BINDING OF PROFLAVINE AND ETHIDIUM BROMIDE TO TWO FORMS OF T2 BACTERIOPHAGE WITH DIFFERENT SEDIMENTATION COEFFICIENTS

Phillip A. Sharp* and Victor A. Bloomfield**

Department of Chemistry and Chemical Engineering
University of Illinois, Urbana, Illinois 61801

Received March 16, 1970

SUMMARY

Equilibrium and kinetic studies of binding of proflavine and ethidium bromide to T2 phage indicate that (1) most of the intraphage DNA is accessible to proflavine; (2) the bulkier ethidium cation binds to a limited amount of DNA within the phage head; (3) intraphage DNA and free DNA in solutions of high ionic strength have similar equilibrium constants for binding to proflavine and ethidium; (4) on a time scale of minutes to hours, there is little difference in the permeabilities of the fast sedimenting (1000 S) and slow sedimenting (700S) forms of T2 phage as measured by proflavine binding.

Bacteriophage T2 has been found[1,2] to have two forms which sediment with different sedimentation coefficients. The 1000 S form is favored by low pH, low temperature, and divalent cations such as Zn^{+2} and Ca^{+2}. The 700 S form is favored by high pH and temperature[3]. Two major explanations have been given of the structural basis of this difference in sedimentation coefficients. Bendet et al[4] suggested that the phage tail fibers were extended in the 700 S form and contracted in the 1000 S form. Cummings and Kozloff[3,5] attributed the difference to a somewhat greater head size, and much greater head permeability, in the 700 S form. The head permeability was measured indirectly by the rate of photoinactivation by

*Present Address: Department of Chemistry, California Institute of Technology.

**Alfred P. Sloan Foundation Fellow. Present address: Department of Biochemistry, University of Minnesota, St. Paul.

methylene blue[5]. Theoretical studies [6,7] have indicated that neither permeability changes nor head size changes of the extent seen electron microscopically[3] can account for the differences in S; and macroscopic model experiments[8] suggest that tail fiber orientation is responsible for most of this difference.

However, the permeability of the T2 head protein coat is still of substantial interest, both because of the implications for the arrangements of protein subunits in the head, and because of the subsequent binding of molecules which, crossing the head membrane to the DNA inside, can give information about the state of DNA in intact virus. We have, therefore, undertaken studies of the binding of the dyes proflavine and ethidium bromide to the 700 S and 1000 S forms of T2. These studies, carried out by difference spectroscopy and fluorimetry, provide a direct measure of permeability and of equilibrium and kinetic binding parameters. They thereby permit checking of the assumption that the rate of photoinactivation is a reflection of the permeability of the protein coat.

Materials and Methods

Ethidium bromide and proflavine dihydrochloride were purchased from Calbiochem, and used without further purification. Calf thymus DNA (Worthington) was twice extracted with distilled phenol before using. T2 DNA was obtained by four extractions of T2 phage with distilled phenol. DNA concentrations were determined spectrophotometrically.

T2 phage was obtained from lysates of E. coli BB infected with virus at a ratio of 1 phage per 5 bacteria. The lysate was treated with DNase overnight at 4° and then centrifuged at 2000 rpm in a Servall at 4° to remove cellular debris. The salt concentration of the lysate was reduced by dialysis against three volumes of water, and the phage was banded on a DEAE cellulose column. The eluted phage were twice treated with DNase and pelleted by centrifuging in a Servall for 1 hour at 10,000 rpm. The

resulting resuspended solution had a spectrum similar to that found for T2 by Herriott and Barlow[9], whose determination of A_{260} for a 10^{11} phage/ml solution was used by us to obtain virus concentrations. T2 ghosts were prepared by the method of Herriott and Barlow[9]; ghost concentrations were determined by comparison with the spectrum they published.

Two buffers were used, both of ionic strength 0.1 M: .0795 M KH_2PO_4, .0069 M Na_2HPO_4, 10^{-3} M Mg^{+2}, pH 5.8; and .00575 M KH_2PO_4, .0314 M Na_2HPO_4, 10^{-3} M Mg^{+2}, pH 7.6. At 9 x 10^{11}/ml, intact phage sedimented at S_{20} = 984S in the pH 5.8 buffer and at 768S in the pH 7.6 buffer, while ghosts sedimented at 276S at pH 5.8. These values agree well with those obtained by others[10].

