Volume 17 Number 17 1989 Nucleic Acids Research

DNA supercoiling determines the activation energy barrier for site specific recombination by Tn*21* resolvase

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Received July 3, 1989; Accepted August 4, 1989

ABSTRACT

A kinetic analysis of site specific recombination by Tn21 resolvase has been carried out using DNA substrates of varying superhelicities. The rates for the formation of the recombinant product increased with increasing superhelicity up to a maximum value, after which further increases in superhelicity caused no further increase in rate. The reactions with DNA of reduced superhelicity were extremely slow, yet they eventually led to virtually all of the substrate being converted to product. Hence, the level of DNA superhelicity must determine the activation energy barrier for at least one of the steps within the reaction pathway that can be rate-limiting. In the presence (but not in the absence) of Mg^{2+} ions, the DNA was fully saturated with resolvase whenever the protein was in stoichiometric excess over resolvase binding sites on the DNA. Thus the process affected by DNA supercoiling cannot be coupled to the binding of resolvase. Instead, the step whose rate is determined by supercoiling seems to be located within the reaction pathway after the synapse. However, these reactions may involve two forms of the synaptic complex that are converted to the recombinant product at different rates.

INTRODUCTION

The transposition of Tn3 and Tn3-like elements involves a protein called resolvase. The resolvase is encoded by the transposon and acts at a DNA sequence within the transposon known as res (1,2). During the transposition of a Tn3-like element, the resolvase converts a circular DNA molecule that contains two copies of the res site into two circles of DNA, by means of a reciprocal recombination between the res sites. This reaction was first characterized in vitro with the interchangeable resolvases from either gamma-delta or Tn3 (1-7). For efficient recombination, the substrate had to contain two res sites in direct repeat on the same molecule of negatively supercoiled DNA. [Under certain conditions, recombination can also be detected on relaxed DNA substrates, but these reactions are inefficient compared to those on supercoiled DNA (5,6)]. In the recombinant product, the two circles of DNA are interlinked

once to form a catenane (4). This conversion alters the linking number of the DNA by + 4 (ref 6). However, the change in linking number between substrate and product is usually greater than + 4 because resolvase is also a type I topoisomerase (4). In certain situations, it functions as a topoisomerase without concomitant recombination (6-8).

Other Tn3-like elements, Tn21 and Tn1721, encode resolvases whose amino acid sequences differ substantially from that of Tn3 resolvase (1,2). The resolvases from Tn21 and Tn1721 have the same function as Tn3 resolvase, but they have different specificities for the DNA sequence at res (8-10). Tn21 resolvase has no activity at res sites from Tn3 nor vice-versa. Nevertheless, the requirements for recombination in vitro by Tn21 resolvase are essentially the same as those for Tn3 resolvase except that the enzyme from Tn21, unlike that from Tn3, shows similar activity in either the presence or absence of Mg^{2+} ions (8). We report here a kinetic analysis of site specific recombination by Tn21 resolvase, with particular reference to its requirement for DNA supercoiling. A preliminary account of part of this study has been given previously (11).

Many other systems for site specific recombination also require supercoiled DNA substrates. These include DNA inversion by either Hin or Gin (12,13), though mutants of Gin have been isolated that no longer need supercoiled DNA (14). For the integrase from phage lambda, the requirement for DNA supercoiling is limited to only one of the recombinational partners, attP (15). In this case, supercoiling facilitates the formation of a higher order nuclear protein complex at attP which then binds the free DNA at attB (16). Similarly, for resolvase, supercoiling assists in synapsis between the DNA-protein complexes at the two res sites (17), but we show here that this is not its only function.

MATERIALS AND METHODS

Proteins. Purification of Tn21 resolvase to homogeneity and the determination of protein concentrations by amino acid analysis were as described previously (9). Molarities of resolvase are given for the dimeric protein of M_r 42,600. EcoRI was purified as before (18). Calf thymus topoisomerase I was from Gibco-BRL.

DNA. The plasmid pEAK9 is a 7.1kb derivative of pBR325 that contains two res sites from Tn21 oriented in direct repeat (9). Transformants of E. coli HB101 with pEAK9 were grown in minimal media containing

[methyl^{3}H] thymidine and the plasmid isolated, through two CsCl/EtBr (ethidium bromide) density gradients, as before (8).

