A Sign Inversion Mechanism for Enzymatic Supercoiling of DNA

Abstract. *Both the introduction and the removal of supertwists by DNA gyrase change the linking number of DNA in steps of two. This surprising finding provides strong evidence that gyrase acts by a mechanism, called sign inversion, whereby a positive supercoil is directly inverted into a negative one via a transient double-strand break.*

Superimposed on the right-handed coiling of the two strands of the DNA double helix around their axis is a higher order coiling of the helix axis itself, called supercoiling (*1*). Supercoils can be positive (right-handed) or negative (left-handed). Virtually all naturally occurring duplex DNA is negatively supercoiled, and this supercoiling plays an important role in its replication, transcription, and recombination. The algebraic sum of the number of supercoils and the number of double-helical turns in a closed circular duplex DNA, that is, the total number of revolutions of one single strand about the other, is an integer called the linking number (*2*). The linking number cannot be changed without breaking one or both strands of the double helix. DNA molecules that differ only in linking number are called topoisomers, and enzymes that alter the linking number of DNA are called topoisomerases (*3, 4*).

DNA gyrase (*5*) is a unique topoisomerase that reduces the linking number of DNA and thus introduces negative supercoils, in a reaction coupled to the hydrolysis of adenosine triphosphate (ATP). The gyrase of *Escherichia coli*, the subject of this report, is composed of two copies each of the *gyrA* and *gyrB* gene products, and plays a major role in maintaining the negative supercoiling of *E. coli* DNA (*6*). Most models (*7–9*) for the mechanism of DNA gyrase postulate an energy-dependent segregation of DNA into positively and negatively supercoiled domains (with no change in linking number), followed by a selective relaxation of the positive supercoils (which reduces the linking number). This relaxation has usually been viewed in terms of a topoisomerase model for which the ω protein of *E. coli* (*10*) is the archetype, entailing nicking of one strand of the double helix, rotation of the free ends around the helix axis to relieve the supercoiling, and resealing of the nick (*11*).

We propose that gyrase acts by a categorically different mechanism. This model was initially developed to explain two observations. First, when a complex of gyrase with DNA is treated under certain conditions with a protein denaturant, double-strand breaks are introduced in the DNA with a gyrase protomer attached covalently to each 5′ phosphoryl end (*7, 12, 13*). This implicates a transient double-strand break in the gyrase reaction mechanism, in contrast to the proposed nicked intermediate in the reactions of the prokaryotic ω protein (*14*) and the eukaryotic topoisomerases (*4*). Second, when gyrase binds to DNA in the absense of ATP, positively supercoiled regions are generated in the DNA (*8, 15*). These positive supercoils must be stabilized by the bound enzyme, since they cannot be relaxed.

Our model, called sign inversion, is illustrated in Fig. 1 and consists of the following steps.

1) Gyrase binds to a DNA molecule at two points such that the two bound segments cross to form a right-handed node (the upper of the two nodes depicted in Fig. 1). This is sufficient to stabilize a positive supercoil and induce a counterposing negative supercoil [represented by the lower (−) node].

2) Gyrase introduces a double-strand break in the DNA at the back of the right-handed node and passes the front segment through the break, inverting the handedness and thus the sign of the node.

3) The break is resealed on the front side of the now left-handed node.

The net result of this procedure is to reduce the linking number of the DNA by two (*16*). Reversal of these steps, that is, starting with a left-handed node and inverting it to a right-handed one, would lead to an increase of two in the linking number. We propose that this reverse process is the path by which gyrase, in the absense of ATP, relaxes negatively supercoiled DNA. The simplest way to convert the sequence shown in Fig. 1 into a cycle is to release one of the segments of DNA from the enzyme after the third step and return to the initial configuration, but with the linking number reduced by two.

The sign inversion model provides a natural explanation for critical existing data and makes a unique prediction that we have verified experimentally—both supercoiling and relaxation by gyrase change the linking number of DNA in steps of two. Another essential prediction, that gyrase can introduce a double-strand break, pass DNA through the break, and reseal the break, in an efficient, concerted reaction, has also been verified—gyrase can catenate (interlock) and uncatenate DNA circles (*17*). Liu *et al.* (*18*) have also obtained evidence for transient double-strand breaks in some topoisomerase reactions.

