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A HELIX-DESTABILIZING PROTEIN SUBSTRATE DEVOID OF HETEROCYCLIC BASES

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SUMMARY

A polynucleotide analog devoid of heterocyclic bases, poly(ribosylurea phosphate), was prepared by $KMnO_4$ oxidation of poly(C). This analog binds effectively to several nucleic acid helix-destabilizing proteins, including gene 32 protein from T4 bacteriophage, UP1 from calf thymus, a protein from rat liver, and RNase A, which is a DNA helix-destabilizing protein. Binding was demonstrated by the ability of poly(ribosylurea phosphate) to inhibit protein-induced depression of polyd(A-T) Tm, as well as, in the case of the T4 and rat liver proteins, the quenching of intrinsic protein tryptophan fluorescence upon interaction with this polynucleotide analog. This substrate may prove useful in assessing the role that protein-ribose phosphate backbone interactions play in the binding specificity of helix-destabilizing proteins.

INTRODUCTION

The lowering of nucleic acid melting temperature (Tm) by helix-destabilizing proteins (HDPs) is a consequence of their selective affinity for nucleic acid single strands relative to double helices. One potential chemical mechanism by which this could be achieved is direct interaction of HDP amino acids with heterocyclic base groups which are inaccessible in the nucleic acid double helix. This mechanism, however, does not appear to be applicable for an HDP from calf thymus, UP1 (1). The reactivity of adenine residues toward chloroacetaldehyde in UP1-denatured DNA complexes is comparable to the reactivity of free denatured DNA (2), suggesting that this protein interacts solely with the polynucleotide backbone. Furthermore, in a recent study we have shown that a polynucleotide analog devoid of heterocyclic bases, poly(ribosylurea phosphate), PRUP, is an effective inhibitor of UP1-induced yeast $tRNA_3^{Leu}$

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renaturation (3). Since this protein-facilitated tRNA renaturation is a consequence of the partial destruction of RNA secondary structure by the HDP (4), the inhibition of the process by PRUP is another indication that UP1 does not interact with the heterocyclic bases.

In this report we show that, in addition to UP1, a number of other HDPs bind PRUP. This polymer was prepared by potassium permanganate oxidation of poly(C), which based on the analogous reaction of cytosine (5), likely yields a product with a urea or biuret moiety at the ribose 1′ position. We have surveyed the effect of PRUP on polyd(A-T) Tm depression by HDPs from T4 bacteriophage (gene 32 protein (6)), rat liver (7,8) and calf thymus (UP1), as well as by pancreatic ribonuclease A, which lowers DNA Tm (9). In addition, the binding of PRUP to the T4 and rat liver HDPs was monitored by the quenching of intrinsic tryptophan fluorescence upon interaction with this polymer.

MATERIALS AND METHODS

Proteins. The T4 HDP was prepared by the method of Bittner et al. (10). Essentially similar results were observed with samples obtained from Bethesda Research Laboratories and Dr. Peter H. von Hippel. Calf thymus UP1 was prepared according to Herrick and Alberts (1), and the rat liver HDP according to Patel (7). Lyophilized, phosphate-free pancreatic ribonuclease A was purchased from Worthington Biochemical.

Nucleic Acids. Polyd(A-T), poly(C) and poly(U) were obtained from P/L Biochemicals. Polyd(T) was purchased from Sigma Chemical Co.

Poly(ribosylurea phosphate), PRUP. The procedures of Jones and co-workers for the oxidation of cytosine (5) and RNA (11) were adapted for poly(C). A 34 mM aqueous solution of $KMnO_4$ (1.5 ml) was slowly added to a stirred solution of equal volume of poly(C) (16 mM) in 0.6 M sodium bicarbonate, pH 9. The solution was allowed to incubate for 48 hrs at 37°C, after which it was successively dialyzed against three changes each of 0.1 M Na_2EDTA, pH 7.8, 1M NaCl, and doubly distilled H_2O; total time of dialysis, $\sim$ 100 hr. The resultant solution was stored at -20°C until use. Its concentration was determined via the orcinol reaction (12). The non-dialyzable material showed virtually no absorbance above 240 nm, indicating that the extent of the reaction was >99%. The yield of this material varied, and was generally about 50%. Based on the reaction products of cytosine and its derivatives (5,13), poly(C) is likely oxidized to a product which possesses a urea or biuret group at the ribose 1′ position. Evidence for this was provided by a positive color test for urea (or biuret) residues using a dimethylaminobenzaldehyde/HCl fume staining procedure (3,14). The product of $KMnO_4$ oxidation of poly(C) is therefore a 3′- 5′ ribose-phosphate backbone with urea or biuret residues as the likely substituents in place of cytosine.

