T_4 Polynucleotide Ligase Catalyzed Joining on Triple-Stranded Nucleic Acids†

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ABSTRACT: dT_{10} will form triple-stranded complexes with dA_n and these complexes can serve as substrate for T_4 polynucleotide ligase (EC 6.5.1.1). The rate of phosphodiester formation was found to be approximately the same as for the double-stranded complex and, furthermore, the rate appears to be similar on the two strands in the complex. Joining of dT_{10} also took place in the presence of the double-stranded complexes $dA_n \cdot dT_n$ and $dA_n \cdot rU_n$. Polyamines increase the rate of joining catalyzed by T_4 polynucleotide ligase under certain conditions.

The existence of triple-stranded nucleic acid complexes has now been thoroughly demonstrated by a variety of different techniques (Cassani & Bollum, 1969; Riley et al., 1966; De Clerck et al., 1975). Arnott & Bond (1973) have suggested that triple-stranded complexes can form wherever there exists a continuous sequence of purine nucleotides in a nucleic acid chain. Triple-stranded deoxyribonucleic acids as well as double-stranded ribonucleotide structures are known to be in the A-type conformation, having 11 or 12 base pairs per turn (Arnott & Selsing, 1974). In contrast, double-stranded DNAs exist in the B form with 10 base pairs per turn. Another significant difference between the A and B forms is the conformation of the furanose ring in the two structures. In the A form the furanose ring is in the C-3-endo form; in the B form it has the C-3-exo conformation (Arnott & Hukins, 1972).

The biological roles of triple-stranded structures are still obscure; however, some possibilities have been suggested. Britten & Davidson (1969) have proposed that the interaction between a double-stranded DNA and a single-stranded RNA could have a regulatory function concerning the control of gene expression. Arnott et al. (1968) have suggested that A-type helices are needed for the transcription process. Such a mechanism assumes that transition from B to A form occurs in DNA, and this indeed seems to be possible in DNA structures rich in dA and dT (Arnott & Selsing, 1974). Triple-stranded DNAs have recently been shown to act as inhibitors of various DNA polymerases (Tamblyn & Wells, 1975).

The difference between 3′-exo and 3′-endo furanose ring structures clearly affects the orientation of the 3′-OH group and its neighboring 5′-phosphate group. Enzymes such as T_4 polynucleotide ligase (EC 6.5.1.1), which bind to "nicks" and catalyze the joining of phosphodiester linkages, might be affected by this change in orientation of the groups involved in going from a double-stranded to a triple-stranded nucleic acid. In the present work, the action of T_4 polynucleotide ligase on synthetic triple-stranded DNAs has been investigated. In a previous publication from this laboratory, the mechanism of action of T_4 polynucleotide ligase on synthetic double-stranded DNA has been described in detail (Raae et al., 1975a). The results of the present work suggest that this enzyme can also carry out joining of phosphodiester linkages on some triple-stranded nucleic acids.

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Experimental Section

Materials

Chemicals. All inorganic salts were purchased from Merck or Baker and were of reagent or analytical grade. The polyamines were purchased from the Sigma Chemical Co. Oligo- and polynucleotides dT_{10}, dA_n, and dT_n were obtained from P-L Biochemicals; rU_n was from Miles Laboratories, while dA_{10} was a product from Collaborative Research Inc. Radioactive [γ-^{32}P]ATP was prepared according to a slightly modified procedure of Weiss et al. (1968), the specific activity being approximately 100 Ci/nmol. [5′-^{32}P]dT_{10} and [5′-^{32}P]dA_{10} were prepared as previously described (Raae et al., 1975b). Radiochemical analysis showed that the oligonucleotides were essentially completely phosphorylated.

Enzymes. T_4 polynucleotide ligase and kinase were prepared according to the procedure of Panet et al. (1973). The ligase preparation, however, was taken a step further to eliminate contaminating nuclease activity (Raae et al., 1975a). One unit of T_4 polynucleotide ligase is defined as the amount which catalyzes the transformation of 1 pmol in 1 min at 20 °C of 5′-phosphate termini of pdT_{10} to a form insusceptible to bacterial alkaline phosphatase, the dT_{10} being bound to a dA_n strand in a double-stranded complex.

Bacterial alkaline phosphatase was a product of Worthington Biochemical Co.

