Comparison of the Kinetics of Ribooligonucleotide, Deoxyribooligonucleotide, and Hybrid Oligonucleotide Double-Strand Formation by Temperature-Jump Kinetics†

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ABSTRACT: The kinetics of double-strand formation were measured by using temperature-jump kinetic techniques for the DNA oligonucleotides dCA_5G + dCT_5G, the analogous RNA oligonucleotides rCA_5G + rCU_5G, and the hybrid rCA_5G + dCT_5G. The DNA oligonucleotides have a faster rate of recombination and a slower rate of dissociation at 12.0 °C than the RNA oligonucleotides; the hybrid has about the same recombination rate and a slightly faster dissociation rate than the RNA oligonucleotides. The activation energies for recombination for the DNA and RNA oligonucleotides are both near 0 kcal/mol. The difference in dissociation and recombination activation energies is consistent with the thermodynamic results obtained earlier. The relaxation process is composed of two exponential components for the RNA and hybrid oligonucleotides at temperatures of 12.0 °C and lower. One exponential component is observed for these oligonucleotides above 12.0 °C and for the DNA oligonucleotides at all temperatures.

The kinetics of double-strand formation for a number of ribooligonucleotides have been studied by using the temperature-jump technique (Craig et al., 1971; Podder, 1971; Pörschke & Eigen, 1971; Pörschke et al., 1973; Ravetch et al., 1974; Breslauer & Bina-Stein, 1977). Studies on deoxyribooligonucleotides are more limited (Drobnies, 1979; S. M. Freier, D. D. Albergo, and D. H. Turner, unpublished experiments). One RNA–DNA hybrid oligonucleotide double helix has been studied (Hoggett & Maass, 1971). However, no thorough study has been made comparing the kinetics of the same sequence for RNA, DNA, and hybrid oligonucleotides. Such knowledge is important in determining how chemical differences between DNA and RNA manifest themselves in the dynamic properties of the double strands.

In this report, we present results of temperature-jump kinetic studies of double-strand formation of the RNA oligonucleotides rCA_5G + rCU_5G, the analogous DNA oligonucleotides dCA_5G + dCT_5G, and a hybrid double helix composed of rCA_5G + dCT_5G. The other hybrid dCA_5G + rCU_5G does not form stable double strands (Martin & Tinoco, 1980).

Materials and Methods

The ribooligonucleotides rCA_5G and rCU_5G were synthesized enzymatically by using polynucleotide phosphorylase as described earlier (Uhlenbeck et al., 1971). The deoxyribooligonucleotides dCA_5G and dCT_5G were chemically synthesized by using the diester method (Khorana, 1968). Extinction coefficients for the ribooligonucleotides were determined by base hydrolysis and the extinction coefficients of the mononucleotides. Extinction coefficients for the deoxyribooligonucleotides were estimated by using dinucleotide and mononucleotide extinction coefficients. The extinction coefficients in terms of L cm^{-1} (mol of strands)$^{-1}$ at 25 °C were the following: rCA_5G, 7.1×10^4; rCU_5G, 6.3×10^4; dCA_5G, 7.9×10^4; dCT_5G, 5.8×10^4.

Concentrations of all mixtures capable of forming double strands were determined by measuring absorbances at 50 °C, where the strands exist as single strands. The oligonucleotides are subject to degradaton due to the high intensity of the UV lamp used in temperature-jump experiments. Therefore, concentrations were determined before and after the temperature-jump measurements and were always within 3%. Additionally, absorbances were recorded at 0 °C before and after to check the hypochromicity. The hypochromicity, $(A_{50°C} - A_{0°C})/A_{50°C}$, generally decreased from 0.5 to 2% after the measurements. Samples with larger discrepancies were not used in the analysis.

The buffer used throughout these studies, 1 M NaCl, 0.01 M sodium phosphate buffer, pH 7, and 0. 1 mM ethylenediaminetetraacetate (EDTA),[1] was filtered by using Uni-Pore polycarbonate membranes (Bio-Rad) with a pore size of 3 μm. Samples were degassed prior to temperature-jump measurements by purging the buffer with helium for 3–5 min before mixing with a small volume of a stock solution of oligonucleotides.

Measurements were taken at the mercury line near 267 nm for the ribooligonucleotides and deoxyribooligonucleotides. Due to problems developing from the sample cell, the hybrid oligonucleotides were more conveniently measured at 250 nm. The temperature was measured to an accuracy of 0.2 °C by using a copper–constantan digital thermocouple (Fluke Model 2100A) in contact with the upper electrode.

Temperature-Jump Instrument. The temperature-jump instrument was manufactured by DiaLog (West Germany) and has been previously described (Rigler et al., 1974; Drobnies, 1979). The data were collected digitally by a Biomation Model 805 transient recorder, using 2048 data points. A PET microcomputer was used to transfer the data from the Biomation to a VAX 11/780 computer, where the data were analyzed.

The program DISCRETE written by S. W. Provencher (Provencher, 1976a,b) was used to analyze the data. The program determines, from the data, what number of exponential components results in the best fit. The program was allowed to

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[1] Abbreviations: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetate.

