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ARTICLE *in* BIOCHEMISTRY · DECEMBER 1976

Impact Factor: 3.02 · DOI: 10.1021/bi00668a017 · Source: PubMed

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Studies on the Noncooperative Binding of the *Escherichia coli* DNA Unwinding Protein to Single-Stranded Nucleic Acids†

William T. Ruyechan and James G. Wetmur*

ABSTRACT: The noncooperative binding of the *Escherichia coli* DNA unwinding protein to single-stranded DNA oligomers has been studied by means of equilibrium dialysis. Dialyses were performed under a number of solution and temperature conditions using oligomers of varying length and base composition. The results of these studies, which include a Scatchard analysis of the binding, have allowed us to propose a model for the cooperative binding of the protein to single-stranded DNA. The results of experiments dealing with the interaction of the protein with single-stranded RNA are also presented.

During the past several years a number of proteins which bind preferentially and cooperatively to single-stranded DNA have been isolated from viral, prokaryotic, and eukaryotic sources utilizing the DNA-cellulose chromatography method of Alberts and Herrick (1971). T4 gene 32 product, the first such protein isolated (Alberts and Frey, 1970), was shown through genetic analysis (Tomizawa et al., 1966; Kozinski and Felgenhauer, 1967; Alberts et al., 1968; Sinha and Snustad, 1971) to be required for both replication and recombination. Subsequent studies (Huberman et al., 1971; Delius et al., 1972) determined that gene 32 product (a) holds single-stranded DNA in an extended conformation, (b) specifically stimulates the activity of T4 DNA polymerase, and (c) forms a weak complex with T4 DNA polymerase in the absence of DNA.

Of the other DNA-binding proteins isolated, the one which has shown properties similar to nearly all of those catalogued for T4 gene 32 product is the *E. coli* DNA unwinding protein (Sigal et al., 1972). The protein holds single-stranded DNA in an extended conformation and stimulates the activity of *E. coli* DNA polymerases II and III* but not *E. coli* DNA polymerase I or T4 DNA polymerase (Sigal et al., 1972; Molineux and Gefter, 1974; Weiner et al., 1975). It has also been shown to form a weak complex with *E. coli* DNA polymerase II (Molineux et al., 1974). While no mutations in the gene coding for the protein have been found, the stimulation of the two *E. coli* DNA polymerases indicates that it is involved in both replication and repair.

The native form of the protein in solution is a tetramer composed of four identical 19 000–20 000-dalton monomers (Molineux et al., 1974; Weiner et al., 1975). The initial studies of Sigal et al. (1972) indicated that the protein bound to single-stranded DNA in a ratio of one protein monomer per eight bases. Electron microscopy of DNA–protein complexes by the

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same authors showed that the protein holds DNA in a rigid configuration with a base spacing of 1.8 Å and suggested that the protein binds cooperatively as a tetramer. Anderson and Coleman (1975), however, determined a binding ratio of 13–14 bases per monomer, using circular dichroism to follow the formation of DNA–protein complexes. In subsequent investigations, Weiner et al. (1975), using a filter binding assay, obtained a stoichiometry of 30–36 bases per tetramer which agrees with the results of Sigal et al. (1972).

In previous work (Ruyechan and Wetmur, 1975) we showed that the cooperative binding of the *E. coli* unwinding protein to DNA could be analyzed by means of a simple statistical mechanical model which broke the binding phenomenon into a relatively weak initiation interaction between a single protein molecule and a relatively small number of base pairs, followed by a strong, cooperative process. It was shown that the binding was maximal under conditions near physiological pH and salt. The cooperative binding constant was determined to be 3.8–7.6 $\times 10^{10}$ l./mol.

In the work presented below, we have investigated the initiation interaction by means of equilibrium dialysis between the unwinding protein and single-stranded DNA oligomers of varying size and base composition. These studies have been done under a variety of salt, pH, and temperature conditions. Scatchard analyses have been carried out under the solution conditions which gave maximal binding. Analytical sedimentation velocity was used to determine gross structural changes in the protein at various salt concentrations and in the presence of oligomers.

In addition to these studies we have developed an assay for the activity of the protein based on its ability to inhibit the action of DNase I and snake venom phosphodiesterase. Finally we offer the results of a study concerning the binding of the unwinding protein to RNA.

Materials and Methods

Preparation of the E. coli DNA Unwinding Protein. The *E. coli* DNA unwinding protein was prepared from *E. coli* K12 frozen cell paste (Grain Processing Corp., Muscatine, Iowa) using previously described methods (Ruyechan and Wetmur, 1975).

Quantitation of the Unwinding Protein. The protein was quantitated using the method of Lowry (Lowry et al., 1951) and the micro-biuret method of Goa (1953). The molar absorbtivity of the protein at 280 nm was determined to be 3.0 $\times 10^4$ based on a molecular weight of 20 000.

Radioiodination of Single-Stranded DNA Oligomers. The single-stranded DNA oligomers $d(pC)_6$, $d(pCpG)_3$, $d(pCpA)_3$, $d(pCpA)_{6-9}$, $d(pCpT)_2$, $d(pCpT)_3$, $d(pCpT)_4$, and $d(pCpT)_{6-9}$ were purchased from Collaborative Research Inc., Waltham, Mass. The molecular weights of the oligomers were spot-checked in a Beckman Model E analytical ultracentrifuge and were found to be correct.

