Published in final edited form as:

J Mol Biol. 2007 July 6; 370(2): 303–314.

***Trans* cooperativity by a split DNA recombinase: the central and catalytic domains of bacteriophage lambda integrase cooperate in cleaving DNA substrates when the two domains are not covalently linked**

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Summary

Site-specific recombinases of the λ -integrase family recognize and cleave their cognate DNA sites through cooperative binding to opposite sides of the DNA substrate by a C-terminal catalytic domain and a flexibly linked 'core-binding' domain; regulation of this cleavage is achieved via the formation of higher-order complexes. We report that the core-binding domain of λ -integrase is able to stimulate the activity of the catalytic domain even when the two domains are not linked. This *trans* stimulation is accomplished without significantly increasing the affinity of the catalytic domain for its DNA substrate. Moreover, we show that mutations in the DNA substrate can abrogate this effect while retaining specificity determinants for cleavage. Since the domains do not significantly interact directly, this finding implies that *trans* activation is achieved via the DNA substrate in a manner that may be mechanistically important in this and similar DNA binding and cleaving enzymes.

Keywords

lambda integrase; DNA recombinase; tyrosine recombinase; covalent intermediate; suicide substrate; substrate binding; DNA binding; NMR; single turnover kinetics; fluorescence anisotropy

Introduction

Bacteriophage λ integrase (λ -Int)¹ is the prototypical member of a family of enzymes that mediate site-specific DNA recombination via the formation of a covalent 3' phosphotyrosyl linkage at the site of DNA cleavage in a manner similar to that of type I eukaryotic and viral topoisomerases². DNA cleavage and recombination by λ -Int at its cognate "core" sites proceeds upon assembly of four protomers of the enzyme at a pair of T-rich semi-symmetrical inverted sequences on the phage and bacterial genomic DNA³. Although the enzyme functions as a tetramer during recombination, the enzyme can also bind and cleave DNA without tetramer assembly⁴, as experiments in vitro show it exhibits topoisomerase activity⁵ and is capable of cleaving small DNA substrates consisting of core half-sites⁶.

The 356-residue λ -Int enzyme consists of three structurally independent domains connected by extended peptide linkers: the amino-terminal "arm binding" domain (residues 1–64), the

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central “core-binding” domain (residues 65–169), and the C-terminal catalytic domain (residues 170–356)^{7; 8; 9}. Although the catalytic domain is capable of sequence-specific cleavage of core DNA sites on its own, maximal cleavage activity requires a construct containing both the catalytic and “core-binding” domains of the protein; this construct is as active as the full-length protein in cleaving DNA^{8; 9}. The “arm binding” domain and other protein factors are not directly involved in core site recognition and cleavage, but are required for efficient recombination^{9; 10; 11; 12; 13; 14; 15}.

Crystallographic¹⁶ and solution¹⁷ structural studies of the isolated catalytic domain (residues 170–356) revealed that in the absence of DNA the tyrosine nucleophile (Y342) resides on a flexible C-terminal segment that adopts an average conformation far from that required for substrate cleavage. This observation led to the proposal that a concerted structural rearrangement plays a regulatory role and is required for generation of an “active” conformation with the tyrosine residue suitably positioned for DNA cleavage^{16; 18}. Intriguingly, although stoichiometric DNA binding by the catalytic domain was found to stabilize the protein structure¹⁹, this interaction was insufficient to significantly populate the active conformation in solution¹⁷.

On the other hand, the crystal structures of a λ -Int construct containing the core-binding and catalytic domains of the enzyme, residues 75–356, trapped in covalent complexes with half-site²⁰ or full core site¹⁵ DNA substrates show the tyrosine residue properly positioned for DNA cleavage. In a fashion similar to that of two other members of the family, Cre and Flp recombinases^{21; 22}, the structures show that the two domains bind to opposite sides of the substrate DNA and are connected by an extended linker (Figure 1)^{15; 20}. In the tetrameric complexes with full sites¹⁵, modest protein-protein interactions are observed between the core binding domains of adjacent protomers, while more extensive interactions between the catalytic domains may be important for regulating activity. In particular, inter-protomer interactions mediated by the C-terminal segment of the protein are believed to have an important regulatory role^{15; 20; 23; 24}. In each of these structures, the DNA is distorted from canonical B-form, with a widening of the major groove at the sites of helix insertion and a narrowing of the minor groove at the site of cleavage. The arrangement of the core-binding domain on the opposite side of the DNA as the site of cleavage is consistent with a role for this domain in increasing the affinity of the enzyme for its substrate by anchoring the catalytic domain on the DNA.