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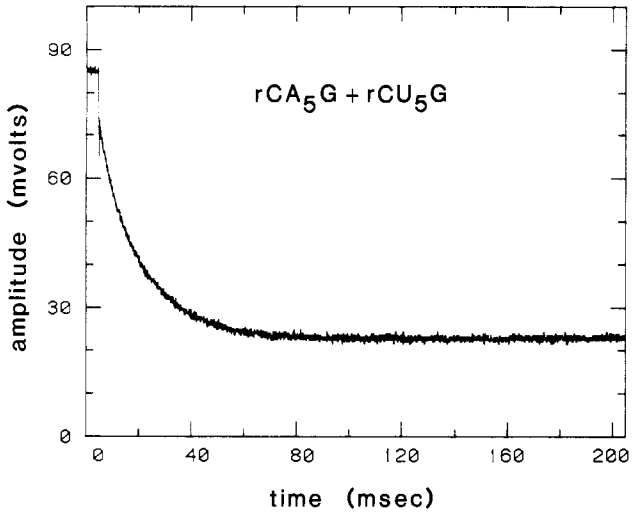

FIGURE 1: Trace of a temperature-jump experiment on 14.2 μM $rCA_5G + rCU_5G$ in 1 M NaCl, 0.01 M phosphate buffer, pH 7, and 0.1 mM EDTA, at a final temperature of 6.7 °C. The temperature jump was 1.8 °C; λ = 267 nm. The initial signal was 5 V. The first 5 ms shows the signal prior to the temperature jump.

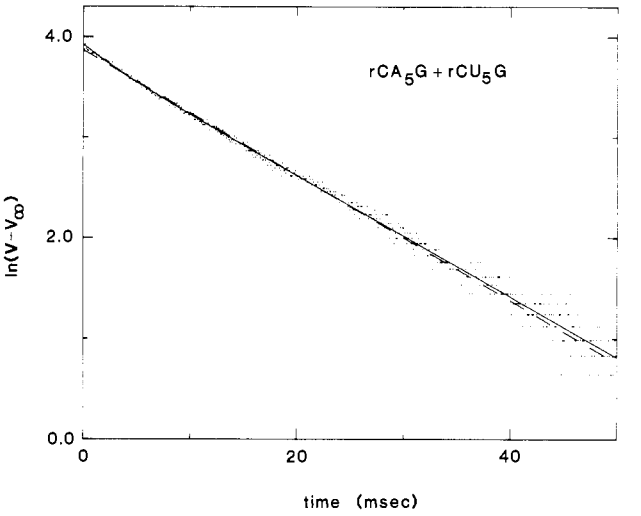

FIGURE 2: Semilog plot of the trace in Figure 1. The amplitude at long times, V_∞, was determined from the two-component fit of the program DISCRETE. The two lines show the two-component fit (—) (τ_1 = 16.6 ms, a_1 = 45.8 mV; τ_2 = 2.87 ms, a_2 = 4.92 mV) and the one-component fit (---) (τ_1 = 16.1 ms, a_1 = 47.8 mV).

search for three exponential components. Two exponentials were reproducibly found for the ribooligonucleotides and hybrid oligonucleotides at 6.7 and 12.0 °C. One exponential was found above this temperature and for the deoxyribooligonucleotides.

Analysis of the Temperature-Jump Kinetic Data. For the reaction scheme given by eq 1, k_r corresponds to the recom-

$$A + B \underset{k_d}{\overset{k_r}{\rightleftharpoons}} AB \qquad (1)$$

bination rate constant for the formation of double strands AB from the non-self-complementary strands A and B whereas k_d corresponds to the dissociation rate constant. When the system at equilibrium is perturbed by producing a fast jump in temperature, the system relaxes exponentially to its equilibrium at the final temperature according to eq 2. C_s is the

$$1/\tau = 2k_rC_s + k_d \qquad (2)$$

equilibrium concentraton of A at the final temperature, and the total concentrations of A and B are assumed to be equal. τ is the relaxation time characterizing the process.

We have considered three methods to determine k_r and k_d by measuring $1/\tau$ at several concentrations of strands. The first is to calculate C_s at each total concentration by using the equilibrium constant determined thermodynamically and plotting $1/\tau$ vs. C_s. The slope gives k_r; the intercept gives k_d.

The second method is to use the kinetic equilibrium constant determined by the first method, $K_{kinetic} = k_r/k_d$, and calculate new values for C_s, k_r, and k_d. Iteration will result in the determination of a kinetically determined equilibrium constant consistent with the data.

The third method is to square eq 2 and eliminate C_s from the equation to get eq 3:

$$1/\tau^2 = 4k_rk_dC_{tot} + k_d^2 \qquad (3)$$

A plot of $1/\tau^2$ vs. C_{tot}, the total concentration, gives an intercept = k_d^2 and a slope = $4k_rk_d$.

The second and third analysis methods have the advantage that previous thermodynamic knowledge is not used in the final determination of the kinetics. A comparison of the results of the kinetics and thermodynamics allows a check of the consistency of the reaction scheme. We used all three methods of analysis.

Results

Figure 1 shows a trace of voltage vs. time for the ribooligonucleotides $rCA_5G + rCU_5G$ at 14.2 μM concentration and a final temeprature of 6.7 °C. The size of the temperature jump was 1.8 °C; the initial signal was 5 V. Figure 2 shows a semilog plot of the trace in Figure 1. The small amount of curvature indicates that there are two exponential components. The two lines indicate the best fits by assuming one exponential (---) and two exponentials (—). Due to the small amplitude of the second component, the relaxation times for the predominant relaxation for both fits are within 3%. Since the second component is seen only at low temperature, the one-component fit is used to characterize the bimolecular reaction. Using the two exponentials, when observed, does not change the results. The second component is seen only for the ribooligonucleotides and hybrid oligonucleotides at temperatures at or below 12 °C. It is not observed at higher temperatures or for the deoxyribooligonucleotides at all temperatures.