Radioiodination of the oligomers was carried out using the in vitro iodination procedure described by Orosz and Wetmur (1974). ^{125}I (0.2 to 1.0 mCi; Amersham) was added to a freshly made solution containing 0.1 M acetate buffer (pH 5.0), 6×10^{-4} M $TlCl_3$, 2×10^{-4} M KI, and one of the above oligomers present at a phosphate concentration of about 2×10^{-4} M. This mixture was heated at 60 °C for 20–30 min.

Following the heating step, the reactions were ice quenched, and the solution was passed over a Sephadex G-10 (Pharmacia) column (internal diameter = 1.0 cm; height = 40.0 cm). The chromatography was carried out at 4 °C, and elution of the oligomer was monitored by means of ultraviolet absorbance at 260 nm. This step gives a clean separation of the oligomer from the inorganic salts present in the original reaction mixture. The eluted oligomer was then made 0.1 M in phosphate buffer (pH 6.0) and heated for an additional 3–5 h at 60 °C in order to complete the iodination reaction. Upon completion of the heating step, the oligomer was rechromatographed at 4 °C on a fresh G-10 column. The oligomer-containing fractions from this column were pooled and stored at 4 °C. Specific activities of 10^{12} to 10^{13} cpm/mol of oligomer were regularly obtained using this procedure.

Higher oligomer concentrations than those obtained from the second Sephadex column were achieved by lyophilization of the product followed by redissolving the resulting solid in a small volume. Lower concentratinns were obtained by dilution.

Preparation of Apurinic Oligomers. An apurinic oligomer was prepared from $d(pCpA)_3$ which had been iodinated using the procedure outlined above. The conditions used for the depurination reaction and subsequent isolation of the product are essentially those used by Burton (1967).

The $d(pCpA)_3$ solution was desalted by elution with water from a Sephadex G-10 column. The oligomer solution was mixed with 2.8 volumes of 90% formic acid and incubated for 17 h. It was then extracted three times with 0.5 volume of water and 4 volumes of ether, with the ether layers being discarded. This solution was passed over a Sephadex G-10 column. The apurinic oligomer cleanly separated from other components in the extraction mixture. The specific activities of the original $d(pCpA)_3$ and depurinated $d(pCpA)_3$ were found to be equivalent when dilution factors were taken into account.

Equilibrium Dialysis. Equilibrium dialysis experiments were carried out in cells made from a pair of Plexiglas acrylic blocks with six shallow cylindrical depressions (6 × 2 mm) cut into each. Dialysis membranes purchased from Sartorius Inc. (Gottigen, West Germany) were cut into pieces approximately 0.5 cm on a side and placed between the two depressions. The contact areas were lightly coated with silicon grease, and the two Plexiglas blocks were tightly screwed together. Thus each pair of depressions resulted in two 50-μl chambers separated by the dialysis membrane. A narrow hole from each depression to the top of the block allowed access to each side.

Dialysis experiments were begun by adding 50 μl of an unwinding protein solution to one side of a pair of chambers and 50 μl of a radioactively labeled oligomer solution to the other. Concentrations of both protein and oligomer as well as the overall solution conditions varied depending upon the experiment in question. Paraffin was used to seal all access ports and junctions between the two Plexiglas blocks. The protein–oligomer system was then allowed to come to equilibrium. Most of the equilibrations were carried out at 4 °C; other temperature conditions will be indicated in Results and Discussion (see below).

Attainment of equilibrium was first determined by dialysis of labeled oligomers vs. buffer. The minimum time for equilibration was determined to be 72 h using this method. All protein–oligomer experiments were allowed to proceed for 1 week. Control experiments using the same concentrations of protein and oligomer which were allowed to proceed for 1, 2, and 3 weeks showed no variation in the amount of binding within experimental error ($\sigma \simeq 10$–20%).

Once equilibrium was reached, the paraffin seal was stripped off the cells, and 30–40-μl aliquots were removed from each chamber. The samples were placed into 10 ml of a dioxane based scintillation cocktail and counted in a Packard 3320 Tri-Carb scintillation counter.

Nuclease Protection Assay. Calf thymus DNA (Sigma) was iodinated using the procedure of Orosz and Wetmur (1974). Following iodination the DNA solution was placed in a boiling water bath for 3 min, ice quenched, and used as a stock solution. The final concentration of the denatured DNA was 4×10^{-5} M in phosphate residues. The DNA was remelted and quenched prior to each assay experiment.

A stock solution containing 0.05 M Tris[1]-HCl (pH 7.0), 0.01 M $MgCl_2$, 100 μg/ml of DNase I (Sigma), and 10 μg/ml of snake venom phosphodiesterase (Sigma) was prepared and used throughout the experiments involving protection of DNA from nuclease activity by the unwinding protein.

A second stock solution containing 0.05 M Tris (pH 7), 0.001 M $MgCl_2$, and 50 μg/ml RNase A (Sigma) was prepared and used in experiments dealing with nuclease protection of RNA.

Ribosomal RNA isolated from *Drosophila melanogaster* larva and labeled with ^{125}I using the method of Orosz and Wetmur (1974) was the generous gift of Ms. Carole Sprouse. The concentration of the iodinated RNA stock solution was 3.7×10^{-4} M phosphate. The RNA and DNA solutions were stored at −20 °C.

The general procedure for the nuclease protection experiments may be outlined as follows. A constant amount of single-stranded DNA or RNA was mixed with varying amounts of unwinding protein. The mixture was made 0.1 ml with the addition of 0.05 M Tris-HCl (pH 7.0). This solution was then incubated at 4 °C for 10 min. Following incubation, the sample was diluted 1 to 10 into the appropriate nuclease solution and placed in a 37 °C bath for 1.5 h. The nuclease reactions were quenched by addition of 0.2 ml of cold 0.1 M EDTA to the mixtures.

