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Surface plasmon resonance analysis of topoisomerase I–DNA binding: effect of Mg^{2+} and DNA sequence

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Surface plasmon resonance detection was used to characterize interactions of human topoisomerase I and DNA. The disassociation of topoisomerase I and DNA is characterized by two rate constants. This suggests two parallel but independent pathways of release. DNA association appears to be complex. Mg^{2+} was found to increase both disassociation rate constants and may have a detectable effect on topoisomerase I DNA association. DNA base content did not affect disassociation rate constants or the rate of association.

Key words: BIAcore, DNA binding, kinetics, surface plasmon resonance, topoisomerase I.

Introduction

Surface plasmon resonance technology allows 'real-time' measurements of protein-DNA binding, and thus offers the potential for facile and direct description of binding and release kinetics.[1–4] The work presented here describes topoisomerase I–DNA interactions using a surface plasmon resonance anlalyzer, the Pharmacia BIAcore 2000 biosensor.

Topoisomerases are enzymes critical to DNA metabolism.[5] Among other functions they facilitate replication and transcription by relieving the topological strain introduced into DNA by these processes. Topoisomerases are also key targets of anti-cancer and anti-microbial drugs.[6,7] In broad terms the mechanisms by which topoisomerases catalyze DNA unwinding are generally agreed. The enzymes bind DNA, introduce transient strand breaks, allow strand passage or rotations of DNA unwinding and then seal the break.[5,8,9] There are at least three topoisomerases encoded in mammalian cells. Topoisomerase I exists as a monomer, it introduces only single strand breaks in the DNA substrate and it does not require ATP or Mg^{2+} (although it is maximally active in the presence of 10 mM MG^{2+}).[10] It is the cellular target of the newly approved anti-cancer drugs CPT-11 and topotecan.[11] Initial kinetic analyses of topoisomerase I catalysis described the relaxation of supercoiled DNA and the production of DNA isomers with reduced linking number.[12,13] Domain and substrate analysis reveal potential bipartite interaction with DNA.[14,15] Other factors which have been implicated as affecting topoisomerase I–DNA interactions include cation type and concentration, and DNA structure (curve or writhe) or supercoiling.[16–20] A detailed description of the kinetic mechanism of type I topoisomerase has been approached using the 32 kDa vaccinia virus protein, rapid quench technology and acrylamide gel analysis of cleaved or ligated DNA substrates.[21] This work has provided the first overall kinetic scheme for topoisomerase catalysis and provides a starting point for the description of such a scheme for human topoisomerase. Nevertheless, the precise nature of topoisomerase I–DNA–drug interactions is poorly understood and remains the subject of intense research.

The objective of the work presented here is to evaluate the potential of the BIAcore biosensor for description of topoisomerase I–DNA interactions. This approach has obtained disassociation and association rate constants for human topoisomerase I interactions using various DNA substrates and conditions. Mg^{2+} has a dramatic effect on topoisomerase I catalysis.[18–20] Therefore, the effect of Mg^{2+} on topoisomerase–DNA binding was determined through a range of concentrations. It was found that rates of topoisomerase I–DNA disassociation increase at least 3- to 4-fold as Mg^{2+} concentrations increase from 0 to 10 mM. The effect of DNA sequence on topoisomerase binding was also evaluated. In these experiments DNAs of vastly different GC content were compared as substrates for topoisomerase I binding. Similar rates of association and

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disassociation were found for the different DNAs. This suggests that while topoisomerase I has definite sequence specificity for the cleavage step of catalysis, it associates with DNA in a non-specific manner.

Materials and methods

Reagents

The BIAcore 2000 Biosensor substrate DNAs, *Micrococcus lysodeikticus, Clostridium perfringins (welchii)* and salmon testis were obtained from Sigma (St Louis, MO), as were the chemical reagents listed below. *Mbo*I restriction endonuclease, Klenow fragment DNA polymerase and pNEB DNA were obtained from New England Biolabs (Beverly, MA). The CM5 sensor chip and biotin-14-dATP were obtained from Pharmacia Biotech (Alameda, CA). Streptavidin was obtained from Pierce Life Science (Rockford, IL). Topoisomerase I was isolated as described elsewhere.[22,23]

Biotinylation of DNA

DNAs were digested overnight to completion with *Mbo*I. The restriction enzyme-digested DNAs were extracted with phenol:isoamylalcohol:chloroform (24:1:25), precipitated with ethanol and redissolved in TE (10 mM Tris, pH 7.4, 1 mM EDTA). DNAs were biotinylated using Klenow buffer (50 mM Tris, pH 7.2, 100 mM $MgSO_4$, 0.1 mM dithiotheitol, and 1 mM dCTP, dGTP, dTTP and biotinylated dATP) for 2 h at 37°C. The DNA was ethanol precipitated as above.

