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Vanadate-based transition-state analog inhibitors of Cre–LoxP recombination

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Abstract

Cre recombinase exchanges DNA strands at the LoxP recognition site via transphosphorylation reactions that involve pentacoordinate transition states. We demonstrate that meta-vanadate ion (VO_3^-) and appropriate DNA substrates assemble a transition-state analog-like complex in the Cre active site. Meta-vanadate inhibits recombination of LoxP-derived oligonucleotide substrates that contain a gap at either or both scissile phosphates, but does not inhibit reactions with intact LoxP. The 3′-hydroxyl group of the gapped substrate is required for inhibition, suggesting that vanadate is ligated by three oxo ligands. Assembly of the inhibited complex is slow ($t_{1/2} = 19$ min at 4 mM $NaVO_3$) and requires Cre, substrates, and meta-vanadate. Holliday junction intermediates accumulated at lower meta-vanadate concentrations, suggesting that the second strand exchange is inhibited more readily than the first. The apparent K_D for meta-vanadate is 1.5–2 mM and binding shows positive cooperativity. This methodology may have general application for mechanistic studies of recombinase/topoisomerase-mediated strand exchange reactions.

Keywords: Integrase family; Tyrosine recombinase; Type 1B topoisomerase; Site-specific recombination; Half-sites recombination; Cre recombinase; Meta-vanadate transition-state analogs; DNA cleavage and religation; Pentacoordinate transition state; Phosphotransfer reaction

Bacteriophage P1 Cre protein catalyzes recombination by promoting a double-strand cleavage and religation of DNA at the 34 bp LoxP sequence [1,2]. Cre-mediated recombination is a fundamental tool for bioengineering because chromosomal rearrangements can be controllably induced in living cells in vitro and in vivo [3]. Cre belongs to the Integrase (Int) family of tyrosine recombinases and Type 1B topoisomerases, which includes FLP recombinase and human and Vaccinia virus Topoisomerase I. Int proteins share a common active site structure and chemical mechanism for reversible DNA cleavage [4,5], in which a tyrosine nucleophile attacks the "scissile" phosphate, displacing the 5′-hydroxyl group of the S1′ nucleotide and forming a covalent protein–DNA intermediate (Fig. 1A). In topoisomerases, the strand is resealed after one supercoil is relaxed when the 5′-hydroxyl generated by the cleavage event displaces the tyrosine. In recombinases, strand exchange takes place when the 5′-hydroxyl generated in a neighboring active site displaces the tyrosine, creating a Holliday junction intermediate (Fig. 1B). A subsequent conformational change induces an analogous cleavage and religation on the second strand, resolving the Holliday junction and leading to the crossover products [6,7]. One function of the Int active site is to stabilize geometry and charge distribution of high energy pentacoordinate phosphoryl transition states. In Cre–LoxP crystal structures, Arg173, Arg292, and Trp315 seem to be positioned for this role. However, recent biochemical studies of Vaccinia virus Topoisomerase 1B have suggested alternate roles for these residues. His289 (Cre numbering), previously proposed to act as a general base for Tyr324 activation, is instead proposed to stabilize the transition state along with Arg292, while Arg173 and Lys201 are proposed to act as part of a proton shuttle [8,9]. To perform these functions, the pK_as must be highly perturbed, and precisely