The relaxation times for the predominant relaxation were averages of 5–10 relaxations for each sample. The standard deviation of the relaxation times was generally 2–6%. These standard deviatons were used in linear regression to give the errors for the recombination and dissociation rate constants reported. Using these errors in the linear regression to determine the activation energies yielded the errors reported for the activation energies.

The temperature range of the instrument is limited by the sample cell to initial temperatures no lower than 5 °C. The upper end of the temperature range is reached when the concentration of double strands becomes too low and the signal becomes too small to measure. Thus, the temperature ranges studied were 6.7–21.1 °C for the ribooligonucleotides and 8.0–24.8 °C for the deoxyribooligonucleotides.

Table I lists the rate constants and activation energies determined for the ribooligonucleotides $rCA_5G + rCU_5G$ at four temperatures, using the three methods of analysis discussed above. The first uses thermodynamic equilibrium constants to calculate the equilibrium single strand concentratons, C_s, in eq 2; the recombination rate constant, k_r, and the dissociation rate constant, k_d, are determined from the slope and intercept, respectively, of a plot of $1/\tau$ vs. C_s. This procedure has the disadvantage of requiring previous thermodynamic knowledge, which might bias the kinetic results. The second

Table I: Kinetic Results for $rCA_5G + rCU_5G$ Using Different Analytical Methods

	recombination rate constant ($\times 10^{-6}$) (L mol^{-1} s^{-1})			dissociation rate constant (s^{-1})		
	$1/\tau$ vs. C_s	iterating $1/\tau$ vs. C_s	$1/\tau^2$ vs. C_{tot}	$1/\tau$ vs. C_s	iterating $1/\tau$ vs. C_s	$1/\tau^2$ vs. C_{tot}
6.7 °C	6.2 ± 0.7	5.2 ± 0.6	5.6 ± 6.4	11 ± 4	12 ± 4	11 ± 1
12.0 °C	6.4 ± 0.4	6.8 ± 0.4	6.8 ± 1.9	38 ± 4	37 ± 4	37 ± 8
16.4 °C	5.9 ± 0.6	6.0 ± 0.6	6.0 ± 1.3	108 ± 9	107 ± 9	107 ± 12
21.1 °C	4.6 ± 1.1	4.4 ± 1.0	4.5 ± 1.3	330 ± 20	330 ± 20	330 ± 20
k (12.0 °C)	6.2 × 10^6	6.2 × 10^6	6.7 × 10^6	38	38	37
E_a (kcal/mol)	−2 ± 2	0 ± 2	−6 ± 6	39 ± 2	39 ± 2	40 ± 3
$\Delta S^{\ddagger}$ (eu)	−36 ± 7	−29 ± 7	−50 ± 20	84 ± 6	84 ± 6	87 ± 10

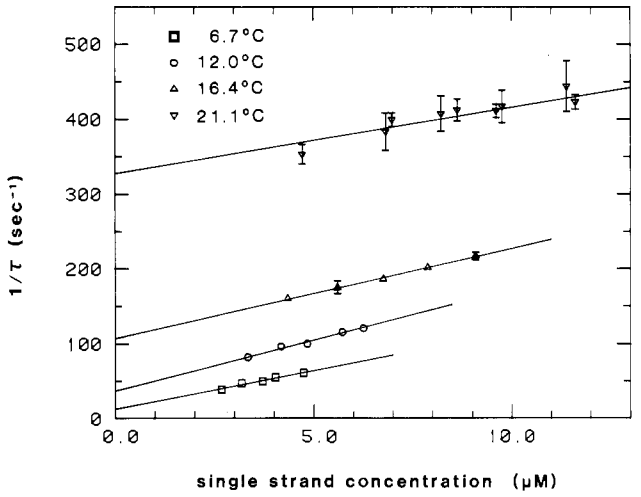

FIGURE 3: $1/\tau$ as a function of the equilibrium single-strand concentration of $rCA_5G + rCU_5G$, determined by the second method (see text). The four temperatures are (□) 6.7, (○) 12.0, (△) 16.4, and (▽) 21.1 °C. The error bars not shown are on the order of the symbol size. The results are tabulated in Table I.

method avoids this problem by calculating the equilibrium constant determined from kinetics, $K_{kinetic} = k_r/k_d$. This equilibrium constant is then used to calculate the next set of rate constants. Iteration produces rate constants which give a consistent equilibrium constant determined kinetically; previous thermodynamic parameters do not bias the results. The third procdure uses eq 3, from which a plot of $1/\tau^2$ vs. C_{tot}, the total concentration, gives directly k_d^2 from the intercept and $4k_rk_d$ from the slope. For our data, the intercept of such a plot is small, and hence k_d has a large uncertainty. This also contributes to the error of the recombination rate constant, k_r. Thus, although this procedure requires no thermodynamic knowledge, it is less accurate than the second procedure.

Of course, if the mechanism is correct, and the kinetics and thermodynamics are consistent, the three methods should give consistent results. The data in Table I show the three analytical methods give consistent results within experimental error. The second method is preferred, since it requires no previous thermodynamic results, and is more accurate than the third method. It will be used for the remainder of the kinetic results reported in this paper. Figure 3 shows plots of $1/\tau$ vs. C_s for the ribooligonucleotides, using the second method of analysis.