The usual DNA substrate for gyrase reactions is a heterogeneous set of topoisomers with a roughly Gaussian distribution of linking numbers (*19*). The observed effect of gyrase is simply to shift the center of this broad distribution such that the unit of change in linking number cannot be discerned. To determine this unit, we therefore used a substrate that was uniform with respect to linking number, a purified relaxed topoisomer of the plasmid p15A (*20*). The small size of this plasmid enhanced the electrophoretic resolution of its topoisomers. DNA supercoiling by gyrase under optimal conditions is both processive and fast. Therefore, to observe the initial reaction products, the rate was reduced by limiting the ATP concentration to 30 μM, about one-tenth the K_m (*21*), and the reaction products were sampled at early times. To synchronize the reaction, gyrase, in roughly twofold molar excess over DNA, was bound to the DNA in a 3-minute preliminary incubation at 30°C before the addition of ATP. Reaction products were deproteinized (*22*) and displayed by electrophoresis through a 1.2 percent agarose gel (*5*).

The only products of the reaction were topoisomers differing in linking number (Lk) from the starting material by multiples of 2 (Fig. 2). This was most clearly seen after 5 seconds (lane c), when the major product had a linking number reduced by 2 ($\Delta Lk = -2$), and products with $\Delta Lk = -4$ and -6 were also visible. Bands corresponding to odd-numbered ΔLk's, which are present in the reference set (lanes d, g, and l), were strikingly absent. At later times (lanes e, f, and h to j), the linking number of the products was progressively reduced, but the every-other-band distribution was maintained. In another experiment (not shown), with the ATP concentration further reduced to 10 μM, the $\Delta Lk = -2$ topoisomer was virtually the only discernible product of a 10-second reaction. Thus, when DNA is supercoiled by gyrase, the quantum of change in the linking number is 2.

The quantum of change in linking number is also 2 for relaxation by gyrase. After a 5-minute incubation with gyrase in the presence of ATP, the DNA was highly supercoiled (lane j). At this time, novobiocin, which inhibits supertwisting but not relaxation by gyrase (*7, 12*) was added to the reaction mixture, and the incubation was continued for 30 minutes.

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The DNA was relaxed, giving a set of topoisomers differing in linking number from the starting material by multiples of 2 (lane k). This "recall" experiment also showed that the unresolved topoisomers in the highly supercoiled band differed in linking number from the starting material by multiples of 2. The consequence of the double-strand break in the sign inversion mechanism is a quasi-linear DNA molecule maintained as a circle only by noncovalent bonds between gyrase protomers, two of which are covalently attached to the 5′ termini. The DNA ends must be prevented from rotating relative to one another during sign inversion, lest supercoils be dissipated. The recall experiment is a stringent test for such slippage. It clearly showed that none occurred (unless by steps of 2), since there were no bands differing in linking number from the starting material by an odd number even after a cycle of supertwisting and relaxation.

As a control (not shown), the supertwisted DNA shown in lane j was relaxed by adding *E. coli* ω protein after heat-inactivating gyrase. The product consisted of a distribution of relaxed topoisomers differing in linking number by 1 rather than 2.

Gyrase changes the linking number of DNA in steps of two. This property, the observation that gyrase makes double-strand breaks rather than nicks in DNA, and the demonstration that gyrase can catenate DNA and therefore can pass a DNA duplex through a transient double-strand break constitute strong evidence that sign inversion is the essential feature of the mechanism of DNA gyrase. Catenation follows directly from sign inversion when the two crossing segments of the node are contributed by different DNA molecules. Alternative explanations for supertwisting in steps of two, such as the segregation of two positive twists followed by their relaxation, fail to explain why relaxation of negative supercoils should alter the linking number only by multiples of 2 and do not account for double-strand breaks or catenation.

The critical first step in the sign inversion mechanism is the generation of a right-handed node. We consider two nonexclusive ways of ensuring that the node has the proper handedness. The first is positive wrapping of the DNA around the enzyme; the wrapped DNA could be the upper loop in Fig. 1. Such a structure has been proposed by Liu and Wang (*8*) to account for the stabilization of positive supercoils by bound gyrase. Supporting evidence for the coiling of DNA around gyrase is provided by the similarity in the pattern of protection from nucleases afforded by gyrase to that obtained with nucleosomes where wrapping has been established (*23*).