CM5 chip activation

The CM5 flow-cell chip was activated with a mixture of *N*-ethyl-*N'*(dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinamide (NHS) as recommended by BIAcore, Pharmacia. Streptavidin then spontaneously reacted with the activated flow-cell surface when applied at 100 μg/ml in 10 mM sodium acetate, pH 6.0, at a flow rate of 10 μg/min. The surface was then deactivated with 1 M ethanolamine, pH 8.0. Biotinylated DNA, 100 μg/ml in HEPES buffered saline (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% p 20 detergent) was applied to the flow cell until the desired level of resonance units (RUs) was achieved.

Topoisomerase application to flow-cell

Topoisomerase I (greater than 95% pure by coomassie staining of polyacrylamide gel analysis) was diluted in running buffer (HBS, 150 mM NaCl, varying Mg^{2+}, 3.7 mM EDTA, 10 mM HEPES, pH 7.4, and 0.005% p 20 detergent) and applied to the biosensor flow cell in (usually) 50 s injections. Flow-cells were regenerated between experiments by 5 min washing with 0.5 M NaCl.

Results

Generation of sensograms

Three types of DNA, salmon testis, *M. lysodeikticus* and *C. perfringins (welchii)*, were derivatized with biotin and then immobilized to a streptavidin activated chip, each DNA being applied to a separate flow cell (channel). To control for non-specific binding, a streptavidin-only channel was used as a blank. Topoisomerase I was injected as the mobile analyte into all four channels at concentrations varying from 50 to 800 nM (an example injection is shown in Figure 1A). The curve from the fourth flow cell was subtracted from the curves generated from DNA bound channels before the data were analyzed (an example is shown in Figure 1B). In a subsequent experimental series with the same design, topoisomerase I in similar concentrations was applied in buffer varying in Mg^{2+} concentration (0.0, 5.0, 6.3 and 10 mM). These experiments were repeated two to three times, each repeat using two or three concentrations of topoisomerase I above diffusion limited concentrations (Table 1).

Analysis of data

Data were analyzed using BIAcore evaluation software. Disassociation rate constants were determined for data analyzed from the cut-off of the topoisomerase injection and extending over an interval of 50 s. The data were not adequately fit by a formula modeling a single disassociation event. Therefore the best fit obtained with this formula was compared to the alternative formula representing two independent rates of disassociation. The latter model was always preferred with a probability of 1 (utilizing the BIAevaluation software which 'calculates an *F*-test probability value based on the ratio of the χ^2 values for two consecutive fits to the same selected