To reconcile the observed properties of the C-terminal segment of the protein in the isolated catalytic domain (Int^{Cat})^{16; 17} with those observed in a construct also containing the core-binding domain (Int^{CB})^{15; 20}, we investigated the effect of isolated Int^{CB} on the catalytic and DNA binding properties of Int^{Cat}. Here we report the surprising finding that Int^{CB} and Int^{Cat} act cooperatively even when they are not covalently linked. Since crystallographic^{15; 20} and solution data (this work) show that the two domains do not significantly interact directly, this cooperativity must be accomplished in an indirect manner. The mechanistic implications for such an indirect means of cooperation between catalytic and accessory DNA binding domains of enzymes are discussed.

Results

The core-binding domain activates catalysis in trans

Proteolytic mapping experiments previously identified the three structural domains of λ -Int^{9; 25; 26}, and crystallographic and NMR studies established the structural independence of the catalytic¹⁶ and arm-binding domains²⁷. We expressed and purified four different protein constructs comprising fragments of the enzyme involved in core site binding and cleavage: (1) Int^{CB}, a protein construct containing the core-binding domain of λ -Int, (2) Int^{CB+Cat},

containing both the central and catalytic domains, (3) Int^{Cat}, the isolated catalytic domain, and (4) Int^{CatΔ349C197S}, a fragment of the catalytic domain bearing a Cys-Ser substitution at position 197 to improve sample behavior and lacking the last seven residues from the C-terminus; these residues have been implicated in mediating inter-protomer interactions important for coordinated cleavage^{23; 24; 28; 29}. All constructs containing the catalytic domain, (i.e., Int^{CB+Cat}, Int^{Cat} and Int^{CatΔ349C197S}) were competent for site-specific cleavage of DNA half-site substrates^{6; 26}, with the Int^{CatΔ349C197S} construct exhibiting slightly increased activity²⁴ and improved stability compared to Int^{Cat}.

Incubation of Int^{Cat} with a suicide core half-site DNA substrate^{6; 17; 30} results in site-specific DNA cleavage and generates a covalent complex with Int^{Cat} attached to the DNA via a 3'-phosphotyrosine linkage that is electrophoretically stable under denaturing conditions (Figure 2, lane 1). As previously noted⁸, the cleavage activity of the catalytic domain alone (Int^{Cat}) is several fold lower than that of a construct containing both the catalytic and core-binding domains (Int^{CB+Cat}; Figure 2, lane 3). Surprisingly, we found that Int^{CB} stimulates the activity of Int^{Cat} in *trans*, i.e., even when the two domains are not covalently linked (Figure 2, lanes 1 and 2). This result indicates that Int^{CB} plays a role in DNA cleavage beyond enhancing the affinity of the catalytic domain for its substrate DNA.

Because of the low activity and relatively low affinity of Int^{Cat} for DNA⁸, several controls were performed to establish whether the observed enhancement was due to nonspecific or chaperone-like effects³¹. Bovine serum albumin was used in place of Int^{CB} to show that the increased activity was not due to solution stabilization³². The core-binding domain of Cre recombinase (Cre^{CB}; residues 1–130)³³ was used in place of Int^{CB} to show that nonspecific DNA binding cannot account for the effect. Excess salmon sperm DNA was used as a nonspecific competitor to show that *trans* complementation could not be eliminated by dilution of weakly binding proteins. The bottom strand was ³²P-labeled to show that the observed DNA cleavage activity by Int^{Cat} is not due to nonspecific topoisomerase-like activity,⁵ which would be expected to result in cleavage of either strand. In each of these controls, catalytic enhancement of Int^{Cat} was only observed when Int^{CB} was added, nonspecific competitor DNA had no effect, and only site-specific cleavage was observed³¹. The results of these control experiments effectively rule out nonspecific effects as being responsible for the observed *trans* complementarity between Int^{CB} and Int^{Cat}.

