Electrostatic potential modulations on polynucleotides as a function of ionic content: potentiometric determinations.

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ABSTRACT

pK values of ionisation of cytidine residues, in poly C and poly I. poly C, have been determined by potentiometric titration as a function of either ionic strength (Na^+) or Mg^{2+} concentration. The results are interpreted as a consequence of the existence of a local pH, prevailing in the vicinity of the polynucleotides, smaller than that of the bulk solution. This local pH is dependent upon the electrostatic potential Ψ due to the negatively charged phosphate groups of the polynucleotide. Moreover Ψ can be modulated through variation of either ionic strength or Mg^{2+} concentration. At a given pH Mg^{2+} addition leads to the release of protons which, in the case of complex systems of limited stability, might be detected by the use of the pH-stat method. Possible functional implications for genetic translation systems are examined.

INTRODUCTION

It is well established that the pK values of the ionizing groups of nucleic acid bases as well as the melting temperatures of these compounds depend on the ionic strength; in most of these investigations univalent cations only have been employed (1) (2) (3) (4) (5).

This general behaviour results from the fact that nucleic acids are polyelectrolytes with a strong electrostatic potential acting on the apparent pK of ionizing groups of the heterocyclic bases and influenced by the ionic strength as a direct consequence of a screening effect explained by the Debye-Huckel theory (6).

A similar process has been observed in the case of the enzyme activity in polyanionic environments, due to the influence of the electrostatic potential and of its variations on the pK of the ionizing groups of proteins involved in catalysis (7) (8), and may be applied to enzyme systems involved in genetic trans-

lation (8).

In such systems, the Mg^{2+} ions play an important role, since it is known that these divalent ions bind specifically to the phosphate groups of polynucleotides and thus should modify their electrostatic potentials. We therefore decided to study the influence of Mg^{2+} ions on the pK of the ionizing groups of the nucleic bases as a possible consequence of the modification of the electrostatic potential Ψ by these ions.

The present paper reports the results obtained on the synthetic polynucleotides poly C and poly I. poly C, in which the ionization of the cytosine is a two step process leading respectively to the compounds poly C. poly C^+ and poly I. poly C. poly C^+ , and then to poly C^+ . and poly I. poly C^+ . The two corresponding pK values can be easily distinguished, and we recorded only the values of the first one as a "probe" of the variations of the electrostatic potential as a function of the ionic content established with Na^+ as ionic strength or Mg^{2+} as specific cation.

It is shown that Mg^{2+} is more efficient than Na^+ and the observed pK variations are interpreted by the polyelectrolyte theory, according to which the local pH is dependent on Ψ and lower than the pH of the bulk phase.

pH-stat measurements at selected pH values show proton release upon increase in Mg^{2+} concentration as a consequence of deprotonation of cytosine. This experimental method might be applied to more complex structures too pH sensitive to be titrated over a suitable range of pH values.

EXPERIMENTAL

Poly I. Poly C, Poly C and Poly U were purchased from Miles Chemical and were dissolved in NaCl solutions of various concentrations and then dialyzed several times against the same solution. Water was bi-distilled and dissolved gas was removed under vacuum, all solutions were prepared under nitrogen to prevent carbon dioxide contamination. Solutions were unbuffered but the pH value was always near 6.

Polynucleotide concentrations were determined as $[P]$ spectrophotometrically using ϵ_p of 9100 for poly U (9), 6300 for poly C (3) and 5400 for poly I. poly C as determined by the classical method. Experimental concentrations were near $2 \times 10^{-3} M$ P .

Mg²⁺ solutions were prepared from MgCl₂ (Merck) in CO₂ free water and concentrations were measured by the Eriochrome Black T method (10). KOH solutions were prepared from solid KOH (Merck) before each experiment and HCl diluted from Merck Titrisol 0.1 M.

Radiometer Instruments were used for titration curves and pH stat measurements : PHM63 digital pH-meter, Titrator TTT 60, Autoburette ABU 12, Recorder REC 6I with titrigraph module REA 160, and a semi-micro combined electrode GK 232I C. Flow of KCl through the porous pin was estimated from conductivity measurements and correction was made at low ionic strength.

Radiometer standard buffers (pH 7.00 and 4.01 at 25°C) were used to calibrate the pH scale after temperature corrections.