Absorbance - Temperature Profiles. Teflon-stoppered micro quartz cuvettes containing 100 µl of test solutions were placed in a Gilford 2400-2 spectrophotometer designed to raise the temperature from 0-95°C at a constant rate, which was 25°C/hr. Temperature was continually monitored by means of a calibrated thermistor (Yellow Springs Instruments) inserted through a narrow hole

Table I: Inhibition of UP1- and RNase A-Induced Polyd(A-T) Tm Depression by PRUP and Polyd(T).

Protein	Substrate	$\frac{[\text{Substrate}]}{[\text{Polyd(A-T)}]_p}$	ΔTm(+substrate)[a] °C	ΔTm(-substrate)[a] °C
UP1[b]	PRUP	5.0	10	12
UP1	PRUP	10	7	12
UP1	Polyd(T)	0.25	7	12
UP1	Polyd(T)	1.0	4	12
RNase A[c]	PRUP	2.5	14	21
RNase A	PRUP	5.0	10	21
RNase A	Polyd(T)	8.0	6	21
RNase A	Polyd(T)	3.4	5	21

[a] $\Delta Tm = |Tm \text{ of } [polyd(A-T)] - Tm \text{ of } [polyd(A-T) + HDP + (if\ applicable)\ substrate]|$.

[b] 3.0×10^{-5}M (p) polyd(A-T), $[UP1]:[polyd(A-T)]_p = 0.13$, in 10.6 mM Tris·HCl, 6 mM NaCl, 0.6 mM phosphate (K^+), 0.2 mM Na_2EDTA, 0.01 mM dithiothreitol, 0.6% (v/v) glycerol, pH 8.1.

[c] 6.0×10^{-5}M (p) polyd(A-T), $[RNase\ A]:[polyd(A-T)]_p = 0.23$, in 8.8 mM Tris·HCl, 1.2 mM phosphate (K^+), 0.1 mM Na_2EDTA, pH 8.0.

in the stopper of the reference cuvette; absorbance was measured at 260 nm. Fluorescence Titrations. Quenching of HDP intrinsic tryptophan fluorescence was monitored in a JASCO FP-4 spectrofluorimeter at room temperature, with excitation at 290 nm, emission at 340 nm. The data used in the titration plots represent volume change-corrected protein fluorescence relative to that of N-acetyltryptophan amide.

RESULTS

Inhibition of HDP-Induced Tm Depression by PRUP. Each of the four HDPs examined lowers the melting temperature of polyd(A-T). The results for calf thymus UP1 (cf ref. 15) and RNase A (cf ref. 8,16) are summarized in Table I. Increasing the $[HDP]:[polyd(A-T)]_p$ produces monophasic melting profiles with decreasing Tm. Thus, in a buffer of low ionic strength at an $[UP1]:[polyd(A-T)]_p$ of 0.13, the Tm of polyd(A-T) is depressed by 12°C. Addition of PRUP reduces the Tm depression, so that at $[PRUP]:[polyd(A-T)]_p$ of 5 and 10, Tm is lowered by, respectively, 10°C and 7°C (Table I). These mixtures could be melted, cooled and re-melted with Tm reproduced. Polyd(T) is a more effective inhibitor than PRUP; at the same concentration as polyd(A-T), this single-stranded DNA reduces the UP1-induced Tm depression to only 4°C.

PRUP is similarly effective in reversing the depression of polyd(A-T) Tm by RNase A. Thus, under conditions where this protein brings about a ΔTm of 21°C (Table I; 16), an 8-fold excess of PRUP (relative to polyd(A-T)) reduced ΔTm to 6°C. Polyd(T) at a 3.4-fold excess reduced ΔTm to only 5°C, while poly(U) under similar conditions had no effect on the Tm-depression. (The amount of RNase present was more than enough to totally degrade the poly(U)). Significantly, RNase A-polyd(A-T)-PRUP mixtures could be melted, cooled and re-melted with the Tm reproduced. Thus, PRUP appears not to be a substrate for the enzyme, which is in accord with the general view of the action of pyrimidine-specific RNase A (17).

