement — dependent on the concentration and type of the counterions — of the structure of the nucleic acid occurring between the first and the second conformation detected.

Scatchard plots (14) of the binding of DAPI to ccDNA in the presence of CaCl₂ and KCl (Fig. 4) give the numbers of binding sites per nucleotide, r, and the values of the dissociation constants, K_d, of the three DAPI-ccDNA complexes. A value of r of 0.05 is found for the first conformation, detected in the KCl concentration range below 5.3 x 10⁻² M and in the CaCl₂ concentration range below 1 x 10⁻² M; for the second one, r is found to be 0.025 in a concentration range from 4.2 x 10⁻¹ M to 1.2 M for KCl and from 2.8 x 10⁻¹ M to 5.8 x 10⁻¹ M for CaCl₂; and for the third conformation, detected in the concentration range from 2.9 to 3.2 M for KCl and from 9.8 x 10⁻¹ M to 3.2 M for CaCl₂, r is found to be 0.020. Consequently, assuming an average mol. wt. of 660 for each nucleotide pair, to each molecule of ccDNA (whose mol. wt. is 6 x 10⁶) 909, 454, or 364 molecules of DAPI are bound when the nucleic acid assumes the first, the second, or the third conformation, respectively. The corresponding K_dS are 2.7 x 10⁻⁷ M, 2.5 x 10⁻⁷ M, and 6 x 10⁻⁷ M in the presence of CaCl₂.
Fig. 2. Dependence of fluorescence of DAPI-ccDNA complex on the concentration of alkaline (a) and alkaline-earth (b) ions, added as chlorides, in the range between $8 \times 10^{-4}$ M and 3.2 M.
Fig. 3. Dependence of fluorescence of DAPI-ccDNA complexes on the concentration of alkaline (a) and alkaline-earth (b) ions in the 13-250 mM range.
The influence of anions, in inducing conformational changes in ccDNA, has also been tested. To this end CH$_3$COO$^-$, Cl$^-$, NO$_3^-$, SO$_4^{2-}$, and H$_2$PO$_4^-$ were used. Under the same experimental conditions, no effect of the anion concentration on the fluorescence intensity was observed, leading to the conclusion that anions do not influence the structure of the nucleic acid.
The data clearly show that salt concentration changes are able to induce different conformational states in ccDNA. Moreover, such conformations, imposed on these molecules by their covalent closure, are peculiar to ccDNA, since linear DNA conformations are not affected by the same experimental conditions (12). In the three steps the number of DAPI molecules bound to the nucleic acid varies, but the differences in stability of the complexes, while measurable, do not seem to be large. The effectiveness of counterions in inducing the structural transitions observed seems to be independent of either ionic radii or ion charge, if considered separately, since alkaline and alkaline-earth ions produce the same fluorescence changes. Only Ca$^{2+}$ shows striking differences: in its presence the first structural transition occurs in the concentration range 0.013 M to 0.150 M and the second between 0.6 M and 1.02 M, while with all the other counterions the first transition is detected in the range 0.05 M to 0.20 M and the second one in a molarity range from 1.8 M to 2.8 M. It would seem that only Ca$^{2+}$ combines the required dimensions and electric properties that make the interactions between the nucleic acid and the ion unique. Furthermore, of the two transitional states shown, the one detected in the salt-concentration range between $5.2 \times 10^{-2}$ M and $2.2 \times 10^{-1}$ M for the monovalent counterions is of biological relevance, occurring at salt concentrations required for the functional role of ccDNA.

References