Methods

Enzyme Assay. The standard assay mixture for T_4 polynucleotide ligase contained in a total volume of 0.1 mL: 66 mM Tris-HCl (pH 8.0), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 100 μM ATP. The concentrations of oligodeoxynucleotides, polydeoxynucleotides, and polyribonucleotides are as given in the legends to the figures.

The amount of polynucleotide ligase added varied from 0.25 to 5 units and the reaction temperature was 20 °C. The enzyme assays were carried out as previously described (Raae et al., 1975a).

Determination of t_m. The melting temperature of the nucleotide complexes was determined spectrophotometrically using a Zeiss PMQII spectrophotometer equipped with temperature cuvette holder.

Sucrose Gradient Centrifugations. The reaction mixtures (0.2 mL) were layered onto 4.8 mL of 5–30% (w/v) sucrose gradients, prepared in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, and centrifuged for 23 h at 5 °C in a Spinco ultracen-

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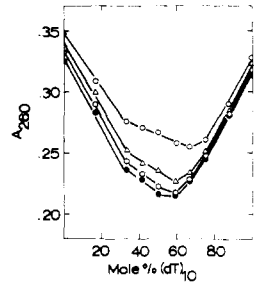

FIGURE 1: Ultraviolet absorbance of dT_{10} and dA_n mixtures at various temperatures. The total nucleotide concentration was kept constant and was 28.3 μM dissolved in 66 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$. The reaction mixtures were first kept at 65 °C for 30 min and then at 5 °C for 12 h. Before the absorbance was read, the solution was equilibrated at each temperature for at least 30 min: (●—●) 16 °C; (○—○) 21 °C; (△—△) 29 °C; (○—○) 37 °C.

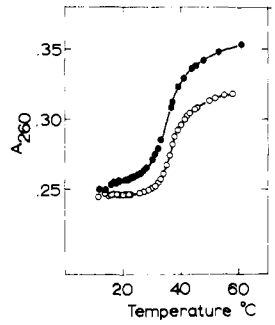

FIGURE 2: Ultraviolet absorbance temperature profiles of 1:1 (●—●) and 1:2 (○—○) mixtures of dA_n and dT_{10}. The buffer and melting conditions were as described in legend to Figure 1.

trifuge, using a SW 50.1 rotor at 40 000 rpm. After centrifugation the tubes were harvested directly from the bottom onto glass filter paper discs (3MM) and analyzed for radioactivity.

Results

Mixing Curve Studies. There is some disagreement in the literature concerning the ability of dT_{10} to form triple-strand helices when annealed to a dA_n strand. Thus, Cassani & Bollum (1969) concluded from mixing studies that these nucleic acids did not form a triple-stranded DNA. Tamblyn & Wells (1975), on the other hand, found that dT_{10} annealed to a dA_n strand and in a proper ratio inhibited the DNA polymerase reaction. Closer investigation of this phenomenon gave good evidence for the idea that such nucleic acids indeed could form triple-stranded structures. Our studies, carried out using optimal joining conditions for T_4 polynucleotide ligase, give results analogous to those of Tamblyn & Wells (1975) concerning the structure of the dA_n·dT_{10} complexes.

Figure 1 shows the absorbance at 260 nm and at different temperatures for various complexes of dA_n and dT_{10}, keeping the total nucleotide concentration constant. All curves had two breaking points, the first one between 30 and 40 mol % and the second at approximately 60%, except that at 37 °C the latter point was at approximately 67 mol %. The theoretical breaking point for a triple-stranded nucleic acid is at 66.6%. The meaning of the first breaking point is not known. One possibility, however, is that a certain ratio of dT_{10} to dA_n is needed to bring about an ordered, partly double-stranded structure from the random coil structure of free dA_n. Addition of more dT_{10} will therefore not cause a decrease in absorbance to the same extent as when added initially. The shapes of the mixing curves at the lower temperatures suggest the presence of both