Proflavine binding studies were done by difference spectroscopy at 430 mμ, using a Cary 15 with constant temperature cell block. Ethidium binding was measured by fluorescence emission at 595 mμ upon excitation at 360 mμ, using a Baird-Atomic Fluorispec SF-1. Binding equilibrium was reached in 4-8 hours. Equilibrium binding data were analyzed with the Scatchard[11] equation to obtain the association constant K and the percentage N of nucleotides available as binding sites. The analysis assumed that the spectral properties of the dyes were the same when bound to free DNA or intraphage DNA.

Results and Discussion

Table I summarizes the binding parameters determined for the interaction of proflavine and ethidium bromide with free DNA and with the two forms of T2. Temperatures were 20-21° in all cases. It is apparent that, for proflavine, there is no gross difference between the equilibrium binding parameters of the two forms of the virus, or between virus and free DNA. The fraction of nucleotides available for binding proflavine seems to be reduced by at most a factor of two by the encapsulation of the DNA within the phage head, and the dye-nucleotide association constant is comparable to that measured for free DNA in high salt[12].

TABLE I

EQUILIBRIUM BINDING PARAMETERS
FOR T2-DYE INTERACTION

	N, %	K x 10^{-5}, M^{-1}
Proflavine		
Free DNA[a] (pH 5.8)	10-20[12]	1-2[b]
T2, pH 7.6	10	1.1
T2, pH 5.8	9	.94
Ethidium Bromide		
Free DNA[a], pH 7.6	17	11±1
Free T2 DNA, pH 5.8	15	7.2
T2, pH 7.6	0.5	3.0
T2, pH 5.8	0.35	3.0

a. T2 DNA and calf thymus DNA gave nearly identical results.

b. Obtained from $NK = 2.3 \times 10^4$ M^{-1} determined by the method of Benesi and Hildebrand[13], and N=10-20% found by Peacocke and Skerrett[12]

It can be argued that the proflavine binding is not due to a disruption of the phage during incubation. If this were the case, 50% of the phage would have to be disrupted and liberate DNA in order to obtain the amount of binding observed. This would entail a 50% decrease in phage titer during incubation of dye with phage in the dark. No evidence of such dark phage inactivation has been observed under our experimental conditions.

The situation is somewhat different for ethidium binding. The binding constant with intraphage DNA is somewhat lower than that with free DNA. The values of K are in reasonable agreement with those found by LePecq and Paoletti[14] for ethidium bromide-free DNA binding in high salt media. The most striking difference, however, is in the number of binding sites in intraphage DNA which is reduced by a factor of 30-50 relative to free DNA. Intraphage DNA seems to be markedly less accessible to ethidium than to proflavine.

Since the amount of ethidium bound is small, the possibility was

investigated that the observed binding was due to contaminating phage ghosts, free DNA, E. coli bodies, or defective or unusual phage. The ethidium binding reported in Table I cannot be attributed to the presence of any of these.

The kinetics of uptake of proflavine by T2 phage also appears to be roughly independent (to within a factor of two) of the state of the virus, as shown in Fig. 1. The rate of diffusion of the dye into and through the phage head is similar, on a time scale of several minutes to several hours, at pH 7.6 and pH 5.8. This rate of dye uptake was also independent of the contaminants listed in the preceding paragraph.

