Molecular mechanics and DNA replication regulation

Arturo Falaschi¹ and Gulnara Abdurashidova²

¹Scuola Normale Superiore, Piazza dei Cavalieri 7, I-56126, Pisa, Italy and International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34012, Trieste, Italy

²International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34012, Trieste, Italy

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Magnetic and optical tweezers are providing novel insights on the structure, energetics, and functional dynamics of biological macromolecules. The modulation of DNA topology has provided very appropriate opportunities to study with these technologies the energetic and mechanistic features of the action of DNA topoisomerases, the enzymes that maintain the physiological level of negative superhelicity in the genome. Modulation of the superhelical state of the DNA replication origins is essential for the initiation of DNA synthesis in prokaryotes and eukaryotes. The results obtained recently from independent studies of two different groups combine to give new insights on the topological aspects of this process. With magnetic tweezers it was shown that the inhibition of human topoisomerase I by camptothecin freezes the drug-enzyme-DNA complex and specifically forbids the relaxation of positive supercoils; a study of the *in vivo* role of topoisomerase I on the activation of a human origin showed that this process is forbidden when the enzyme is frozen on the origin DNA by camptothecin. The inhibition of the relaxation of positive supercoils, probably introduced by the proteins performing origin activation, is therefore lethal for this process. Thus, the use of advanced physical technologies provides insights on an essential biological process. [DOI: 10.2976/1.2812798]

CORRESPONDENCE

Arturo Falaschi:
falaschi@icgeb.org

DNA topology nicely exemplifies the reasons why molecular biologists claim to feel particularly attracted by the aesthetic aspects of their discipline: the DNA duplex, this elegant all-pervasive icon of the postindustrial society, undergoes elaborate and rigorously described modulations in which the two helices wind around each other less times (negative supercoiling) or more times (positive supercoiling) than requested by the B structure (Bates and Maxwell, 2005); these variations cause (if the ends of the DNA molecule are held fixed, either because they are present in a circular molecule or because they are bound to other fixed nuclear structures) a supercoiling of the duplex; this consists, in the first case (see Fig. 1), in the molding of the duplex into a linear left-handed superhelix (or into a right-handed double-duplex superhelix in a circular structure), and into

the opposite (right-handed linear single-duplex superhelix, left-handed circular double-duplex superhelix) in the second case. These structural modulations are visually gratifying and correspond nicely to functional modulations. Thus, negative supercoiling, characterized by less turns of the helices than requested by the B structure, tends to weaken the bonds between the bases and expose them to the action of other molecules, typically for transcription and replication. Actually, the circular chromosomes and plasmids of bacteria are normally negatively supercoiled; this is true also for the eukaryotic chromosome, with the proviso that in these organisms the nucleosome structure, with the left-handed wrapping of the duplex around the histone octamer, nicely adapts to this supercoiling and relaxes the tension.

Figure 1. Negative superhelicity. If the two helices wind around each other less times than required by the B structure, a tension arises in the duplex; if the duplex is maintained in a linear form and the ends are tethered to a physical structure (bottom panel), this tension causes the creation of a left-handed superhelix. If the molecule is in the form of a covalently closed circle, the same tension causes the formation of a right-handed double-duplex superhelix (top panel).

SINGLE-MOLECULE MECHANICAL ANALYSIS OF DNA TOPOLOGY MODULATION

The aesthetic attractiveness of the DNA structural representations and of the appealing structure/function correspondences is accompanied by the exquisite amenability of the latter to innovative single-molecule biophysical approaches for their study. In general, these methodologies are based on the tethering of one end of a DNA duplex molecule to a fixed surface coupled to the binding of a force sensor/effector on the other end (Fig. 2, Charvin *et al.*, 2005). The molecule can be twisted or untwisted by applying a rotating force to the sensor (typically, a plastic bead of 1 μm diameter) with a magnetic or optical agent and measuring the induced variations in the position of the end of the molecule, including bending, shortening, or stretching.