Comparison of $rCA_5G + rCU_5G$, $dCA_5G + dCT_5G$, and $rCA_5G + dCT_5G$. Table II shows the kinetic results for the ribooligonucleotides, deoxyribooligonucleotides, and hybrid oligonucleotides. Activation energies were determined for the first two; rate constants at 12.0 °C were determined for the third. The rate constants are all compared at 12.0 °C. The recombination rate for the deoxyribooligonucleotides (8.3 × 10^6 L mol^{-1} s^{-1}) is 34% faster than that for the ribooligonucleotides (6.2 × 10^6 L mol^{-1} s^{-1}); the hybrid recombination rate is essentially the same as that for the ribooligonucleotides (6.6 × 10^6 L mol^{-1} s^{-1}).

Table II: Kinetic Results for $rCA_5G + rCU_5G$, $dCA_5G + dCT_5G$, and $rCA_5G + dCT_5G$

	$rCA_5G + rCU_5G$	$dCA_5G + dCT_5G$	$rCA_5G + dCT_5G$
k_r (12.0 °C, L mol^{-1} s^{-1})	6.2 × 10^6	8.3 × 10^6	6.6 × 10^6
$E_{a,r}$ (kcal/mol)	0 ± 2	−0.5 ± 2	
$\Delta S^{\ddagger}_r$ (eu)	−29 ± 7	−30 ± 4	
k_d (12.0 °C, s^{-1})	38	9.6	50
$E_{a,d}$ (kcal/mol)	39 ± 2	43 ± 3	
$\Delta S^{\ddagger}_d$ (eu)	84 ± 6	95 ± 9	
$\Delta H°$ (kcal/mol)[a]	−41	−49	
$\Delta S°$ (eu)[a]	−120	−145	

[a] Thermodynamic results from Nelson et al. (1981).

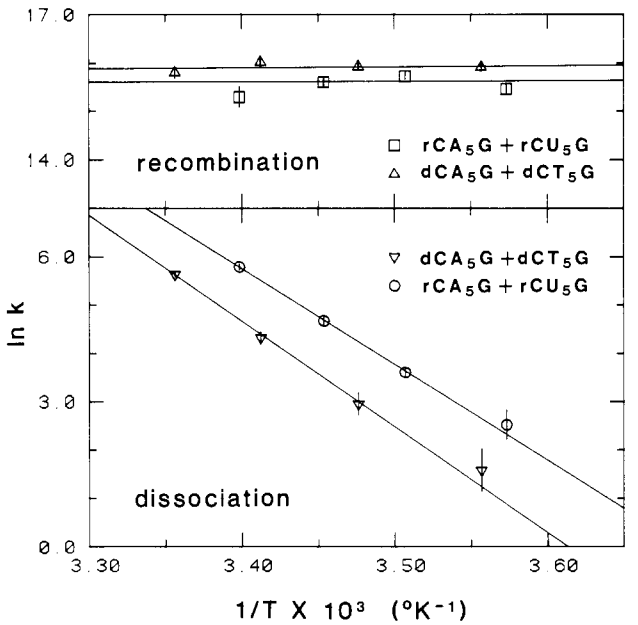

FIGURE 4: Arrhenius plot of the natural logarithm of the rate constants as a function of the inverse temperature for the recombination and dissociation rate constants. The slope of such a plot is $-E_a/R$. (△) $dCA_5G + dCT_5G$ recombination rate; (□) $rCA_5G + rCU_5G$ recombination rate; (▽) $dCA_5G + dCT_5G$ dissociation rate; (○) $rCA_5G + rCU_5G$ dissociation rate. The results are given in Table II.

The activation energy, E_a, and the activation entropy, $\Delta S^{\ddagger}$, are obtained from the temperature dependence of the rate constants. Figure 4 shows an Arrhenius plot for the recombination and dissociation rate constants for the ribooligonucleotides and deoxyribooligonucleotides. The recombination activation energies for the ribooligonucleotides and deoxyribooligonucleotides are small and the same within experi-

Table III: Apparent Rate Constants and Activation Energies for the Second Exponential Component

	$k_{app} = 1/\tau_2$ (s^{-1})	
	$rCA_5G + rCU_5G$	$rCA_5G + dCT_5G$
6.7 °C	440 ± 230	470 ± 150
12.0 °C	2000 ± 1500	1900 ± 1100
$E_{a(app)}$ (kcal/mol)	45	41

mental error: 0 ± 2 and −0.5 ± 2 kcal/mol, respectively. The activation entropies are negative and nearly equal, −29 and −30 eu, respectively. The activation parameters were not measured for the hybrid oligonucleotides; they are assumed to be consistent with the ribooligonucleotide and deoxyribooligonucleotide data since the recombination activation energies are the same in the ribooligonucleotides and deoxyribooligonucleotides.

The dissociation rate constant at 12.0 °C for the deoxyribooligonucleotides (9.6 s^{-1}) is several times smaller than that for the ribooligonucleotides (38 s^{-1}). The hybrid dissociation rate constant (50 s^{-1}) is slightly faster than that for the ribooligonucleotides. The dissociation activation energies are large. The deoxyribooligonucleotide activation energy (43 kcal/mol) is somewhat larger than that for the ribooligonucleotide (39 kcal/mol). The activation entropies for the deoxyribooligonucleotides and ribooligonucleotides (95 and 84 eu, respectively) are both large and positive.