In order to determine the extent of protection, the samples were placed on a Sephadex G-100 (Pharmacia) column and eluted with distilled water. Fractions (1.6 ml) were collected and counted as described above. Sephadex chromatography of nuclease-treated materials has, in our hands, shown itself to be more sensitive and reproducible than trichloroacetic acid precipitation techniques. The chromatographic method gives unambiguous separation of digested and undigested material whereas Cl_3CCOOH-precipitated materials sometimes act as carriers for soluble counts, thus leading to results which could be seriously in error (Sprouse and Wetmur, unpublished observations).

Velocity Sedimentation. Boundary velocity sedimentation studies of the unwinding protein were performed in a Beckman Model E ultracentrifuge equipped with ultraviolet optics. Sedimentation runs were done at room temperature under a variety of solution conditions. (See Results and Discussion.)

Electron Microscopy. Preparation of poly(rA)- and poly(rU)-unwinding protein complexes and visualization of these complexes by electron microscopy were essentially the same as that described for DNA-unwinding protein complexes (Ruyechan and Wetmur, 1975).

Results

Ionic-Strength Dependence of Binding. Equilibrium dialysis experiments using iodinated $d(pC)_6$, $d(pCpT)_3$, $d(pCpG)_3$, and $d(pCpA)_3$ were done at a variety of NaCl concentrations in order to determine the ionic-strength dependence of the binding interaction. The oligomer concentrations were 1.3×10^{-6} M in all cases. The unwinding protein concentration was 1.5×10^{-6} M tetramer. The buffer used was 0.01 M Tris-HCl (pH 7.8)–10^{-4} M EDTA. The NaCl concentrations used were 0.04, 0.2, 0.5, and 1.0 M. Dialyses were carried out at 4 °C.

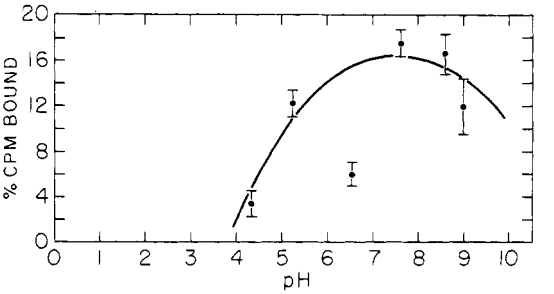

FIGURE 1: pH dependence of oligomer binding.

The behavior of all four oligomers was the same: a plateau of binding was seen from 0.04 to 0.2 M NaCl, with a precipitous loss of binding occurring above 0.2 M. The lack of binding indicates that the apparent binding constant for oligomers at salt concentrations above 0.2 M is less than 10^3 l./mol, which is the limit of detection for the dialysis system.

Binding to DNA at high salt concentrations is not completely inhibited since DNA-protein complexes at NaCl concentrations ranging from 0.29 to 1.0 M have been observed (Ruyechan and Wetmur, 1975; Weiner et al., 1975).

pH Dependence of Oligomer Binding. pH dependence experiments were done by dialyzing 10^{-7} M $d(pCpT)_4$ against unwinding protein at a concentration of 2×10^{-6} M tetramer. The NaCl and EDTA concentrtions were 0.2 and 2×10^{-4} M throughout the experiments. The buffers used were 0.01 M acetate (pH 4.36 and 5.25), 0.01 M PO_4 (pH 6.62), and 0.01 M Tris (pH 7.62, 8.53, and 8.94). The unwinding protein solutions used were dialyzed against these buffers and the oligomer solutions were prepared by dilution. All pH values were checked with a Beckman pH meter. The results of these experiments are shown in Figure 1. A broad pH optimum from pH 7.5 to 8.5 is indicated. The drop in binding at pH 6.6 may be due to precipitation of the protein near its isoelectric point. Unwinding proteins isolated from *E. coli* D10 (Sigal et al., 1972) and *E. coli* B (Weiner et al., 1975) have isoelectric points of 7 and 6, respectively.

Mg^{2+} Dependence of Oligomer Binding. Equilibrium dialyses of $d(pCpT)_4$ against unwinding protein were performed at pH 7.8 and 0.2 M NaCl in the presence of $MgCl_2$ concentrations ranging from 10^{-7} to 10^{-1} M. Oligomer concentrations were 1.3×10^{-6} M, and the protein concentration was 1.5×10^{-6} M tetramer throughout these experiments. The results indicate that Mg^{2+} affects the oligomer-protein equilibrium in a manner similar to that of NaCl. There is a broad plateau of binding showing a decrease in binding at 10^{-3} M followed by an abrupt drop to no observable binding at Mg^{2+} concentrations above 10^{-2} M.

Effect of Spermidine on Oligomer Binding. Weiner et al. (1975) reported a reduction in binding of the unwinding protein to single-stranded DNA in the presence of spermidine. In order to determine whether the inhibition was due primarily to a change in the secondary structure of the DNA or to a direct interaction between spermidine and the unwinding protein, the following experiment was done.

Iodinated $d(pCpT)_{6-9}$, 1.1×10^{-7} M, was dialyzed against unwinding protein at a concentration of 1.6×10^{-6} M tetramer at 4 °C. The buffer used in control experiments contained 0.2 M NaCl, 0.01 M Tris-HCl (pH 7.8), and 2×10^{-4} M EDTA. The experiment itself was carried out with a concentration of

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

TABLE I: Binding of Oligomer by 37 °C Treated Unwinding Protein.