Int^{CB} and Int^{Cat} do not interact in the absence of DNA

Comparison of the two-dimensional ¹H-¹⁵N correlation NMR spectrum of the larger Int^{CB+Cat} with that of Int^{Cat} (Figure 3) reveals the positions of resonances from the catalytic domain in Int^{CB+Cat} are nearly identical to those in spectra of catalytic domain alone¹⁷. This finding indicates that in the absence of DNA, except for the linker connecting them the two domains do not significantly interact. Biophysical and biochemical analysis of Int^{CB} alone showed that the isolated domain, a helical bundle²⁰, is monomeric but not well folded when free in solution, becoming more structured upon binding to its cognate DNA³⁸. The NMR spectra of Int^{CB+Cat} indicate that the core-binding domain is also not well structured in the context of the larger construct, as most of the resonances not attributable to Int^{Cat} are clustered in the random coil region of the spectrum, with amide proton shifts of 8–8.5 ppm (Figure 3).

Trans activation is DNA sequence dependent

Since the catalytic and core binding domains do not significantly interact in the absence of DNA, nor do they exhibit significant inter-domain contacts in the presence of DNA, we examined whether the observed *trans*-activating effect might be mediated via the DNA substrate. This hypothesis was tested by examining the effect of alterations of the DNA substrate on the DNA cleavage activity of Int^{Cat} in the absence and presence of Int^{CB}.

Examination of the crystal structure of Int^{CB+Cat} (residues 75–356) covalently bound to DNA (Figure 4A) reveals that the only bases contacted by both domains are two adenines at positions 5 and 6 downstream from the center of the overlap region¹⁷, and one nucleotide from the site of cleavage; these residues are two of three invariant adenines in the consensus λ -Int recognition sites. Inspired by studies of cooperativity between the homeodomain and POU-specific domain of the Oct-1 transcription factor³⁴, we reasoned that if cooperativity between the two domains were mediated by the DNA substrate, alteration of these two nucleotides would interfere with *trans* activation.

As shown in Figure 4B, a substrate with the AT basepair at position +5 altered to TA was recognized and cleaved by Int^{Cat}, and addition of Int^{CB} resulted in a strong enhancement in cleavage activity (lanes 3, 4). Alteration of the AT basepair at position +6 to TA resulted in decreased cleavage by Int^{Cat}, but addition of Int^{CB} nevertheless enhanced cleavage (lanes 5, 6). However, cleavage of a substrate variant in which both AT basepairs are changed to TA is no longer enhanced by addition of Int^{CB} although it is still cleaved specifically by the catalytic domain (lanes 7, 8). As an additional control, altering both AT basepairs to either GC or CG (lanes 9–12), completely abrogated recognition and cleavage by Int^{Cat}. These results suggest that contacts between Int^{CB} and these nucleotides are important for the observed *trans* activation and are consistent with a DNA-mediated interaction between the domains.

Int^{CB} has a large effect on k_{cat}

Trans activation of an enzyme can be accomplished by increasing the affinity of the enzyme for its substrate (i.e., lowering K_M) and/or by promoting catalytic efficiency (i.e., increasing k_{cat}). Low solubility and a tendency to aggregate complicate detailed kinetic and thermodynamic analyses of DNA binding by λ -Int, and Int^{Cat} in particular. Nevertheless, we endeavored to characterize this *trans* effect by measuring the effect of Int^{CB} on the kinetics of DNA cleavage/covalent complex formation.

The efficiency of Int^{Cat} for DNA cleavage is strongly dependent on solution conditions³¹. Furthermore, since the reaction catalyzed by the domain is slow, degradation (i.e., denaturation, precipitation) of the protein during the reaction can be a complicating factor. To minimize these complications, we sampled a large array of reaction conditions and arrived at a set that enabled collection of interpretable data (see Materials and Methods). Most notably, measurements of initial rates of product formation were performed at 4 °C under the same solution conditions we routinely use for long-term storage (2–3 months), which we have found both from kinetic and spectroscopic analyses to preserve the integrity of Int^{Cat} over timescales relevant to the kinetic measurements (hours). Representative time course data are shown in Figure 5.