The activation energies determined from kinetics can be used to calculate the enthalpy and entropy for the reaction scheme. Comparison of these values with those determined thermodynamically provides a useful check for consistency of the reaction scheme. The thermodynamic parameters for these oligonucleotides have been previously determined from melting curve analysis (Nelson et al., 1981) and are included in Table II. The kinetically determined enthalpy is given by $\Delta H_{kinetic} = E_{a,r} - E_{a,d}$; the entropy is given by $\Delta S_{kinetic} = \Delta S^{\ddagger}_{r} - \Delta S^{\ddagger}_{d}$. For the ribooligonucleotides, the values of the enthalpy determined from thermodynamics and kinetics are −41 and −39 kcal/mol, respectively, in excellent agreement. The corresponding values for the entropy are −120 and −113 eu, respectively. For the deoxyribooligonucleotides, the comparisons for the enthalpy are −49 and −44 kcal/mol; for the entropy, they are −145 and −125 eu. The consistency of these values derived from independent techniques lends confidence to the procedures used to characterize the double-strand formation of these oligonucleotides.

Second Exponential Component. Table III summarizes the relaxation times found for the faster second component for the ribooligonucleotides and hybrid oligonucleotides at 6.7 and 12.0 °C. For these molecules, the second component was not observed above 12 °C; no second component was observed for the deoxyribooligonucleotides at any temperature. There was no trend of the relaxation time with concentration. The data shown are averages of 16–23 relaxations at several concentrations for each temperature. Due to the small amplitude of the effect (5–10% of the total amplitude), the errors in determining the relaxation times are large. This relaxation was 7–12 times faster at 6.7 °C and 15–25 times faster at 12.0 °C than the predominant bimolecular component. No trend in the amplitude with concentration or temperature was determinable.

The relaxaton times for the ribooligonucleotides and hybrid oligonucleotides are the same within experimental error at both temperatures: 440 and 470 s^{-1}, respectively, at 6.7 °C and 2000 and 1900 s^{-1} at 12.0 °C. The apparent activation energies determined were large and nearly equal for the ribooligonucleotides and hybrid oligonucleotides: 45 and 42 kcal/mol, respectively. Since we do not know what the process is, we cannot resolve the apparent rate into forward and reverse components.

The predominant relaxation is due to the bimolecular single-strand to double-strand transition. The fast minor component may be a rearrangement between two double-helical species (Frier et al., unpublished experiments) (see Discussion).

Discussion

Comparison of the Ribooligonucleotides, Deoxyribooligonucleotides, and Hybrid Oligonucleotides. These studies allow us to make direct comparisons of the kinetics of ribooligonucleotides, deoxyribooligonucleotides, and hybrid oligonucleotides of equivalent sequences under identical conditions. Thermodynamic results on these same oligonucleotides showed the order of stability was $dCA_5G + dCT_5G > rCA_5G + dCT_5G > rCA_5G + rCU_5G$ (Martin & Tinoco, 1980). The hybrid $dCA_5G + rCU_5G$ was very unstable and formed triple strands at low temperatures (Martin & Tinoco, 1980; Pardi et al., 1981); it was not studied here.

The recombination rate constant for the deoxyribooligonucleotides at 12.0 °C is faster than that for the ribooligonucleotides (Table II). The hybrid recombination rate constant is essentially the same as that for the ribooligonucleotides. The dissociation rate constant for the deoxyribooligonucleotides at 12.0 °C is much smaller than that for the ribooligonucleotides; the hybrid dissociation rate constant was slightly larger than that for the ribooligonucleotides. From this, we can conclude that the deoxyribooligonucleotide double helix is more stable than the ribooligonucleotide double helix because of both a greater recombination rate and a smaller dissociation rate. However, the difference in dissociation rates is the more important factor. The data for the hybrid double helix are only at one temperature, and thus comparisons with it are not as meaningful.

The activation energies for recombination of the deoxyribooligonucleotides and ribooligonucleotides were both small, 0 and −0.5 kcal/mol, respectively. This suggests that although the rates are different, the mechanism is nearly the same for both oligonucleotides. The dissociation activation energies were both large, with the deoxyribooligonucleotide activation energy being greater than that of the ribooligonucleotides. The values of the activation energies are consistent with the greater enthalpy of double-strand formation for the deoxyribooligonucleotides (Nelson et al., 1981). The activation energies for the hybrid oligonucleotides were not determined but are presumably consistent with those for the deoxyribooligonucleotides and ribooligonucleotides.

The kinetics of exchange with water for the base-pairing imino protons of the three helices in this work were studied by NMR (Pardi & Tinoco, 1982). For $dCA_5G + dCT_5G$ in 0.18 M NaCl, the rate constant and activation energy for exchange at 12.0 °C were 7 s^{-1} and 47 ± 7 kcal/mol, respectively, for the interior A-T base pairs. The similarity to the values in the present work, 9.6 s^{-1} and 43 ± 3 kcal/mol, led the authors to conclude that the rate-limiting step for exchange of the imino protons was the dissociation of the double strands to single strands. The exchange from the single strands was fast. The agreement of kinetic studies using techniques as diverse as temperature jump and NMR lends valuable support to the interpretations of the results from both techniques.

Second Exponential Component. As reported under Results, the relaxation data are best fit by using a two-exponential

fit for the ribooligonucleotides and hybrid oligonucleotides at temperatures of 12.0 °C and below. One-exponential fits are observed above 12.0 °C and for the deoxyribooligonucleotides at all temperatures.

Freier et al. (unpublished experiments) obtained two-exponential fits for the deoxyribooligonucleotide dG-C-G-C-G-C at temperatures below the melting temperature (T_m). They attribute the second relaxation to a reaction between two different double-helical species. Since the oligonucleotides in this study are less stable than dG-C-G-C-G-C, we obtained less data below the T_m. They found that the relaxation time of the second component was independent of concentration. The amplitude of their signal was also indpendent of concentration and temperature. Our data also indicate no trend of relaxation time with concentration; the data do not allow a conclusion to be drawn concerning the behavior of the amplitude.