The polynucleotide solution was placed in a closed cell (2 ml) under a stream of nitrogen and stirred magnetically. A thermostated jacket gave the selected temperature ($\pm 0.1^\circ\text{C}$) measured with a thermocouple in the solution. The burette volume was 0.25 ml and the precision on titrant volume was 10^{-3} ml. The pH value was read with a precision of 0.02 unit.

Titration curves were recorded for various concentrations of Na⁺ or Mg²⁺, the pH was brought to 7.50 by addition of small quantities of KOH and then titrated with HCl to pH 4.00. Titration curves were obtained by the difference between the titration curves of the polynucleotide and that of the solvent. For each curve the titrant volume (HCl 10^{-2}M) was of the order of 50 μl .

During pH-stat experiments, the pH was kept constant during the stepwise addition of Mg²⁺ solution by a titrigraph controlled addition of KOH solution (10^{-2}M) and the pH of the added of Mg²⁺ solution was also at the pH-stat value.

RESULTS AND DISCUSSION

1) Ionic strength induced pK variations

Titration curves of both poly I. poly C and poly C, represented on fig.1 only for poly I. poly C, are characteristic of a cooperative process and the apparent pK can be determined with a precision of ± 0.05 .

These pK values are associated with the following equilibria
(3) (11) :

It can be seen from fig.1 that, as expected from these two equations, a plateau is reached for values of the ratio $[\text{H}^+] / [\text{P}]$ of the order of 0.25.

Curve 1 in fig.1, which corresponds to poly I. poly C with $[\text{Na}^+] = 3.4 \times 10^{-2}$, is a typical example of what occurs at low ionic strength : at high pH, a second pK appears which could be ascribed to single strand poly C, the double strand poly I. poly C being presumably dissociated in these conditions. In order to avoid this effect, Na^+ concentrations higher than 5×10^{-2} , were used throughout this work.

Figure 1 : Titration of poly I. poly C : protons added by nucleic phosphorus ($[\text{H}^+] \text{ a} / [\text{P}]$) as a function of pH at various Na^+ concentration : respectively, 1- $3.4 \times 10^{-2} \text{M}$, 2- $5.4 \times 10^{-2} \text{M}$, 3- $8.6 \times 10^{-2} \text{M}$, 4- $1.1 \times 10^{-1} \text{M}$, 5- $4.8 \times 10^{-1} \text{M}$, 6- $9.7 \times 10^{-1} \text{M}$.

As Na^+ concentration is increased, the titration curves are shifted towards acid pH's (fig.1), the corresponding pK variations of poly C and poly I. poly C being linearly dependent on the logarithm of Na^+ concentration (fig.2), as long as Na^+ is lower than 10^{-1}M . These results are in good agreement with those reported elsewhere (3).

From thermodynamics, the apparent pK (pK_a) of any equilibrium is related to the corresponding free energy change by the equation:

$$\Delta G = 2.3 RT \text{ pK}_a$$

In this case, ΔG can be expressed as the sum of three terms (11):

$$\Delta G = \Delta G_i + \Delta G_D + \Delta G_e \quad (3)$$

- a) ΔG_D is the non electrostatic free energy change principally associated with the breaking of hydrogen bonds ;
- b) ΔG_i is the free energy change associated with deprotonation.
 $\Delta G_i = 2.3 RT \text{ pK}_i$, pK_i being the intrinsic pK of the ionisable group.
- c) ΔG_e is the free energy change associated with electrostatic interactions. It depends on ionic strength (12) as expressed by the semi-empirical equation

$$\Delta G_e = RT (C_1 - C_2 \log I) \quad (4)$$

Such a dependence was classically ascribed to the fact that ionic strength decreases the repulsive electrostatic energy between the phosphate groups of two associated strands; in these conditions an increase of ionic strength should favour association into double strands. However the opposite effect was observed on poly A (5), as well as in this work on poly C (fig.1).

An alternative interpretation was then proposed (13), based on X Ray studies, according to which poly A^+ . poly A^+ association was promoted through attractive forces between PO_4^- and H^+N . However, while such bonds are not suspected in poly C^+ . poly C^+ , the variation of ΔG_e we observe as $\log I$ varies is identical with that occurring with poly A^+ . poly A^+ (11).

We therefore deduce from these observations that :

- a) the electrostatic repulsions between phosphate groups are not of significant importance with respect to the total electrostatic

energy of the system ;

b) the main contribution to the electrostatic term ΔG_e is the indirect interaction between the phosphate groups and the protonation sites via the local hydrogen ion concentration.