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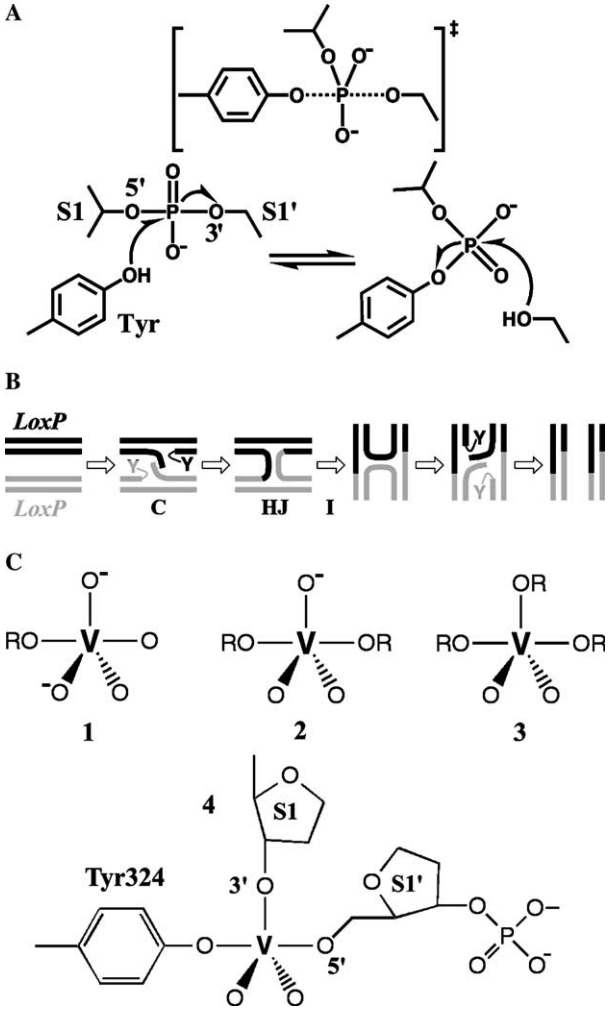

Fig. 1. (A) General mechanism of transesterification reactions catalyzed by Int tyrosine recombinase/Type 1B topoisomerase family. The nucleotides on the 5′ and 3′ sites of the cleavage phosphate are denoted "S1" and "S1′." The pentacoordinate structure ("‡" in brackets, above) is thought to represent the highest energy point along the reaction pathway (the "transition state"). (B) The strand exchange mechanism for the Int recombinases. The DNA strands of a pair of 34 bp LoxP recognition sequences (see Fig. 2A), shown in gray and black, undergo two sets of cleavage and religation reactions. A covalent bond is formed between the 3′-end of the exchanged DNA strands and an active site tyrosine residue ("C"). The first exchange produces a Holliday junction intermediate bound complex ("HJ"), which isomerizes to activate cleavage on the second set of strands ("I"). (C) Pentacoordination scheme for vanadates to mimic phosphotransfer reactions. Typically, ortho-vanadate forms monovalent phosphatase transition state analog complexes (**1**), while meta-vanadate forms divalent diesterase transition state analogs (**2**), with axial coordination. Recently, both ortho- and meta-vanadates were demonstrated to form trivalent complexes as analogs for transesterification reactions (**3**) [17,19]. For Cre recombinase, trivalent complexes are expected (**4**), with the Tyr324 and S1′ base 5′ hydroxyl groups acting as axial ligands, and the S1 base 3′-hydroxyl group potentially participating as an equatorial ligand.

how the Int active site promotes strand exchange remains somewhat controversial.

A direct method to identify protein ligands involved in phosphoryl transfer or hydrolysis transition-state stabilization is through crystallographic visualization of complexes containing oxo-vanadate inhibitors in enzyme active sites [10–19]. Vanadates replace the phosphate group and readily achieve trigonal-bipyramidal geometry through axial coordination by substrate and/or protein hydroxyl groups [20–22] (Fig. 1C). In crystal structures of such complexes, the amino acid side chains that stabilize the transition state negative charge and pentacoordinate geometry interact with the vanadate equatorial oxo groups via hydrogen bonds and salt bridges. For phosphatases, generally ortho-vanadate (vanadium(VI), VO_4^{2-}) and the product alcohol assemble the active site inhibitor with the axial attacking water being mimicked by a vanadate oxygen (structure **1** in Fig. 1C) [11,13,15,16,22]. In phosphoryl-transfer reactions, meta-vanadate (vanadium(V), VO_3^-) becomes sandwiched between the leaving and attacking hydroxyl groups (structure **2** in Fig. 1C) [10,12,14]. More recently, both ortho- and meta-vanadates have been demonstrated to assemble trivalent complexes containing a third equatorial ligand [17,19]. Particularly relevant to Int family strand exchange reactions, Rupert et al. [17] crystallized a meta-vanadate transition-state complex of the hairpin ribozyme catalytic RNA, which cleaves RNA by promoting an internal transphosphorylation. In the X-ray structure, the attacking 2′- and leaving 5′-hydroxyls coordinated the vanadium at axial positions and the remaining 3′-hydroxyl coordinated an equatorial position (structure **3** in Fig. 1C). By analogy, we reasoned Cre might assemble similar trivalent complexes with meta-vanadate (structure **4** in Fig. 1C).