Single turnover DNA cleavage kinetics were monitored by measuring the time-dependent formation of covalent Int^{Cat}-DNA complexes as a function of enzyme concentration while using a double-stranded oligonucleotide suicide substrate radiolabeled on the 5' end of the cleaved strand¹⁷. Although the reactions were quite slow under the conditions of the experiments (i.e., 4 °C), timepoints recorded in triplicate showed the data to be reasonably reproducible (Figure 5), allowing determination by nonlinear regression (Figure 6) of the initial rates of DNA cleavage by Int^{Cat}, in both the absence and presence of Int^{CB}. Although auto-inhibition at higher enzyme concentrations increased uncertainty in the extracted parameters, analysis of the initial rates allowed determination of the corresponding k_{cat} and K_M values. Int^{Cat} was found to have a K_M of $25 \pm 23 \mu\text{M}$ in the absence of Int^{CB}, and $5 \pm 4 \mu\text{M}$ in the presence, while k_{cat} increased from $0.08 \pm 0.04 \text{ s}^{-1}$ to $3 \pm 1 \text{ s}^{-1}$. Although the uncertainty in the kinetic parameters is large, the data indicate that addition of Int^{CB} strongly enhances the turnover rate of the enzyme (k_{cat}) by a factor of ~40, while there appears to be a less significant effect on K_M (Figure 6). Overall, the efficiency of DNA cleavage (k_{cat}/K_M) is enhanced by ~200 fold in the presence of Int^{CB}.

Int^{CB} does not enhance DNA binding by Int^{Cat}

Because of the uncertainties in the extracted K_M values, the affinity of Int^{CB} for its substrate was determined independently. Both Int^{Cat} and Int^{CB} demonstrate relatively weak affinities for DNA, with neither forming electrophoretically stable (non-covalent) complexes in non-denaturing electrophoresis experiments³¹. Because the specific activity of the enzyme is quite low at 4 °C, by performing binding assays quickly (relative to the enzyme's activity), equilibrium affinity for substrate could be measured directly without significant contribution from covalent product formation.

To measure DNA binding affinity by the various λ -Int constructs, a duplex DNA substrate matching the recognition sequence used for the kinetic experiments (see Methods) was labeled at the 3'-end of one strand with 6-carboxy-Fluorescein, and protein binding was monitored by changes in fluorescence anisotropy (Figure 7). These measurements yielded equilibrium dissociation constants (K_D) for this substrate of $17 \pm 2 \mu\text{M}$ for Int^{CB}, $11 \pm 1 \mu\text{M}$ for Int^{Cat} and $34 \pm 3 \text{ nM}$ for Int^{CB+Cat}. Notably, if the two domains were perfectly cooperative in binding to DNA, one would expect the affinity of Int^{CB+Cat} to correspond to the product of the dissociation constants of the individual domains, i.e., 0.19 nM. The ratio of the affinities of the separate and linked constructs, $(K_{D, \text{CB}} K_{D, \text{Cat}} / K_{D, \text{CB+Cat}})$ yields the effective concentration of one linked domain in the presence of the other³⁵, which in this case is 5.5 mM; this result is similar to that obtained for the cooperating domains of Oct-1³⁴. Importantly, the affinity of Int^{Cat} for the DNA was not significantly enhanced by the presence of near-saturating Int^{CB}. Although the binding curve could be fit to a K_D of $60 \pm 10 \mu\text{M}$, the small amplitude change in anisotropy renders this result qualitative, not quantitative: that is, the effect on binding is well below the effect on catalysis (i.e., much less than the ~200 fold enhancement in k_{cat}/K_M). This finding indicates that the strong *trans* activating effect of Int^{CB} is accomplished by enhancing catalytic efficiency, not by promoting substrate binding by Int^{Cat}.

Discussion

Although the isolated catalytic domain is capable of specific recognition and cleavage of half-core DNA substrates, maximal cleavage activity requires the core binding domain. The poor activity of isolated Int^{Cat} is attributable in part to a reduced binding affinity for DNA: in the absence of a covalent bond to the DNA it does not produce electrophoretically stable complexes in the presence of nonspecific competitor DNA^{8; 9; 31}. The difference in activity between Int^{CB+Cat} and Int^{Cat} could be potentially be interpreted in a straightforward manner by invoking the "chelate effect"³⁵: Int^{CB}, in the context of Int^{CB+Cat} serves as an anchor to increase the effective concentration of the catalytic domain in the vicinity of the DNA cleavage site. However, the NMR data indicate there is little or no interaction between the two domains in the *absence* of DNA (Figure 3), consistent with the observed absence of interdomain contacts in the Int^{CB+Cat}-DNA crystal structures (Figure 1). Thus, although the chelate effect clearly contributes to the DNA cleavage activity of the bipartite protein (Int^{CB+Cat}), the observed *trans* complementation indicates there's more to the story.

