Correlation of Structural Transitions in Coliphage R17 with its Loss of Infectivity

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SUMMARY

A circular dichroism comparative study of isolated and in situ phage R17 RNA reveals in both cases the same degree of base pairing. However, thermal circular dichroism melting profiles exhibit the presence of free energy of interaction between RNA and capsid protein. It is apparent that the capsid stabilizes the RNA structure with and without the addition of Mg$^{2+}$. A close RNA capsid association is also derived from pH titration circular dichroism studies. The pH melting of the RNA in situ starts to occur about 0.5 pH unit higher with and without the addition of Mg$^{2+}$ than the acid denaturation of isolated RNA. A direct correlation between bathochromic CD peak shift of the main position band and loss of survivors is noted for the thermal melting as well as pH titration experiments. It is suggested that the heat and pH induced conformational alterations of R17 RNA in situ coinciding with loss of infectivity occur after an in situ alteration of nucleic acid-capsid protein interaction.

INTRODUCTION

Although the smaller RNA viruses exhibit some morphological simplicity, the interactions among the constituent components are still subject to extensive research. In the spherical f2 phage, an RNA phage of the same mol. wt. and dimensions as the phages belonging to the f2-group, it was observed that the circular dichroism (CD) of the virus is close to the sum of the CD contributions from the isolated RNA and protein components (Isenberg, Cotter & Gratzer, 1971). U.v. melting studies on f2 seemed also to rule out interactions between the protein shell and the double-stranded part of the RNA (Isenberg et al. 1971). However, some evidence for a possible protein-RNA interaction has been given for the small spherical phages R17 and MS2 (Remsen, Muller & Cerutti, 1970; Hartman, Clayton & Thomas, 1973; Slegers & Fiers, 1973; Turchinsky, Kusova & Budowsky, 1973), both belonging to the f2-group.

In the present study isolated and in situ R17 RNA were investigated by CD spectroscopy at variable temperature and as a function of pH. Direct evidence for some interaction between R17 RNA and its capsid is presented. In addition, comparing melting and pH titration CD curves obtained on R17 to survival plots of the phage revealed an interesting correlation, namely, at the temperature or pH where CD data indicate a loss of base pairs of R17 RNA in situ, a rapid decrease in the number of survivors occurs. It is likely that our conclusions based on R17 data are applicable to all phages in the f2-group.
METHODS

Bacteriophage growth, isolation, and purification. The host cell for growth and assay of the phage was *Escherichia coli* K 12 Hfr RNase- D10. The cells were grown in defined medium (Gesteland & Spahr, 1970) to a cell density of $4 \times 10^8$/ml, then infected with R17 at a multiplicity of 10 phage/bacterium. The phage was purified by the method of Gesteland & Spahr (1970).

Preparation of R17 RNA. RNA was prepared according to the procedure of Gesteland & Spahr, 1970. To check the absence of hidden breaks a fraction of each preparation was sedimented through a sucrose density gradient containing formaldehyde, as described by Boedtker (1968). The protein shell was obtained through short alkaline treatment of the R17 phage following the procedure of Samuelson & Kaeberg (1970).

Circular dichroism measurements. Ultraviolet circular dichroism measurements were made on a Cary Model 6002 circular dichroism attachment to a Cary 60 recording spectropolarimeter, equipped with a thermostated cylindrical cell holder. Results are reported either in terms of specific ellipticity, $[\gamma]$, or in terms of mean residue ellipticity, $[\theta]$, which is calculated for the bacteriophage on the basis of nucleotide residue molar concentration. We made use of the fact that R17 phage contains 30.6% RNA by weight (Gesteland & Boedtker, 1964). A mean residue weight of 342 was used for the RNA building blocks. The measurements were made in 1 or 0.5 cm cells and the concentration was about 0.1 mM (nucleotide residues). Constant nitrogen flushing was employed over the wavelength range examined (200 to 320 nm).

