Thermal denaturation of *B. subtilis* DNA in H₂O and D₂O observed by electron microscopy

Vincenzo Izzo, Sandro L. Fornili and Lorenzo Cordone

Istituto di Fisica dell'Universita and Gruppi Nazionali Struttura della Materia
of the Consiglio Nazionale delle Ricerche, Via Archirafi 36, 90123 Palermo, Italy¹

Received 11 August 1975

ABSTRACT

In the present work we studied the initial part of thermal denaturation curves of *B. subtilis* DNA in normal and heavy water, observing, by electron microscopy, the "opening" of DNA as a function of temperature. The results support the hypothesis that D₂O plays a stabilizing role on strands separation during thermal denaturation of DNA.

INTRODUCTION

The effects of thermal denaturation due to substitution of D₂O for H₂O as a solvent have been studied both for artificial and naturally occurring nucleic acids by several authors.

For artificial double stranded polyA.polyU, Klump² reported spectrophotometric and calorimetric data indicating that thermal denaturation curves in H₂O and D₂O were superimposable in shape, being the D₂O curve shifted by about 2°C upwards in temperature. Cupane *et al.*³, using spectrophotometric techniques, extended these investigations to solutions with different D₂O to H₂O ratios, finding also in this case superimposable melting curves, shifted in the temperature scale. The magnitude of the shift increased as a function of the relative D₂O concentration, up to about 2°C for 100% D₂O.

For naturally occurring DNA's, Maslova and Varshavsky⁴, using an elution technique, found about 2°C difference between elution profiles of DNA-DNA hybrids when H₂O was substituted by D₂O, whereas Crespi and Katz⁵ and Mahler *et al.*⁶, using spectrophotometric techniques, found no difference in thermal denaturation. Recently, however, Blake⁷ reexamined the loss of U.V. hypochromicity near 260 nm. for various nucleic acids, using computer assisted methods, and found a 1.6°C difference between melting temperatures in D₂O and H₂O.

In the present paper we have studied by electron microscopy the "opening" of DNA during thermal denaturation in H_2O and in D_2O , in order to see how isotopic substitution influences the appearance of partially denatured regions on B. subtilis DNA.

MATERIALS AND METHODS

Media - H_2O was ultrapure water (Super Q, Millipore). D_2O , 99.8%, was purchased from Prochem (London).

20xSE medium was 0.4 M NaCl, 0.1 M Na_2EDTA , adjusted to a pH value such that its twentyfold dilution into H_2O (1xSE) had pH 7.4; heavy 1xSE was made by dilution of 20xSE twenty times into D_2O . Light and heavy SEF media were obtained through twentyfold dilution of 20xSE into 4.6% formaldehyde (R.G., Ruidipont) solution in H_2O or D_2O respectively. Formaldehyde was previously neutralized (pH 7.8) with NaOH and heated for 10 min. at $100^\circ C$. The pH value of light SEF medium (7.5 at $25^\circ C$), measured as a function of temperature, did not show relevant changes up to $70^\circ C$.

DNA Isolation - B. subtilis 168 M was grown, under good aeration at $37^\circ C$, to the end of exponential phase in Spizizen's salts⁸ supplemented by 0.5% glucose, 0.02% Vitamin free Casamino Acids (Difco) and 50 $\mu g/ml$ tryptophan. Bacteria were then harvested, washed three times with cold Tris EDTA buffer (0.1 M Tris (Sigma), 0.01 Na_2EDTA (Sigma), pH 8.0) and resuspended into the same buffer with 0.3 M sucrose and 1 mg/ml lysozyme (Egg White, 3x, Sigma). This mixture was incubated at $37^\circ C$ for 10 min. and lysed with 0.3% sarcosyl (Geigy Industrial Chemicals, Ardley, N.Y.). The lysate was then incubated overnight at $40^\circ C$ with 1 mg/ml pronase (S. griseus, Serva, Heidelberg). DNA purification was carried out by isopycnic separation in CsCl (Suprapur, Merck) gradient. Fractions containing DNA were pooled and extensively dialyzed against 1xSE medium.

Preparation and Temperature Treatment of DNA Samples - Light and heavy DNA samples were prepared by dilution, into light and heavy 1xSE respectively, of a concentrated DNA solution to a final concentration of about 5 $\mu g/ml$. These samples were then incubated overnight at $40^\circ C$ in order to allow DNA in heavy me-

dium to undergo a complete hydrogen exchange⁹. The above DNA samples were then diluted four times into light or heavy SEF medium respectively in thin-walled glass vials. The final concentration of D₂O in heavy samples so obtained was about 87% and that of formaldehyde was about 3.5% in both heavy and light samples. The vials were then sealed and heated for 15 minutes at various temperatures (constant to 0.05°C). After heating samples were rapidly chilled to 0°C.

