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Processivity of T4 Endonuclease V Is Sensitive to NaCl Concentration†

Ann K. Ganesan,*,‡ Patricia C. Seawell,‡ Roger J. Lewis,§ and Philip C. Hanawalt‡
Department of Biological Sciences and Biophysics Program, Stanford University, Stanford, California 94305
Received March 4, 1986; Revised Manuscript Received May 29, 1986

ABSTRACT: We previously reported that endonuclease V of bacteriophage T4 reacts processively with pyrimidine dimers in UV-irradiated DNA, tending to react with all of the dimers on one DNA molecule before reacting with any dimers on another DNA molecule [Lloyd, R. S., Hanawalt, P. C., & Dodson, M. L. (1980) *Nucleic Acids Res. 8*, 5113–5127]. In this paper we show that this processivity depends upon salt concentration: it can be detected in 10 mM NaCl but not, by our methods, in 100 mM NaCl. In addition, we show that endonuclease V binds to unirradiated DNA in 10 mM NaCl but not in 100 mM NaCl. We conclude that T4 endonuclease V binds to pyrimidine dimers in a two-step process in 10 mM NaCl. It first binds electrostatically to undamaged sections of DNA, and it remains bound during the second step in which it "searches" for pyrimidine dimers. Our conclusion is analogous to the expanded target theory developed for *Lac* repressor [Berg, O. G., Winter, R. B., & von Hippel, P. H. (1981) *Biochemistry 20*, 6929–6948].

T4 endonuclease V, coded by the *denV* gene of bacteriophage T4, comprises a DNA glycosylase that specifically recognizes pyrimidine dimers in DNA and an AP[1] endonuclease that recognizes apurinic and apyrimidinic sites. DNA containing pyrimidine dimers is incised by the sequential action of the glycosylase and the AP endonuclease (Gordon & Haseltine, 1980; Radany & Friedberg, 1980; Seawell et al., 1980; Nakabeppu & Sekiguchi, 1981).

Previously we reported that when limiting amounts of endonuclease V were incubated under appropriate conditions with superhelical DNA containing several pyrimidine dimers per molecule, partial digests contained a mixture of intact, unnicked DNA molecules and completely digested molecules

† Supported by Project Agreement DE-AT03-76EV70007 from the U.S. Department of Energy and Grant GM O9901-24 from the National Institute of General Medical Sciences. R.J.L. was supported by the NIH Medical Scientist Training Program at Stanford University School of Medicine.
* Address correspondence to this author.
‡ Department of Biological Sciences.
§ Biophysics Program.

[1] Abbreviations: AP, apurinic or apyrimidinic; N-AAAF, 2-[*N*-(acetoxyacetyl)amino]fluorene; 4-NQO, 4-nitroquinoline 1-oxide.

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(Lloyd et al., 1980). This result was interpreted to mean that endonuclease V can act processively on substrate DNA. A processive reaction could result if an enzyme molecule remained bound to a DNA molecule between catalytic events and thus tended to react with all the dimers in one DNA molecule before reacting with dimers in another DNA molecule. Alternatively, under certain conditions, simple diffusion might produce the same phenomenon.

In this paper we show that the processivity previously reported (Lloyd et al., 1980) can be observed in 10 mM but not in 100 mM NaCl and that endonuclease V binds to unirradiated DNA in 10 mM but not in 100 mM NaCl. On the basis of our observations we suggest that the processivity of endonuclease V is mediated by electrostatic binding to undamaged regions of irradiated DNA and that the enzyme remains bound as it "searches" for pyrimidine dimers. This behavior would be analogous to that of several other proteins that interact with DNA including the restriction endonuclease *Eco*RI (Jack et al., 1982; Ehbrecht et al., 1985; Terry et al., 1985) and consistent with the expanded target theory of von Hippel and co-workers (Berg et al., 1981).