As one might imagine, these refined technologies, usually referred to as “magnetic” or “optical tweezers,” are particularly fit for the study, at the single molecule level, of the action of the enzymes specialized in the modulation of the supercoiled status of DNA, the DNA topoisomerases. These are present in all living organisms and are, basically, of two types: Type I, which cuts and reseals one helix of the duplex and releases negative or positive supercoils present in it; and Type II, which introduces double-stranded cuts in the duplex, lets another intact duplex cross the cut and then reseals it. Only Type II topoisomerases require the hydrolysis of ATP for their action and are able, either alone (bacterial DNA gyrase) or in combination with the wrapping of the duplex around the histone octamer (eukaryotic topoisomerase II) to introduce negative supercoils.

As Type II topoisomerases handle double cuts in the DNA molecule and “magically” operate the crossing of a duplex by another one, their effects on the described experimental setup are more visible and, to a certain extent, these enzymes have been privileged by this kind of experimentation. Thus, for instance, Charvin and collaborators

Figure 2. A magnetic trap (a) and an optical trap (b). In the first case, the magnets stretch and twist the molecule and the relative forces can be measured from the fluctuation of the beads; in the second case, the rotation of the dielectric bead is prevented by a laser beam and the relative forces are deduced from the deflection of the beam by the bead. [Reprinted from Charvin *et al.*, (Fig. 2), with permission, from the Annual Review of Biophysics and Biomolecular Structure, Vol. 34 C2005 by Annual Reviews, www.annualreviews.org]

(2005), with a very elegant approach, rotated a tethered 1,000 base pairs (10 kb) duplex with 50 turns of the bead, causing a supercoiling of the structure that resulted in a clearly measurable shortening of the molecule, as a consequence of the buckling of the DNA in response to the induced tension. The addition of eukaryotic topoisomerase II, promptly released the supercoiling and allowed again the complete extension of the duplex in a very efficient and processive way, i.e., it was able to bring the uncoiling (and unbuckling) to completion in a single burst of successive duplex crossings.

Subsequently, the laboratory of C. Bustamante, in collaboration with the late N. Cozzarelli (Gore *et al.*, 2006), published a very accurate study of the mode of action of the bacterial Type II topoisomerase, DNA gyrase. Contrary to the eukaryotic topoisomerase II, which can release both positive and negative supercoiling but cannot introduce, alone, negative supercoiling in relaxed structures, gyrase performs this process very efficiently, and this property is essential for life. For this study, one end of a 9 kb duplex was tethered to a coverslip and the other end to a magnetic bead; the molecule was held in an extended form by rare earth magnets acting on the bead; at about 1 kb from the coverslip tethered end, a sequence with high affinity for gyrase was present in the DNA; above this, a fluorescent bead was attached, the time, direction, and extent of whose angular movements could be finely recorded. Further, a nick was present between the fluorescent bead and the magnetic bead to act as a swivel that did not allow the transmission of the rotation of the fluorescent bead to the magnetic one. With this elegant contraption the action of gyrase was detectable by following the rotation of the fluorescent bead caused by the introduction of superhelical turns in the duplex portion between the coverslip attachment and the site of the fluorescent bead. In this way the authors were able to follow directly, from the direction of the rotation

caused by the action of gyrase, the introduction of negative turns in the duplex; further, they could see that the number of rotations imposed by the enzyme was always even, two or a multiple, as expected from the known chemical mechanism of action of Type II topoisomerases, that cut and reseal two phosphodiester bonds [4 base pairs (bp) displaced in the two complementary strands] and reduce by two the so-called linking number (the number of times a helix has to be rotated around the other for attaining complete duplex unwinding). The same experimental setup also provided more hints into the mechanism of action of this enzyme: the action of gyrase is envisaged to require, at a given moment, a certain degree of “wrapping” of the duplex around the enzyme, prerequisite to the crossing of an intact segment through an open one; these authors observed that, as might be expected, this hypothesized wrapping is actually reflected in a momentary shortening of the end-to-end distance of the duplex, a shortening hampered by the application of increasing forces (of the order of a piconewton) to the magnetic bead that tend to maintain the duplex in an extended form, with a nice direct confirmation of the model.