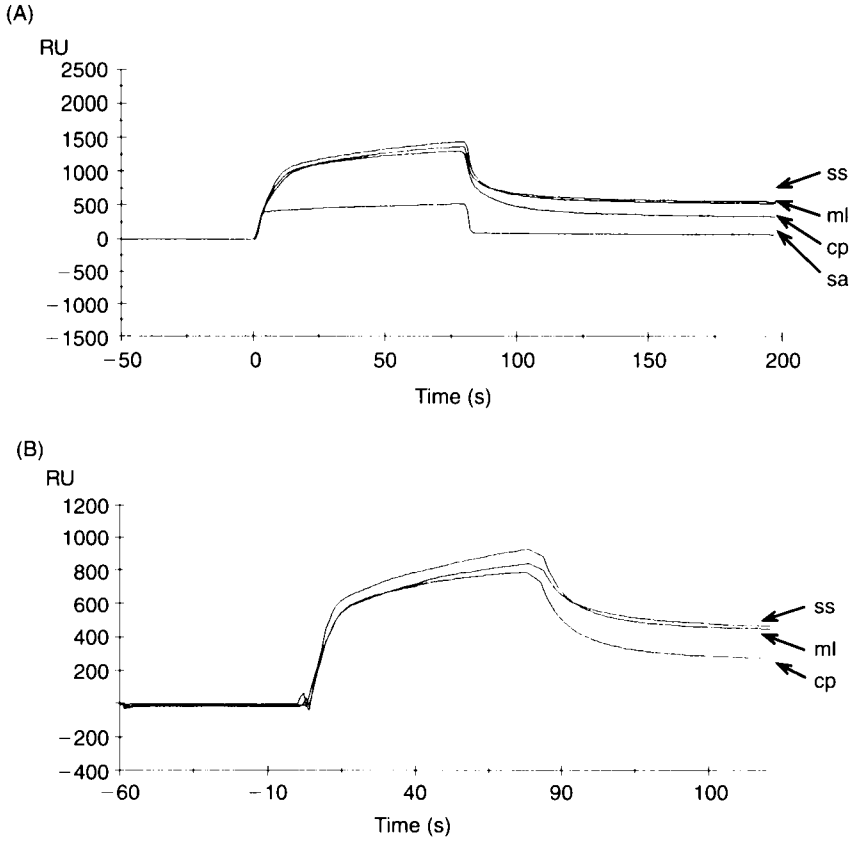


Figure 1. (A) Sensogram generated by an injection of topoisomerase I over CM5 chip activated with three different types of DNA. The curves generated by the four flow-cells in the chip are labeled ss (salmon testis DNA), ml (*Micrococcus* DNA), cp (*Clostridium* DNA) and sa (control streptavidin). (B) The same data with the non-specific binding (the streptavidin curve) subtracted.

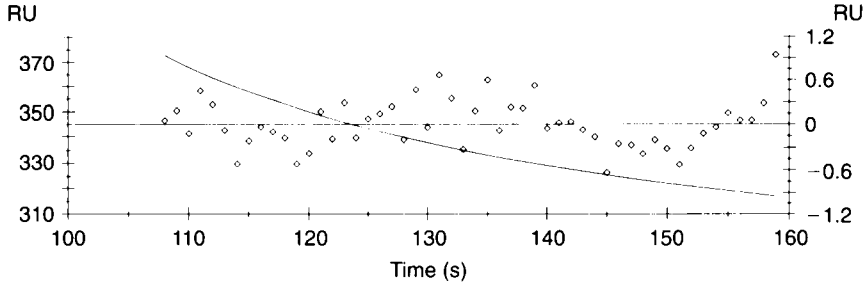
Table 1.

Mg^{2+} (mM)		Experiment					
		1[a]		2[a]		3[a]	
		k_d1	k_d2	k_d1	k_d2	k_d1	k_d2
0	ss	0.0476	2.07E − 03	0.0362	1.66E − 03	0.0396	1.43E − 03
	ml	0.0346	2.88E − 03	0.0326	1.38E − 03	0.0498	1.27E − 03
	cp	0.0545	2.05E − 03	0.0281	1.99E − 03		
5	ss			0.0615	3.21E − 03	0.0572	1.70E − 03
	ml			0.0515	3.45E − 03	0.0591	1.87E − 03
	cp			0.0472	3.03E − 03		
6.3	ss	0.134	2.95E − 03				
	ml	0.125	5.29E − 03				
	cp	0.134	3.09E − 03				
10	ss			0.096	4.96E − 03	0.118	4.63E − 03
	ml			0.095	4.93E − 03	0.113	5.81E − 03
	cp			0.11	5.01E − 03		

[a]Values presented for each experiment are the averages determined from several different non-diffusion limited concentrations of topoisomerase I. For experiment 1 (0 mM Mg^{2+}) values from five topoisomerase injections were averaged, for 6.3 mM Mg^{2+}, three injections were averaged. For experiment 2 (0 mM Mg^{2+}) four topoisomerase injections were averaged, for 5 and 10 mM Mg^{2+}, two injections were averaged. For experiment 3 each result is from a single non-diffusion limited concentration of topoisomerase I.

data').[24] Representative error determinations for these 'fits' are also shown in Figure 2.