Experimental Procedures

Two preparations of T4 endonuclease V were used: one was purified through phosphocellulose (Seawell et al., 1981) and the other through CM-Sephadex by a modification of a previously described method (Yasuda & Sekiguchi, 1976; Nakabeppu et al., 1982). The method of preparation did not affect the behavior of the enzyme.

Radioactive plasmid (*Col*E1 or pSV2-*gpt*) was prepared and irradiated as previously described (Seawell & Ganesan, 1981). Nonradioactive plasmid (pSV2-*gpt*) was prepared in essentially the same manner.

Linear *Col*E1 was produced by incubating superhelical plasmid with *Eco*RI (New England Biolabs, Inc.) at 37 °C in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5 mM $MgCl_2$. The DNA was precipitated and washed in ethanol before being treated with T4 endonuclease V.

Reaction products were analyzed by sedimentation in 5–20% (w/v) alkaline sucrose gradients (Lloyd et al., 1980). This method of analysis measures DNA glycosylase activity, detecting not only strand breaks but also AP sites, which are converted to strand breaks in alkali.

Results

A limiting amount of endonuclease V was incubated with linear *Col*E1 DNA containing approximately 20 pyrimidine dimers per molecule under the conditions used by Lloyd et al. (1980). Analysis on alkaline sucrose gradients of samples removed at 15, 30, and 60 min revealed mixtures of intact (unnicked) DNA molecules and fragments of the size expected if incisions had occurred at every dimer site. The relative amount of the latter increased as the reaction progressed (Figure 1A). In contrast, when the same procedure was followed with a different reaction buffer, few or no full-length DNA molecules were observed. Instead, we found randomly nicked *Col*E1 molecules that decreased in size as the reaction progressed (Figure 1B). The critical component of the buffer was found to be the NaCl concentration (Figure 2). In "low" concentrations of NaCl (0 or 10 mM) endonuclease V acted processively, while in "high" concentrations (100 mM) it acted randomly. This was observed for substrate DNA that was initially in the linear (Figure 1) or the superhelical (Figure 2) form.

Table I shows the results of competition experiments designed to detect the binding of endonuclease V to undamaged