Circular dichroism melting studies. For the melting of R17 and R17 RNA the same method as described earlier for R17 RNA (Phillips & Bobst, 1972; Bobst, Pan & Phillips, 1974) was used.

Circular dichroism (CD) acid titration studies. The R17 and R17 RNA solutions (0.1 × SSC, pH 7.0) were mixed on a vortex after the addition of samples of 1 to 2 μl of 0.5 N HCl and then allowed to equilibrate for 10 to 30 min before pH determination. The pH was measured with an IL thin combination glass electrode and a Beckman expandomatic SS-2 pH meter. The pH was lowered until u.v. scans between 340 and 300 nm revealed the formation of turbidity. The CD of the solutions as a function of pH was monitored between 310 and 245 nm at 28 °C.

Determination of infectivity. The infectivity of R17 phage suspensions was determined as described by Remsen *et al.* (1970). The values for fractional survival as a function of temperature or pH were means of three experiments. For the pH study samples of the phage solutions used for the CD measurements were taken for infectivity determination. In the case of the thermal study R17 solutions of the same concentration were heated either in a CD cell located in a thermostated cylindrical cell holder for the CD measurement or in a separate test tube for the infectivity determination.

RESULTS

CD of R17 and R17 RNA

Some CD data on R17 RNA have already been reported (Phillips & Bobst, 1972; Bobst *et al.* 1974). In Fig. 1 the mean residue ellipticity based upon the RNA building blocks is given for both the R17 phage and the R17 RNA. Typical non-conservative CD curves are observed for both the isolated and *in situ* RNA. Addition of Mg$^{2+}$ increases the intensity of the main positive band significantly, the change being slightly greater for R17 RNA than for
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Fig. 1. Circular dichroism in 0.1 x SSC, 4 x 10^{-3} M-Mg^{2+}, pH 7.0, 28 °C. ▲—▲, R17 phage; △—△, R17 RNA. [θ] is the mean residual ellipticity of the RNA components.

Table 1. Circular dichroism data of R17 and its RNA with and without addition of 4 x 10^{-3} M-MgCl_2 at 28 °C

<table>
<thead>
<tr>
<th>System</th>
<th>R17</th>
<th>R17 RNA</th>
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<tbody>
<tr>
<td>0.1 x SSC, pH 7.0</td>
<td>λ(nm)</td>
<td>[θ]</td>
</tr>
<tr>
<td></td>
<td>266</td>
<td>22700 ± 500</td>
</tr>
<tr>
<td>0.1 x SSC, pH 7.0, 4 x 10^{-3} M-MgCl_2</td>
<td>266</td>
<td>23800 ± 500</td>
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R17 (Table 1). It is also apparent from Fig. 1 that the CD spectra of R17 phage and its RNA differ significantly below 250 nm. This difference is due to the protein contribution to the CD spectrum. As in the case of μ2 (Isenberg et al. 1971) and f2 (Henkens & Middlebrook, 1973) the partial specific ellipticities of the R17 components were determined (not shown). It was observed that the CD of the R17 virus can be mimicked by the sum of the RNA and capsid CD contribution as reported earlier for μ2 and f2.
CD melting studies

The intensity and position of the peak of the main positive band were monitored as shown in Fig. 2 and 3. In Fig. 2 [θ] at 266 nm of free and in situ R17 RNA is reported on the left ordinate as a function of temperature in 0.1 × SSC without the addition of Mg²⁺, whereas Fig. 3 gives the data in the presence of 4 × 10⁻³ M-Mg²⁺. On the right ordinate of these two figures the shift of the peak ellipticity is plotted. Without the addition of Mg²⁺ the loss in circular dichroism as well as the red shift are of sigmoidal character for the isolated RNA. Both curves reveal a Tₘ at about 46 °C. In case of in situ RNA the variation of the mean residue ellipticity shows several melting regions with different co-operativity. The effect of the temperature on the position of the peak also reveals a marked difference for the RNA in situ. No bathochromic shift of the peak occurs up to about 37 °C.