Buffers. Buffer A is 50mM Hepes, 100mM NaCl, 10mM $MgCl_2$, 0.1 mg/ml gelatin, pH 8.2. Buffer B is as A except for 1mM EDTA in place of $MgCl_2$. Buffer T is 50mM Tris, 50mM KCl, 10mM $MgCl_2$, 0.5mM dithiothreitol, 0.1mM EDTA, 5mg/ml bovine serum albumin, pH 7.5.

Superhelicity. Preparations of pEAK9 with altered superhelicity were generated by reactions to equilibrium with topoisomerase I in the presence of EtBr (19). Preparative scale reactions with topoisomerase I were typically carried out with 25 units of the enzyme and 0.1 mg pEAK9 in 0.8 ml buffer T (with the requisite concentration of EtBr) for 4 h at 37°C. The covalently closed DNA was recovered from each reaction by density gradient centrifugation.

Linking Differences. The difference between the mean linking number of each preparation of pEAK9 and that of the relaxed form of this DNA (after reaction with topoisomerase I in the absence of EtBr) was measured by band counting (20). A calibration series of topoisomers were produced by reactions of topoisomerase I on 10µg pEAK9 in 100 µl buffer T at 37°C: each reaction also contained EtBr at a concentration in the range 0-20 µM. After washing with phenol, the DNA was analysed by electrophoresis through 1% agarose in 50 mM Tris-phosphate containing chloroquine phosphate (21), at 2 V/cm for 42 h at 4°C. (To separate the topoisomers throughout the range of superhelicities generated here, the calibration series was run at 2, 5 and 10 µg/ml chloroquine). The gels were then washed with 1 mM $MgSO_4$, stained with EtBr, photographed over u.v. illumination and the negatives scanned by densitometry. Aliquots from the preparative scale reactions were run alongside the calibration series, and the number of topoisomers between the centre of the spread of topoisomers in these samples and that of the relaxed form of pEAK9 was counted. These values for linking differences were then converted to superhelical densities ($\Delta Lk/Lk_o$) by using a fixed value of 670 for Lk_o, calculated for a 7.1kb DNA with 10.6 bp/helical turn (22).

Recombination. Site specific recombination between the two res sites on pEAK9 converts the DNA from a 7.1kb circle to a catenane of two circles at 3.8 and 3.3 kb, and this alters the relative locations of two EcoRI sites on this DNA (9). Kinetics were typically monitored by adding Tn21 resolvase to 10µg pEAK9 (^{3}H-labelled) in 0.4 ml buffer A (or B) at 20°C, then withdrawing 10µl aliquots at timed intervals and immediately vortexing