2) The local pH concept

It is well known (14) that in the neighbourhood of a poly-anion, the cation concentration is higher than that of the bulk solution. Such a situation also applies to hydrogen ions and the local pH (pH_L) is lower than that of the bulk solution

$$pH_L = pH + \frac{\epsilon \Psi}{kT} \quad (5)$$

This equation holds for any point where the electrostatic potential is Ψ (negative) ; ϵ is the unit charge, and kT has its usual significance. Ψ is obviously dependent on all the ionic species existing in the solution, responsible for the actual ionic strength. For instance it can be calculated that on the surface of the double helix of a DNA model (14), the ration $[H^+]_L / [H^+]$ is about 20 for $[Na^+] = 0.1M$; in these conditions the pH difference between bulk and local pH is, as a rough estimation $\Delta pH = 1.3$.

This theory has allowed a satisfactory understanding of the large shifts in pH-activity profiles induced by ionic strength variations, in the case of enzymic systems in polyanionic environments : that of trypsin covalently bound to insoluble polyanions (7), that of lysozyme acting on bacterial (*M.luteus*) cell walls (8) and that of RNase in the hydrolysis of RNA (8).

However this theory is generally not considered in studies on the pK of polynucleotides, although by equation 5, the apparent pK of any ionisable group (15) is given by :

$$pK_a = pK_o - \frac{\epsilon \bar{\Psi}}{kT}$$

$\bar{\Psi}$ being the average electrostatic potential around the ionisable group under consideration, so that the corresponding free energy change is

$$\Delta G = \Delta G_i + \Delta G_D + \left(\Delta G'_e - \frac{\epsilon \bar{\Psi}}{kT} \right) \quad (6)$$

$\Delta G'_e$ in this equation is associated with the interactions between charged groups of the polynucleotides ; as already discussed its contribution may be negligible in comparison with $\epsilon\bar{\Psi}/kT$. As a consequence of ion screening, $\bar{\Psi}$ linearly decreases as a function of $\log I$ (12) (14) so that the observed variations in pK_a can then be understood in terms of local pH.

When the ionic strength becomes very high, the linear variation of $\bar{\Psi}$ as a function of $\log I$ is no longer observed (fig.2), the approximations on which it is based being presumably incorrect, Ψ then vanishes and

$$\Delta G \simeq \Delta G_i + \Delta G_D$$

In the case of poly I. poly C at high ionic strength the pK_a becomes very similar to the pK_i of cytidine ($pK = 4.2$ at 20° (16)) so that ΔG_D must be quite small as expected from the absence of any variation in the number of hydrogen bonds under the equili-

Figure 2 : Variation of experimental pK versus Na^+ concentration (logarithm scale) $T = 20^\circ C$, O : poly I. poly C, x : poly C.

brum depicted by eq. 2.

On the other hand with poly C, ΔG_D is not negligible since three hydrogen bonds are broken in the process summarized in eq. 1, and the pK'_a at high ionic strength is indeed greater than pK_i . However it has been shown (11) that when a correction is made for ΔG_e by equation 4 and as the temperature is increased to the point where ΔG_D is negligible, pK_a tends to pK_i which is near to the pK of cytidine.

In more complex structures, like RNAs, where both single and double strands as well as loops are involved, local values of Ψ can vary widely and thus account for the abnormal pK reported in the literature in the case of t-RNA (17).

3) Mg^{2+} induced pK variations

As Mg^{2+} concentration increases, in a solution of poly I. poly C, the titration curves are shifted (fig.3) the pK varying as

Figure 3 : Titration curves of poly I. poly C at 20°C for various $[Mg^{2+}] / [P]$ ratios respectively : 1- 0, 2-0.28, 3-1.12, 4-3.36, $[Na^+] = 5.10^{-2}M$, $[P] = 2.10^{-3}M$.

shown in fig.4. Whereas this phenomenon is identical with that just mentioned at a qualitative level, fundamental quantitative differences occur : the pK variation actually depends on the ratio of Mg^{2+} /polynucleotide concentration $[\text{Mg}^{2+}] / [\text{P}]$, as a consequence of specific binding of Mg^{2+} on polynucleotides.