Addition of 4 × 10⁻³ M-Mg²⁺ to the system alters the melting profiles significantly. None of the curves have sigmoidal character and the transitions are shifted towards considerably higher temperature. Analysing the variation of the mean residue ellipticity at 266 nm for free RNA, two melting regions are apparent (one below 50 °C having less of a slope than the one above 50 °C). For the RNA in situ the [θ] at 266 nm remains virtually unchanged up to 50 °C. Above 60 °C a broken line is given for the melting profile, because the solution starts to become turbid at this temperature. The bathochromic shift of the peak begins for the in situ RNA at about the same temperature as the drop in [θ]266. The free RNA exhibits at lower temperature two small cooperative peak shifts with transition midpoints at about 37 and 46 °C which are followed by a larger one with a transition midpoint greater than 65 °C. Thus, the CD melting profiles shown in Fig. 2 and 3 indicate unequivocally that the RNA denatures differently in and out of the virus. This is true in a buffer system which lacks or contains a well-defined amount of Mg²⁺.

In order to detect possible heat-induced conformational changes in the protein, the 250 to 200 nm CD region of the phage was monitored as a function of temperature. The thermal profiles are shown in Fig. 4 without the addition of Mg²⁺ and in Fig. 5 in the presence of 4 × 10⁻³ M-Mg²⁺. If Mg²⁺ is not added to the solution, the large negative band at about 215 to 217 nm undergoes no significant changes in the temperature range of 27 to 72 °C except for a small loss of intensity (Fig. 4). Addition of Mg²⁺ does not reveal any cooperative CD transitions allowing a correlation with previously seen R17 RNA transitions, or a marked loss of infectivity which occurs at well-defined temperatures, as will be shown subsequently. The Mg²⁺ seems to promote precipitation since it was noticed that above 60 °C the protein band vanished and the solution became turbid.

CD pH titration

The pH was lowered until precipitation of the whole phage could be detected. Turbidity began to form about pH 4.3 without the addition of Mg²⁺, whereas the presence of 4 × 10⁻³ M-Mg²⁺ allowed the phage solution to be titrated slightly below pH 4 before apparent absorption between 340 and 300 nm occurred. Fig. 6 and 7 give the results of [θ]266 versus pH on the left ordinate and peak of main positive band versus pH on the right ordinate with and without the addition of 4 × 10⁻³ M-Mg²⁺. The [θ]266 of R17 RNA in 4 × 10⁻³ M-Mg²⁺ does not change until pH 4.8 is reached. Only thereafter a strong decrease of the main positive band occurs. The peak shift will take place at an even lower pH of about 4.3. If the titration is carried out under the same condition for the RNA in situ, the overall shape of the two curves is similar to the ones recorded for the free RNA except for the pH range at which the CD spectrum starts to change. Both the change in [θ]266 as well
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Fig. 2. Circular dichroism melting curves of R17 phage and R17 RNA in 0.1 × SSC, pH 7. Two parameters are plotted: on the left-hand ordinate the peak residue ellipticity at 266 nm, for R17 phage (●●●) and R17 RNA (▲▲▲); on the right-hand ordinate the red shift of the peak of the intense positive band, for R17 phage (○○○) and R17 RNA (△△△).

Fig. 3. Circular dichroism melting curves of R17 phage and R17 RNA in 0.1 × SSC, 4 × 10⁻³ mM-Mg²⁺, pH 7. Two parameters are plotted: on the left-hand ordinate the peak residue ellipticity at 266 nm, for R17 phage (●●●) and R17 RNA (▲▲▲); on the right-hand ordinate the red shift of the peak of the intense positive band, for R17 phage (○○○) and R17 RNA (△△△).
Fig. 4. Circular dichroism spectra of R17 phage in 0.1 × SSC, pH 7, at various temperatures between 210 and 250 nm. •——•, 27; O——O, 33 °; ▲——▲, 37 °; △——△, 44 °; ▼——▼, 72 °C.