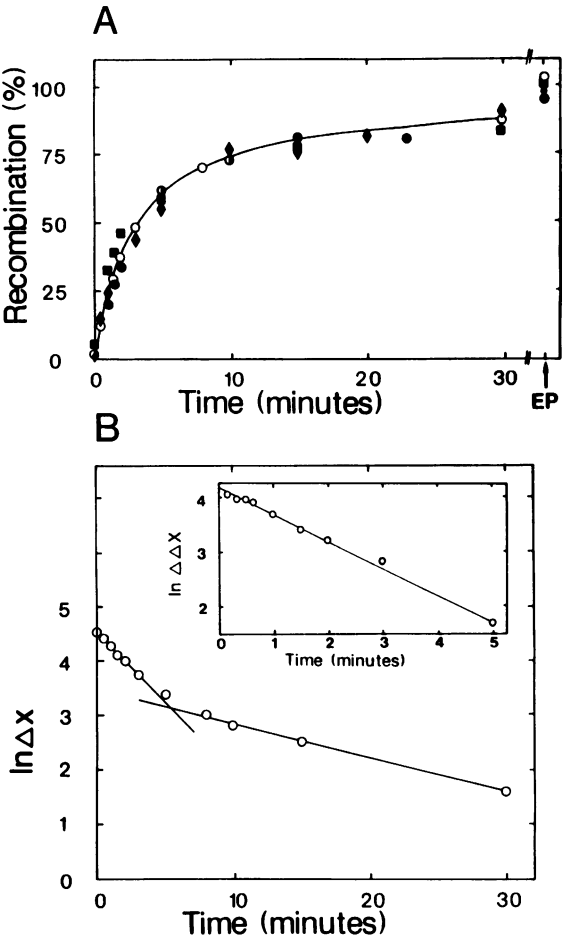

FIGURE 1 (A): Reactions in buffer A at 20° contained 5 nM pEAK9 (native superhelicity) and Tn21 resolvase at either 42 nM (♦), 85 nM (■), 305 nM (●) or 415 nM (○). Samples were taken from each reaction at timed intervals and % recombination determined as in Materials and Methods. Reaction end points (noted as EP on the abscissa) were evaluated from all data at >20 h after the start of the reaction. (B): Semi-logarithmic plot of the data in (A) at 415 nM resolvase: ΔX is the difference between % recombination at the reaction end point and that at time t. The insert is a semi-log plot of the residuals (ΔΔX) at each time point between % recombination evaluated by back extrapolation of the slow phase and the observed % recombination.

these with phenol. The zero time point was taken before the addition of resolvase. The aqueous layer was extracted with ether, warmed to 67°C for 10 min and the DNA then digested with 250 units EcoRI at 37°C.

(For reactions in buffer B, $MgCl_2$ was added with the EcoRI). Subsequent analysis of the DNA by electrophoresis through agarose, and scintillation counting on each DNA fragment at each time point during the reaction, was as described previously (8). Parallel with each reaction, a sample of pEAK9 was incubated in the same buffer for the duration of the reaction and then analysed for the amounts of open circle and covalently closed DNA (8). Values for % recombination were calculated from the relative amounts of DNA in the fragments that derive from the catenane, and are given here as a % of the covalently closed form of pEAK9 that had undergone recombination: i.e. with a correction for open circle DNA, typically 10%. Reaction end points are average values from all data taken when % recombination no longer varied systematically with time.

RESULTS

Kinetics of Recombination

The plasmid pEAK9 contains two res sites from Tn21 oriented in direct repeat (9). These sites have the same organization as res from other Tn3-like elements (1,2): each contains three separate locations for binding the cognate resolvase (10). Recombination between two res sites seems to need a minimum of about 6 molecules of resolvase (dimer) per molecule of DNA (8).

The rate at which Tn21 resolvase catalysed recombination between the two res sites on pEAK9 was measured over a range of protein concentrations. Across a range where the molar ratio of protein to DNA varied from 8 to 80, no variation in rate was observed (Fig 1A). For a reaction of the type,

$$E + S \rightleftharpoons ES \rightarrow EP \qquad (1)$$

analysed under single turnover conditions ([E] > [S]), the apparent rate constant (k_o) for the formation of product is given by

$$k_o = k_2(1 + K_1/[E])^{-1} \qquad (2)$$

where K_1 is the equilibrium dissociation constant for the first step and k_2 the rate constant for the second step. For the rate to be invariant with the enzyme concentration, [E] must be much greater than K_1. Hence, under the reaction conditions used in Fig 1, the DNA must be fully saturated with resolvase whenever the protein is in molar excess over resolvase binding sites on the DNA.

[The apparent requirement for a stoichiometric excess of resolvase

over DNA is not due to any irreversible change in the protein during the reaction. Reactions that contained Tn21 resolvase at lower concentrations than that of resolvase binding sites on the DNA still led to recombination between the res sites, but at rates that were very much slower than those in Fig. 1 (23). Hence, resolvase must be able to catalyse repetitive turnovers. However, at any one time during a reaction with a low concentration of the enzyme, comparatively few of the DNA molecules will have bound simultaneously 6 dimers of the protein].

The reactions in Fig.1 are effectively single turnovers of an enzyme that generate a single product. Thus, one first order rate constant should be adequate to describe the progress of each of these reactions. However, these reactions cannot be fitted to a single exponential (Fig. 1B). Instead they involve at least two phases: a fast phase over the first 5 min of the reaction, during which about 70% of the DNA is recombined, followed by a slow phase that eventually leads to close to 100% recombination after several hours. The fast phase can by itself be fitted to a single exponential (Insert to Fig 1B) and this yields a first order rate constant of about 0.4 min^{-1}. The second phase is more than ten times slower than this.