A more speculative alternative means for determining node handedness requires two asymmetric sequences in the substrate DNA—one to orient the segment of the node that will be transiently broken and the other to orient the segment that will be passed through the break. Gyrase purified from diverse bacterial species binds to and can cleave DNA at the same limited set of asymmetric sequences (*24*). The use of DNA sequences in determining handedness would offer a reasonable explanation for this puzzling site specificity. A consequence of this scheme for determining handedness is that catenated products would have a unique handedness. Furthermore, this scheme predicts that reversing the relative orientation on a DNA molecule of the two node-orienting sequences could "trick" gyrase into introducing positive supercoils by inverting a left-handed node.

The initial positive node is converted by sign inversion into a negative node. There are at least two possible routes by which the gyrase-DNA complex could return to its original conformation. The simplest is for gyrase to release one of the two segments comprising the node before returning to the positive node-binding conformation. Since gyrase binds stably at the sites at which it can cleave DNA (*25*) the segment that was passed through the break would likely be the one released. An alternative is to regenerate a positive node directly by rotating the front segment of the negative node 180° counterclockwise relative to the back segment, about the axis of the node (*26*). Regardless of the reset mecha-

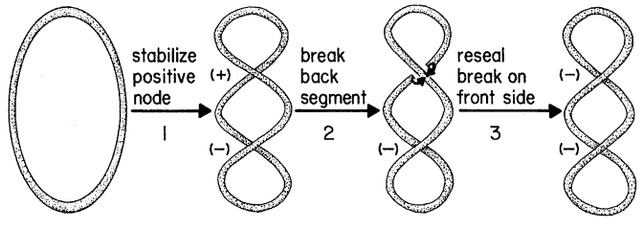

Fig. 1. The sign inversion model for enzymatic supercoiling of DNA. The enzyme maintains and acts at the upper node; the (+) and (−) symbols refer to the signs of the nodes (*27*). Since the second and fourth structures in the sequence each have a vertical dyad axis of symmetry which passes through the two nodes, breaking the front segment and resealing it on the back side is an operation equivalent to the one shown.

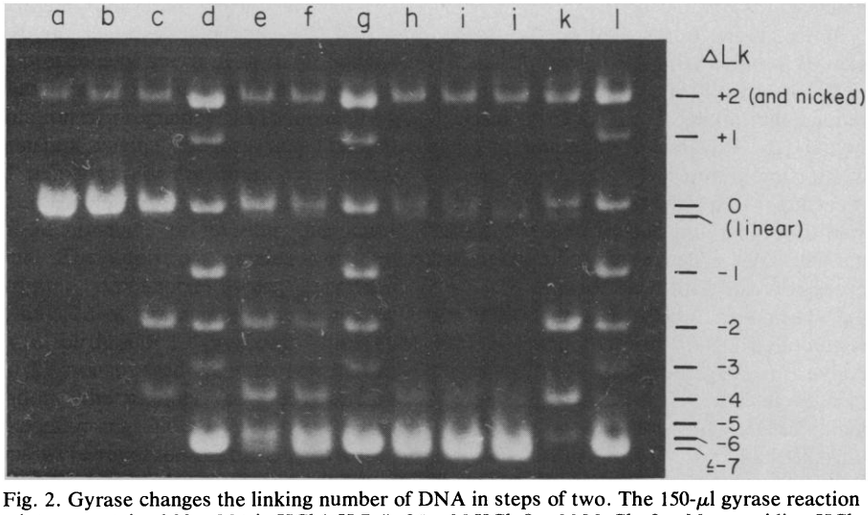

Fig. 2. Gyrase changes the linking number of DNA in steps of two. The 150-μl gyrase reaction mixture contained 30 m*M* tris-HCl (*p*H 7.4), 25 m*M* KCl, 8 m*M* $MgCl_2$, 2 m*M* spermidine-HCl, 5 m*M* dithiothreitol, 50 μg of bovine serum albumin per milliliter, DNA gyrase reconstituted from 300 units each of subunits A and B of *E. coli* DNA gyrase (the *gyrA* and *gyrB* gene products, respectively), and 1 μg of the purified p15A topoisomer. After 3 minutes at 30°C (a) to allow gyrase to bind to the DNA, ATP was added, and 17-μl samples were removed at the start (b), at 5 (c), 10 (e), 20 (f), 40 (h), 80 (i), and 300 seconds (j). After 300 seconds, 10 μ*M* novobiocin was added and the incubation was continued for 30 minutes to allow gyrase to relax the DNA (k). The reactions were stopped, deproteinized, and subjected to electrophoresis through a 1.2 percent agarose gel to resolve topoisomers. As a reference, a mixture of topoisomers of p15A ranging from fully relaxed to highly negatively supercoiled (*28*) was run in lanes d, g, and l. Adjacent bands in these channels represent topoisomers differing in linking number by one. The difference in linking number (ΔLk) relative to the substrate topoisomer is indicated to the right of lane l. Small amounts of linear and nicked circular DNA were produced during the purification of the substrate.