Fig. 5. Circular dichroism spectra of R17 phage in 0.1 × SSC, 4 × 10⁻⁸ M-Mg²⁺, pH 7, at various temperatures between 210 and 250 nm. •——•, 29; O——O, 33 °; ▲——▲, 37 °; △——△, 45 °; □——□, 50; ▼——▼, 54 °; ▼——▼, 72 °C.
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Fig. 6. Circular dichroism pH titration curves of R17 RNA and R17 phage in 0.1 x SSC. Two parameters are plotted: on the left-hand ordinate the peak residue ellipticity at 266 nm, for R17 RNA (△ — △) and R17 phage (● — ●); on the right-hand ordinate the red shift of the peak of the intense positive band for R17 RNA (△ — △) and for R17 phage (○ — ○).

Fig. 7. Circular dichroism pH titration curves of R17 RNA and R17 phage in 0.1 x SSC with 4 x 10⁻³ M-Mg²⁺. Two parameters are plotted: on the left-hand ordinate the peak residue ellipticity at 266 nm, for R17 RNA (△ — △) and for R17 phage (● — ●); on the right-hand ordinate the red shift of the peak of the intense positive band for R17 RNA (△ — △) and for R17 phage (○ — ○).
Fig. 8. Effect of heating R17 phage on its circular dichroism and its infectivity in 0·1 x SSC, pH 7, (a) without and (b) with the addition of 4 x 10^{-3} M-Mg^{2+}. ▲—▲, ●—●, log fraction of R17 survivors present at the time corresponding to the circular dichroism recordings taken at the various temperatures. ▲—▲, ○—○, red shift of the intensive positive circular dichroism peak.

as the peak shift occur approx. 0·5 pH units earlier. Without the addition of Mg^{2+} a completely different set of curves is obtained, although the same characteristic phenomenon is observed as before, namely the CD changes resulting from lowering the pH occur again for R17 RNA in situ earlier than for the free RNA. Monitoring [θ]_{266} for R17 as well as R17 RNA reveals multiphased curves. R17 RNA exhibits a small transition at about pH 5·8. Below pH 5·2 the 266 nm CD band strongly diminishes with a simultaneous bathochromic shift of its peak. With respect to R17 RNA in situ, two small transitions of [θ]_{266} at about pH 6·4 and 5·8 are measured. The rapid loss of the rotation at 266 nm occurs after pH 5·4. As with the isolated R17 RNA the sharp decrease in intensity is accompanied by a bathochromic shift of the peak.
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In order to check if some of the CD characteristics are related to the viability of the phage, its thermal and acid inactivations were determined by the agar layer method.

For thermal inactivation non-linear semi-logarithmic survival plots were observed with and without the addition of Mg$^{2+}$ (Fig. 8). Until 37 °C there was virtually no loss in the number of survivors without the addition of Mg$^{2+}$, whereas $4 \times 10^{-3}$ M-Mg$^{2+}$ extended the range of no loss to about 45 °C. Above 37 and 45 °C a rapid linear decrease in infectivity was observed with approx. the same slope without and with addition of Mg$^{2+}$, respectively. Taking into account the peak shift CD data shown in Fig. 2 and 3 one obtains an interesting relationship. In both cases the decrease in the amount of survivors starts to take place at the temperature at which one observes the beginning of the bathochromic shift of the CD peak.

A similar correlation between CD peak shift and loss of survivors was noted with the acid inactivation of R17 (not shown). In the presence of $4 \times 10^{-3}$ M-Mg$^{2+}$ there was virtually no change in survivors between pH 7.2 and 4.7. At pH 4.7 a steep linear decrease in infectivity would set in. Comparing this inactivation result to the CD pH titration curve of R17 with $4 \times 10^{-3}$ M-Mg$^{2+}$ (Fig. 7) reveals that the abrupt bathochromic shift of the peak of the main positive band begins at the pH of rapid loss of infectivity, namely around pH 4.7.