Time Inc. at 37 °C (h)	% cpm Bound
0	14.9
3	14.1
6	18.3
11	11.1
21	13.9
52	6.7

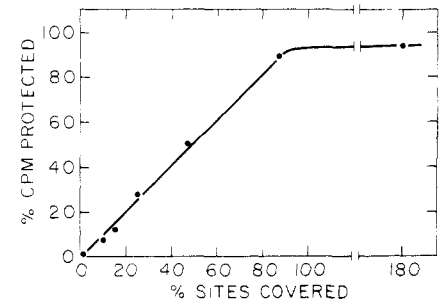

FIGURE 2: Protection of ^{125}I-labeled calf thymus DNA by the unwinding protein against nuclease action.

10^{-3} M spermidine present in both the oligomer and protein solutions.

Analysis of the data obtained upon equilibration indicated that the amount of binding seen in the presence of spermidine was 80% of that seen in control experiments. Owing to the relatively weak ($K_B = 10^5$) binding of the oligomers (see below), this decrease corresponds to only a few tenths of a kilocalorie in binding free energy. Thus our results suggest that the intrinsic interaction is affected slightly if at all by the presence of spermidine.

Thermal Stability of the Unwinding Protein and the Protein–Oligomer Interaction. The *E. coli* unwinding protein has been shown to be remarkably stable to boiling for a short period of time. This property has led to a technique for bulk purification of the protein (Weiner et al., 1975). Retention of activity by the protein was monitored by its ability to bind cooperatively to single-stranded DNA.

In order to determine whether the intrinsic interaction was affected by boiling, unwinding protein at a concentration of 4×10^{-7} M tetramer in 0.2 M NaCl, 0.01 M Tris-HCl (pH 7.7), and 0.001 M EDTA was divided into three portions. One portion was untreated and used as a control. The other two were boiled vigorously for 3 min and either ice-quenched or allowed to cool slowly to room temperature. The three samples were then dialyzed against iodinated $d(pCpT)_4$ at a concentration of 9.7×10^{-8} M in the above buffer. No difference in the amount of binding was seen among the three samples, indicating a total return to the native configuration.

The stability of the protein at 37 °C was investigated by incubating unwinding protein, 5×10^{-7} M tetramer, for various lengths of time at 37 °C. The protein was then dialyzed against iodinated $d(pCpT)_4$ at 4 °C. Solution conditions were identical with those described above. The results of this study are presented in Table I. These results show that the unwinding protein is relatively stable when kept at 37 °C for protracted periods of time, with a plateau of binding from 0 to 21 h of heat treatment and 50% of the original binding capacity remaining after a 52-h incubation.

Thermal stability of the protein–oligomer interaction was determined by allowing equilibration to take place at elevated temperatures. Samples of unwinding protein at a concentration of 2×10^{-6} M tetramer and 10^{-7} M $d(pCpT)_4$ in 0.2 M NaCl, 0.01 M Tris (pH 7.7), and 10^{-3} M EDTA were set up for dialysis in two equilibrium dialysis cells. One cell was incubated at 4 °C and used as a control. The other was incubated at 37 °C. Analysis of the data indicated that the binding manifested by the protein–oligomer system at 37 °C was 75–80% of that found at 4 °C. As mentioned above, this decrease corresponds to a drop of only a few tenths of a kilocalorie in binding free energy. Thus the information gathered at 4 °C appears to be an accurate picture of the unwinding protein's action at physiological temperatures.

Experiments performed at 37 °C and tenfold less protein showed less binding than expected and a great deal of scatter. This seems to indicate that the unwinding protein, not surprisingly, undergoes denaturation more rapidly at lower concentrations. Studies in which ovalbumin was added to a final concentration of 0.1 mg/ml to the low concentration protein solution showed, in general, more binding. Once again, however, the data showed a good deal of scatter.

Finally, an equilibrium dialysis cell containing 10^{-6} M unwinding protein tetramer in the presence of 0.1 mg/ml ovalbumin and 10^{-7} M iodinated $d(pCpH)_3$ was incubated at 45 °C for 16 h. Following this high-temperature incubation, the cell was shifted to 4 °C and incubated for an additional 4 days. The amount of binding seen was approximately 25% of that expected. Experiments in which ovalbumin was not present gave similar results. These experiments show that thermal denaturation of the protein proceeds a good deal more rapidly at 45 °C than at 37 °C, the denaturation process is irreversible and denaturation seems to be independent of carrier proteins added to the unwinding protein solution.

Determination of Binding Stoichiometry by Means of Nuclease Protection. Experiments monitoring protection from nuclease action afforded to single-stranded calf thymus DNA by the unwinding protein were carried out as described in Materials and Methods. Stock solutions of iodinated single-stranded DNA and unwinding protein contained 4×10^{-5} M phosphate and 1.2×10^{-6} M tetramer, respectively. Both solutions contained 0.1 M NaCl, 0.01 M Tris (pH 7.7), and 2×10^{-4} M EDTA.

Assays were conducted at DNA/protein ratios corresponding to 6, 15, 27, 46, 92, and 180% coverage of the DNA based on the 8/1 DNA to protein weight ratio established by Sigal et al. (1972) and Weiner et al. (1975). The results are presented in Figure 2. The protection appears to be quite linear between 6 and 92% coverage. The 180% coverage experiment was repeated several times and no protection greater than 93% was ever seen. Since control experiments in which undigested, nonprotein treated DNA was eluted over a Sephadex G-100 column showed no counts at the position of digested DNA, we feel that the lack of total protection is due to a combination of snap back sequences present in the calf thymus DNA and the action of proteolytic contaminants in the snake venom phosphodiesterase. These results are similar to those obtained by Molineux and Gefter (1975) who investigated the action of DNase I and snake venom phosphodiesterase separately.