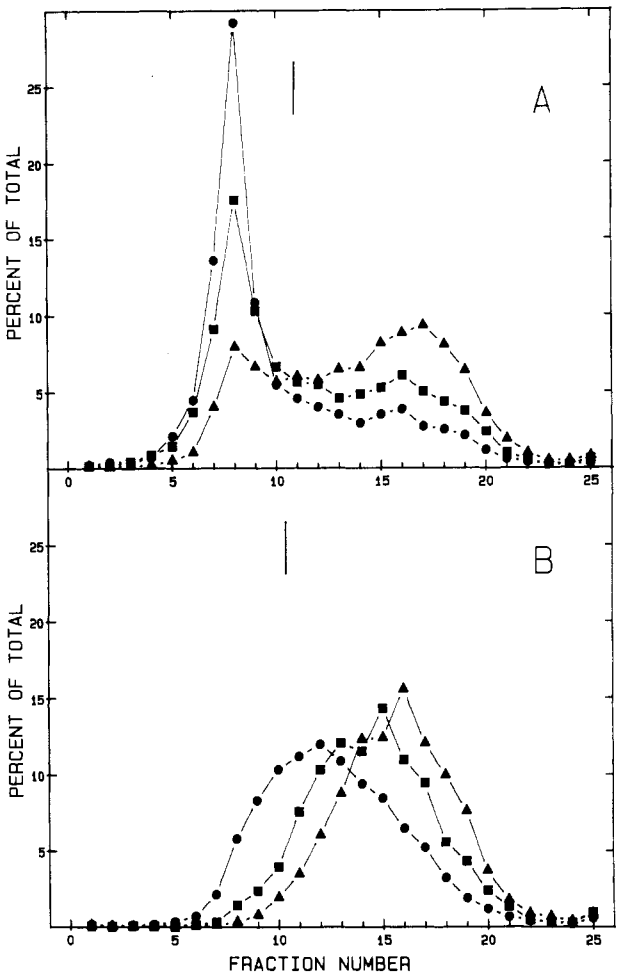

FIGURE 1: Effect of NaCl concentration on the action of T4 endonuclease V on linear DNA. Linear ^{14}C-labeled *Col*E1 (270 ng) containing approximately 20 pyrimidine dimers per molecule was incubated with limiting amounts of T4 endonuclease V at 37 °C in a 300-μL reaction containing (A) 10 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, pH 8, and BSA (1 mg/mL) or (B) 100 mM NaCl, 10 mM Tris-HCl, 10 mM EDTA, pH 8, and BSA (1 mg/mL). Samples were removed at 15 (●), 30 (■), and 60 min (▲) into tubes containing enough Sarkosyl to give a final concentration of 1%. Linear ^{3}H-labeled pBR322 DNA was added to each sample as marker (bar marks the position of the peak) and the samples were loaded atop 5-mL alkaline sucrose gradients (5–20% sucrose in 0.1 N NaOH, w/v). After 30 min the gradients were centrifuged in an SW50.1 rotor for 5.5 h at 45 000 rpm at 20 °C in a Beckman L2-65B ultracentrifuge. Fractions were collected through the bottom of the tubes onto strips of Whatman 17 filter paper, which were then washed in trichloroacetic acid, ethanol, and acetone and dried. Radioactivity was measured by scintillation spectrometry.

DNA. In these experiments the substrate DNA was irradiated ^{14}C-labeled pSV2-*gpt* and the competing DNA was nonradioactive pSV2-*gpt* either irradiated with the same UV dose as the substrate or not irradiated. Competing DNA was mixed with substrate prior to addition of the enzyme. Because of the nonrandom distribution of incisions made in low concentrations of NaCl, we calculated the total number of incisions in a reaction by assuming that incisions occurred at all 20 dimers in each substrate molecule incised. For high concentrations of NaCl we calculated the frequency of incisions with a nomograph (Litwin, 1969; Litwin et al., 1969). In both 10 mM and 100 mM NaCl we observed that an equal quantity of UV-irradiated competing DNA inhibited the incision of irradiated radioactive substrate DNA by 41% and 42%, respectively. Under ideal conditions both values are expected to be 50%. The slightly lower values observed suggest that the quantity of enzyme used was almost, but not entirely,