DNA Supercoiling

A possible explanation for the biphasic reaction is the heterogeneity of the substrate. Plasmid DNA, as isolated from E. coli in its covalently closed form, consists of a series of topoisomers: the distribution of the DNA between the topoisomers is gaussian, and the mean superhelical density is defined by the centre of the gaussian (20). Since resolvase (under standard conditions) needs the DNA substrate to be supercoiled (3-6), perhaps the fast phase corresponds to reactions on the more supercoiled topoisomers and the slow phase to the less supercoiled topoisomers. To test this model, samples of pEAK9 were generated with mean superhelical densities in the range from -0.028 to - 0.10 (Materials and Methods). When isolated from E. coli, the native form of pEAK9 was found by analysis in chloroquine gels (not shown) to have a superhelical density of -0.062. The kinetics of site specific recombination by Tn21 resolvase were measured on each of these preparations of pEAK9 (Fig. 2).

At superhelical densities between - 0.10 and - 0.044, the kinetics were invariant with the level of DNA supercoiling (Fig. 2A). The reactions remained biphasic and the rates for both fast and slow phases were the same as those with the native form of the DNA, as were also the relative

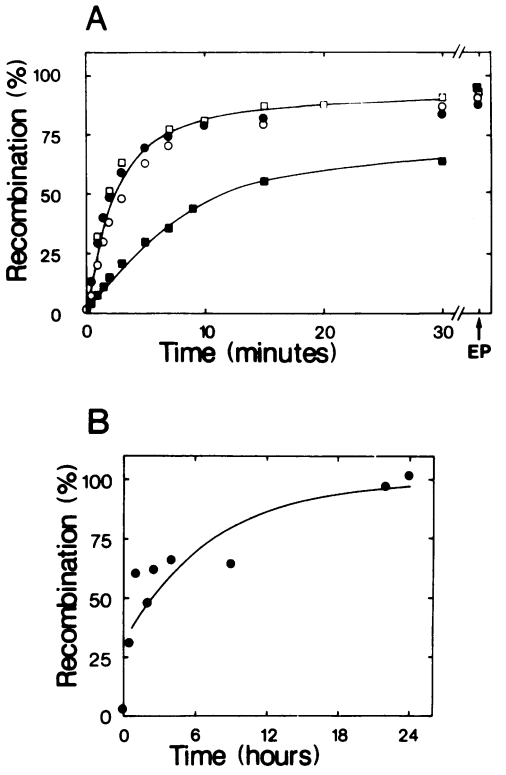

FIGURE 2 (A): Reactions in buffer A at 20°C contained 360 nM Tn21 resolvase and 5 nM pEAK9 at one of the following superhelical densities: - 0.1 (□), - 0.062 (●), - 0.044 (○), - 0.035 (■). The reaction end points are noted as EP. (B): as (A) except that the superhelical density of pEAK9 was - 0.028, and that the reaction was monitored over a longer time base. The line drawn in (B) is that for a single exponential fitted to all data points except that at zero time.

amplitudes of the two phases. Hence, the biphasicity observed with the native form of pEAK9 cannot be due to its distribution of topoisomers around a mean superhelical density of - 0.062.

In contrast, reductions in the superhelical density below - 0.04 caused large and progressive reductions in the rate of recombination (Figs 2A and 2B). The reactions on DNA of low superhelicity were still biphasic, with both rates reduced relative to native DNA, but progressively less of the DNA in these samples underwent recombination in the initial phase. Nevertheless, with DNA that was less than half as supercoiled as native, Tn21 resolvase still converted virtually all of the substrate to product,