In a separate experiment DNA/protein mixtures corresponding to 92 and 180% coverage were prepared and incubated at 4 °C. The samples were then placed into a boiling water bath for 3 min, ice quenched, and immediately diluted into the nuclease mixture. Incubation at 37 °C and column chromatography were carried out as described above. Analysis of the data showed that 70% of the counts were protected in

TABLE II: Sequence Dependence of Oligomer Binding.

Oligomer	$\Delta G°$(app) (kcal/mol)
$d(pC)_6$	5.97
$d(pCpT)_3$	5.97
$d(pCpG)_3$	5.86
$d(pCpA)_3$	5.53

the 90% coverage case and 93% of the counts were protected in the 180% coverage experiment. These results indicate that renaturation of the unwinding protein and reformation of stable protein-DNA complexes is quite rapid. The time elapsed between removal of the samples from the 100 °C bath to their dilution into the nuclease mixture was approximately 1 min.

Sequence Dependence of the Protein-Oligomer Interaction. Unwinding protein at 1.5×10^{-6} M tetramer and $d(pC)_6$, $d(pCpT)_3$, $d(pCpG)_3$, and $d(pCpA)_3$ were equilibrated at 4 °C. All oligomers were at a concentration of 1.3×10^{-6} M. Solution conditions were 0.2 M NaCl, 10^{-2} M Tris-HCl (pH 7.8), and 10^{-3} M EDTA. Apparent equilibrium constants, K(app), and free energies of binding, ΔG(app), were calculated using eq 1 and 2 below.

$$K(\text{app}) = \frac{(\text{UO})}{(\text{U})_{\text{free}}(\text{O})_{\text{free}}} \quad (1)$$

where U ≡ unwinding protein, O ≡ oligomer, and UO ≡ oligomer-unwinding protein complex. The apparent free energy of binding is calculated from

$$\Delta G°(\text{app}) = -RT \ln K(\text{app}) \quad (2)$$

A summary of the apparent binding free energies is presented in Table II. These results indicate that the binding of the protein is essentially independent of base sequence and is probably centered on the sugar-phosphate backbone. The slight but reproducible drop in binding free energy in going from oligomers containing only pyrimidines to those containing alternating purines and pyrimidines could be due to steric effects engendered by the bulkier guanine and adenine residues.

As a further confirmation of the fact that the unwinding protein binds to the sugar-phosphate backbone, apurinic oligomer was prepared from iodinated $d(pCpA)_3$, as described in Materials and Methods, and dialyzed against unwinding protein at 4 °C. Dialyses against $d(pCpA)_3$ were used as controls. Unwinding protein was present at a concentration of 1.75×10^{-6} M tetramer. Iodinated $d(pCpA)_3$ and apurinic oligomer were present at a concentration of 10^{-7} M. Analysis of the data indicated a drop of only 0.3 kcal in the binding free energy for apurinic oligomer as compared with $d(pCpA)_3$. Thus even with alternate bases removed, the binding interaction undergoes only a minor perturbation.

Length Dependence of Oligomer Binding. Experiments in which unwinding protein was dialyzed against oligomers of varying length were carried out for two reasons. First, such studies would determine the specificity of the binding site with regard to oligomer size. Second such experiments should also show whether the monomeric or oligomeric form of the protein is the cooperatively binding species under the given solution conditions.

Unwinding protein at a concentration of 8.7×10^{-6} M was dialyzed against $d(pCpT)_2$, $d(pCpT)_3$, $d(pCpT)_4$, and $d(pCpT)_{6-9}$ all at a concentration of 10^{-7} M. Salt and buffer concentrations were 0.2 M NaCl, 10^{-2} M Tris (pH 7.6), and 10^{-3} M EDTA. Apparent binding constants were calculated and are presented in Table III. These results indicate that, after a jump of approximately an order of magnitude between $d(pCpT)_2$ and $d(pCpT)_3$, the observed binding increases gradually with increasing oligomer length. This small increase is probably due to the increase in the number of ways the protein can bind to a longer oligomer. No strong cooperative effect was seen on going from $d(pCpT)_4$ to $d(pCpT)_{6-9}$ which would have been the case if protein monomers bound cooperatively to adjacent sites. These results are in contrast to those of Molineux et al. (1975) who did observe a cooperative effect on going from $d(pT)_8$ to $d(pT)_{16}$. These authors, however, carried out their study at very low protein and salt concentrations (see Discussion).

TABLE III: Length Dependence of Oligomer Binding.

Oligomer	K_B(app)
$d(pCpT)_2$	$0.5\text{-}0.6 \times 10^4$
$d(pCpT)_3$	$3\text{-}5 \times 10^4$
$d(pCpT)_4$	$5\text{-}9 \times 10^4$
$d(pCpT)_{6-9}$	$8\text{-}14 \times 10^4$

TABLE IV: Results of Sedimentation Studies on the Unwinding Protein.