It should be emphasized that Fig. 8 does not imply that the log fraction of survivors is linked through a linear relationship to the CD peak shift, only that a decrease in the number of survivors is noticeable at the same temperature or pH at which CD reveals the beginning of a bathochromic peak shift. Nothing further can presently be deduced from the experiments, since it is well known that a considerable amount of R17 particle infectivity is lost during the isolation procedure. Although it could be argued that only the damaged particles contribute to the CD effect, this seems to be unlikely in view of the correlation shown in Fig. 8, which is observed with and without the addition of Mg$^{2+}$.

**DISCUSSION**

It is well established that CD is a sensitive probe to gain insight into the forces stabilizing an RNA structure (Brahms & Mommaerts, 1964). The optical rotatory dispersion studies of Tinoco and his co-workers have made it feasible to analyse RNA conformations (Cantor, Jaskunas & Tinoco, 1966; Warshaw & Tinoco, 1966). It is assumed that the separation of paired bases causes a red shift in the position of the Cotton effect with only a minor change in its magnitude. The decrease in magnitude can on the other hand be attributed to the disruption of stacking interactions.

The fact that the algebraic addition of the partial specific ellipticity of R17 RNA and its capsid results in a CD spectrum virtually identical to the R17 phage spectrum corroborates previous observations on υ2 and f2 (Isenberg et al. 1971; Henkens & Middlebrook, 1973). It can be concluded that there are no major conformational alterations of the macromolecular components during assembly of the phage of the f-2 group. However, the dissimilarity of the CD melting and pH titration profile found between isolated and *in situ* R17 RNA suggests the presence of protein-RNA interactions in this phage. For MS2 some evidence was presented indicating an interaction of the A protein with capsid proteins or RNA, the latter possibility being favoured (Verbraeken & Fiers, 1972a). The bathochromic shift observed for R17 at about 37 °C could reflect the loss of partial stabilization by the A protein of double-stranded RNA regions. The addition of $4 \times 10^{-3}$ M-Mg$^{2+}$ to the R17 solution (Fig. 3) extends the temperature region of R17 RNA stabilization to about 50 °C.
Studies concerning the swelling of the cowpea chlorotic mottle virus at various pH suggested that the RNA structure may play a role in maintaining the stability of this virus particle (Adolph, 1975). Titration of R17 RNA in situ without the addition of Mg\(^{2+}\) reveals an additional small [\(\theta\)]\(_{266}\) change at about pH 6.4 which is missing in the absence of phage protein (Fig. 6). It is likely that the CD change is caused by abnormal proton binding between juxtaposed cytidine and amino acid carboxylate ions in the phage. Lowering the pH further results in a small CD intensity change at about pH 5.8, which is seen for the RNA in and out of the virus. The small [\(\theta\)]\(_{266}\) decreases are suppressed when the R17 RNA solution contains 4 x 10\(^{-3}\) M-Mg\(^{2+}\) (Fig. 7). The most apparent difference in the titration pattern of free and in situ R17 RNA, however, is noticeable in the pH range of the strong CD changes. To wit, lowering the pH further causes a sharp decrease of the main positive bands (Fig. 6 and 7). Fig. 6 shows that the changes in situ occur at a pH which is about 0.5 pH units higher than in the case of free RNA. The presence of 4 x 10\(^{-3}\) M-Mg\(^{2+}\) (Fig. 7) does not suppress this phenomenon of early RNA ‘pH melting’ ascribed to RNA–protein interaction. The sharp loss in intensity as well as the bathochromic shift is believed to reflect the opening of A-U base pairs. Slegers & Fiers (1973) have shown that poly (A, U) loses its Watson–Crick type secondary structure upon lowering the pH below pH 4.8 in 10\(^{-2}\) M-NaCl and 6 x 10\(^{-5}\) M-MgCl\(_2\).