nism used, there is evidence that gyrase remains bound at the same site on the DNA, acting processively through cycles of sign inversion until binding is sufficiently weakened by the increased supertwist density that the enzyme is released (*25*).

In contrast to other models, supercoiling is necessarily quantized in the sign inversion scheme. The change in linking number per cycle is always two, even though the work required to change it increases with increasing supertwist density. Therefore, if the number of ATP molecules bound per cycle is constant, the thermodynamic efficiency of the reaction must progressively increase. A lower limit on the number of ATP molecules hydrolyzed per cycle can be estimated from the work (*19*) required to reduce by two the linking number of the most negatively supercoiled DNA molecule which can be further supercoiled by gyrase. Such an analysis suggests that at least two ATP's must be hydrolyzed for each round of sign inversion.

Most other models for supertwisting invoke an initial energy-requiring segregation of positive supercoils followed by their passive relaxation. Sign inversion is an active process in which breakage-and-rejoining is intrinsically coupled with the mechanical energy-requiring step of DNA traversal. The following is presented as a plausible sequence of events linking ATP binding and hydrolysis (*6*) to supertwisting. Gyrase binds to DNA forming a right-handed node. The subsequent binding of ATP by the *gyrB* protomers changes the conformation of gyrase (*21*) to one that stabilizes instead a left-handed node. To relieve the resultant strain, gyrase performs the remarkable feat of passing the front segment of DNA through a double-strand break in the back segment, and perhaps through the enzyme itself, while holding both ends of the break so that they cannot rotate. The energy of the broken phosphodiester bonds, conserved as a protein-DNA bond, is used to reseal the break after sign inversion. After resealing the break, gyrase catalyzes the hydrolysis of ATP. Release of adenosine diphosphate (ADP) and P_i allows the enzyme to return to its initial conformation so that a new cycle of sign inversion can begin.