Protein	Solution Conditions	$s_{20,w}$ (S)
Ovalbumin		3.5
Unwinding protein	0.2 M NaCl	4.9
Unwinding protein	0.5 M NaCl	4.5
Unwinding protein	1.0 M NaCl	4.9
Unwinding protein	0.2 M NaCl, 4×10^{-6} M $d(pCpT)_{6-9}$	5.0

Competition experiments were also done in which the protein was equilibrated against 5×10^{-7} M iodinated $d(pCpT)_9$, a mixture of iodinated $d(pCpT)_{6-9}$ and cold $d(pCpT)_{6-9}$, both at 5×10^{-7} M, and iodinated $d(pCpT)_{6-9}$ and cold $d(pCpT)_4$, once again both at 5×10^{-7} M. Solution conditions were the same as those above. The slightly lower binding seen in the experiments where cold oligomer had been added was consistent with the binding constant calculated from the control experiment and the Scatchard plot data presented below. The iodinated oligomers behave no differently from the cold oligomers. Once again, there was no evidence for a cooperative interaction with $d(pCpT)_{6-9}$.

Sedimentation Velocity Studies of the Unwinding Protein. Sedimentation velocity studies were carried out in order to determine whether gross structural changes occurred in the unwinding protein in either the presence of high salt or oligomers. Boundary velocity sedimentation runs were carried out in a Beckman Model E analytical ultracentrifuge. The runs were performed at room temperature at a rotor speed of 40 000 rpm. Progress of the sedimentation was followed by ultraviolet optics. Photographs were traced on a Canalco densitometer.

Unwinding protein was present at concentrations between 1.0 and 1.5×10^6 M tetramer. The buffer used throughout was 0.01 M Tris-HCl (pH 7.8) and 10^{-4} M EDTA. Ovalbumin was used as a standard. The results are summarized in Table IV. The $s_{20,w}$ value for ovalbumin is in good agreement with the value of 3.55 S previously reported (Tanford, 1961). Sigal

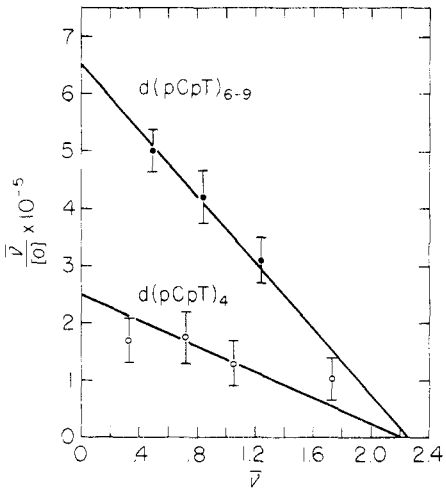

FIGURE 3: Scatchard plots of the binding of the unwinding protein to $d(pCpT)_4$ (○) and $d(pCpT)_{6-9}$ (●).

et al. (1972) and Weiner et al. (1975) have respectively determined the $s_{20,w}$ value of the unwinding protein to be 4.7 and 4.9 S.

Our results indicate that there is no substantial change in the gross morphology of the protein when it is in the presence of salt concentrations greater than 0.2 M NaCl. Thus the sharp drop in binding seen above this ionic strength is not due to dissociation of the tetramer and may be the result of an allosteric change (see Discussion).

Sedimentation in the presence of $d(pCpT)_{6-9}$ also shows no gross change in protein structure, indicating that the protein species which binds to oligomers of this length is tetrameric. No hint of other forms (i.e., monomeric, dimeric) of the protein were seen in this study. These data are in contrast to those of Molineaux et al. (1975) who found that $d(pT)_{16}$ cosedimented with a dimeric protein complex. Once again, however, the solution conditions which these authors employed were markedly different than those used here.

Scatchard Analysis of Oligomer Binding. Unwinding protein at a concentration of 5×10^{-7} M tetramer was dialyzed against several concentrations of $d(pCpT)_4$ and $d(pCpT)_{6-9}$. Solution conditions were 0.2 M NaCl, 0.01 M Tris-HCl (pH 7.8), and 0.001 M EDTA throughout. Dialysis cells were set up in which dialyses against $d(pCpT)_4$ and $d(pCpT)_{6-9}$ were done in quintuplicate and quadruplicate, respectively. The data obtained were analyzed using the Scatchard equation (Scatchard, 1951):

$$\bar{\nu}/[O] = nK - \bar{\nu}K \qquad (3)$$

where [O] is the free oligomer concentration, K is the binding constant for the interaction, $\bar{\nu}$ is the number of binding sites occupied, and n is the total number of binding sites. The results are shown in Figure 3. Binding constants of 3.25×10^5 and 1.25×10^5 l./mol per site were obtained for $d(pCpT)_{6-9}$ and $d(pCpT)_4$, respectively. Both oligomers appear to have two binding sites per tetramer. This rather surprising result will be discussed below along with a possible model for the cooperative binding of the protein (see Discussion).

Binding of the Unwinding Protein to Single-Stranded RNA. Binding of the unwinding protein to single-stranded RNA was investigated in two ways. In the first the nuclease protection assay system was modified as described in Materials and Methods to monitor any protection from ribonuclease activity afforded to iodinated rRNA by the unwinding protein. In the second poly(rA) and poly(rU) were incubated with unwinding protein, and the resulting complexes were observed electron microscopically following fixation with glutaraldehyde.

No protection of the RNA was seen with RNA/protein ratios of 4/1 and 8/1 mol of phosphate per protein monomer, conditions which would have given 50 and 92% protection in the DNA nuclease assay. Some protection was seen at RNA/protein ratios of 100/1 following a 10-min nuclease treatment at 37 °C. Longer incubations (20 min and above) abolished this effect.