Methods and materials

Homogeneous Cre recombinase was purified as previously described [23]. RPC-purified oligonucleotide substrates were obtained from DNA Express, quantified by UV absorbance, and mixed in 10 mM Tris–Cl, 1 mM EDTA, pH 8.0, to yield the specified duplexes. The substrate plasmid containing a single LoxP site, pLT38(+)–LoxP [24], was purified using a Qiagen Midiprep Purification Kit. *Ban*I/*Stu*I fragments were prepared and ^{32}P 5′-labeled with polynucleotide kinase as previously described [24]. The substrate LoxP-3′dd was prepared by separately incubating 6 μM of each half-arm of LoxP-ΔS1 with 60 μM ddGTP or ddATP and 125 U/ml DNA Pol I Klenow fragment (NEB) in the supplied buffer for 40 min at 21 °C, followed by a treatment at 70 °C for 10 min, and then combining the two reactions to make a 3 μM stock. All recombination reactions were carried out using optimized Cre reaction buffer [23] at 21 °C. For non-preincubation experiments, the reactions were initiated by mixing equal volumes of twofold concentrated solutions of Cre recombinase and oligonucleotide substrates and with twofold concentrated reaction buffer and plasmid or labeled substrate. For preincubation experiments, Cre and oligonucleotides, with or without $NaVO_3$, were mixed in 10 mM Tris–Cl, 1 mM EDTA, and 30 mM NaCl, allowed to stand on ice for the indicated times, and then rapidly mixed with a twofold concentrated stock of reaction buffer containing the same vanadate concentrations and the indicator substrate. Reactions were terminated after 30 min with 1% SDS, 0.3 mM EDTA, 5 mM DTT, 6% glycerol, and 0.025% bromophenol blue. Radiolabeled reactions were additionally digested

with 1 mg/ml proteinase K in 20 mM EDTA. Electrophoresis was carried out using either 1% agarose/TAE buffer for plasmid, or 8–10% Lamelli SDS polyacrylamide gels [25]. DNA was visualized using either ethidium bromide or autoradiography [26]. Reactions with labeled substrate typically yield 30–40% turnover, 80–90% products, and 10–20% intermediate.

Results and discussion

LoxP consists of two inverted 13 bp repeats, which recruit one Cre molecule each [2]. Two Cre_2LoxP dimers then associate to form the active tetrameric recombination synapse [7]. DNA cleavage occurs within the "8 bp spacer" segment between the two 13 bp repeats (Fig. 2A) [27]. Vanadate complexes are generally labile in solution and are stabilized in active sites by favorable contacts with residues that interact with the reaction transition-state [10,20]. At least two hydroxyl groups must be present to supply axial ligands for the meta-vanadate anion, VO_3^- (Fig. 1C, **2** and **3**). LoxP derivatives were constructed (Fig. 2B) in which the scissile phosphate was removed from both strands (LoxP-cl) or each strand individually (LoxP-hcl1 and -hcl2), leaving free 3′- and 5′-hydroxyl groups to act as potential meta-vanadate ligands. Although these gapped molecules cannot form covalent intermediates with Cre, they can resolve covalent LoxP-derived intermediates because of the free S1′ 5′-hydroxyl groups (Fig. 2C, lanes 4, 6, and 8). This property allows these DNAs to be used both as substrates in so-called "half-site recombination" [28] and as potential inhibitors when complexed with vanadate.

After preincubation with 5 mM sodium meta-vanadate ($NaVO_3$), reactions with the gapped substrates are markedly inhibited (Fig. 2C, lanes 5, 7, and 9, and Fig. 2D, lane 3), while the reaction with LoxP, in which the scissile phosphate would be expected to block the assembly of the meta-vanadate complex, is essentially unaffected (Fig. 2C, lanes 2 and 3). At high concentrations of $NaVO_3$, greater than 10 mM, some inhibition (10–20%) is observed in the LoxP reactions, but this contribution is negligible at the concentrations used here