Such specific binding has been extensively studied on a large number of nucleic acids as well as polynucleotides, (18) (19) (20) (21). While some disagreement exists either in experimental data or as to the heterogeneity of fixation sites, the following points are unambiguously accepted :

a) The binding involves the electrostatic interaction of one Mg^{2+} ion with two negatively charged phosphate groups, i.e. 0.5 Mg^{2+} per P.

b) The magnitudes of stability constants imply the filling of all the binding sites, as long as the ratio $[\text{Mg}^{2+}] / [\text{P}]$ is higher than 2.

Figure 4 : Variation of the experimental pK of poly I. poly C as a function of $[\text{Mg}^{2+}] / [\text{P}]$ for different temperatures. x : 3°C, ● : 20°C, ○ : 37°C, $[\text{Na}^+] = 5 \cdot 10^{-2} \text{ M}$.

The observed pK variation can then be ascribed to the binding of Mg^{2+} on the phosphate groups, such a process involving both partial neutralisation and decrease of Ψ . The variation of pK and thus of Ψ with Mg^{2+} concentration is not a linear function of $\log [Mg^{2+}]$, but a decreasing exponential one, so that Mg^{2+} appears to be a powerful modulator of Ψ and pK, (for instance, with $[P] = 2 \times 10^{-3} M$ and $[Na^+] = 5 \times 10^{-2} M$, pK_a falls from 5.1 to 4.7 as Mg^{2+} changes from 0 to $2 \times 10^{-3} M$, whereas to reach the same result a solution 0.15 M in Na^+ would have been necessary).

As previously mentioned, the structural complexity can involve different local effects. In this respect, the strong binding sites for Mg^{2+} detected in both t-RNA (19) and r-RNA (22), might correspond to molecular regions under the influence of a very strong local electrostatic potential.

Fig.4 shows that temperature variations induce a translation of the whole curves $pK - Mg^{2+}/P$, while the electrostatic effect is apparently independent of temperature. In this connection it should be remembered that $\epsilon\Psi/kT$ actually varies as a function of DT , where D is the dielectric constant of the solvent, the product DT being a constant whatever the value of T (23), so that, either slight or negligible variation in the electrostatic contribution is not a priori inconsistent. Such an analysis, as well as our results fig.4, are in good agreement with the finding by several authors (24) (25) that the Mg^{2+} fixation constants are only slightly affected by temperature. The temperature induced pK variation is therefore controlled by that of $\Delta G_i + \Delta G_D$ of eq.3 written for $[Mg^{2+}] = 0$.

4) pH-stat experiments

At a fixed pH, located within the range of variation in pK observed on changing both the Na^+ and Mg^{2+} concentration, addition of Mg^{2+} involves, as a consequence of the lowering of their pK, a deprotonation of cytosine residues i.e. protons are released into the bulk solution from the polynucleotide. These protons can for instance be titrated by determining the amount of KOH necessary to restore the initial pH, i.e., the pH-stat method.

We show in fig.5 the results gathered from such experiments. In the first part of the experiment titration curves (1) and (2) of poly I. poly C are recorded at 20° for $[Na^+] = 5 \times 10^{-2} M$ and

$[P] = 2 \times 10^{-3} M$, respectively with $[Mg^{2+}] / [P] = 0$ and 0.5 . In the second step, to the solution (1) whose pH has been adjusted to 4.9 (point A), Mg^{2+} is added and the hydrogen ions released titrated by pH-stat. When $0.5 [Mg^{2+}] / [P]$ have been added it can be seen that the number of protons released corresponds quite closely to the difference AB on the titration curves. Such an experimental approach only used here for verification, could be a very useful one applied to complex systems involving inresolved pK, which are only stable over a very narrow pH range.

Figure 5 : Titration curves of poly I. poly C, $T = 20^{\circ}C$, $[Na^+] = 5 \times 10^{-2} M$ for : 1- $[Mg^{2+}] / [P] = 0$ and 2- $[Mg^{2+}] / [P] = 0.52$. pH-stat curve 3 : H^+ release as a function of $[Mg^{2+}] / [P]$ at pH 4.9 between $[Mg^{2+}] / [P] = 0$ (point A) and 0.52 (point B).

We previously showed that as a consequence of Ψ , the local pH in the neighbourhood of a polyanion, is lower than that of the bulk solution. Under the action of Mg^{2+} , Ψ vanishes so that the local pH becomes equal to the bulk pH, and the corresponding protons are released together with those coming from the bases. In order to detect them, we used poly U, a polynucleotide which is not protonated in the pH range 4 to 7. At pH 6 addition of Mg^{2+} did not involve any proton release $\frac{[H^+]}{[P]} < 10^{-3}$ as determined by the pH-stat ; their contribution is then negligible.