The melting behavior of polyd(A-T) with T4 gene 32 protein and the rat liver HDP is more complex; apparent biphasic absorbance-temperature profiles are observed, with the higher transition identical (or nearly so) to the Tm seen in the absence of protein. At low ionic strength, the lower transition apparently occurs below 0°C (18). The relative amount of the helix melting at the lower transition is proportional to $[HDP]:[polyd(A-T)]_p$. For gene 32 protein, the effect of PRUP on the absorbance-temperature profile was assessed under conditions where about 60% of the polyd(A-T) melts at the higher transition (40% saturation). Thus, in the absence of added PRUP, a hyperchromic change of 60% of the total expected for polyd(A-T) is seen with a Tm of 43°C in the 10 mM phosphate buffer used. With the addition of PRUP, a lower transition appears, and increasing $[PRUP]_p:[polyd(A-T)]_p$ increases this lower Tm without altering the Tm or ΔA_{260} of the higher transition (Fig. 1). The absorbance change of the lower transition accounts for the remaining 40% of polyd(A-T) hyperchromicity. Apparently, PRUP can compete effectively at low temperatures with polyd(A-T) for gene 32 protein. However, as the temperature increases, the shift in the helix-coil equilibrium of polyd(A-T) toward the coil results in the transfer of HDP to the nucleic acid, with concurrent melting. Polyd(T) is a much more effective inhibitor than PRUP; at a $[polyd(T)]_p:[polyd(A-T)]_p$ of 0.5, no effect of gene 32 protein (40% saturation) on Tm is observed (Fig. 1). Clearly, polyd(T) binds this HDP stoichiometrically

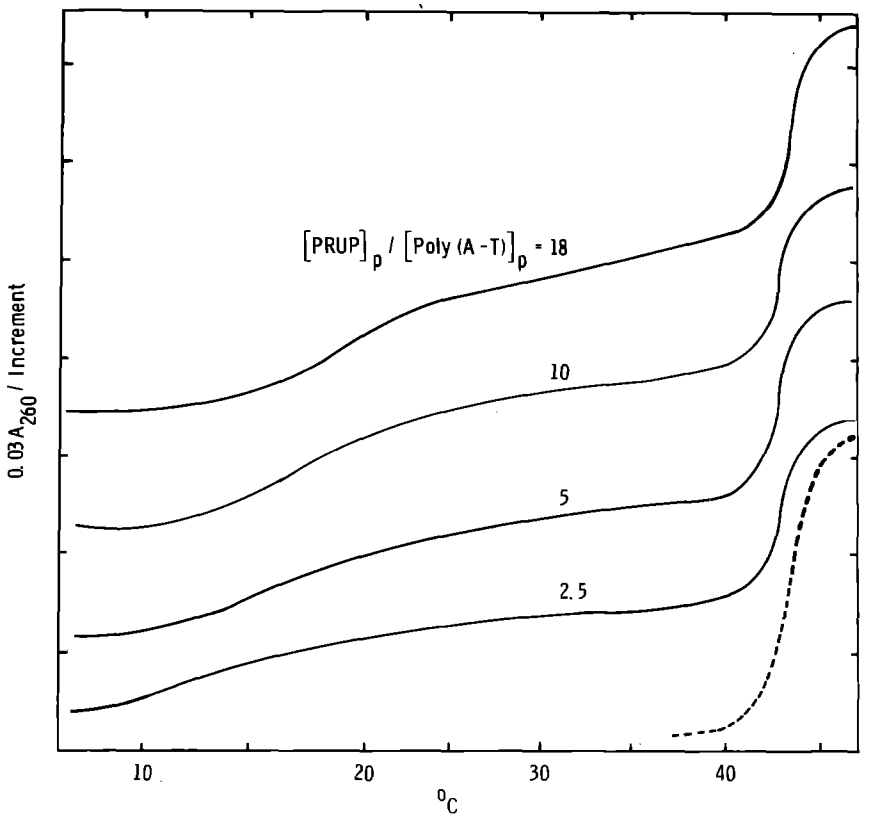

Figure 1: Effect of PRUP (——) and polyd(T) (---) on T4 gene 32 protein-induced Tm depression. $[Polyd(A-T]_p$ = 3.0 x 10^{-5} M; [Gene 32 protein] = 1.8 x 10^{-6} M. $[PRUP]_p$:$[polyd(A-T)]_p$ as indicated; $[polyd(T)]_p$:$[polyd(A-T)]_p$ = 1. 9.1 mM phosphate (K^+), 4 mM NaCl, 0.1 mM Na_2EDTA, 0.03 mM dithiothreitol, pH 7.0.

up to the point where polyd(A-T) thermally denatures, so that no protein is available to melt the double helical DNA.