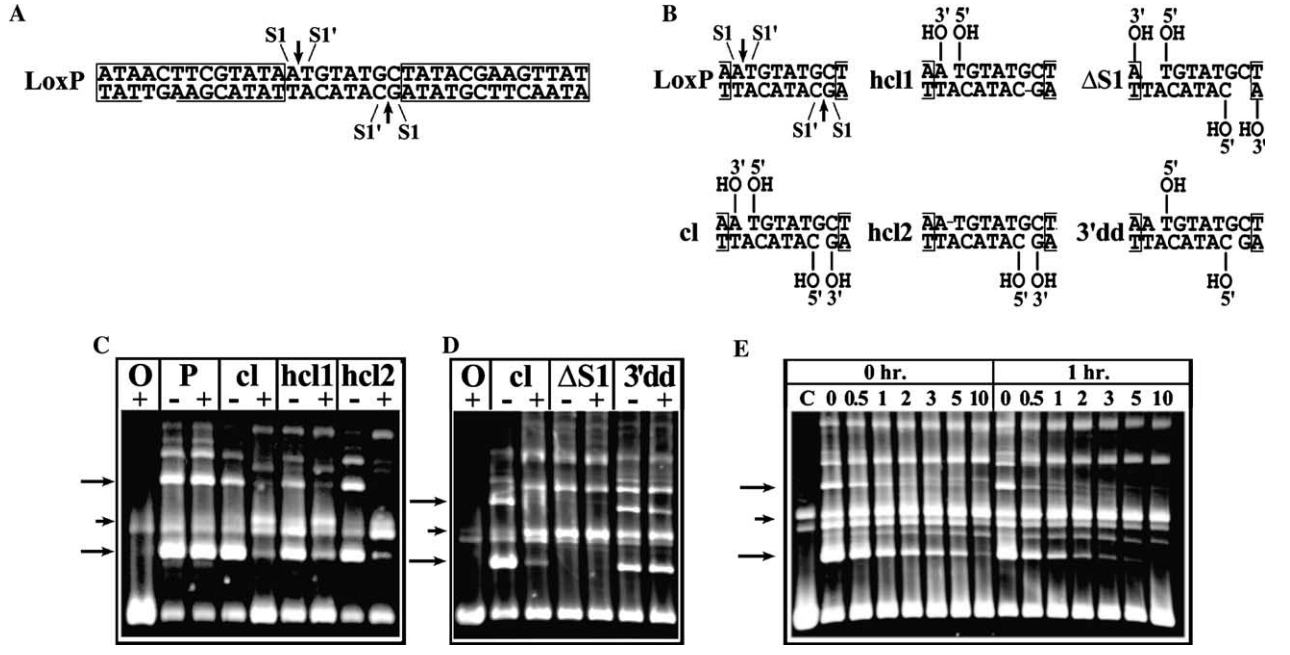

Fig. 2. (A) 34 bp LoxP site. The 13 bp inverted repeat Cre binding sequences are boxed. Cleavage occurs within the 8 bp spacer between the S1 and S1′ bases (black vertical arrows). (B) Sequences of the 8 bp spacer regions of gapped LoxP derivatives used in this study. LoxP-hcl1, LoxP-hcl2, and LoxP-cl lack scissile phosphates at the cleavage sites on the upper, lower, or both strands, respectively. Meta-vanadate would be expected to replace the phosphate in these gaps. (C) Meta-vanadate inhibits Cre-mediated half sites recombination with scissile gap-containing substrates. Recombination-induced plasmid cleavage was carried out after 60 min preincubation of 1200 nM Cre and 300 nM oligonucleotide substrates with (lanes 3, 5, 7, and 9, marked "+") or without (lanes 2, 4, 6, and 8, marked "−") 5 mM $NaVO_3$ (see Methods and materials for details). Cre-mediated recombination inserts the synthetic duplex into the plasmid LoxP site and converts the plasmid to the linear form (long arrows, the upper band is linear plasmid dimer) and reaction intermediate(s) (short arrow), probably Holliday junctions. Reactions with LoxP-cl ("cl," lanes 4 and 5), LoxP-hcl1 ("hcl2," lanes 6 and 7), and LoxP-hcl2 ("hcl2," lanes 8 and 9) were substantially inhibited, while the reaction with LoxP ("P," compare lanes 2 and 3) was unaffected. (D) Inhibition requires the presence of the 3′-hydroxyl group of the S1 base. Experiments were performed essentially as in (C). Reaction with LoxP-cl ("cl") was inhibited with 5 mM $NaVO_3$ ("+") compared to the control ("−"). However, reactions with LoxP-3′dd, a substrate containing a 3′-dideoxy nucleotide at the S1 position, were not inhibited ("3′dd" lanes, compare "−" and "+"). The precursor LoxP-ΔS1 is not an active substrate (Lane "ΔS1"). (E) Dose-dependent inhibition and effect of one hour preincubation of the inhibited complex in the Cre-mediated reaction with synthetic LoxP-cl and plasmid substrates. Left panel: titration with 0–10 mM $NaVO_3$, no preincubation; right panel: preincubation of 1200 nM Cre, 300 nM synthetic LoxP-cl with 0–10 mM $NaVO_3$ for 60 min prior to reaction with LoxP-containing plasmid.