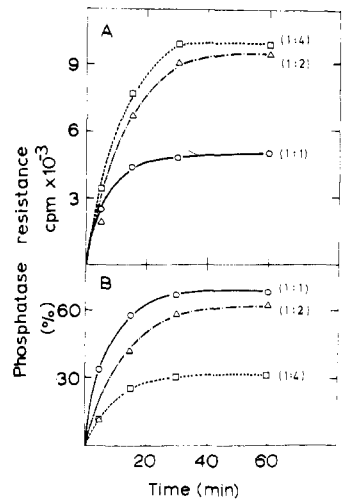

FIGURE 3: Time course of the T_4 polynucleotide ligase joining reaction using various mixtures of dA_n and dT_{10}. (A) Plotted as total phosphatase resistant counts; (B) plotted as % counts which were phosphatase resistant. The compositions of the reaction mixtures were as described in Methods and contained 1.5 μM dA_n and 1.5–6.0 μM dT_{10} (total phosphate) in a total volume of 0.2 mL. The reaction mixtures were heated for 10 min at 65 °C and then kept at 5 °C for 12 h. The reactions were initiated by adding 0.6 unit of T_4 polynucleotide ligase, ATP, and dithiothreitol after the reaction mixtures had been equilibrated at the reaction temperature of 20 °C for 15 min.

triple- and double-stranded complexes, the major structure being the triple-stranded structure for a 1:2 mixture. However, with the same mixture at 37 °C, essentially all structures are triple stranded. The melting point profiles for the 1:1 and 1:2 complexes, Figure 2, indicated a single transition in both cases with t_m of 35 and 37 °C, respectively. The melting point interval, σ, was found to be slightly smaller for the 1:2 complex than the 1:1, suggesting a more homogeneous class of complexes where the triple-stranded structure predominates. The fact that single transitions are found for both complexes suggests 2 → 1 and 3 → 1 strand transitions. Similar results have been presented by other workers (Blake et al., 1967; Howard et al., 1971; Torrence et al., 1973).

Joining of dT_{10} on Different Triple-Stranded DNAs. The time course of a typical joining reaction catalyzed by T_4 polynucleotide ligase with various ratios of [5′-^{32}P]dT_{10} to dA_n is shown in Figure 3. The phosphatase resistant radioactivity obtained for the mixtures of dA_n and dT_{10} of 1:4 and 1:2 was twice that found for the 1:1 mixture, or the dA_n·$(dT_{10})_{n/10}$ DNA. The percentages of phosphodiester bonds formed, on the other hand, were 31, 62, and 68%, respectively. Taken together with the fact that the optimal joining for a 1:1 mixture usually is 65–75%, these results indicate that T_4 polynucleotide ligase can catalyze joining of dT_{10} when in a triple-stranded DNA and, further, the rate of joining is approximately the same with double- and triple-stranded DNAs.

The sizes of the DNAs formed in the 1:2 and 1:1 mixtures were investigated using neutral sucrose gradient centrifugation and the results are shown in Figure 4. In the case of both reaction mixtures one sedimenting peak was seen. The sedimentation coefficients were estimated for the peak fractions, fractions 9 and 11, and found to be 11.9 and 10.8 S, respectively. Using the equation obtained for native double-stranded DNA, relating sedimentation coefficient to molecular weight, $s_{20,w} = 0.0882M^{0.346}$ (Studier, 1965), a molecular weight of 1.08×10^6 was obtained for the peak fraction of the 1:1 complex. Assuming that the same equation also is valid for a triple-stranded DNA, the molecular weight of the peak fraction

TABLE I: Slab Gel Analysis of Reaction Products from a 1:1 and a 1:2 Mixture of dA_n and dT_{10}.[a]

fraction no.	dT_x	dA_n:dT_{10} = 1:1 cpm	% of total cpm	dA_n:dT_{10} = 1:2 cpm	% of total cpm
1	10	4 442	22.4	11 844	17.6
2	20	766	3.9	2 984	4.4
3	30	1 355	6.9	4 191	6.2
4	40	1 149	5.8	4 832	7.2
5	50	1 206	6.4	3 778	5.6
6	>50	10 801	54.7	39 556	58.9

[a] The analyses were carried out essentially as described by Raae et al. (1975a).