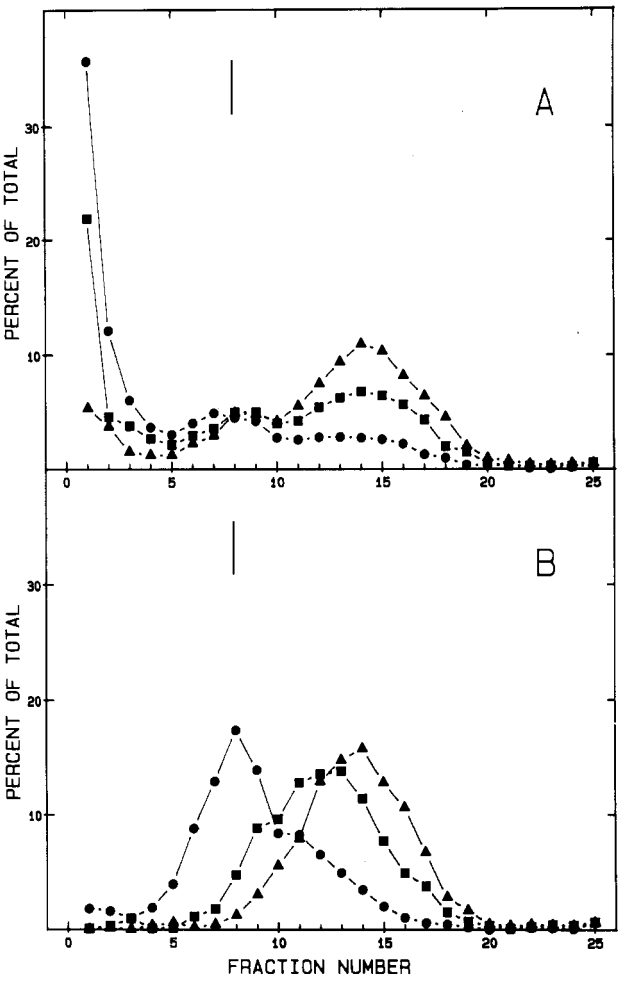

FIGURE 2: Effect of NaCl concentration on the action of T4 endonuclease V on superhelical DNA. Superhelical ^{3}H-labeled *Col*E1 (480 ng) containing approximately 20 pyrimidine dimers per molecule was incubated with limiting amounts of T4 endonuclease V at 37 °C in a 300-μL reaction containing (A) 10 mM NaCl, 10 mM Tris-HCl, 10 mM EDTA, pH 8, and BSA (1 mg/mL) or (B) 100 mM NaCl, 10 mM Tris-HCl, 10 mM EDTA, pH 8, and BSA (1 mg/mL). At 5 (●), 15 (■), and 30 min (▲) samples were removed and analyzed as described in the legend to Figure 1 except that linear ^{14}C-labeled *Col*E1 was added as marker (bar marks position of the peak).

Table I: Effect of Nonradioactive Competing DNA on Incision of Radioactive Substrate DNA by T4 Endonuclease V[a]

NaCl concn (mM)	competitor	dimers incised (×10^{-11})	inhibition (%)
10	0	3.8[b]	0
	undamaged (1 μg)	3.3[b]	13
	undamaged (2 μg)	2.8[b]	26
	UV irradiated (0.2 μg)	2.2[b]	42
100	0	3.4[c]	0
	undamaged (1 μg)	3.4[c]	0
	UV irradiated (0.2 μg)	2.0[c]	41

[a] Superhelical ^{14}C-labeled pSV2-*gpt* (200 ng) containing approximately 20 pyrimidine dimers per molecule was incubated with a limiting amount of T4 endonuclease V in 50-μL reactions under the same conditions used in Figure 2. Where indicated, nonradioactive competing pSV2-*gpt* was mixed with the ^{14}C-labeled substrate before addition of the enzyme. Irradiated competing DNA received the same UV dose as did irradiated substrate. Reactions were incubated for 5 min at 37 °C before analysis on alkaline sucrose gradients. [b] Calculated from percent DNA incised, assuming that 20 incisions were produced in each DNA molecule incised. [c] Calculated from a nomograph (Litwin, 1969; Litwin et al., 1969).

limiting. The near equality of the two values obtained provides a check on the methods used to calculate the number of incisions under the two conditions. Consistent with previous reports (Seawell et al., 1980; McMillan et al., 1981), in 100 mM NaCl we detected no inhibition of incision of UV-irradiated (radioactive) substrate DNA by a fivefold excess of unirradiated (nonradioactive) DNA. In contrast, in 10 mM NaCl a five- and tenfold excess of unirradiated (nonradioactive) DNA caused a 13% and 26% inhibition, respectively. We infer that the inhibition was caused by binding of the enzyme to unirradiated DNA. The rate of incision in these experiments was only slightly (approximately 10%) slower in 100 mM than in 10 mM NaCl.

Discussion

Several proteins that bind to specific sites in DNA, for example the RNA polymerase and the *Lac* repressor protein of *Escherichia coli*, also bind nonspecifically to DNA. The nonspecific binding is sensitive to ionic strength and can be completely suppressed at salt concentrations greater than 1 M. From this it has been inferred that nonspecific binding is due to electrostatic interaction. It has been proposed that after binding nonspecifically the protein can "slide" or be translocated along the DNA to sites where it can bind specifically (Berg et al., 1981; Winter & von Hippel, 1981; Winter et al., 1981; von Hippel et al., 1984).