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References and Notes

1. W. R. Bauer, *Annu. Rev. Biophys. Bioeng.* **7**, 287 (1978).
2. For a more thorough discussion of linking number, see F. H. C. Crick, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2939 (1976).
3. J. C. Wang and L. F. Liu, in *Molecular Genetics*, J. M. Taylor, Ed. (Academic Press, New York, 1979), part 3, pp. 65–88.
4. J. J. Champoux, *Annu. Rev. Biochem.* **7**, 449 (1978).
5. M. Gellert, K. Mizuuchi, M. H. O'Dea, H. A. Nash, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3872 (1976).
6. For review, see D. T. Denhardt [*Nature (London)* **280**, 196 (1979)] and N. R. Cozzarelli (in preparation).
7. A. Sugino, C. L. Peebles, K. N. Kreuzer, N. R. Cozzarelli, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4767 (1977).
8. L. F. Liu and J. C. Wang, *ibid.* **75**, 2098 (1978).
9. K. Mizuuchi, M. H. O'Dea, M. Gellert, *ibid.*, p. 5960.
10. J. C. Wang, *J. Mol. Biol.* **55**, 523 (1971).
11. An interesting exception to this general form is suggested by P. Forterre (in preparation).
12. M. Gellert, K. Mizuuchi, M. H. O'Dea, T. Itoh, J. Tomizawa, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4772 (1977).
13. A. Morrison and N. R. Cozzarelli, *Cell* **17**, 175 (1979).
14. R. E. Depew, L. F. Liu, J. C. Wang, *J. Biol. Chem.* **253**, 511 (1978).
15. C. L. Peebles, N. P. Higgins, K. N. Kreuzer, A. Morrison, P. O. Brown, A. Sugino, N. R. Cozzarelli, *Cold Spring Harbor Symp. Quant. Biol.* **43**, 41 (1978).
16. The topology of such a transition is discussed by F. B. Fuller, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3557 (1978).
17. K. N. Kreuzer and N. R. Cozzarelli, in preparation.
18. L. F. Liu, C. C. Liu, B. M. Alberts, personal communication.
19. R. E. Depew and J. C. Wang, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4275 (19 → D. E. Pulleyblank, M. Shure, D. Tang, J. Vinograd, H.-P. Vosberg, *ibid.*, p. 4280.
20. Fifty micrograms of p15A DI → [N. R. Cozzarelli, R. B. Kelly, A. Kornberg, *ibid.* **60**, 992 (1968)] was relaxed with purified *E. coli* ω protein (provided by J. C. Wang), and topoisomers of the relaxed plasmid were resolved by electrophoresis through a 1.2-mm-thick, 1 percent agarose gel (*4*). After electrophoresis, the gel was stained with ethidium bromide and photographed under ultraviolet light. With the photograph as a guide, a section of the gel containing the predominant topoisomer was cut out. The DNA was eluted from the gel (*17*) and the ethidium bromide extracted with 2-butanol.
21. A. Sugino, N. P. Higgins, P. O. Brown, C. L. Peebles, N. R. Cozzarelli, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4838 (1978).
22. Reactions were stopped by mixing the 17-μl sample with 5 μl of a solution containing 5 percent sodium dodecyl sulfate, 25 percent glycerol, 0.25 percent bromophenol blue, and 1 μg of proteinase K and then incubating for 15 minutes at 37°C to allow proteolysis.
23. L. F. Liu and J. C. Wang, *Cell* **15**, 979 (1978).
24. P. O. Brown, C. L. Peebles, N. R. Cozzarelli, *Proc. Natl. Acad. Sci. U.S.A.*, in press; A. Sugino and K. Bott, unpublished data.
25. A. Morrison, N. P. Higgins, N. R. Cozzarelli, in preparation.
26. In Fig. 1, the axis is a line through the node perpendicular to the plane of the paper.
27. The size and structure of the loops of DNA determined by the node have no effect on the change in linking number by sign inversion. However, if the loops are intertwined so that they are topologically linked, a knot is produced in the DNA by the process of sign inversion.
28. Topoisomer standards were prepared by relaxing native p15A DNA in two separate reactions, each containing 0.5 μg of DNA and either 5 or 10 ng of ω protein. After incubation for 25 minutes at 37°C, the reactions were stopped and combined with 0.1 μg of native p15A DNA, to yield a mixture of relaxed and negatively supercoiled topoisomers covering the range resolvable by electrophoresis through a 1.2 percent agarose gel.
29. The sign inversion model was developed during conversations with our former colleague N. Patrick Higgins; we thank him for his help. Supported by NIH grant GM-21397 and (to P.O.B.) NIH fellowship GM-07281.

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Cerebral Vessels Have the Capacity to Transport Sodium and Potassium

Abstract. *The activity of Na^+,K^+-activated adenosinetriphosphatase and the uptake of a potassium analog, rubidium, were found to be similar in cerebral microvessels and choroid plexus when measured in vitro. This similarity suggests that sodium and potassium concentrations in the nascent brain extracellular fluid are determined by the same active process that regulates their concentration in nascent cerebrospinal fluid. The brain microvessels may thereby play an active role in brain potassium homeostasis and brain extracellular fluid formation.*

The endothelial cells lining the cerebral blood vessels are joined by tight junctions and contain few, if any, pinocytotic vesicles (*1*). This continuous endothelial membrane forms a limiting barrier, restricting the movement of many solutes between blood and brain. The study of movement of solutes through this barrier has been advanced by the recent development of techniques for isolation of metabolically active brain microvessels (*2, 3*). Experiments with preparations of these isolated vessels have shown that they contain specific transport systems for certain amino acids and sugars (*4, 5*). The mechanism by which the major cations Na^+ and K^+ cross this barrier has not been experimentally defined; however, the presence of a specific transport system may be inferred.

Cerebrospinal fluid (CSF) and brain extracellular fluid (ECF) are identical or virtually identical (*6*). The concentration of Na^+ and K^+ in the nascent CSF secreted by the choroid plexus has been shown to be dependent on a ouabain-sensitive process, indicating that Na^+, K^+-adenosinetriphosphatase is involved in the transport (*7, 8*) and that transport of Na^+ is a major energy-dependent step in the formation of the fluid. Although the CSF and brain ECF are contiguous at the ventricular wall, where movement of even large solutes is not impeded (*9*), it is unlikely that the secretory mechanisms

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