In this work we report three critical observations: 1) Int^{CB} activates Int^{Cat} even when the two domains are not linked, 2) the *trans* activation can be abrogated through mutations in the DNA substrate, and 3) the effect is manifested through increased catalytic efficiency (k_{cat}), as opposed to substrate binding (K_M). These observations invite intriguing mechanistic hypotheses.

One plausible explanation for the observed activation derives from the native functional form of the enzyme, in a tetrameric "intasome"³⁶ structure, implicating Int^{CB}-promoted tetramerization. The crystal structure of Int^{CB+Cat} in a tetrameric complex with two core recognition full-sites (Figure 1)¹⁵ reveals protein-protein interactions that must be important

for coordinated cleavage of two strands at a time. Close inspection of those structures reveals that packing of Int^{CB} domains appears to be somewhat loose, with only a few interprotomer contacts (only ~ 600 Å² buried) involving residues Ala¹²⁵, Thr¹⁶⁸ and Arg¹⁶⁹ on one protomer and Ala¹⁵⁶, Arg¹⁵², Asp¹⁴⁹ and Glu¹⁵³ on the other. On the catalytic domain, residues at the C-terminus of the protein (347–356) rearrange relative to their observed position¹⁶ in the isolated Int^{Cat} structure and pack against strands β₁–β₃ on the adjacent protomer. Indeed, it has been proposed that these interactions between adjacent catalytic domains is a dominant contributor to stabilization of the “active” conformation of the enzyme^{16; 18; 23}. Additional close Int^{Cat} contacts were observed between Lys³³⁴ and Ser³³⁵ on one protomer, and Asp³⁴⁴ and Ser³⁴⁰ on the Tyr³⁴²-bearing helix (α_M) on the other. Consequently, if *trans* addition of Int^{CB} were to promote the formation of higher-order complexes that bring together Int^{Cat} domains, this protein-protein interaction could lead to an increase in *k*_{cat} without affecting DNA binding.

Although attractive in some respects, this model is not well supported by other data. First, the DNA substrate used for these studies, a “half-core site” is not long enough to support assembly of two protomers on the same DNA strand in a geometry that would favor stimulatory protein-protein interactions. Second, full-length λ-integrase, although prone to aggregation, has been shown to be monomeric in the absence of DNA³⁷, indicating that it is not a strong promoter of higher-order complexes. The monomeric nature of Int^{Cat} and Int^{CB} are also well supported by experimental evidence: size exclusion chromatography and NMR resonance linewidths (Int^{Cat})^{17; 31}, and NMR and analytical ultracentrifugation data (Int^{CB})³⁸, which show both constructs to be monomeric under a wide range of solution conditions. Importantly, the construct of the catalytic domain used to obtain the kinetic parameters, Int^{Cat}Δ349C197S, lacks most of the C-terminal residues, including Trp³⁵⁰ and Ile³⁵³, which are attributed with making stimulatory protein-protein interactions in the recombining intasome^{23; 24}. The fact that deletion of these residues does not diminish *trans* activation by Int^{CB} argues against multimerization playing an important role in the process. Although it is difficult to rule out the possibility that transient formation of protein-protein contacts are formed that could be stimulatory, it is not clear how isolated Int^{CB} could promote, in a DNA sequence-dependent fashion, productive interactions between Int^{Cat} protomers.

An more compelling explanation for these observations is that the *trans* activating effect of Int^{CB} on Int^{Cat} is mediated through the DNA substrate, by inducing a conformational change in the substrate so that it adopts a structure that is more readily acted upon by the catalytic domain. Indeed, the fact that sequence alterations at the Int^{CB} binding site can specifically abrogate *trans* activation without affecting cleavage specificity strongly argues for a DNA substrate-mediated effect. In each of the available structures of Int-DNA complexes the DNA duplex is distorted away from its B-form structure with a widening of the major groove at the site of Int^{CB} binding and a narrowing of the minor groove at the site of cleavage^{15; 20}; this is also a feature of the DNA cleavage sites of the related recombinases, Cre^{22; 39; 40} and Flp²¹. Although the structure of the Int^{CB}-DNA complex has not been determined, it seems likely that the domain is responsible for at least some of the observed distortions in the cognate DNA structure.