The analysis employed by BIAevaluation software calculates association rate constants once disassociation rate constants have been determined. The data obtained yielded average disassociation rate constants for topoisomerase I and DNA of approximately 4×10^{-2} and 2×10^{-3} (average from Table 1) in the absence of Mg^{2+}. The association appears to be complex and inadequately modeled by the available BIAevaluation software or by an additional formula written for this work representing a model in which one association event could result in two separate rates of disassociation. A characteristic inflection in the association curve was suggestive of a cooperative influence on topoisomerase binding (Figure 3). Association rate constants determined using the simplest model of DNA and topoisomerase association are presented in Table 2 soley to provide a means of comparison between experiments.



R_1	SE(R_1)	k_d1	SE(k_d1)	R_0	k_d2	SE(k_d2)	t_0	Offset	SE(Offset)	chi^2
338	2.42	0.0557	3.99e−4	3.72e3	1.41e−3	1.2e−5	108	−0.0542	0.201	0.112

Figure 2. Data analysis using the formula in which disassociation is characterized by two rate constants. The model curve is superimposed on the actual data. The scatter plot shows the variation of the actual data from the 'ideal' curve (the right-hand scale). The tabular data shown were obtained from this 'fit'. R_1 is the fraction of the decrease in resonance units (R_0) due the rate characterized by k_d1 over this period of disassociation. SE(R_1) is the standard error for R_1. k_d1 is the fastest disassociation rate constant, k_d2 is the slower disassociation rate constant. SE(k_d1) and (k_d2) are the standard errors for those values. t_0 is the time at which disassociation began. Offset is the estimated response a $t = 0$, its standard error equals SE(Offset). χ^2 represents the goodness of fit between the experimental data and the fitted curve.[24]

Table 2.

Mg^{2+} (mM)		Experiment					
		1[a]		2[a]		3[a]	
		k_a	R_1/R_0	k_a	R_1/R_0	k_a	R_1/R_0
0	ss	1.18E + 05	1.18	1.35E + 05	1.13	2.16E + 05	0.216
	ml	1.46E + 05	0.12	1.16E + 05	0.095	3.83E + 05	0.201
	cp	9.50E + 04	0.13	1.00E + 05	0.09		
5	ss			1.82E + 05	0.13	4.86E + 05	0.258
	ml			1.50E + 05	0.13	5.31E + 05	0.22
	cp			1.75E + 05	0.13		
6.3	ss	2.32E + 05	0.37				
	ml	2.56E + 05	0.46				
	cp	2.20E + 05	0.27				
10	ss			1.84E + 05	0.19	5.55E + 05	0.51
	ml			2.19E + 05	0.18	6.54E + 05	0.58
	cp			5.57E + 05	0.17		

[a]Values presented for each experiment are the averages determined from several different non-diffusion limited concentrations of topoisomerase I. For experiment 1 (0 mM Mg^{2+}) values from five topoisomerase injections were averaged, for 6.3 mM Mg^{2+}, three injections were averaged. For experiment 2 (0 mM Mg^{2+}) four topoisomerase injections were averaged, for 5 and 10 mM Mg^{2+}, two injections were averaged. For experiment 3 each result is from a single non-diffusion limited concentration of topoisomerase I.

Effect of DNA sequence

The effect of DNA sequence on association and disassociation rates was approached by comparing DNAs derived from three different sources. *M. lysodeikticus*, *C. perfringens (welchii)* and salmon testis. *M. lysodeikticus* DNA is approximately 72% GC, *C. perfringens (welchii)* DNA is about 31% GC and salmon testis DNA is about 50% GC. The data obtained with the different DNAs are presented in Tables 1 and 2.

Discussion

Surface plasmon resonance analysis is a relatively new approach for measuring molecular interactions. Determinations of association and dissociation constants can be made without the need of quantification of product formation or the use of labeled substrates. Furthermore, disassociation constants can be determined independent of concentration. Inherent difficulties with BIAcore use and data interpretation have been discussed in the literature.[25] Problems with BIAcore use are addressed here in two parts. First, limitations due to inappropriate experimental design, i.e. problems in aquiring data that accurately reflect the molecular interactions in question. Second, limitations due to data interpretation and analysis of data by inappropriate mathematical models.