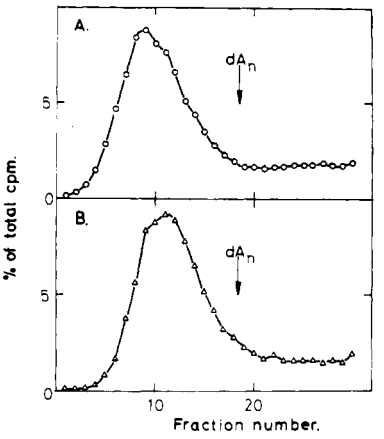

FIGURE 4: Sucrose gradient centrifugation of the reaction products of 1:1 and 1:2 mixtures of dA_n and dT_{10}. The reaction conditions were essentially as described in legends to Figure 3. The reaction mixtures contained 3 μM dA_n and 3-6 μM dT_{10}. At the end of the reaction periods, the reaction mixtures were layered on top of 4.8 mL of sucrose gradient (5-30%) and centrifuged for 23 h at 40 000 rpm. For further details, see Methods. (A) The 1:2 mixture; the reaction mixture showed 73% phosphatase resistance. (B) The 1:1 mixture which was 78% phosphatase resistant. The average sedimentation coefficient for dA_n was estimated from analytical ultracentrifugation data to be 6.5 S.

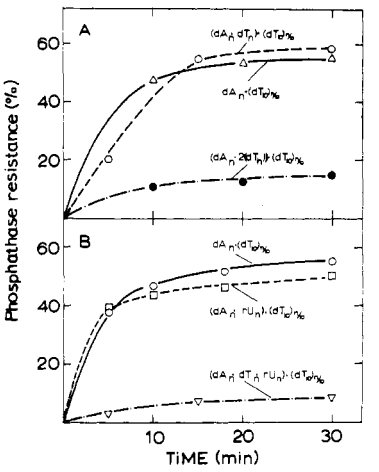

FIGURE 5: Time course of joining of dT_{10} annealed on different polyoligonucleotide complexes. The reactants within the parentheses were mixed simultaneously, heated to 90 °C for 10 min, and then kept at room temperature for 4 to 6 h. dT_{10} was added 10 min prior to the enzyme. The nucleotide concentration used was 1.5 μM and 0.25 unit of enzyme was employed. Fur further details, see Methods.

of the 1:2 complex was estimated to be 1.62×10^6. Thus, it would appear that the difference in the rate of sedimentation obtained corresponds well to that expected between a double- and a triple-stranded structure.

The molecular weight of the single-stranded products formed by joining of dT_{10}, $dT_{10\text{-}m}$, where m is 1, 2, 3 . . . 20, was estimated by slab gel electrophoresis as previously described (Raae et al., 1975b). The results shown in Table I indicate that there is no significant difference in the distribution in size of the oligo(dT) products formed on a double- and triple-stranded DNA.

Oligo(dT) will also form triple-stranded DNAs upon annealing to previously formed double-helical DNAs such as dA_n·dT_n. Numerous joining experiments with such substrates were performed and the results for some are given in Figure 5. The rate of joining of dT_{10} annealed on dA_n·dT_n was approximately the same as when annealed on dA_n alone. When dT_{10} was added to the triple-stranded complex, dA_n·$2dT_n$, there was as expected little or no joining. In similar experiments (Figure 5B), rU_n was used instead of dT_n and the results obtained for this system were analogous to those using combinations of dA_n and dT_n. Thus, these observations add further evidence to the idea that T_4 polynucleotide ligase is able to carry out enzymatic joining of dT_{10} when present in triple-stranded DNA.

Effect of Polyamines. We have previously shown that low concentrations of the polyamines putrescine, cadaverine, and spermidine (up to 1 mM) give a small activation of T_4 polynucleotide ligase catalyzed joining of dT_{10} in a double-stranded substrate (Raae et al., 1975a). At higher concentrations, however, a marked inhibition was obtained. The influence of polyamines has also been investigated with regard to joining of dT_{10} on triple-stranded DNAs. Melting point determination indicated clearly as expected that addition of polyamines increased the t_m of triple-stranded complexes (results not shown). The time course of joining of dT_{10} in the presence and absence of spermidine, using triple-stranded complexes, is shown in Figure 6. When dT_{10} and dA_n were annealed for a relatively short period (2.5 h), addition of 1 mM spermidine prior to the enzyme resulted in an increase in the initial rate of up to 50%. When the substrate was allowed to anneal for longer periods (18 h), addition of spermidine had no effect. Thus, it is evident that low concentrations of polyamines in the present case increase the rate of formation of the correct triple-stranded structure for joining. High concentrations of polyamines (5 mM) inhibited the reaction to the same extent as for double-stranded DNAs (Raae et al., 1975a), probably due to some aggregation of the DNA substrate (Osland & Kleppe, 1977).