The action of gyrase is essential and irreplaceable for bacterial life in different ways. In the first place, as mentioned earlier, the bacterial chromosome is organized into a rather compact “nucleoid” that contains a relatively constant degree of negative supercoiling, essential for its maintenance and function; this state is assured by the continuous presence of gyrase, allowing and easing different DNA transactions, like transcription, recombination, and repair. But, most important, gyrase is absolutely required for bacterial DNA replication, at two crucial levels. First, one has to consider that the advancement of a semiconservative growing fork requires the unwinding of the parental duplex, but this cannot possibly be accompanied by the rotation of the two newly copied arms as many times as required for unwinding a whole chromosome (i.e., approximately one turn every 10 bp). Thus, positive supercoiling accumulates rapidly ahead of the growing fork and the corresponding physical stress on the molecule has to be relaxed continuously for the fork to proceed. This is accomplished by the continuous action of gyrase, as the bacterial topoisomerase I cannot relax positive supercoiling (contrary to the corresponding eukaryotic counterpart, as we shall see in the following). But gyrase is believed to do actually more: by acting ahead of the growing fork and introducing negative stress on the unreplicated parental DNA, it eases the unwinding required for the advancement of the fork and reduces the formation of positive supercoiling this would cause.

DNA TOPOLOGY AND REPLICATION ORIGIN FUNCTION

Further, and no less essentially, gyrase is instrumental for the initiation of chromosome replication; the regulation of DNA replication occurs by the activation of specific sequences (the

replication origins) recognized by specific “initiator” proteins, that cause, at the right moment of the cell cycle, the organization and start of two oppositely moving replicative forks. In *Escherichia coli*, the initiator protein is the product of the *dnaA* gene, a 52 kDa protein that has to bind in several copies at the single origin present in the bacterial circular chromosome. Now, in order to bind all those copies of the *dnaA* protein, the origin sequence has to be negatively supercoiled; the combination of *dnaA* binding and negative supercoiling is believed to induce the unwinding of a nearby AT-rich region (the so-called DNA unwinding element) where the bidirectional replication machinery assembles. In absence of gyrase, this crucial process for genome replication (and life) of the bacterium cannot occur. Thus, the refined single-molecule physical techniques described earlier provide essential insights on the mechanism (and mechanics) of a biological process of the utmost importance.

In eukaryotic cells the regulation of DNA replication follows the same basic pattern as in bacteria, but each chromosome contains many specific replication origins (approximately one every 100 kb) and the initiator protein is actually a multiprotein replicative complex whose structure and composition change along the cell cycle, a complication certainly related to the inescapable need of these organisms to accurately couple cell division and genome duplication. The origin activation/deactivation cycle (each origin must absolutely fire once, and only once, every cell cycle) is still far from being defined in its molecular (chemical and mechanistic) details, because of its complexity and the consequent lack of a physiological *in vitro* system, contrary to the situation in bacteria. Yet, recently, the application of the magnetic tweezers technique to a human topoisomerase and the independent acquisition of an understanding of the role of topoisomerases in the process of activation of a human replication origin combine to offer novel insights on this process in the human organism.

In the issue of *Nature* of July 12 are reported the results of a very elegant study performed by the group of N. Dekker and collaborators (in Delft, The Netherlands and Memphis, Tenn.) (Koster *et al.*, 2007) applying the magnetic tweezers approach to human DNA topoisomerase I. This enzyme relaxes both positive and negative supercoils by cutting one phosphodiester bond, remaining covalently linked to the 3' phosphate, allowing the intact strand to pass through the cut and then reforming the phosphodiester bond leaving an intact duplex with a linking number modified by ± 1 . This enzyme is the target of a specific and efficient poison, camptothecin (and derivatives), that binds the enzyme and freezes the covalent topoisomerase-DNA intermediate forbidding the subsequent resealing step. In this way, when a growing fork reaches this single stranded cut, the DNA polymerase molecule cannot bypass it and a double stranded cut is induced, blocking the continuation of replication on that particular fork, but also inducing a cell cycle checkpoint re-

sponse that blocks the continuation of DNA replication everywhere in the cell. Thus, camptothecin is essentially an inhibitor of DNA replication, and this explains why it is one of the most utilized drugs in cancer chemotherapy.