Pure human topoisomerase I (kDa 67 000) isolated from placenta was used for these experiments. Catalytic activity was characterized during isolation and in DNA cleavage experiments carried out coincidentally with this work in unrelated projects in the laboratory. Low non-specific binding was obtained in HEPES buffered saline (see Methods) as compared to other reaction/running buffers, so it was adopted as running buffer for the generation of the data presented here. The critical conditions for topoisomerase I activity (150 mM monovalent cation and physiological pH) were preserved in the HBS running buffer. Thus, the topoisomerase I analyzed in these experiments was fully active, based on the reaction conditions, the previously verified topoisomerase I isolation procedure and parallel experiments demonstrating activity in DNA cleavage assays.

A relatively fast flow rate of 30 μl/min was used to minimize mass transport limitation (diffusion limitation) of protein–DNA association.[24] Also, experiments were conducted using several different concentrations of injected topoisomerase I. This allowed determination of diffusing limiting conditions. Diffusion limitation of topoisomerase I–DNA association was observed at low concentrations depending on the flow-cell DNA concentration. Nonspecific binding of topoisomerase I was controlled for by subtraction of a control channel curve (biotinylated streptavidin with no DNA) from the DNA curves prior to analysis. To minimize the theoretical contribution of analyte (topoisomerase) rebinding to the disassociation constants,[24] curves were fit to data through time ranges for 50 s beginning immediately after cut-off of topoisomerase. At the initiation of this interval all mobile phase-derived topoisomerase had cleared the flow cell but the binding sites on the immobilized ligand (DNA) were still maximally occupied, thereby minimizing any rebinding effect.

Analysis of data was dependent upon acquisition of sufficient signal (RUs) due to topoisomerase I (mobile) and DNA (stationary) interactions. Minimally acceptable RU levels were arbitrarily set at 200. Furthermore, to insure sufficient data for statistical validity, curves analyzed for determination of disassociation rates were fit with 100 data points (over 50 s). Association rate constants were determined for the entire association phase of the experiment.

The BIAevaluation software is based on a Langmurian model of molecular interaction which assumes that the ligands are homogeneous and pure, and that they interact in a stoichiometric manner.[24,25] The simple equation of $dR/dt = R^{-k_d}$ describes the single disassociation formula (R = bound analyte as defined by resonance units, t =time in seconds and k_d =disassociation constant). The BIAevaluation formula to model two independent disassociation components contributing to the disassociation curve is $dR/dt = R_1{}^{-k_d1(t-t_0)} + (R_0 - R_1)^{-k_d2(t-t_0)}$. For these formulae to be applied appropriately it is important that there are not multivalent interactions nor steric cooperativity between molecules of ligand/analyte. (Association data presented here suggest that this might not be the correct assumption for topoisomerase I DNA binding in this model.) Nevertheless, for this initial work, these formulae are assumed to adequately describe the release of topoisomerase I from DNA. The association of topoisomerase I and DNA in this experimental design appears to be complex and has not yet been adequately modeled. It has been suggested that topoisomerase I binding induces a bend in the substrate DNA.[16,17] Topoisomerase I has also been reported to bind more avidly to protein-bound and protein bent DNA.[26,27] Thus is not

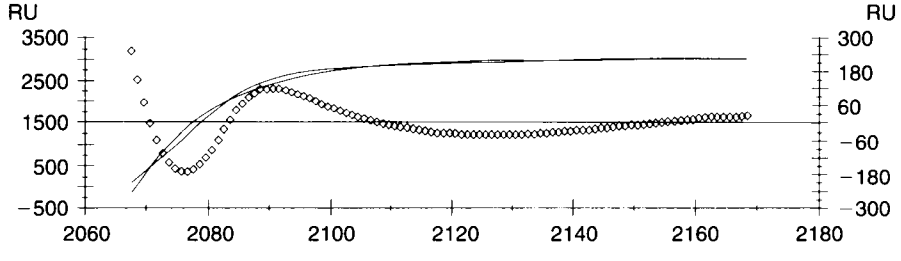
inconceivable that topoisomerase I binding could appear cooperative in the model of topoisomerase–DNA interaction used here. A different curve for topoisomerase I–DNA association was obtained in an alternative protocol measuring association (Figure 4B).