Electron Microscopy - A volume of 10 μ l of a freshly prepared 0.1 M Na₂CO₃ solution was added to 0.1 ml formamide (R.G., Matheson Coleman and Bell) at 0°C. The pH of this solution was 7.4 and did not change more than 0.2 units within 30 minutes (maximum time interval needed in our experiment to accomplish all e.m. operations but shadowing). To a 5- μ l volume of this mixture were added, at 0°C, 5 μ l of treated DNA solution and 1 μ l of 0.1% solution of cytochrome c (Type VI, Sigma). Spreading was carried out as described by Inman and Schnöbs¹⁰, and DNA-protein films were picked up using collodion (Serva) coated grids, stained with uranyl acetate and rotary shadowed at 6° angle with Pt-Pd (80/20). A Zeiss EM9 electron microscope was used. The micrographs, enlarged 100,000 times, were measured using a curvimeter.

RESULTS AND DISCUSSION

Plates 1 and 2 show partially denatured DNA molecules, in presence of 3.5% formaldehyde (see Materials and Methods), in H₂O and D₂O, at 59°C. In Fig.1 we report the percent fraction of open DNA, in H₂O and D₂O, as a function of temperature.

If the curve which fits experimental points for denaturation in light medium is shifted about 1.5°C upward in temperature, it also fits very well the experimental points for denaturation in heavy medium. These results are in agreement on one side with data previously reported by Maslova and Varshavsky⁴ on strands separation during thermal denaturation of DNA-DNA hybrids, and on the other with the results recently reported by Blake⁷. Although the microscopic processes involved may be different, this fact parallels the stabilizing role played by deuterium during thermal denaturation of proteins^{11,12} and

Plate 1 - Electron micrograph of partially denatured DNA in H_2O at $59^\circ C$.

Plate 2 - Electron micrograph of partially denatured DNA in D_2O at $59^\circ C$.

the effects of D_2O on the association-dissociation equilibrium in subunits proteins¹³.

Fig. 1 - Initial part of thermal denaturation curves of B. subtilis DNA in H₂O and D₂O (87%) in presence of 3.5% of formaldehyde.

As described in Materials and Methods, in our experiments we have used formaldehyde (needed for e.m. visualization) which blocks the "open" DNA regions caused by thermally induced "breathing"¹⁴. In order to ascertain whether the isotope effect we found is to be related to the presence of formaldehyde, spectrophotometric measurements are being carried out in our laboratory on thermal denaturation of B. subtilis DNA in H₂O and D₂O in presence and in absence of formaldehyde.

ACKNOWLEDGEMENTS

We like to thank Prof. M.U. Palma and Dr. R.M. Sperandio-Mineo for useful discussions and criticism. We like also to thank Prof. G. Giudice for having allowed us to use the electron microscope at Istituto di Anatomia Comparata (Palermo) and Dr. I. Pucci-Minafra for having introduced us to the use of the Zeiss EM9 electron microscope.

REFERENCES AND NOTES

- 1 General indirect support to this work was provided by Comitato Regionale Ricerche Nucleari, Palermo.
- 2 Klump, H. (1972) Biopolymers 11, 2331 - 2336
- 3 Cupane, A., Palma, M.U. and Vitrano, E. (1973) in Atti I Riunione Scientifica Plenaria - Società Italiana di Biofisica Pura ed Applicata, pp. 91 - 103
- 4 Maslova, R.N. and Varshavsky, Ya.M. (1966) Biochim.Biophys.Acta 119, 633 - 636
- 5 Crespi, H.L. and Katz, J.J. (1962) J.Mol.Biol. 4, 65 - 68
- 6 Mahler, H.R., Dutton, G. and Mehrotra, B.D. (1963) Biochim. Biophys.Acta 68, 199 - 210
- 7 Blake, R.D. (1975) Abstract 19th Annual Meeting of Biophysical Society, p. 136a
- 8 Spizizen, J. (1958) Proc.Nat.Acad.Sci. USA 44, 1072 - 1078
- 9 Englander, J.J. and von Hippel, P.H. (1972) J.Mol.Biol. 63, 171 - 177
- 10 Inman, R.B. and Schnöls, M. (1970) J.Mol.Biol. 49, 93 - 98
- 11 Maybury, R.H. and Katz, J.J. (1956) Nature 177, 629 - 630
- 12 Hermans, J.jr. and Scheraga, H.A. (1959) Biochim.Biophys.Acta 36, 534 - 535
- 13 Henderson, R.F., Henderson, T.R. and Woodfin, B.M. (1970) J.Biol.Chemistry 245, 3733 - 3737
- 14 von Hippel, P.H. and Wong, K.Y. (1971) J.Mol.Biol. 61, 587 - 613.