(data not shown). To assemble an inhibited complex, a free 3′-hydroxyl from the S1 base must also be present. LoxP-ΔS1 is a LoxP derivative that lacks the S1 scissile base (Fig. 2B) and is inactive as a substrate (Fig. 2D, lanes 4 and 5). LoxP-ΔS1 was extended to yield a LoxP-cl derivative, LoxP-3′dd (Fig. 2B), that contained the S1 nucleotide but lacked scissile 3′-hydroxyl groups. While LoxP-3′dd acted as a substrate because of the free 5′-hydroxyl (Fig. 2D, lane 6), the reaction was not affected by $NaVO_3$ (Fig. 2D, lane 7).

The level of inhibition is concentration-dependent between 0 and 10 mM (Fig. 2E, left panel) with maximal inhibition occurring at greater than 5 mM. In addition, the efficacy of inhibition is enhanced by preincubation of Cre, LoxP-cl, and $NaVO_3$ prior to adding the indicator substrate (Fig. 2E, right panel). To quantitate these effects on the overall reaction and on each of the cleavage steps, inhibition experiments were performed using a 200 bp radiolabeled LoxP-containing restriction fragment as an indicator substrate (Fig. 3A) [24,26]. With this methodology, the disappearance of substrate, the level of Holliday junction intermediate (the product of the first strand exchange), and the level of recombination products (the product of the second strand exchange) could be simultaneously monitored. Under these conditions, the formation of recombination products is inhibited to nearly 90% by $NaVO_3$ at 10 mM, with a concentration of half-maximal inhibition (C_{50}) of ~2 mM when no preincubation is employed (Fig. 3B, open circles). With a 90 min preincubation, lower concentrations of $NaVO_3$ are required for half-maximal and maximal levels of inhibition, 1.5 and ~6 mM, respectively (Fig. 3B, filled squares). The shapes of the inhibition curves are also dramatically different. The curvature of the non-preincubation curves cannot be fit to a simple binding isotherm. More homogeneous inhibition is observed with 90 min preincubation and the inhibition curve can be modeled as a single cooperative binding process (Fig. 3D). Interestingly, the accumulation of products and Holliday junction intermediate is differentially sensitive to $NaVO_3$ concentration