Topoisomerase I exhibits sequence-specific cleavage of DNA. In a random 100 bp stretch of DNA it would be expected that topoisomerase I–DNA cleavage could easily be detected (by DNA sequencing technology) at several sites. Greater than 90% of the cleavage has been found to occur 3′ to thymine.[28,29] Furthermore, several reported topoisomerase I cleavage sites are characterized by high AT content and curved AT-rich DNA is reported to be a preferred topoisomerase I substrate.[12,16,17] It was therefore hypothesized that the site-specific cleavage of DNA by topoisomerase I would be reflected in DNA sequence-specific binding of topoisomerase I. To test this hypothesis DNAs of vastly different GC contents were linked to the chip and tested for topoisomerase I binding. It was hypothesized that DNA with higher AT content would contain more high-affinity binding sites than low AT DNA. Suprisingly, the rates of association and disassociation were not different amongst these substrates. The rate constants were not influenced by DNA sequence.

The results obtained here suggest that human topoisomerase I binds linear DNA with an affinity rate constant of approximately 4×10^5 mol^{-1} s^{-1} and that it releases DNA with a k_d1 of approximately 0.15 s^{-1} and a k_d2 of approximately 5×10^{-3} s^{-1} in 10 μM Mg^{2+}. In the absense of Mg^{2+}, topoisomerase I may bind DNA with a slightly slower rate, and it releases DNA with a k_d1 of approximately 0.04 s^{-1} and a k_d2 of approximately 2×10^{-3} s^{-1}. Consistent with this observation is the increased contribution of k_d1 to the total disassociation in high Mg^{2+} (see R_1/R_0 ratios in Table 2). Overall R_0s are decreased in high Mg^{2+} (data not shown).

The divalent cation Mg^{2+} is thought to bind DNA in a site-specific manner to twist the curvature of intrinsically curved (e.g. AT-rich) sequences out of 'optimal planarity'.[30,31] The data suggest that increasing Mg^{2+} may slightly increase the rate of association of topoisomerase I and DNA. However, no sequence-specific enhancement was observed with any one of the three DNAs used (Table 2). Therefore, it is concluded that the effect of Mg^{2+} on the DNAs was approximately uniform across the ranges of GC content tested. The reported ability of Mg^{2+} to increase the catalytic activity of topoisomerase I is consistent with the observed increases in the disassociation constants provided the increased k_ds reflect increased topoisomerase turnover.

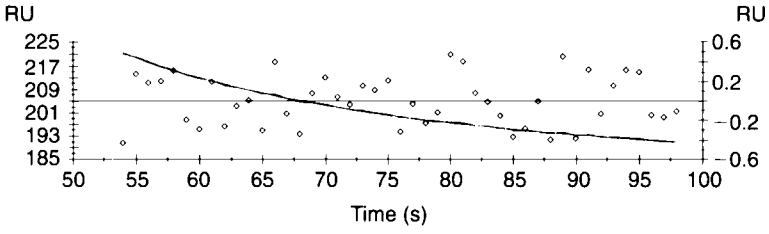
Experiments using a second protocol to model topoisomerase I–DNA interaction were conducted to confirm the results reported above. In this experimental design human placenta topoisomerase I was biotinylated and linked to the streptavidin coated biosensor flow cell. Plasmid (pNEB) DNA (approximately 80% supercoiled) was used as the mobile analtyte. Data were analyzed as described



k_a	SE(k_a)	R_{eq}	SE(R_{eq})	C	n	t_0	k_d	Offset	SE(Offset)	chi^2
1.1.33e5	2.87e3	3.15e3	35	5.2e−7	1	2.07e3	4.09e−3−	134	36	4.81e3

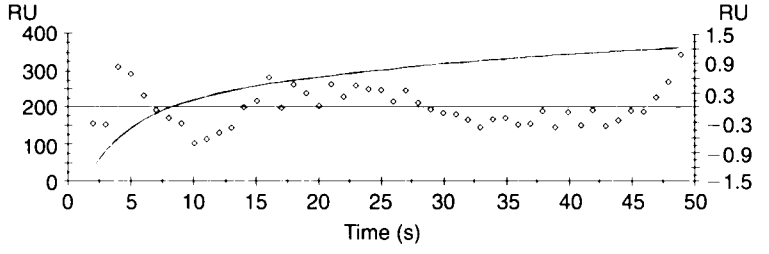
Figure 3. Data analysis using the formula in which association is characterized by a single rate constant. The model curve is superimposed on the actual data. The scatter plot shows the variation of the actual data from the 'ideal' curve (the right-hand scale). The tabular data shown were obtained from this 'fit'. The k_a is the association rate constant determined by this analysis, the standard error for this value is SE(k_a). R_{eq} is the estimated resonance units at equilibrium, SE(R_{eq}) is the standard error of that estimate. C is the concentration of topoisomerase I during this injection. N is defined as the steric interference factor (1 assumes no steric interference). t_0 is the time at the beginning of injection. k_d is the disassociation constant used to calculate k_a. Offset is the estimated response a $t = 0$, its standard error equals SE(Offset). χ^2 represents the goodness of fit between the experimental data and the fitted curve.[24]