Discussion

In the triple-stranded DNAs of the homopurine–homopyrimidine type such as dA_n·$2(dT_n)$, one of the homopyrimidine strands is thought to have the classical Watson–Crick type base pairing to the homopurine polymer, whereas the other strand is bound by Hoogsteen base pairing to the homopurine strand

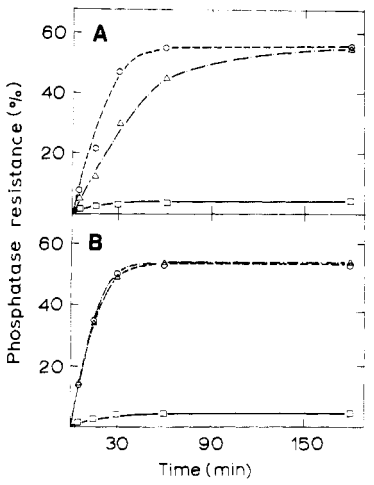

FIGURE 6: Time course of joining of dT_{10} in 1:2 mixture of dA_n and dT_{10} in the presence of 1 mM (○—○) and 5 mM (□—□) spermidine; (△—△) control. The concentrations of dA_n and dT_{10} were 1.5 and 3 μM, respectively. Further details are given in Methods and in the legend to Figure 3. (A) The substrate was annealed for 2 h at 5 °C and (B) 18 h. Spermidine was added 5 min prior to the enzyme in both cases.

(Arnott et al., 1976). Similar hydrogen bonding is also predicted for the homopurine-homopyrimidine-oligonucleotide DNA such as $dA_n \cdot 2(dT_{10})_{n/10}$. The conformation of such triple-stranded nucleic acids is always the A form (Arnott & Selsing, 1974). Moreover, Arnott & Selsing have also shown that the structures of the two polypyrimidine strands in $dA_n \cdot 2(dT_n)$ are not only of the same type but also nearly identical despite the difference in their hydrogen bondings to dA_n. The present results, which showed that the rate of T_4 polynucleotide catalyzed joining on the two strands in the triple-stranded complex is approximately the same, therefore, are in agreement with this model.

The existence of triple-stranded complexes in vivo has not yet been established. Arnott & Selsing (1974) have, however, suggested that homopurine-homopyrimidine stretches in DNA might have an important function by their ability to participate in triple-stranded complexes with an additional oligopyrimidine or oligopurine nucleotide. An oligopyrimidine nucleotide of approximately 10 base pairs in length might not only lead to the formation of A type conformations in the local triple helix but could also induce the same conformations in longer neighboring double-helical stretches. Both RNA and DNA polymerases have been reported to be inhibited by triple-stranded complexes (Tamblyn & Wells, 1975). Since T_4 polynucleotide ligase also can carry out efficient joining on a number of naturally occurring DNAs (Lehman, 1974) which presumably must be in the B conformation, it is likely that the conformational specificity for this enzyme is not so strict as for the two others mentioned above. It should also be pointed out that T_4 polynucleotide ligase can join dT_{10} on rA_n at a rapid rate (Kleppe et al., 1970) and this substrate has the A conformation. Polynucleotide ligase from uninfected *E. coli* cannot use a ribotemplate for joining of oligo-dT, (Olivera & Lehman, 1968) and therefore its specificity both with regard to conformation of substrate as well as cofactor is different from that of T_4 polynucleotide ligase.

It has previously been shown that the rate of T_4 polynucleotide ligase catalyzed joining of dA_{10} on dT_n template is very slow compared with that of the substrate $dA_n \cdot (dT_{10})_{n/10}$ (Kleppe et al., 1970). We have continued this work, but it is not possible at the present time to draw any definite conclusions as to whether or not joining occurs on triple-stranded complexes. A triple-stranded complex of these nucleic acids would have the following composition $2(dT_n) \cdot (dA_{10})_{n/10}$, and thus it would be quite different from the dT_{10}-dA_n system with regard to enzyme binding. Steric hindrance could explain partly the low rate of joining since even in a 1:1 complex triple-stranded DNA might be the predominating species under certain reaction conditions (Howard et al., 1971). Another reason for the low rate of joining could be the larger stacking effects observed in dA_{10} than in dT_{10} (Arnott & Bond, 1973). Further experiments are currently in progress to throw some more light on this problem.

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