Proton Relay Mechanism of General Acid Catalysis by DNA Topoisomerse IB*

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Berit Olsen Krogh and Stewart Shuman†‡

From the Molecular Biology Program,
Sloan-Kettering Institute, New York, New York 10021

Type IB topoisomerases cleave and religate DNA by a mechanism involving a DNA-(5’-phosphotyrosyl)-enzyme intermediate. A constellation of conserved amino acids (Arg-130, Lys-167, Arg-223, and His-265 in vaccinia topoisomerase IB) catalyzes the attack of the tyrosine nucleophile (Tyr-274) on the scissile phosphodiester (10–12). Mutational, stereochemical, and structural data for vaccinia and nuclear topoisomerase IB and tyrosine recombinases suggest that the two arginines and the histidine contact the nonbridging oxygens of the scissile phosphodiester and that these interactions serve to stabilize a proposed pentacoordinate phosphorane transition state (3, 5, 9, 10–13).

Recently we used 5’-bridging phosphorothiolate-modified DNAs to implicate Lys-167 of vaccinia topoisomerase as a general acid catalyst of the DNA cleavage reaction (14). The hypothesis was that if the expulsion of the 5’-oxygen of the leaving DNA strand was indeed catalyzed by a general acid on the topoisomerase, then the requirement for the general acid ought to be alleviated by introducing a 5’-bridging phosphorothiolate at the scissile phosphodiester because the pK_a of the DNA 5’-SH is ~5 log units lower than that of the DNA 5’-OH. Accordingly the diagnostic feature of a topoisomerase mutant defective in general acid catalysis would be a significant increase in the cleavage rate on a 5’-S substrate compared with a 5’-O substrate. The “sulfur/oxygen ratio” ought not to increase for topoisomerase mutants that are impaired in other aspects of transesterification chemistry (e.g. transition state stabilization). Our findings were that the 5’-S group restored activity to the catalytically defective K167A mutant, whereas there was no positive thiolate effect for mutants R223A and H265A (14). Thus, we concluded that Lys-167 functions in proton transfer to the leaving strand. Lys-167 is located in a flexible interstrand hairpin loop of vaccinia topoisomerase and is conserved in all known type IB topoisomerases and tyrosine recombinases.

Our earlier experiments did not address whether Arg-130 plays any role in leaving strand expulsion. Arg-130, which is invariant among type IB topoisomerases and tyrosine recombinases, enhances the transesterification rate of vaccinia topoisomerase by 5 orders of magnitude as gauged by the effect of the R130A mutation (10). Because a conservative R130K substitution had little restorative effect, it was surmised that multivalent contacts of the guanidinium nitrogens of Arg-130 with the scissile phosphodiester are essential for catalysis. The occurrence of either one or two hydrogen bonding contacts between this conserved arginine side chain and the DNA backbone in various crystal structures of topoisomerase IB and tyrosine recombinases (3, 5, 9) was broadly consonant with the mutational data for the vaccinia enzyme (11). Here we report the surprising observation that Arg-130 plays a major role in proton transfer to the 5’-leaving DNA strand.

EXPERIMENTAL PROCEDURES

Topoisomerase Mutants—Recombinant wild-type vaccinia topoisomerase and mutants K167A, R130A, and R130K were produced in Escherichia coli and purified by phosphocellulose chromatography as described previously (15). The double mutant R130/K167A was constructed for the present study and was produced and purified using the same protocol.

Equilibrium Cleavage Assay—Reaction mixtures containing (per 20 µl) 50 mM Tris-HCl (pH 7.5), 0.3 µmol of 34-mer/60-mer DNA (Fig. 1), and 75 µg of purified topoisomerase were incubated at 37 °C. Aliquots (20 µl) were withdrawn at the times indicated and quenched immediately with SDS. The mixtures were digested with 10 µg of proteinase K for 60 min at 37 °C, then adjusted to 50% formamide, and heat-denatured. The samples were analyzed by electrophoresis through a 17% polyacrylamide gel containing 7 M urea in 90 mM Tris borate, 2.5 mM

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† To whom correspondence should be addressed. E-mail: s-shuman@ski.mskcc.org.
‡ The abbreviation used is: topo, topoisomerase.

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Alkaline Phosphatase Assay—Reactions were performed as above except the DNA was double-stranded, an excess of alkaline phosphatase was added, and the reactions were incubated at 37 °C. The 5’-S substrates were additionally treated with 5’-exonuclease to remove the 5’-terminal phosphorothiolate group. The reactions were quenched as above and analyzed by polyacrylamide gel electrophoresis.
EDTA. The cleavage product, a $^{32}$P-labeled 12-mer oligonucleotide bound to a short peptide, was resolved from the 34-mer substrate. The yield of covalent adduct at equilibrium was quantitated by scanning the gel with a PhosphorImager. Rate constants for approach to equilibrium on the 5'-S substrate (Fig. 1). We refer to this as an equilibrium substrate because the 22-mer leaving strand generated upon cleavage at CCCTT remains associated with the topoisomerase-DNA complex via base pairing to the noncleavable strand. The yield of covalent adduct at equilibrium in reactions containing saturating levels of the wild-type vaccinia topoisomerase was increased from 28% for the 5'-O substrate ($K_d = 0.35$) to 96% for the 5'-S substrate (Fig. 1A). An explanation for the observed altered equilibrium is that the 5'-S-sulfhydryl leaving strand in the cleavage reaction is an extremely poor nucleophile in the religation step (16, 17). The reaction rates of wild-type topoisomerase on the 5'-O and 5'-S equilibrium substrates were identical within our limits of detection; both reactions were effectively complete in 5 s (the earliest time examined).