Although it has been well established that the A protein is required for infectivity (Roberts & Steitz, 1967; Steitz, 1968a), monitoring the protein part of the CD spectrum gave no conclusive results with respect to possible conformational changes in the protein shell as a function of temperature (cf. Fig. 4 and 5). Since the A protein is only about 1 to 2 % of the total phage protein, its contribution to the overall CD spectrum is relatively small. However, monitoring the RNA part of the CD spectrum led us to discover an interesting relationship with and without the addition of 4 x 10\(^{-3}\) M-Mg\(^{2+}\). In both cases, the decrease in the amount of survivors starts to take place at the temperature at which the CD peak undergoes a bathochromic shift (Fig. 8). As mentioned earlier, a red shift of the peak position of the main positive band is indicative of base pair breakage. Thus, the CD data imply a direct relationship between base pair melting of in situ RNA and phage viability. The observed correlation between temperature induced CD peak shift and loss of infectivity is of interest in view of reports indicating the cooperative formation of expanded phage particles. Based on sedimentation studies a conversion to an expanded phage was noticed to take place within a narrow temperature range for R17 (Steitz, 1968c) as well as for MS2 (Verbraeken & Fiers, 1972b). It is also known that the A protein, always present in infective particles, remains on the expanded particles (Steitz, 1968c; Verbraeken & Fiers, 1972b). Fig. 8 shows that the temperature at which loss of infectivity starts to be noticeable coincides with RNA base pair cleavage. It is possible that the CD data reflect partial extrusion of RNA occurring concomitantly with its loss of double helical regions making the RNA vulnerable to the action of traces of nucleases, leading thereby to non-infective particles. The melting of double helical RNA regions could be due to a loss of stabilization by the A protein. An A protein RNA interaction has been postulated, based on the observation that RNA as well as associated A protein can penetrate into a host cell (Kozak & Nathans, 1971; Paranchych et al. 1971). Thus, the temperature at which bathochromic shift and loss of infectivity (Fig. 8) occur could be the result of a loss of A protein-RNA interaction producing nuclease sensitive particles. It is known that amber mutants in the A protein cistron have an intact RNA which is very sensitive to nuclease (Steitz, 1968a, b).

The problem of understanding the acid inactivation in molecular terms is complex, too. It is known that the range of phage resistance to changes in the pH of the medium lies
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between about pH 5·0 to 8·0 (Adams, 1959). In contrast to thermal inactivation f2-group phage particles seem not to expand upon acidification. Schubert (1969) observed with electron microscopy and ultracentrifugation that fR phage as well as the reaggregated protein shells are stable against diluted HCl of pH 1·8. Our observation that loss of R17 infectivity occurs simultaneously with 'pH melting' of base pairs should be discussed in view of data reported by Kaper (1969) for TYMV. There, a loss of RNA–RNA links in favour of the formation of protein–RNA complexes at about pH 4·5 was measured by ultracentrifugation. A similar process could also take place in R17 as discussed earlier in the RNA–protein interaction section. Such structural rearrangement or a subsequent irreversible change taking place as a consequence of the breakage of RNA–RNA interactions seems to induce the formation of non-infective particles.

In summary, CD temperature melting curves as well as CD titration data on R17 reveal the existence of some subtle interactions between R17 RNA and its capsid. While the present study does not provide detailed information on the nature of the interaction, it is evident that the capsid affects RNA base pair melting. Furthermore, the onset of base pair melting in situ is followed by a loss of infectivity. What the exact cause for the loss of infectivity could be is not proven, although it is suggested that the RNA becomes nuclease sensitive as a result of its unwinding. It is postulated that proteins of the capsid, in particular the A protein, interact with R17 RNA and thereby play an important role in stabilizing its in situ structure.

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