Although the dependence of PRUP inhibition on this analog's length was not studied in detail, material co-eluting with tRNA on a Sephacryl S-200 column produced the same effect on gene 32 protein-polyd(A-T) melting profiles as did unfractionated PRUP. Preliminary results also indicate no significant dependence of the quenching of gene 32 protein intrinsic fluorescence on PRUP length.

The effect of PRUP on rat liver HDP depression of polyd(A-T) Tm is similar to the results seen with gene 32 protein. At low (∿0.005) ionic strength and a one-third saturation of the DNA helix by the rat liver protein, a ten-fold excess of PRUP (relative to polyd(A-T)) brings about a biphasic melting profile

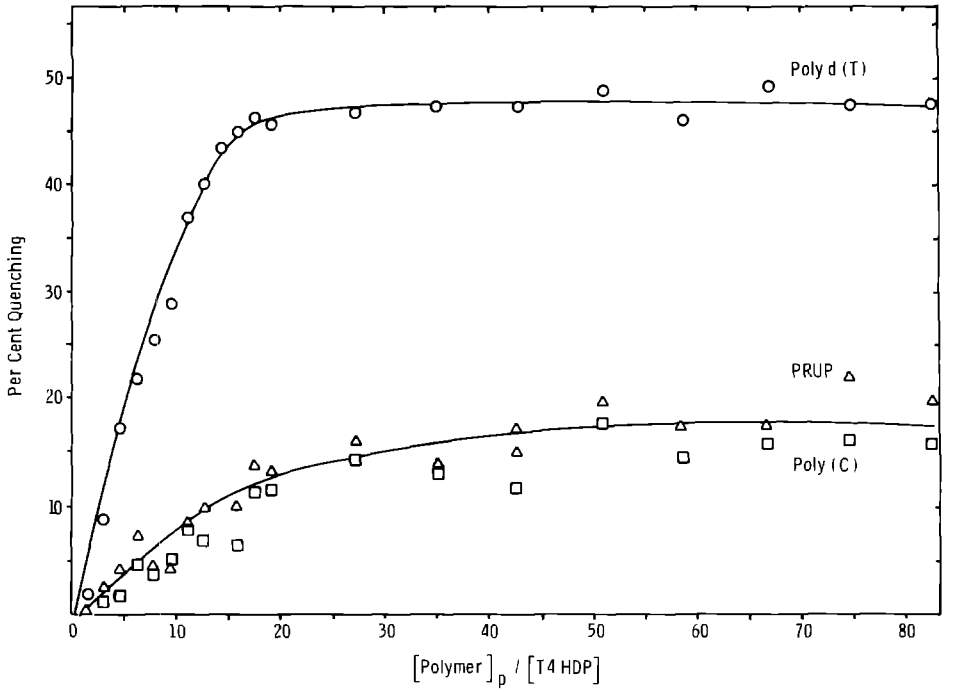

Figure 2: Quenching of intrinsic gene 32 protein fluorescence by PRUP (—△—), poly (C) (—□—) and polyd(T) (—○—). [Gene 32 protein] = 1.6 x 10^{-7} M, in 50 mM phosphate (K^+), 1 mM Na_2EDTA, 1 mM β-mercaptoethanol, pH 7.7.

with the two transitions separated by 20°C, whereas an equimolar level of polyd(T) totally eliminates the effect of this HDP on Tm depression.

Quenching of Intrinsic HDP Fluorescence by PRUP. The quenching of the intrinsic fluorescence of gene 32 protein by PRUP, and for comparison, by poly(C) and polyd(T), is shown in Fig. 2. The effect of PRUP is very similar to that of poly(C), suggesting that these two polymers have similar affinities for the T4 HDP. In contrast, the saturation level of quenching seen with polyd(T) is higher, and the sharper plot is indicative of the higher affinity (relative to poly(C)) of this substrate for the protein (19). A similar experiment with the rat liver HDP is shown in Fig. 3. Here, as with gene 32 protein, the quenching plot, and hence, affinity of PRUP for the protein is comparable to that of poly(C).