The second component found by Freier et al. (unpublished experiments) had an amplitude of 10–15% of the maximum signal; it comprised a larger fraction of the signal at low temperatures, where the signal from the bimolecular process was small. The second component observed in this study was never more than 5–10% of the total amplitude. The second component of Freier et al. (unpublished experiments) was 4–10 times faster than the predominant component; in this study, it was 7–25 times faster, depending on the temperature (see Results).

The rates for the fast process in dG-C-G-C-G-C are calculated to be 9.6 and 24 s^{-1} at 6.7 and 12.0 °C, respectively, with an activation energy of 23 kcal/mol (Freier et al., unpublished experiments). For the ribooligonucleotides in this study, the corresponding rates are 440 and 2000 s^{-1} at 6.7 and 12.0 °C, respectively, with an activation energy of 45 kcal/mol. The process observed in this study behaves very differently from that observed by Freier et al. (unpublihsed experiments). They observe the effect in the deoxyribooligonucleotide; we observe it for the ribooligonucleotides and hybrid oligonucleotides, but not for the deoxyribooligonucleotides. It is not clear that the process we observe is similar to the one observed by Freier et al. (unpublished experiments). Further studies on this effect will be necessary before the process can be characterized to any extent.

Hybrid Behaves Similarly to the Ribooligonucleotide. The rate constants of the hybrid oligonucleotides at 12.0 °C are closer to those for the ribooligonucleotides than those for the deoxyribooligonucleotides. This suggests that the properties of the hybrid more closely resemble the RNA than the DNA. More convincing evidence comes from the behavior of the fast second component. The rates and activation energies were essentially the same for the ribooligonucleotides and hybrid oligonucleotides, and the effect was not observed in the deoxyriboolingonucleotides (Table III).

NMR studies of the chemical shifts of the base-pairing imino protons and the nonexchangeable base and sugar protons of the helices used in this study (Pardi et al., 1981) indicate that the structure of the hybrid is close to the structure of the ribooligonucleotide. The NMR study on exchange rates of base-pairing imino protons mentioned earlier (Pardi & Tinoco, 1982) showed that at 5 °C the dissociation rates of the hybrid and ribooligonucleotides are nearly equal and an order of magnitude faster than that for the deoxyribooligonucleotide.

The evidence seems to indicate that the properties of the hybrid double helices are governed mainly by the ribooligonucleotide strand. RNA gererally exhibits less structural diversity than DNA. This lower freedom of flexibility of the RNA might dictate the properties of the more flexible DNA strand in the hybrid double helix.

Table IV: Recombination Activation Energies for Several Oligonucleotides

oligonucleotide	[NaCl] (M)	E_a (kcal/mol)	ref
$rA_n + rU_n$, $n = 8$–18	0.05	−9	*a*
rA_nU_n, $n = 4$–7	0.25–1	−4 to −6	*b, c*
rA_nGCU_n, $n = 2$–4 $rA_nCG + rCGU_n$, $n = 6, 8$ $rA_nG_2 + rC_2U_n$, $n = 4, 5$	0.05–1	+6 to +9	*d*
$rCG_3 + rG_3C$	0.18	+4.5	*e*
dG-C-G-C-G-C	1	+1	*f*
dA-T-G-C-A-T	1	−1	*g*
$dA_8 + dT_8$	0.05	−2	*g*
$rA_n + dT_n$, $n = 7$–9	1	−1	*h*

[a] Pörschke & Eigen (1971). [b] Breslauer & Bina-Stein (1977). [c] Craig et al. (1971). [d] Pörschke et al. (1973). [e] Podder (1971). [f] Freier et al. (unpublished experiments). [g] Drobnies (1979). [h] Hoggett & Maass (1971).

Comparison with Previous Results. The kinetics of double-strand formation have been studied for a number of ribooligonucleotide sequences containing only A·U base pairs (Craig et al., 1971; Pörschke & Eigen, 1971; Breslauer & Bina-Stein, 1977), both A·U and G·C base pairs (Pörschke et al., 1973; Ravetch et al., 1974), and one sequence containing only G·C base pairs (Podder, 1971). The data for deoxyribooligonucleotides are much more limited (Drobnies, 1979; Freier et al., unpublished experiments). One hybrid has been studied (Hoggett & Maass, 1971).

The rate of recombination increases considerably as the ionic strength is increased. The dissociation rate is roughly independent of ionic strength (Pörschke et al., 1973). Both recombination and dissociation activation energies are independent of salt concentrations between 0.05 and 1 M NaCl (Pörschke et al., 1973). Thus, we can compare activation energies and dissociation rate constants from experiments done at different ionic strengths; recombination rate constants can be compared only when measured at the same ionic strength. Table IV summarizes the results for the activation energy for recombination for several oligonucleotides.

Ribooligonucleotides containing only A·U base pairs recombine with a negative activation energy: −9 kcal/mol for $rA_n + rU_n$ and −4 to −6 kcal/mol for rA_nU_n (Table IV). Rate constants and activation energies were essentially independent of chain length for both systems. The negative activation energy suggests that the mechanism of recombination requires the formation of a stable nucleus with two or three base pairs; the rate-determining step is the formation of the next base pair (Craig et al., 1971; Pörschke & Eigen, 1971) (see below).