Our results show that T4 endonuclease V acts processively in reactions containing 10 mM NaCl but nonprocessively in reactions containing 100 mM NaCl. Our results also show that the enzyme's affinity for undamaged DNA is greater in 10 mM NaCl than in 100 mM NaCl. Taken together these observations provide strong evidence for a processive mechanism that relies upon electrostatic binding to undamaged sections of DNA. Similar results have recently been obtained by Gruskin and Lloyd (1986).

In order to quantify the binding of endonuclease V to undamaged DNA we calculated the apparent affinity constant of the enzyme for undamaged DNA in reactions containing 10 mM NaCl from the data in Table I using a published estimate of the Michaelis constant, K_m. We proceeded by assuming that undamaged DNA acts as a competitive inhibitor in the Michaelis–Menton manner, although the processive action of the enzyme may complicate the interpretation of this calculation because the measured affinity for undamaged DNA may depend upon the length of the competing DNA as well as upon salt concentration. The enzymatic mechanism is assumed to be

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$E + I \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EI \qquad (1)$$

where the ES complex signifies endonuclease V bound to a pyrimidine dimer on an irradiated DNA molecule, P, the dimer after glycosylic incision, and EI, the enzyme bound to undamaged DNA.

For the DNA glycosylase activity of endonuclease V in 100 mM NaCl, Duker and Merkel (1985) have reported a K_m of 3.1×10^{-7} M for pyrimidine dimers in DNA. The Michaelis constant is a measure of the affinity of an enzyme for its substrate and could be affected by a change in the mechanism by which the enzyme finds and binds to its substrate. In our experiments, NaCl concentration apparently affects the binding mechanism of the enzyme and might alter its K_m. However, the rate of reaction of endonuclease V changes by less than a factor of 2 as the NaCl concentration increases from 10 to 100 mM. Nakabeppu et al. (1982) found this to

be the case for substrate concentrations of approximately 10^{-6} M thymine dimers in poly(dA)·poly(dT), and we obtained similar results for 10^{-10} and 10^{-9} M pyrimidine dimers in DNA (unpublished data). From this we conclude that the apparent value of K_m is not significantly affected by NaCl concentrations between 10 and 100 mM, allowing us to use the published value for K_m in our calculations. Solving mechanism 1 for the reaction rate (see, for example, Laidler, 1978), we obtain

$$\frac{-d[S]}{dt} = \frac{k_2[E_t][S]}{[S] + K_m(1 + [I]/K_I)} \quad (2)$$

where $K_m = (k_{-1} + k_2)/k_1$, $K_I = k_{-3}/k_3$, and $[E_t]$ is the total enzyme concentration. Solving for K_I, the affinity constant for undamaged DNA, with the data from Table I, we obtain 1.8×10^{-4} M, assuming $K_m = 3.1 \times 10^{-7}$ M. McMillan et al. (1981) have reported a much smaller value for K_m than the value used here. Using their value for $K_m(\sim 10^{-9}$ M) would reduce the resulting value for K_I by a factor of 250.

Although our data support the hypothesis that endonuclease V acts processively by a mechanism that depends upon an electrostatic interaction with undamaged regions of DNA, we have also considered an alternative hypothesis. The processivity apparent in 10 mM NaCl might be due to pyrimidine dimers on a DNA molecule being closer to each other than to dimers on other molecules. In this case, if the enzyme tended to react with the dimer closest to the previous site of incision, it would usually react with all the dimers in one DNA molecule before reacting with dimers in other molecules. If this were true, the lack of processivity observed in 100 mM NaCl might reflect a decrease in the specific activity of the enzyme in high concentrations of salt, resulting in an increase in the period between consecutive incisions sufficient to allow the enzyme to diffuse from one DNA molecule to another. It can be shown, however, that the apparent processivity due to the proximity of dimers on a DNA molecule will be small at the dimer frequencies we have used (Berg et al., 1981; O. G. Berg, personal communication).