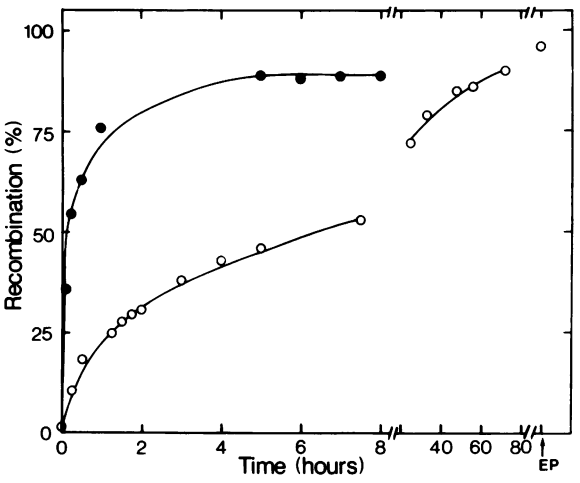

FIGURE 3 Reactions at 20°C contained 5 nM pEAK9 (that had a superhelical density of - 0.035) and 200 nM Tn21 resolvase in either buffer A (●) or buffer B (○). Buffer A contains $MgCl_2$, buffer B contains EDTA (Materials and Methods). Samples were removed from each reaction at timed intervals and % recombination evaluated as before: the time scale shown is discontinuous. The reaction end point noted as EP refers to the reaction in buffer B after 180 h.

even though the reaction took >20 h (Fig. 2B). In a previous study on Tn3 resolvase (24), it had been reported that the fraction of the DNA converted to product varied with the superhelicity of the DNA but, in those experiments, the extent of recombination was measured after only 1 h. Given the extremely slow rate of Tn21 resolvase on DNA of reduced superhelicity (Fig 2B), it seems possible that 1 h may have been insufficent for the reactions with Tn3 resolvase to have reached their end points. With Tn21 resolvase, variations in the superhelical density of the DNA, over the range tested (- 0.028 to - 0.10), affected only the kinetics of the reactions and not the final extent of recombination.

The kinetics of the reaction on the sample of pEAK9 that had a superhelical density of - 0.028 (Fig 2B) were also measured at several different concentrations of Tn21 resolvase, under the same reaction conditions as used in Figs 1 and 2: the protein was always in stoichiometric excess over its binding sites on the DNA. As with the DNA of native superhelicity (Fig. 1A), no variation was observed (23). Therefore the DNA of reduced superhelicity must also be fully saturated with resolvase: i.e., in eqn 2, the value for K_1 must remain far below

that of [E]. The reduction in the reaction rates on DNA of low superhelicity cannot be due to incomplete saturation of the res sites caused by weakened binding of resolvase to this DNA.

Role of Magnesium Ions

All of the reactions described above were carried out in the presence of $MgCl_2$. However, resolvase has no absolute requirement for Mg^{2+} ions though its activity can be stimulated by Mg^{2+} (8). The resolvases from certain transposons have very low activity in the absence of Mg^{2+} and their activities are enhanced considerably by Mg^{2+} (3, 6). However, in the presence of NaCl (as here), the rate for recombination by Tn21 resolvase on DNA of native superhelicity is only slightly faster in the presence of Mg^{2+} than in its absence (8). The stimulation may be due to an effect of Mg^{2+} on the structure of the DNA (8) and, if so, the degree of the stimulation might depend on the superhelicity of the DNA.

The kinetics of recombination by Tn21 resolvase, in either the presence or absence of $MgCl_2$ (buffers A and B respectively), were compared on the same sample of pEAK9 with reduced supercoiling (Fig 3). At this superhelical density, -0.035, the reaction in the presence of Mg^{2+} was complete within 5 h while that in the absence of Mg^{2+} took over 100 h. The difference caused by Mg^{2+} on this DNA is very much larger than that on DNA of native superhelicity (8). A DNA molecule with a fixed linking number will have more negative supercoils in buffer A than in buffer B, due to the effect of Mg^{2+} on duplex winding (25). However, given the magnitude of this effect (25), the superhelical density of pEAK9 will alter by about 0.008 between buffers A and B. By itself, this alteration is not large enough to account for the kinetics on DNA of reduced superhelicity in the absence of Mg^{2+} (Fig 3). In the presence of Mg^{2+} (Fig. 2), an alteration in the superhelical density from -0.035 to -0.028 reduced the rate by less than that caused by omitting $MgCl_2$ (Fig 3).