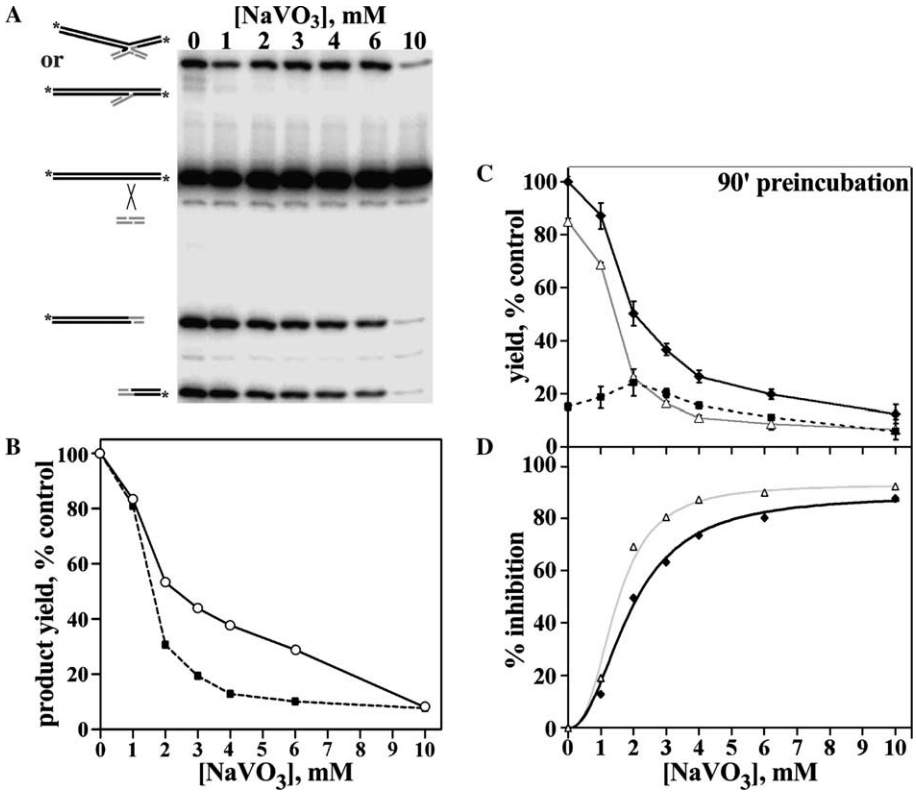

Fig. 3. Quantitation of dose–response and effect of preincubation. (A) Sample SDS–PAGE analysis of the effects of increasing $NaVO_3$ concentrations (0–10 mM) on "half-sites recombination" reactions with no preincubation (See Methods and materials for details). Half-sites recombination of LoxP-cl with a ~220 bp radiolabeled LoxP-containing restriction fragment leads to single exchange HJ intermediate (slow migrating) and full exchange products (fast migrating). (B) Phosphorimaging quantitation of product yield as a function of $NaVO_3$ concentration, relative to an untreated control. Preincubation for 90 min (dashed line, filled squares) increases the inhibitory effect from 1 to 10 mM $NaVO_3$, compared to no preincubation (solid line, open circles). The error bars represent the variation between independent duplicate experiments. (C) Effect of increasing $NaVO_3$ concentration on the amount of substrate turnover (solid black lines, filled diamonds) after preincubation for 90 min. Substrate turnover is further broken down into the levels of intermediates (dashed lines, filled squares) and products (solid gray line, open triangles), expressed as a percentage of the total substrate turned over in the control reaction. The error bars represent the variation between independent duplicate experiments. (D) Data from (C) replotted as the percentage inhibition of substrate turnover (black diamonds) and production of product (open triangles). The fit lines were generated from a modified Hill function, as previously described [26].