The rate of approach to equilibrium by R130A on the 5'-O duplex substrate was extremely slow ($k_{obs} = 7.9 \times 10^{-6} \text{s}^{-1}, K_d = 0.54$) (Fig. 1B). Solving the equations $k_{obs} = k_d + k_{rel}$ and $K_d = k_d/k_{rel}$ yielded a value for $k_d$ of $2.8 \times 10^{-6} \text{s}^{-1}$, which is 5 orders of magnitude slower than the rate of single-turnover cleavage by wild-type topoisomerase ($k_d = 0.3 \text{s}^{-1}$) (10, 18). The instructive finding was that the rate constant for single-turnover cleavage of the 5'-S DNA by R130A ($k_d = 5.3 \times 10^{-4} \text{s}^{-1}$) was 190 times faster than $k_d$ with the 5'-O substrate. Thus, the simple replacement of the 5'-O leaving group by the less nucleophilic 5'-S diminished the requirement for Arg-130 in catalysis. Nonetheless, the R130A cleavage rate on the 5'-S substrate was still much slower (by a factor of ~570) than the single-turnover cleavage rate of wild-type topoisomerase (0.3 s$^{-1}$). These results provide evidence that Arg-130 participates in the step of proton donation to the 5'-O of the leaving strand, but they also suggest additional functions of Arg-130 in transesterification chemistry.

We compared the thiolate effects for R130A with those for the K167A mutant assayed in parallel (Fig. 1C). The approach to equilibrium by K167A on the 5'-O duplex substrate ($k_{obs} = 6.8 \times 10^{-5} \text{s}^{-1}, K_d = 1.04$) was an order of magnitude faster than that of R130A. As reported previously (11, 14), the K167A mutation increased the cleavage equilibrium constant compared with wild-type topoisomerase. We calculated a value for $k_d$ of $3.5 \times 10^{-5} \text{s}^{-1}$. The reaction of K167A with 5'-S DNA ($k_d = 1.3 \times 10^{-2} \text{s}^{-1}$) was 370 times faster than the reaction with the 5'-O substrate and was within a factor of 25 of the $k_d$ of wild-type topoisomerase. These results agree with kinetic data reported previously for K167A (14), and they underscore that the 5'-S modification boosted the activity of K167A closer to the wild-type level than it did the activity of R130A. Again, the implication is that Arg-130 plays two roles in catalysis.

**RESULTS**

**Involvement of Arg-130 in General Acid Catalysis**—The DNA cleavage reactions of wild-type vaccinia topoisomerase and mutant protein R130A were examined using equilibrium substrates containing a 34-mer scissile strand with either a phosphodiester $\text{T}p \rightarrow \text{A}$ scissile phosphodiester (5'-O) or a 5'-bridging phosphorothioate $\text{T}p \rightarrow \text{sA}$ (5'-S). Covalent adduct formation by wild-type (WT) topoisomerase (A), R130A (B), K167A (C), and R130K (D) on the 5'-O and 5'-S substrates is plotted as a function of time.

In the 5'-bridging phosphorothioate effect on equilibrium DNA cleavage by wild-type vaccinia topoisomerase and Arg-130 mutants. The 34-mer/60-mer equilibrium substrate is shown with the cleavage site indicated by the arrow. The 5'- 32P-labeled scissile strand contained either a standard $\text{T}p \rightarrow \text{A}$ scissile phosphodiester (5'-O) or a 5'-bridging phosphorothioate $\text{T}p \rightarrow \text{sA}$ (5'-S). Covalent adduct formation by wild-type (WT) topoisomerase (A), R130A (B), K167A (C), and R130K (D) on the 5'-O and 5'-S substrates is plotted as a function of time.

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**5'-S Effect on the R130K Mutant**—Insights to the dual function of Arg-130 emerged from an analysis of the thio effects on equilibrium cleavage reaction of the conservative mutant R130K (Fig. 1D). From the observed kinetic parameters of the approach to equilibrium by R130K on the 5'-O duplex substrate ($k_{obs} = 3.5 \times 10^{-5} \text{s}^{-1}, K_d = 0.67$), we calculated a value for $k_d$ of $1.4 \times 10^{-5} \text{s}^{-1}$. This rate is 5-fold faster than that of R130A but slower by at least 4 orders of magnitude than wild-type topoisomerase. Reaction of R130K with the 5'-S DNA was much faster than the reaction with the 5'-O substrate (Fig. 1D). 93% of the input 5'-S DNA was bound covalently by R130K, and the apparent $k_d$ was $3.7 \times 10^{-2} \text{s}^{-1}$. This value was within a factor of 10 of the $k_d$ of wild-type topoisomerase and was 2600 times faster than $k_d$ of the reaction of R130K with the 5'-O substrate. Thus, the gain of function by R130K in response to the bridging thiolate modification was comparable to that of the K167A mutation, i.e. R130K phenocopied K167A.