(A)



R_1	SE(R_1)	k_d1	SE(k_d1)	R_0	k_d2	SE(k_d2)	t_0	Offset	SE(Offset)	chi^2
24.9	2.54	0.0584	5.68e−3	221	8.5e−4	2.36e−4	54	0.435	0.167	0.0713

(B)



k_a1	SE(k_a1)	k_a2	SE(k_a2)	k_d1	k_d2	Offset	SE(Offset)	chi^2
5.27e4	738	7.45e3	159	0.0583	4.97e−4	43.0	0.359	0.17

Figure 4. (A) Data analysis using the formula in which disassociation is characterized by two rate constants. This disassociation is that of mobile pNEB supercoiled DNA and biotinylated topoisomerase attached to one channel of the chip. The model curve is superimposed on the actual data. The scatter plot shows the variation of the actual data from the 'ideal' curve (the right-hand scale). The tabular data shown were obtained from this 'fit'. R_1 is the fraction of the decrease in resonance units (R_0) due the rate characterized by k_d1 over this period of disassociation. SE(R_1) is the standard error for R_1. k_d1 is the fastest disassociation rate constant, k_d2 is the slower disassociation rate constant. SE(k_d1) and (k_d2) are the standard errors for those values. t_0 is the time at which disassociation began. Offset is the estimated response a $t = 0$, its standard error equals SE(Offset). χ^2 represents the goodness of fit between the experimental data and the mathematical curve.[24] (B) Data analysis using the formula in which association is characterized by two constants. The model curve is superimposed on the actual data. The scatter plot shows the variation of the actual data from the 'ideal' curve (the right-hand scale). The tabular data shown were obtained from this 'fit'. The k_a1 and k_a2 are the association rate constants determined by this analysis, the standard errors for these values are SE(k_a1) and (k_a2). t_0 is the time at the beginning of injection. k_d1 and k_d2 are the disassociation rate constants used to calculate kas. Offset is the estimated reponse a $t = 0$, its standard error equals SE(Offset). χ^2 represents the goodness of fit between the experimental data and the fitted curve.[24] The association of DNA and topoisomerase I was adequately fit in this protocol.

above. Figure 4(A) presents dissociation rate constants obtained in initial experiments performed using this second protocol. These data clearly show the duality of the disassociation event and yield constants of the same order presented above. The association data obtained with this protocol are shown in Figure 4(B). Interestingly, the association curve was fit well by a formula in which DNA–topoisomerase association is characterized by two rate constants. The k_a1 obtained in these confirmatory experiments was slightly slower to the one determined in the primary model on topoisomerase I–DNA interaction.

Conclusion

The data presented here support the hypothesis that topoisomerase I–DNA interactions can be modeled by surface plasmon resonance analyzers such as the BIAcore 2000. Initial analysis suggests that DNA-bound topoisomerase I releases at either of two independent rates. The mechanism responsible for this duality is unknown. One possibility is that topoisomerase I can bind DNA non-specifically. If binding occurs at a suitable site it could result in subsequent conformational changes before release. Binding that occurs at sites unsuitable for subse-

quent enzyme activity could result directly in release. Alternatively, there could be two types of binding sites in DNA. One type of site could result in prolonged binding and the other could result in abbreviated binding. Topoisomerase I disassociation from these two types of binding sites could be represented by the two disassociation rate constants. Other explanations can be devised involving either or both of the binding domains of topoisomerase I. In any case, it is likely that analysis of topoisomerase I–DNA interactions by surface plasmon resonance will provide invaluable new descriptions of topoisomerase I function. This approach promises relatively facile descriptions of drug effects on topoisomerase I–DNA interactions in the future.

Acknowledgments

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