Ribooligonucleotides containing both G·C and A·U base pairs have positive activation energies for recombination: +6 to +9 kcal/mol for sequences like rA_nGCU_n, $rA_nCG + rCGU_n$, and $rA_nG_2 + rC_2U_n$ (Table IV). The activaiton energy was roughly independent of length or sequence. However, the rate constants decreased with increasing chain length. The rates were faster if the G·C base pairs were at the end rather than the middle of the sequence. It appears recombinaition is faster when formation of the stable nucleus involves more stable G·C base pairs. Adding more A·U base pairs slows the rate due to increased steric hindrance; G·C bases at the end could be more accessible than in the middle of the sequence (Pörschke et al., 1973). The ribooligonucleotides rG-G-G-C + rG-C-C-C, which contain only G·C base pairs, have a recombination activation energy of +4.5 kcal/mol. The positive activation energy of oligonucleotides with G·C base pairs probably means the nucleus involved in the rate-determining

step requires one or two G·C base pairs (Pörschke et al., 1973).

Much less work has been done on deoxyribooligonucleotide kinetics. The recombination activation energy for dG-C-G-C-G-C is +1 kcal/mol (Freier et al., unpublished experiments). For $dA_8 + dT_8$ and dA-T-G-C-A-T, the corresponding values are −2 and −4 kcal/mol, respectively (Drobnies, 1979). The hybrid oligonucleotides $rA_n + dT_n$ have an activation energy of −1 kcal/mol (Hoggett & Maass, 1971).

The trend in activation energies for the ribooligonucleotides is negative values when only A·U base pairs are present (−4 to −9 kcal/mol), zero when isolated G·C base pairs are present, and positive when two or more G·C base pairs are adjacent (+5 to +9 kcal/mol). Due to the greater stability of G·C base pairs, fewer base pairs are required to form a stable nucleus, thus the larger activation energy when G·C base pairs are present. This trend is not as apparent in the deoxyribooligonucleotides. The activation energies vary from −2, −0.5, −4, and +1 kcal/mol for the series $dA_8 + dT_8$, $dCA_5G + dCT_5G$, dA-T-G-C-A-T, and dG-C-G-C-G-C. This might mean the number of bases required for the stable nucleus does not depend on the presence of G·C base pairs. The difference in stability between A·T and G·C base pairs in DNA is not as great as for RNA; thus, in DNA, the stability of the nucleus will not depend as strongly on the presence of G·C base pairs and the number of bases in the nucleus, and thus the activation energies vary less than those for RNA.

Because of the restriction that recombination rate constants must be compared at the same salt concentration, there are fewer results to compare. Values determined in 1 M NaCl at 12.0 °C from earlier work (in units of L mol^{-1} s^{-1}) are the following: rA_7U_7, 2×10^6 (Breslauer & Bina-Stein, 1977); rA_2GCU_2, 5×10^6 (Pörschke et al., 1973); dA-T-G-C-A-T, 8×10^6 (Drobnies, 1979); dG-C-G-C-G-C, 11×10^6 (Freier et al., unpublished experiments). No systematic comparisons may be made from the data available.

Mechanism of Double-Strand Formation. The data presented in Tables II and III allow us to investigate the differences between the kinetics of ribooligonucleotides, deoxyribooligonucleotides, and hybrid oligonucleotides.

The negative activation energy found for the recombination of ribooligonucleotides $rA_n + rU_n$ means there is a preequilibrium step involved in double-strand formation. The rate-determining step of the recombination is hypothesized to be the addition of the next base pair to the nucleus composed of a few base pairs (Craig et al., 1971). The nucleus is the species which adds the next base pair faster than it dissociates. Thereafter the double helix quickly zippers up to the fully base-paired double helix. The nucleus is in a fast equilibrium with the single strands. Thus, the forward rate is given by k_f(nucleus), where k_f is the rate of forming the next base pair. Dissociation of the strands occurs by breaking enough base pairs to get to the nucleus, which then quickly dissociates. The forward rate is then characterized by a small activation energy, positive or negative depending on the number of base pairs in the nucleus. The dissociation rate is characterized by a large activation energy required for breaking several base pairs to get to the nucleus.

Differences in the forward rate will depend on two factors: the concentration of the nucleus and the rate of adding base pairs to an existing nucleus.

It was stated earlier that the recombination of $dCA_5G + dCT_5G$ was faster than $rCA_5G + rCU_5G$. The hybrid $rCA_5G + dCT_5G$ recombines at essentially the same rate as $rCA_5G + rCU_5G$. The dissociation rates went as hybrid oligonucleotide ≳ ribooligonucleotide > deoxyribooligonucleotide. The behavior of the second exponential component also suggests that the behavior of the hybrid is similar to that of the ribooligonucleotide.

The similarity of the recombination activation energies for the deoxyribooligonucleotides and ribooligonucleotides might suggest that the nuclei for double-strand formation are of similar stability. Thus, the increased rate for the deoxyribooligonucleotides might be manifested in a larger k_f than that for the ribooligonucleotides. Freier et al. (unpublished experiments) suggest that the zippering rate, k_f, might be limited by the diffusion of the single-stranded bases into a helical conformation. The faster rate of recombination of the deoxyribooligonucleotides might be a result of faster single-strand stacking. From laser temperature-jump studies, it was determined that single-strand stacking in poly(dA) is about 4 times faster than in poly(rA): k(single strand stacking) = 2.7×10^7 and 0.7×10^7 s^{-1}, respectively, at 25 °C in 0.05 M sodium cacodylate (Dewey & Turner, 1979). This reasoning suggests that the hybrid recombination rate would be similar to that for the ribooligonucleotides, since the helix zippering would be limited by the stacking of the slower rCA_5G strand.