These results imply that the unwinding protein interacts much less strongly with RNA than it does with DNA. There is, however, the possibility that ribonuclease A, owing to its small size, can penetrate the RNA–protein complex, as seems to be the case with micrococcal nuclease (Molineux et al., 1975) and DNA–protein complexes (see Discussion).

In the electron microscopic studies poly(rA) and poly(rU) in 0.01 M EDTA were mixed with unwinding protein in 0.04 M NaCl, 0.01 M Tris-HCl (pH 7.5), and 0.001 M EDTA. The unwinding protein was present in concentrations in three- to fourfold excess of those necessary to saturate an equivalent amount of DNA. Following a 10-min incubation at 4 °C, the ribopolymer–protein complexes were fixed with glutaraldehyde for 10 min at 37 °C, mounted on parlodian-coated copper grids, and observed in the electron microscope. Few extended poly(rA) complexes were seen. The majority of the nucleic acid was present in collapsed, bush-like structures. The molecules containing extended regions very often also contained collapsed areas. Similar observations of poly(rU)–protein complexes indicated that the protein bound regions were more extensive both in number and length. Some of the poly(rU), however, was also seen to be unextended. These findings seem to be in agreement with those of Molineux et al. (1975) and Weiner et al. (1975) both of whom observed a much stronger binding to poly(rU) than to poly(rA). In both cases, however, the binding does not appear to be as strong with RNA as with DNA.

Discussion

Ionic Strength Effects and the Mode of Protein Binding. We have shown that the intrinsic binding of the *E. coli* DNA unwinding protein to a small number of nucleotides is optimal near conditions of physiological salt, temperature, and pH. This result was not surprising since it had been shown in a previous study (Ruyechan and Wetmur, 1975) that the cooperative interaction of the protein with single-stranded DNA was also optimal under these conditions.

Our studies with oligomers of varying base composition, and apurinic oligomer, indicate that the intrinsic binding of the protein is essentially independent of the nucleotide sequence and is centered on the sugar-phosphate backbone. The question remaining concerning the site to which the protein binds is whether the interaction is one between the protein and the sugar residues, the phosphate residues, or a combination of the two.

In order to make a statement concerning this problem in the light of the data presented above, we have made use of results obtained by Dr. R. Thomas Record and his associates (Record et al., 1976, in press) concerning ion effects in protein–nucleic acid interactions. Their investigations have shown that the negative of the partial derivative of the logarithm of the observed binding constant with respect to the logarithm of the sodium ion concentration is equal to the product of the number of electrostatic interactions involved and a constant which is the same for all single-stranded DNA's. The results of the ionic

strength studies presented in which no change in binding was seen in the presence of sodium ion concentrations between 0.04 and 0.2 M NaCl indicate that the binding constant is not changing over this range and that the number of electrostatic interactions contributing to the binding is essentially zero. Thus at concentrations near physiological salt, the binding interaction appears to take place without displacement of counterions from the DNA phosphates.

The drop in binding seen at high ionic strength is less easily understood. Sigal et al. (1972), Molineux et al. (1974, 1975), and Weiner et al. (1975) have all reported seeing similar effects. We have four observations concerning the phenomenon: (a) the intrinsic binding constant drops below 10^3 l./mol at sodium ion concentrations above 0.2 M or Mg^{2+} concentrations above 0.01 M, (b) previous studies (Ruyechan and Wetmur, 1975) showed a drop in the length of single-stranded DNA–protein complexes at $[Na^+]$ above 0.2 M indicating a decrease in both the intrinsic and cooperative interactions, (c) sedimentation studies at Na^+ concentrations as high as 1.0 M show that the protein remains tetrameric in high salt, and (d) as discussed above, the protein–DNA interaction appears to be essentially nonelectrostatic.

A model which takes into account all of the above statements would be one in which cations at high concentrations bind cooperatively to the tetrameric form of the protein and sufficiently distort its internal structure to engender the decreases in both intrinsic and cooperative binding which are observed. This model is, however, purely speculative. Data which could prove or disprove it might possibly be obtained from fluorescence depolarization studies.

The data presented in this study and the electron photomicrographs of Sigal et al. (1972) argue that the cooperatively binding form of the protein is tetrameric. We have not seen any evidence for cooperative binding of oligomers or a breakdown in the tetrameric structure in the presence of DNA oligomers containing 16 or more nucleotides as was observed by Molineux et al. (1975). As was pointed out above (see Results), the study carried by these authors was performed at protein concentrations 100–1000 times below those used in our work and in 0.01 M Tris, 10^{-4} M EDTA. The authors previously showed (Molineux et al., 1974) that under these conditions the protein is predominantly tetrameric, though some monomer is observed on sedimentation through glycerol gradients. It may be that addition of negatively charged DNA oligomers under these conditions shifts the equilibrium away from the tetrameric form and allows charge–charge interactions between protein and oligomer to occur which would not manifest themselves at higher ionic strengths. The authors state that the binding they observe is salt labile with the binding constant for the interaction of the protein with poly(dT) dropping from 10^9 to 2×10^7 on going to 0.32 M NaCl.

Model for the Cooperative Binding of the Protein. We propose the following model for the cooperative binding of the unwinding protein to single-stranded DNA based on the data gathered in this study and by other investigators.

The cooperatively binding species of the protein under the solution conditions used in this work appears to be a tetramer with two identical binding sites only one of which is occupied during the cooperative interaction. The sites apparently cannot be spanned. If they could be, six to eight nucleotides on the oligomers containing 12–18 nucleotides could have bound to one site and the remainder of the oligomer would then be free to bind to the other site. Such a spanning would have produced a cooperative effect with a binding constant on the order of 10^9. No such result was observed.