Dekker and collaborators have studied the action of this drug on human topoisomerase I with a magnetic tweezers contraption, in which, by measuring precisely the shortening and lengthening of a duplex maintained in extended configuration by a magnetic force, they could detect the action of the enzyme in releasing different degrees of negative or positive supercoiling experimentally introduced in the duplex. By adding camptothecin (or equally effective analogs) in the solution, they could measure the effect of this poison on the uncoiling effect of the enzyme. In principle, one would expect that, in an experimental setup like the one under study, the freezing of the topoisomerase-cut DNA complex would certainly impede the release and further action of the enzyme, but should not impede the spontaneous uncoiling of the nicked DNA to reduce the experimentally induced tension, as the intact strand should now be free to rotate and reach the B configuration. But this was not the case: in the presence of camptothecin the rate of uncoiling was reduced by a factor of 20, probably because the interaction of the drug with both topoisomerase I and DNA duplex hinders strongly the rotation of the cut and intact strands around each other. This aspect of camptothecin action had never been suspected before.

But this was not all: surprisingly, it turned out that the uncoiling of positive supercoils is significantly much more hindered than that of negative supercoils. This brings the authors to propose (quite reasonably) that the inhibition of DNA replication brought about by the drug results also from the inability of relaxing the positive supercoils formed ahead of the advancing replicative fork.

The beauty of the results and insights brought by them are further highlighted by recent work from our laboratory [published after the submission of Dekker's work, [Abdurashidova et al. \(2007\)](#)] that addresses the other aspect of human DNA replication distinct from fork advancement, namely the origin activation event. We described the site and time of interaction of the two topoisomerases with a human replication origin along the cell cycle and we observed, in particular, that DNA topoisomerase I binds at two precise sites, on the templates for leading strand synthesis, few tens of nucleotides ahead of the start sites (the same sites are specifically recognized also *in vitro* by topoisomerase I). This binding occurred, *inter alia*, immediately before the activation of the origin and after the multimolecular preinitiation complex (to which also topoisomerase I belongs) was assembled and cocked for firing. The addition of camptothecin at this moment forbids the initiation event. This inhibition is not due to the block of elongation when the DNA polymerase reaches the sites of topoisomerase few tens of nucleotides ahead of the start site, as might be surmised: actually, also

those short fragments are not synthesized and the initiation event is abolished. Also, it cannot be due to the described induction of checkpoint, which has not had the time to be activated: the blockage of initiation must have occurred instantly after drug addition, as it has forbidden even the synthesis of the stretch between start site and topoisomerase I site, which takes less than a second.

This observation fits nicely with Dekker's conclusion and adds a further dimension to the understanding of the drug action and of the mechanism of eukaryotic origin activation: it appears reasonable to hypothesize that the initiation event brought about by the molecules of the preinitiation complex involves the creation of a positive supercoiling stress that has to be released by topoisomerase I; camptothecin inactivates the enzyme and hampers this relaxation, forbidding DNA replication initiation.

Thus basically, the topological requirements for origin activation in prokaryotes and eukaryotes are analogous: in bacteria, negative supercoiling must be introduced directly by gyrase and any consequently induced nearby positive superhelicity cannot be released by the bacterial topoisomerase I; in human cells, the multimolecular preinitiation complex probably introduces an analogous negative superhelical tension (in both cases, presumably, to ease the opening of the DNA unwinding element) that is reflected in the positive supercoiling of a nearby region, which has to be relaxed (in this case, by topoisomerase I) for the attainment of an initiation-proficient origin structure.

CONCLUSION

The integration of the results obtained by the mechanochemical procedures with those obtained with cellular and molecular biology approaches synergize in offering a deeper understanding of the events leading to the activation of human replication origins and its analogy with the situation in prokaryotic organisms. The human (indeed, eukaryotic) situation is certainly much more complex, in view of the multiplicity of proteins necessary to perform a step that in bacteria depends essentially on a single protein. In any case, the observations we have here highlighted demonstrate, once more, that the use of a sophisticated physical nanotechnology may offer essential insights on the mechanisms of crucial biological processes: this should further encourage the (sometimes still difficult) dialogue among different disciplines that appears to be the key for an acceleration in the progress of our knowledge in the life sciences.

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