The concentration of Tn21 resolvase required for recombination on pEAK9, in the absence of $MgCl_2$, was also examined, using in this case DNA of native superhelicity (Fig. 4). In marked contrast to the reactions in the presence of Mg^{2+} (Fig. 1), the rates for these reactions now varied with the concentration of resolvase, even though the enzyme was always at a higher concentration than that of resolvase binding sites on the DNA (Fig. 4). Hence, the affinity of resolvase for its DNA substrate must be lower in the absence of Mg^{2+} than in its presence: i.e., the value of K_1 is no longer far below that of [E] (eqn. 1). At least part

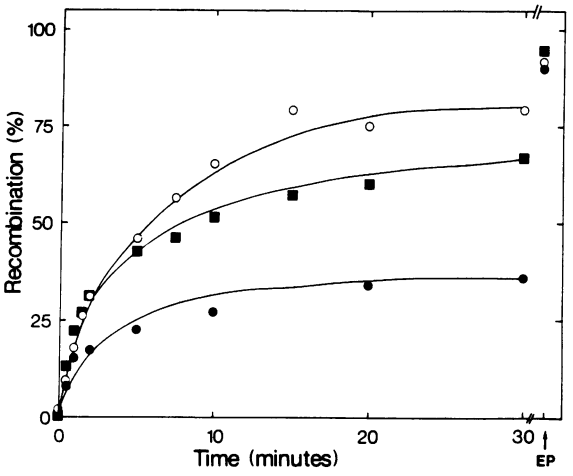

FIGURE 4 Reactions in buffer B at 20°C contained 5 nM pEAK9 (native superhelicity) and Tn21 resolvase at either 42 nM (●), 92 nM (■) or 275 nM (○). Samples were removed from each reaction at timed intervals and % recombination evaluated as before: only the data between 0 - 30 min and the reaction end points (EP) are shown. The end points for each reaction are the average values for all time points >20 h.

of the effect of Mg^{2+} on the kinetics of recombination (ref 8: Fig. 3) must be due to the fractional saturation of the DNA with resolvase being lower without Mg^{2+} than with Mg^{2+}.

DISCUSSION

The reaction pathway for site specific recombination by resolvase must involve several steps: the initial binding of the protein to its sites within *res*; the synapse between the DNA-protein complexes at the two *res* sites; the strand transfer reactions; perhaps also the final dissociation of the protein from the DNA product (alternatively, the product at equilibrium might still be bound to the protein). Moreover, each of these steps contains several events. For example, the strand transfer reactions consist of the cleavage of the DNA at both *res* sites to form covalent enzyme-DNA intermediates, the rotation of the termini to align each with its recombinational partner and finally the religations (2,6). In this study, single turnovers of Tn21 resolvase were monitored on simply the relative concentrations of the circular DNA substrate and the catenated product. Intermediates in which the covalent structure of the DNA differed from either substrate or product were not detected: during the reaction,

they may never have formed a significant fraction of the total DNA. In addition, this assay for recombination makes no distinction between enzyme bound and free DNA though, in the presence of Mg^{2+}, the DNA must have been saturated with resolvase (Fig. 1).

The stages within this overall mechanism that might be responsible for the sensitivity of the kinetics to DNA supercoiling, and for the biphasic reaction records, are discussed below.

DNA Supercoiling

With Tn3 resolvase, a synaptic complex between the res sites can be trapped with glutaraldehyde if the two res sites are on a supercoiled molecule of DNA: if the res sites are on either nicked or linear DNA, no synaptic complexes are detected (17). Thus one role for DNA supercoiling is to facilitate synapsis between the res sites (17). However, the rate limiting step for resolvase is located within the reaction pathway after the synapse (C.N. Parker & S.E. Halford, in preparation). This was shown by using plasmid constructs where the rate of synapsis could be measured independently of recombination : the constructs contained two res sites each from Tn3 and Tn21, located so that synapsis between the Tn3 sites places the Tn21 sites into separate domains in the DNA and thus prevents Tn21 recombination (26). The overall rate of appearance of the catenane product, as measured here, is much slower than the rate for the formation of the synaptic complex.

DNA supercoiling therefore affects not only synapsis (17) but also one or more of the steps within the reaction pathway after synapsis. At superhelical densities below - 0.04, the rate of recombination increased with increasing superhelicity (Fig. 2). The step that is rate limiting for the reaction on the DNA of low superhelicity must therefore have an activation energy barrier whose size depends on DNA supercoiling. This could be due to partial unwinding of the DNA at the transition state, thus reducing the linking difference between this and relaxed DNA, as has been observed during the transition between closed and open complexes with RNA polymerase (27). At superhelical densities above -0.04, the rate of recombination was invariant with the level of supercoiling, so the step that had been rate limiting on DNA of low superhelicity either possesses a maximal rate that cannot be exceeded by further supercoiling, or it is no longer rate limiting and some other process is now the slowest stage of the reaction.