CONCLUSIONS

Taking synthetic polynucleotides, poly C and polyI. poly C, as models we determined the pK variations of the cytosine residues as a function of ionic strength. Such variations are the consequence of a local pH directly controlled by an electrostatic potential Ψ itself modified by changes in ionic strength. Specific ions such as Mg^{2+} are shown powerfully to modify Ψ and thus the pK, this process being presumably amplified in complex structures where high electrostatic potentials can be generated.

At a fixed pH, the pK variations as Mg^{2+} is added involve hydrogen ion release, which in complex systems can appropriately be studied in a pH-stat.

Important functional implications, follow from the action of Mg^{2+} and other specific ions on nucleic acids, according to the process described in this work :

1) variations in the pK of bases under the action of Mg^{2+} could control and regulate via changes in the extent of protonation, interactions between nucleic acids. It is a priori quite hazardous to make any predictions concerning the way in which stability is affected in complex systems involving a large number of pairings between protonated or deprotonated bases. However, it could be thought that in two complementary sequences, base protonation results in decreased stability as suggested by the finding that the melting temperature of DNA is lowered at acidic pHs (26). In this connection deprotonation, of a limited number of bases, could control the association of two complementary sequences, under Mg^{2+} action.

2) pK variations could also appear in the ionising groups of proteins interacting with nucleic acids ; Mg^{2+} in these cases might

affect both the interaction and enzymatic activity of these systems.

Studies on these possible implications are now under way in this laboratory.

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REFERENCES

- (1) Cox R.A. and Peacocke A.R. (1956) J.Chem.Soc., 2499-2512.
- (2) Michelson A.M., Massoulié J. and Guschlbauer W. (1967) Prog.Nucl.Acids Res. 6, 83-140.
- (3) Thiele D. and Guschlbauer W. (1969) Biopolymers 8, 361-378.
- (4) Giannoni G. and Rich A. (1964) Biopolymers 2, 399-413.
- (5) Holcomb D.N. and Timasheff S.N. (1968) Biopolymers 6, 513-529.
- (6) Lifson S. and Katchalsky A. (1954) J.Polym. Sc. 13, 43-53
- (7) Goldstein L., Levin Y. and Katchalski E. (1964) Biochem. 3, 1913-1919.
- (8) Maurel P. and Douzou P. (1976) J.Mol.Biol., in press.
- (9) Simpkins H. and Richards E.G. (1967) Biopolymers 5, 551-560.
- (10) Harvey A.E., Komarmy J.M. and Wyatt G.M. (1953) Anal.Chem. 25, 498-500.
- (11) Guschlbauer W. (1975) Nucl.Acids Res. 2, 353-360.
- (12) Kotin L. (1963) J.Mol.Biol. 7, 309-311.
- (13) Rich A., Davies D.R., Crick F.M.C. and Watson J.D. (1961) J.Mol.Biol. 3, 71-86.
- (14) Daune M. (1969) Biopolymers 7, 659-670.
- (15) Katchalsky A., Shavit N. and Eisenberg H. (1954) J.Polym. Sc. 13, 69-84.
- (16) Handbook of Biochem, C.R.C. Press 2nd Ed, p 98.
- (17) Lynch D.C. and Schimmel P.R. (1974) Biochem. 13, 1852-1860.
- (18) Schreier A.A. and Schimmel P.R. (1974) J.Mol.Biol. 86, 601-620.
- (19) Danchin A. (1972) Biopolymers 11, 1317-1333.
- (20) Römer R. and Hach R. (1975) Eur.J.Biochem. 55, 271-284.
- (21) Choi Y.S. and Carr C.W. (1967) J.Mol.Biol. 25, 331-345.
- (22) Morris D.R., Dahlberg J.E. and Dahlberg A.E. (1974) Nucl. Acids Res. 1, 1249-1258.
- (23) Handbook of Chem. and Phys. CRC Press, 54th Ed., p.E 60.
- (24) Rialdi G., Levy J. and Biltonen R. (1972) Biochem. 11, 2472-2479.
- (25) Ross P.D. and Shapiro J.T. (1974) Biopolymers 13, 415-416.
- (26) Zimmer C. and Venner H. (1966) Biopolymers 4, 1073-1079.