The processive behavior of the enzyme can affect the interpretation of other measurements. Most assays for endonuclease V activity specify buffers containing 100 mM NaCl in which the enzyme has little affinity for undamaged DNA and reacts randomly with pyrimidine dimers in DNA. In these conditions, by using the Poisson expression, the average number of incisions per substrate DNA molecule can be calculated from the proportion of molecules remaining unincised (e.g., Seawell et al., 1980; Bohr et al., 1985). Alternatively, when the frequency of incisions is too large to allow an accurate determination of the proportion of DNA remaining unincised the average number of incisions per molecule can be calculated from the size of the product molecules. When the substrate DNA is initially monodisperse, e.g., phage or plasmid DNA, the Litwin nomograph provides an appropriate method (Litwin, 1969; Litwin et al., 1969), while number-average molecular weight (M_n) values can be used when the size of the substrate DNA forms a random distribution (Lehmann, 1981). We have obtained reasonable agreement between values obtained by both methods.

Under processive conditions, if the substrate DNA contains several dimers, incisions are not randomly distributed among the molecules and neither the Poisson expression nor the size of the product molecules can be used to calculate the number of incisions. In this case we calculated the total number of incisions that occurred from the proportion of DNA sedimenting at the position of completely digested molecules in alkaline sucrose gradients and the number of incisions per molecule required to produce complete digestion.

These constraints should be kept in mind when the activity of an endonuclease V preparation is to be determined, as the analysis will be facilitated by using conditions under which the enzyme acts nonprocessively. In contrast, when the enzyme is used to measure the pyrimidine dimer content of DNA, excess enzyme should be used and an incision should be produced at every dimer site in the DNA. In this case the product is the same whether the enzyme acts processively or not, and nonprocessive conditions are less important.

ACKNOWLEDGMENTS

We are grateful to C. A. Smith for helping to clarify and organize our ideas and to R. S. Lloyd for initiating this project in our laboratory and sharing with us his unpublished results. We greatly appreciate the careful review of this paper by O. G. Berg.

Registry No. Endodeoxyribonuclease V, 52227-85-7.

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Energetics of Internal GU Mismatches in Ribooligonucleotide Helixes†

Naoki Sugimoto,‡ Ryszard Kierzek,§ Susan M. Freier,‡ and Douglas H. Turner*,‡
Department of Chemistry, University of Rochester, Rochester, New York 14627, and Institute of Bioorganic Chemistry, Polish Academy of Sciences, 60-704 Poznan, Noskowskiego 12/14, Poland
Received January 13, 1986; Revised Manuscript Received May 15, 1986

ABSTRACT: Thermodynamic parameters of helix formation were measured spectroscopically for 16 oligoribonucleotides containing either internal GU mismatches or the corresponding AU pairs. Internal GU mismatches stabilize each helix, but not as much as the corresponding AU pairs. The differences in the enthalpy and entropy changes of helix formation associated with replacing AU pairs with GU mismatches are less than previously realized. At both 25 and 37 °C, the decrease in helix stability associated with replacing an AU with a GU is also less than thought previously. Approximations are suggested for predicting the effects of GU mismatches on helix stability.

Internal GU mismatches in helical regions are known to occur in tRNA (Kim et al., 1974; Ladner et al., 1975; Sussman & Kim, 1976; Johnston & Redfield, 1981; Sprinzl et al., 1985) and are proposed to occur in the secondary structures of essentially all large RNA molecules (Fox & Woese, 1975; Noller, 1984; Woese et al., 1983; Steger et al., 1984; Cech et al., 1983). Romaniuk et al. (1979a,b) and Alkema et al. (1982) used NMR to demonstrate the existence of GU mismatches in RNA oligonucleotides. Corresponding GT mismatches in DNA oligomers have been studied by Patel et al. (1982), Aboul-ela et al. (1985), and Brown et al. (1985). Despite the prevalence of internal GU mismatches, there is only one experimental study of their thermodynamic properties (Uhlenbeck et al., 1971). This study was limited to A_4GUU_4, A_4UGU_4, and A_5UGU_5 because of the lack of methods for making defined-sequence oligonucleotides. Recent advances have made it possible to make oligoribonucleotides with more varied sequences (Uhlenbeck & Gumport, 1982; Kierzek et al., 1987; Markiewicz et al., 1984; Matteucci & Caruthers, 1980). This paper reports thermodynamic measurements on 16 oligomers containing either internal GU mismatches or the corresponding AU pairs. The results are important for improving predictions of RNA structure and stability (Tinoco et al., 1973; Borer et al., 1974; Gralla & Crothers, 1973), and the temperature dependence of these properties (Steger et al., 1984). The results indicate internal GU pairs are more stable and more temperature dependent than previously realized.