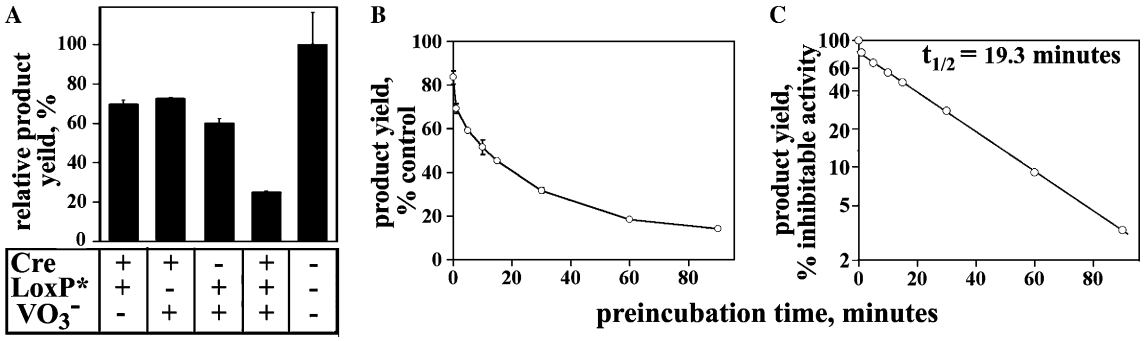

Fig. 4. (A) Requirements for preincubation effect. Various combinations of Cre (2400 nM), LoxP-cl (600 nM), and 5 mM $NaVO_3$, as depicted below the panel, were preincubated for 90 min, diluted twofold and into twofold concentrated buffer and labeled substrate, and reacted. The product levels were normalized to the uninhibited control. Preincubation with any two components gave the same levels of product formation as no-preincubation (see Fig. 3A), while maximal inhibition was achieved only when Cre, LoxP-cl, and $NaVO_3$ were present. (B) Inhibition time course. 1200 nM Cre, 300 nM LoxP-cl, and 4 mM $NaVO_3$ were incubated for the designated times at 4 °C, and then diluted twofold into reactions with buffer, 4 mM $NaVO_3$, and radiolabeled LoxP-containing restriction fragment. Reaction level is expressed as the percentage of the product compared to the zero preincubation time control (about 15% turnover). After 90 min, a control containing no $NaVO_3$ reacted to the same degree as the zero time control (data not shown). The error bars represent the variation between independent duplicate experiments. (C) Semi-log plot of data from panel (A), after normalizing to and correcting for the difference between no and maximal inhibition (12% of the zero time control level). The line represents the fit to the dominant first order process ($k_{app} = 0.035\,min^{-1}$, $t_{1/2} = 19.3$ min, $R^2 > 0.99$).

(Fig. 3C, compare open triangles and filled squares). At low $NaVO_3$ concentrations Holliday junction formation is enhanced, becoming maximal at 2 mM, before also becoming inhibited. Plotting the inhibition binding curves and fitting them in a modified Hill equation [26] yielded apparent dissociation constants and Hill numbers, respectively, of 1.9 mM and 2.2 for turnover of substrate, and 1.5 mM and 2.6, for the production of products. These results suggest that the second strand exchange is somewhat more sensitive to meta-vanadate inhibition than the first.

Cre, the Lox substrate, and $NaVO_3$ are required to observe the preincubation effect (Fig. 4A). Reactions in which two of the three components were preincubated gave similar levels of recombination product as reactions with no preincubation, suggesting that the rate-limiting step for the formation of inhibited species involves requires simultaneous interactions between the Cre active site, meta-vanadate, and the substrate hydroxyls. The time dependence of inhibition at 4 mM $NaVO_3$ was also investigated (Fig. 4B). The majority of the inhibited complex assembled in a first-order process with a half-time of nearly 20 min (Fig. 4C).

The slow onset of inhibition, and its dependence on meta-vanadate and the appropriate DNA substrate, is consistent with the idea that a transition-state complex is assembled. Indeed, slow onset of inhibition is a signature of transition-state analogs [29,30]. In the transition state of phosphotransfer reactions, the attacking and leaving groups, in this case the tyrosine and deoxyribose 5′ hydroxyl groups, are colinear with the phosphate and occupy the axial positions. In the Cre–Lox complex, the requirement for the S1 base 3′-hydroxyl suggests several possible geometries. The most likely geometry, in direct analogy to the hairpin ribozyme/meta-vanadate complex [17], is that the attacking Tyr324 and leaving S1′ 5′-hydroxyl groups occupy the axial positions, and the S1 3′-hydroxyl occupies one of the equatorial positions (Fig. 1C, **4**). Alternatively, meta-vanadate could form a bridge between the 3′-hydroxyl group and either the S1′ 5′-hydroxyl or Tyr324. However, to achieve the required colinear geometry, the 3′-Tyr or 3′–5′ bridges would require a substantial shift in either the DNA or Tyr324, and it is not readily apparent how the active site could productively interact with this arrangement. A second geometric issue is the source of the 5′-hydroxyl ligand, which could originate from a non-bridging or bridging strand, that is from the first cleavage or final resealing events, or creation or resolution of the Holliday junction, respectively (Fig. 1B). The proclivity for Cre to form Holliday junction complexes with DNA [24] suggests that the 5′-hydroxyl likely originates from the bridging strand.

A crystal structure of this complex is expected to reveal the Cre active-site ligands responsible for ligating the vanadate oxo groups, and in turn for stabilizing the transition state of the authentic recombination reaction. Meta-vanadate may also prove useful for studying the mechanisms of other Integrase family proteins, in which the role of active site ligands is still in question.

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