We surmise that Arg-130 and Lys-167 jointly participate in a bridging thiolate modification comparable to that of K167A at the CCCTT cleavage site (Fig. 1). However, they also suggest additional functions of Arg-130 in transesterification chemistry.

**5'-S Effect on a R130K/K167A Double Mutant**—If the guanidinium group at position 130 and the primary amine at position 167 are collaborating in a proton transfer relay, then the catalytic defect of an R130K/K167A double mutation should still be rescued by the 5'-S modification, and indeed that is what we observed (Fig. 2). Cleavage of the 5'-O DNA by purified recombinant R130K/K167A was slower than either of the single mutants. The covalent adduct accumulated steadily over...
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a 4-day incubation period, after which 10% of the input 5'-O DNA had been cleaved (longer times were not tested). Absent an end point, we could not reliably calculate a cleavage rate constant for R130K/K167A on the 5'-O substrate. Nonetheless, it was clear that cleavage by R130K/K167A displayed a pronounced 5'-bridging thiolate effect (Fig. 2). 91% of the input 5'-S DNA was bound covalently by R130K/K167A, and the apparent k_d of 4.3 × 10^{-3} s^{-1} was within a factor of 3 of that of the single K167A mutant on the 5'-S substrate.

**DISCUSSION**

Lys-167 and Arg-130 enhance the rate of transesterification by factors of ~10^4 and ~10^5, respectively. The substantial alleviation of the requirements for Lys-167 and Arg-130 during scission of a 5'-bridging phosphorothiolate-modified substrate indicates that the process of general acid catalysis is more complex than was anticipated initially (14). The present data suggest that Lys-167 and Arg-130 comprise a proton relay from Lys-167 by contributing to a local positive electrostatic environment of the protein-DNA interface before and after transesterification, and electrostatic effects, does not account for the observed mutational and 5'-bridging phosphorothiolate effects on transesterification, insofar as the R130K substitution should have restored a positive electrostatic environment, yet the R130K mutant remains grossly defective in expulsion of the leaving DNA strand as gauged by the 2600-fold thiolate effect.

We speculate that the Arg-130 side chain contacts both the scissile phosphate and Lys-167 in the transition state. The former interaction would help neutralize the developing negative charge on the proposed pentacoordinate phosphorane transition state, while the latter would donate either a hydrogen bond (proton sharing) or fully transfer a proton to Lys-167 as it gives up a proton to the 5'-O of the leaving strand. A model of proton relay from Arg-130 to Lys-167 can account for the 5'-bridging phosphorothiolate effects observed for the R130K and K167A mutants, and it raises the prospect that Arg-130 in turn accepts a proton from an upstream donor, which either directly or indirectly leads to abstraction of a proton from the active site tyrosine.

An answer to the question of how the tyrosine is activated for nucleophilic attack on the scissile phosphodiester has been extremely elusive. A scheme has been proposed whereby a general base on the enzyme accepts a proton from the attacking tyrosine nucleophile during covalent adduct formation (23, 24); however, the available structures of type IB topoisomerases provide no clues to the identity of a putative general base, i.e. there is no “conventional” proton-accepting side chain (e.g. His, Asp, or Glu) in the immediate vicinity of the tyrosine nucleophile that can be shown by mutagenesis to be functionally relevant. Comprehensive alanine scanning of His, Asp, Glu, and Cys residues of vaccinia topoisomerase has failed to uncover any other than His-265 that contributes significantly to
transesterification chemistry; moreover, it has been established by conservative mutagenesis that His-265 is not a general base catalyst (10). Alternatively it has been suggested based on the recent structural data for human topo IB that an ordered water molecule in the active site could act as a specific base to accept a proton from the Tyr O-4 atom (19). We speculate that Arg-130 might accept a proton from the active site tyrosine either directly or via an intervening water molecule as part of a wider proton relay network (conceivably Tyr-274 → Arg-130 → Lys-167 → 5’-O or Tyr-274 → water → Arg-130 → Lys-167 → 5’-O) that links in a concerted fashion the deprotonation of the attacking Tyr nucleophile and the protonation of the 5’-O leaving group during the cleavage reaction. The religation reaction, entailing attack of the 5’-OH of the noncovalently held DNA strand on the covalent DNA-3’-phosphotyrosyl-enzyme intermediate, would presumably entail a reversal of the proposed proton relay whereby Lys-167 accepts a proton from the 5’-OH, transfers its to Arg-130, and then (directly or via water or some other intermediary) to the oxygen of the Tyr-274 leaving group.

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