Materials and Methods

Oligonucleotide Synthesis. Oligomers starting with AUG were synthesized by using T4 RNA ligase to make successive additions of nucleoside 5′,3′-bisphosphates to AUG (Sigma) as described previously (Hickey & Turner, 1985; Freier et al., 1985a). Ligase reactions differed widely in rates, requiring 8 h to 7 days at 37 or 4 °C for 100% yield. Reactions and product purity were monitored by HPLC using a methanol gradient (Petersheim & Turner, 1983). These oligomers have a 3′-terminal phosphate.

AUACGUAU, GCUGGC, GGCGUC, CUGCGG, CGGCUG, and GCUAGC were synthesized chemically on solid support by using phosphoramidite procedures, and purified by anion-exchange chromatography on DEAE-Sephadex (A-25) using NaCl gradients in 7 M urea, 10 mM Tris, pH 8.2. These oligomers do not have a 3′-terminal phosphate.

Melting Curves. Extinction coefficients were calculated with the nearest-neighbor approximation (Richards, 1975). For oligomers studied at 260 nm, the calculated extinction coefficients ($\times 10^{-4}$ cm^{-1} M^{-1}) at 260 nm are as follows: AUGCGUp, 4.89; AUGCAUp, 5.09; AUGCGCGUp, 6.14; AUGCGCAUp, 6.24; AUGCGUAUp, 6.72; AUACGUAU, 7.09; AUGUGCAUp, 6.89; AUGUACAUp, 7.58; GCUGGC, 4.38; GCUAGC, 4.81. For oligomers studied at 280 nm, the calculated extinction coefficients ($\times 10^{-4}$ cm^{-1} M^{-1}) at 280 nm are as follows: GGCGUC, 3.15; CUGCGG and CGGCUG, 3.18. Total strand concentrations, C_T, were determined from the absorbance measured at 90 °C.

Absorbance vs. temperature melting curves were measured at 260 or 280 nm on a Gilford 250 spectrometer (Freier et al., 1983a). The heating rate was 1 °C/min, and the buffer for measurements at 260 nm was 1 M NaCl, 10 mM Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7. For measurements at 280 nm, the buffer was 1 M NaCl, 5 mM Na_2HPO_4, 0.5 mM Na_2EDTA, pH 7. For each oligonucleotide, 13–21 absorbance vs. temperature profiles were measured over a 100-fold range in strand concentration. Melting curves were analyzed with a two-state model as previously described (Petersheim & Turner, 1983; Freier et al., 1983a,b).

Results

Temperature-Independent Thermodynamic Parameters. For a two-state transition, the reciprocal melting temperature, T_m^{-1}, depends linearly on the log of the total strand concentration, C_T (Borer et al., 1974):

$$T_m^{-1} = \frac{2.303R}{\Delta H^\circ}(\log C_T) + \frac{\Delta S^\circ}{\Delta H^\circ} \quad (1)$$

Plots suggested by eq 1 are shown in Figure 1. Good straight

†This work was supported by National Institutes of Health Grant GM 22939.
‡University of Rochester.
§Polish Academy of Sciences.

0006-2960/86/0425-5755$01.50/0