Once one site of a tetramer binds to single-stranded DNA, a strong, cooperative interaction between protein tetramers ensues giving rise to long, linear protein–DNA complexes. These complexes do not involve an interaction of all of the nucleotides with the protein. We say this for two reasons: (a) the Scatchard analysis described above showed only a slight difference in binding constants for $d(pCpT)_4$ and $d(pCpT)_{6-9}$, and (b) the protein does not protect DNA against the action of micrococcal nuclease (Molineaux and Gefter, 1975) probably due to the fact that this low molecular weight nuclease can slip between the unwinding protein tetramers and digest the DNA not specifically in the protein binding site. Once this initial digestion has occurred only the relatively weak intrinsic binding would remain leading rapidly to complete loss of nuclease protection. Thus the stoichiometric value of 32 nucleotides bound per tetramer need not represent a rigid structure.

Summary

We have examined the noncooperative binding of the *E. coli* unwinding protein under a variety of solution and temperature conditions. These studies coupled with Scatchard analyses of the binding and sedimentation studies of the protein's structure have allowed us to offer models for the behavior of the protein at high ionic strength and for its cooperative binding to single-stranded DNA.

The results of studies on the binding of the protein to RNA were also presented.

Acknowledgment

We thank Dr. R. Thomas Record for making his results available to us prior to their publication. We also thank Dr. O. C. Uhlenbeck for providing the design for the equilibrium dialysis cells.

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Purification of a Factor from Ehrlich Ascites Tumor Cells Specifically Stimulating RNA Polymerase II†

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ABSTRACT: A factor stimulating RNA polymerase II from Ehrlich ascites tumor cells was purified. The final preparation appeared almost homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had a molecular weight of 38 000. The endonuclease activity of about 10 μg of purified factor, if any, was well below the 10^{-5} μg equivalent of pancreatic deoxyribonuclease, indicating that the stimulation of RNA synthesis by this factor was not due to contaminating endonuclease. This factor specifically stimulated RNA polymerase II on native DNA as template and did not affect RNA polymerase I at all. The molecular size of RNA synthesized in the presence of this factor increased markedly compared with that synthesized by RNA polymerase II alone.

Multiple forms of DNA-dependent RNA polymerase exist in eukaryotic cells (Roeder and Rutter, 1969). Among these, RNA polymerase II (type B), which is completely inhibited by a low concentration of α-amanitin, is thought to be responsible for the synthesis of messenger RNA in the nucleoplasm (Roeder and Rutter, 1970). It is generally believed that many thousands of genes are actively transcribed in dividing cells by this enzyme. Thus the questions arise of how the enzyme selects the genes required and regulates the synthesis of messenger RNA quantitatively and qualitatively. There are probably many species of regulatory proteins controlling the expressions of specific genes. So the first step in elucidating their effects in regulation of RNA synthesis is to find proteins which affect transcription in vitro. In studies along these lines, many protein factors which stimulate or inhibit mammalian RNA polymerase II have been found (Stein and Hausen, 1970; Seifart et al., 1973; Natori, 1972; Lee and Dahmus, 1973; Sugden and Keller, 1973; Shea and Kleinsmith, 1973; Natori et al., 1974; Kostraba et al., 1975; Kostraba and Wang, 1975; Chuang and Chuang, 1975). However, most of the factors isolated were impure and the possibility was not excluded that their stimulatory or inhibitory effects on RNA synthesis may have been due to contaminatory materials, such as nucleases. Moreover, there have been no reports of quantitative studies on the purification of these factors. Recently we reported two factors which stimulate RNA polymerase II of Ehrlich ascites tumor cells (Natori et al., 1973a). To elucidate the precise mechanism of stimulation of RNA synthesis by these factors, we tried to purify one of them, named S-II. This paper describes a method to purify this factor. We were able to determine the yield and specific activity of this factor at each step of purification by measuring stimulation quantitatively. The amount of endonuclease contaminating the final preparation was negligible, so that stimulation of RNA synthesis by this factor was not due to introduction of random nicks in template DNA by contaminating nucleases.

Materials and Methods

Cells. Male ddY mice, weighing 25 to 30 g, were each inoculated with 3×10^6 Ehrlich ascites tumor cells. Ascites fluid was harvested from the abdominal cavity at 8 days after inoculation. Cells were collected by centrifugation for 10 min at 150*g*. The cells harvested from 5 mice were combined and rapidly suspended in 27 ml of deionized water to lyse erythrocytes. Then 3 ml of 10 × PBS[1](−) was added and the mixture was centrifuged. The resulting white pellet of cells was washed three times with PBS(−) and stored at −80 °C.

Preparation and Assay of RNA Polymerase II. DNA-dependent RNA polymerase II was isolated from Ehrlich ascites tumor cells by the procedure described previously (Natori et al., 1973b) involving the following steps: extraction of RNA polymerase by sonication in solution of high salt concentration, ammonium sulfate precipitation, DEAE-cellulose chromatography, and glycerol gradient centrifugation. The specific activity of the RNA polymerase II used usually 1000–2000 units per mg of protein. One unit of enzyme activity was defined as the amount catalyzing incorporation of 1 pmol of UMP into the acid-insoluble fraction under the standard conditions. The standard assay medium contained, in a total volume of 0.25 ml: 10 μmol of Tris-HCl, pH 7.9, 0.75 μmol of

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[1] Abbreviations used: DEAE, diethlaminoethyl; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; CM, carboxymethyl.