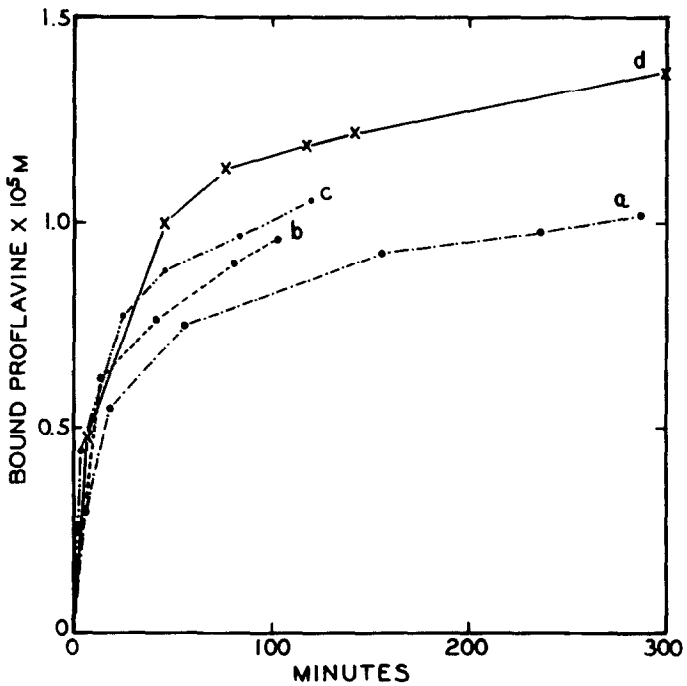

Figure 1: Amount of proflavine bound to T2 phage as a function of time. The small amount of binding due to free DNA and E. coli contaminants has been subtracted from such curve. Total proflavine concentration for each curve was 2.61×10^{-5} M. The virus concentration in particles/ml was: at pH 5.8 (a) 9.8×10^{11}, (b) 10.0×10^{11}; at pH 7.6 (c) 9.2×10^{11}, (d) 9.8×10^{11}.

In conclusion, the equilibrium and kinetics studies reported above indicate that there are no gross differences in the permeabilities of the pH 7.6 and pH 5.8 forms of T2 phage to proflavine and ethidium dyes,

at least on a time scale of minutes to hours. Similar results were reported by Cummings and Kozloff[5] for methylene blue over a somewhat longer time span (4 hours for equilibrium dialysis and 30 hours incubation for photoinactivation). The binding constants are similar in the two forms and in free DNA at high salt. Ethidium bromide binds to a limited amount of DNA within the phage head (about 0.5%, or 2000 bases), presumably because the diffusion of this rather bulky molecule into the head is restricted. The more compact proflavine diffuses into the phage head more readily than ethidium, and most of the DNA is accessible to it.

The results with proflavine are in striking variance with the finding by Cummings and Kozloff[5] that the pH 7.6 form of T2 is photoinactivated at a 20-30 fold greater rate than the pH 5.8 form, after incubation with methylene blue for one hour, if photoinactivation is correlated with binding to DNA. Methylene blue and proflavine are similar in size and shape, and would be expected to enter the phage head at similar rates. The discrepancy may perhaps be explained by hypothesizing that the primary site of photodynamic action of methylene blue is on the protein coat. This explanation is consistent with the observation by Cummings[15] that methylene blue photooxidation alters the imidazole ring of histidine and eliminates the long to short head transition in T2, which has been shown[5] to be necessary for the infectivity of the pH 7.6, long head form of the virus.

Acknowledgements

This research was supported in part by NIH Research Grant GM12555 and NIH Biophysical Training Grant 2G 722. We thank Professor James Wetmur for electron microscopic examination of the phage preparation.

References

1. A. E. Hook, D. Beard, A. R. Taylor, D. G. Sharp, and J. W. Beard, J. Biol. Chem. 165, 241 (1946).
2. D. G. Sharp, A. E. Hook, A. R. Taylor, D. Beard, and J. W. Beard, J. Biol. Chem. 165, 259 (1946).

3. D. J. Cummings and L. M. Kozloff, Biochem. Biophs. Acta 44, 445 (1960).
4. I. J. Bendet, J. L. Allison, and M. A. Lauffer, Virology 6, 571 (1958).
5. D. J. Cummings and L. M. Kozloff, J. Mol. Biol. 5, 50 (1962).
6. V. A. Bloomfield, K. E. Van Holde, and W. O. Dalton, Biopolymers, 5, 149 (1967).
7. D. P. Filson and V. A. Bloomfield, Biochemistry 6, 1650 (1967).
8. R. J. Douthart and V. A. Bloomfield, Biochemistry 7, 3912 (1968).
9. R. M. Herriott and J. L. Barlow, J. Gen. Physiol. 40, 5, 809 (1957).
10. D. J. Cummings, V. A. Chapman, and S. S. Delong, Virology 37, 94 (1969).
11. G. Scatchard, Ann. N. Y. Acad. Sci 51, 660 (1949).
12. A. R. Peacocke and J. H. Skerrett, Tran. Faraday Soc. 52, 261 (1956).
13. H. A. Benesi and J. H. Hildebrand, J. Am. Chem. Soc. 71, 2703 (1949).
14. J. B. LePecq and C. Paoletti, J. Mol. Biol. 27, 87 (1967).
15. D. J. Cummings, Virology 19, 536 (1963).