The data presented in this paper are insufficient to fully justify the explanations for the differences between deoxyribo- and ribooligonucleotide kinetics. More studies comparing deoxyribo- and ribooligonucleotides would greatly clarify the situation. Ribooligonucleotide G·C base pairs are more stable than deoxyribooligonucleotide G·C base pairs. It would be very informative to compare the kinetics of double-strand formation for two analogous G-C-containing deoxyribooligonucleotides and ribooligonucleotides. This would determine if the differences in stability are due to the ribooligonucleotide having a greater rate of recombination, a slower rate of dissociation, or both. Also comparing recombination activation energies and single-strand stacking rates would determine whether the differences in recombination rates are due to a more stable nucleus as manifested by a more negative activation energy, or a faster zippering rate.

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Subunit Composition of Bovine Muscle Acetylcholine Receptor†

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ABSTRACT: Acetylcholine receptors from fetal calf muscle were purified to homogeneity (specific activity up to 7500 nmol/g of protein), in reasonable yields (20–50%), and near-milligram quantity. Purification was by affinity chromatography on *Naja naja siamensis* toxin coupled to agarose by using methods similar to those for receptors from fish electric organs, but with modifications to account for the low concentration of receptor in muscle and the high probability of proteolysis. Immunochemical methods are described for approximating the extent of proteolysis in receptor preparations. Bovine acetylcholine receptor is composed of four glycoprotein subunits designated α ($M_r \simeq 41\,000$), β ($M_r \simeq 50\,000$), γ ($M_r \simeq 53\,000$), and δ ($M_r \simeq 56\,000$) which correspond immunochemically to the four glycoprotein subunits of fish electric organ acetylcholine receptors of the same designations. Electron micrographs of purified bovine receptor show that it has the same size and shape as receptors from fish electric organs. Immunization of rats with receptor from bovine and human muscle is very effective at inducing experimental autoimmune myasthenia gravis. Acetylcholine receptors purified from rat muscle are composed of subunits which correspond immunochemically to the α, β, γ, and δ subunits of receptor from *Torpedo californica*. The evidence presented strongly suggests that acetylcholine receptors from fish electric organ tissue and mammalian muscle share a fundamentally similar shape, antigenic structure, and $\alpha_2\beta\gamma\delta$ subunit structure.

Striated muscle fibers are usually innervated at a single site; thus, although the acetylcholine receptor is a major component of the postsynaptic membrane, the 10^7 (Fambrough, 1979) receptors contained in this patch of membrane a few micrometers long are a very small component of a muscle fiber which might be nearly 100 μm in diameter and several centimeters long. The electric organs found in some fish are thought to have evolved from striated muscle by increasing the number of synapses on the muscle derivative to 10^4, or so, and by eliminating the contractile proteins and sarcoplasmic reticulum that compose the bulk of a muscle cell (Mellinger et al., 1978). The proposed phylogenic link between receptors from electric organs and skeletal muscle and their observed pharmacological and physiological similarity suggest that receptors from these sources should be structurally similar (Changeux, 1981; Karlin, 1980). Receptors from fish electric organs are much better characterized than are those from mammalian muscle because receptors from electric organs are available in much larger amounts and are less subject to proteolytic degradation. In order to put the problems encountered during the purification of mammalian striated muscle acetylcholine receptors into perspective, it is useful to first briefly consider what is known about receptor from fish electric organs.

The various species of the marine electric rays contain receptor at a concentration of 1–2 nmol/g of electric organ tissue (Karlin, 1980; Changeux, 1981). Acetylcholine receptors from the electric organs of *Torpedo californica* have been affinity purified in amounts up to 50 mg/batch and, consequently, have been relatively well characterized (Weill et al., 1974; Raftery et al., 1975; Hucho et al., 1978; Lindstrom et al., 1981). *Torpedo* receptors are composed of four kinds of partly homologous glycoprotein subunits of apparent molecular weights α = 38 000, β = 49 000, γ = 57 000, and δ = 64 000 organized in "monomers" of apparent molecular weight 250 000 consisting of two α subunits and one each of β, γ, and δ (Reynolds & Karlin, 1978; Lindstrom et al., 1979a; Raftery et al., 1980). The receptor monomers contain two acetylcholine binding sites (Damle & Karlin, 1978; Neubig & Cohen, 1979) and the cation-specific channel whose opening they regulate (Anholt et al., 1980; Changeux et al., 1979; Wu & Raftery, 1979; Lindstrom et al., 1980a). The acetylcholine binding sites are formed at least partially by amino acids from the α subunits (Karlin, 1980). However, which subunits form the cation channel is not known. The *Torpedo* acetylcholine receptor is 110 Å long, about 55 Å of which is on the external surface of the membrane and about 15 Å on the cytoplasmic side, and there appears to be a channel running across the membrane through the center of the molecule (Kistler et al., 1982). Initially, in some laboratories, proteolysis of receptor during purification cleaved β, γ, and δ subunits into fragments so that only α subunits were recognized by electrophoresis on acrylamide gels in sodium dodecyl sulfate (Sobel et al., 1977). Despite proteolytic nicking, so that only fragments the size of α and smaller remain, the subunit fragments remain associated and capable of agonist-triggered channel opening (Lindstrom et al., 1980c).

The freshwater teleost *Electrophorus electricus* contains receptor at a concentration of 0.1 nmol/g of electric organ tissue. As a consequence of its lower concentration, it is usually only purified a few milligrams at a time. Initially, only two subunits corresponding to α ($M_r \simeq 41\,000$) and β ($M_r \simeq$

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