Recombination by resolvase alters the linking number of the DNA

by + 4 (ref. 6). Given the quadratic relationship between the free energy of a DNA molecule and its linking difference (20), the change in free energy between substrate and product DNA must become more negative as the number of negative supercoils on the DNA is increased. However, over the range of superhelicities examined here, only the kinetics of the reactions varied and not the final fraction of the total DNA that was converted to product (Figs. 2 & 3). There are at least two reasons why there may be no simple relationship between DNA supercoiling and the free energy change in this reaction. First, resolvase also has a type I topoisomerase activity (4-8). Second, the reactions of resolvase involve the protein in stoichiometric excess over the DNA, and the equilibrium constant between a substrate bound to an enzyme and the product, also bound to the enzyme, can differ by several orders of magnitude from that between the free reactants (28).

The role of Mg^{2+} ions in enhancing the activity of resolvase may appear to be related to its requirement for DNA supercoiling. Recombination in the absence of Mg^{2+} was particularly slow on DNA of low superhelicity (Fig. 3). However, this is at least partly due to a reduction in the affinity of resolvase for its DNA substrate (Fig. 4). Electrostatic interactions between proteins and DNA are weakened by the addition of Mg^{2+} (29). The opposite effect of Mg^{2+} on resolvase implies that some specific requirement for DNA structure outweighs the electrostatic component. One aspect of DNA structure that is affected by Mg^{2+} is duplex winding (20,25) and perhaps resolvase recognizes a particular pitch for the DNA helix.

Biphasic Reactions

In all of the reactions of Tn21 resolvase reported here, the conversion of the circular DNA substrate to the catenane took place in two distinct phases. The time taken to complete the first phase of the reaction (on DNA of native superhelicity : Fig. 1) is about the same as that needed for a single turnover of a type II restriction enzyme : most restriction enzymes have turnover numbers within an order of magnitude of 1 min^{-1} (30). However, in the initial phase, only 70% of the DNA is converted to product and recombination on the remaining 30% is much slower (Fig. 1). A biphasic record of this type cannot be accounted for by any pathway that involves only a linear series of irreversible steps (31) : such a pathway must yield either a single exponential for product formation (if one step is much slower than any other), or a lag phase preceding the

formation of product (if two or more steps are rate limiting).

The biphasic records can be accounted for by mechanisms involving reversible equilibria with two forms of either the enzyme-product complex or the enzyme-substrate complex. One example is given in eqn. 3 :

$$E + S \rightleftharpoons ES \rightleftharpoons EP_1 \rightarrow EP_2 \qquad (3)$$

Provided that the equilibration between ES and EP_1 is rapid compared to the subsequent conversion of EP_1 to EP_2, this scheme yields product (measured as $[EP_1 + EP_2]$) in two phases, with the amplitude of the fast phase being determined by the equilibrium constant between ES and EP_1 (28). However, for resolvase, eqn. 3 is unattractive. It implies that the strand transfer reactions are freely and rapidly reversible yet, if these change the linking number of the DNA by + 4 in the forward direction, they must do so by -4 in the reverse direction and the latter would be unfavoured (6). Alternatively, if the substrate is partitioned between two forms of the enzyme-substrate complex,

$$E + S \begin{array}{l} \rightleftharpoons ES_1 \\ \rightleftharpoons ES_2 \end{array} \rightarrow EP \qquad (4)$$

one of which yields product faster than the other, the reactions will again be biphasic. This scheme (eqn. 4) can be applied to resolvase. The interactions of resolvase with two res sites might yield two forms of the synaptic complex, only one of which (ES_1) is competent for site specific recombination. The slow phase would then correspond to the rate at which ES_2 is converted to ES_1. Perhaps the second complex, ES_2, is responsible for the topoisomerase activity of resolvase.

ACKNOWLEDGEMENTS.

We thank Christian Parker and Martin Boocock for extensive discussions, and Brenda Fowler and RoseMary Musgrave for preparing this manuscript.This work was funded by the Science and Engineering Research Council.

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