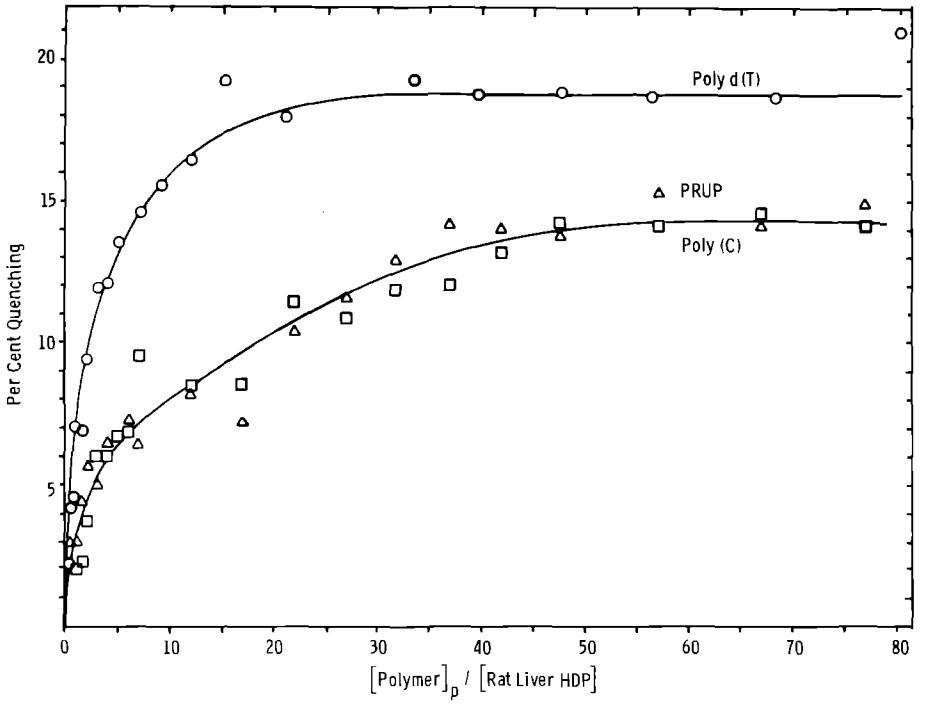

Figure 3: Quenching of intrinsic rat liver HDP fluorescence by PRUP (—△—), poly(C) (—□—) and polyd(T) (—○—). [Rat liver HDP] ≈ 4.0 x 10^{-8} M, in 5 mM phosphate (K^+), 0.5 mM Na_2EDTA, 0.5 mM β-mercaptoethanol, pH 7.7.

DISCUSSION

The results presented above indicate that poly(ribosylurea phosphate) can serve as a substrate for helix-destabilizing proteins. A comparison of the PRUP results with the analogous polynucleotide and polydeoxynucleotide experiments leads to a qualitative assessment of the importance of HDP-(deoxy) ribose phosphate backbone interactions in the overall affinity of these proteins for single strands. Thus, although polyd(T) is a more effective inhibitor of UP1-induced polyd(A T) Tm depression, PRUP was shown to be more effective than poly(C) and poly(A) as an inhibitor of UP1-effected $tRNA_3^{Leu}$ renaturation (3). Along with the chloroacetaldehyde reactivity results (2), it is clear that backbone interactions play a major part in the overall binding affinity. The Tm-depression results with RNase A indicate that interactions with nucleic acid bases are also relatively unimportant in the specificity of this model melting protein for single strands.

In contrast, PRUP is a relatively poor substrate for T4 gene 32 protein and the rat liver HDP. Of all single-stranded polynucleotides and polydeoxynucleotides examined in a recent study (19), poly(C) had the lowest affinity for gene 32 protein. Other single-stranded polynucleotides bound this protein with affinities that were two or more orders of magnitude greater. The comparable affinity of the T4 HDP for PRUP and poly(C) indicates that backbone interactions cannot fully explain the selective binding of this protein to single strands. The similarity of the fluorescence titration plots with these two substrates suggests that the presence of heterocyclic bases is not required for quenching, and that other mechanisms must be operative (20).

Further study of the interaction of PRUP with HDPs would seem to be warranted, since it offers a direct approach to evaluating the contribution of the heterocyclic bases to HDP binding specificity. Application of nucleic acid analogs in general (21) may lead to a better understanding of the molecular basis of these proteins' activities.

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