The notion that Int^{CB} pre-forms the DNA substrate for efficient cleavage in an “induced fit” manner^{41; 42} is in line with biochemical⁴³ and single molecule force⁴⁴ studies arguing for an active role by DNA substrates in cleavage by restriction enzymes. This finding may also help explain important determinants of DNA site recognition and cleavage efficiency: the protein makes many contacts to the DNA phosphate backbone but very few contacts to the bases^{15; 20}; a clear and perhaps critical contact is that between Asn⁹⁹ and Ade+6 (Figure 4a). Nevertheless, the enzyme exhibits a high degree of specificity for its cognate sites and sufficient versatility to recognize both halves of the asymmetric phage and bacterial core sites^{3; 45}. The

enzyme's versatility and fidelity might be explained by the fact that both core half-site sequences are AT-rich, suggesting that shape and/or deformability are important determinants for DNA binding and cleavage by λ -Int. Given the existence of integrase homologs that possess only the catalytic domain (i.e., the *E. coli* FimB, FimE inversion elements^{46; 47}), the findings reported here invite some tantalizing speculation about the evolution and mechanism of recruitment of the core binding domain by a functional catalytic domain.

Conclusion

The activity of λ -Int is regulated through intricately intertwined intra- and inter-molecular couplings; the findings reported here reveal yet a new level of complexity with potentially broad implications. The most interesting observation of this work is that Int^{CB}, an auxiliary domain of an enzyme, serves a novel role in DNA substrate recognition and cleavage, not via protein-protein interactions with the catalytic domain, but via important interactions with the substrate. In addition, considering the prevalence of bipartite structures in DNA cleaving enzymes (e.g., topoisomerases, restriction enzymes, base excision repair enzymes, ligases; see 48; 49 for reviews), it may be worth investigating whether the present means of domain cooperation may be a general feature of such enzymes. These observations yield new insights into the complexities of sequence recognition, induced fit and catalysis.

Methods

Protein samples

The Int^{CB+Cat}, Int^{Cat} and Int^{CB} protein expression constructs used in this study^{8; 9} (generously provided by A. Landy, Brown University) encompass residues 65–356, 170–356 and 62–176 of the wild type λ -Int, respectively. A truncated form of the catalytic domain, Int^{Cat} Δ 349C197S, lacking the seven C-terminal residues and a cysteine to serine mutation at residue 197, was generated by introducing an extra stop codon using the “Quik-Change” procedure (Stratagene, Inc)³¹. Protein expression and purification were performed as described previously^{17; 19}, except that for the DNA cleavage assays the final gel filtration column was omitted; sample purity was judged to be > 90% by SDS-PAGE analysis. After purification, the proteins were exchanged by gel filtration (Sephadex G-25) into cleavage buffer: 25 mM Tris (pH 9.1), 100 mM NaCl, 5 mM DTT, 5 mM EDTA, 10% DMSO, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc; Roche). Uniformly ¹⁵N-labeled Int^{Cat} and Int^{CB+Cat} were obtained by culturing transformed bacterial cells in vitamin-enriched M9 minimal media with ¹⁵N-ammonium chloride as the sole nitrogen source¹⁷; NMR spectra were obtained in 25 mM Tris (pH 7.2), 400 mM NaCl, 1 mM EDTA, 2 mM DTT. The core-binding domain of Cre recombinase (Cre^{CB}; residues 1–130) was obtained by site-directed mutagenesis from a plasmid encoding the full-length protein (obtained from G. van Duyn, University of Pennsylvania). The protein was expressed in *E. coli* using an approach similar to that used for Int^{CB} and transferred into cleavage buffer (C. Amero and M. Foster, unpublished).

DNA samples

DNA oligonucleotides were obtained from MWG Biotech, Inc. or IDT, Inc. For DNA cleavage experiments, a hybrid C/C' hairpin “flap” substrate was used (Figure 7)^{17; 18; 20}, with a top strand (5'-*pCCC TGC CAA CTT T↓TT T-3') radiolabeled with ³²P at the 5'-end⁵⁰. The bottom strand (5'-pTTT GCG AAG CAA AAA AGT TGG CAG GG-3') was chemically phosphorylated at the 5' end (or enzymatically labeled with ³²P as a control). The site of cleavage of the top strand between the −3 and −4 nucleotides is indicated by “↓”, and the +5 and +6 adenines and their corresponding (−6, −5) thymines are bold. Nucleotides incorporated for increased stability are italicized, and the three-nucleotide hairpin in the bottom strand is

underlined. Sequence specificity of the trans activating effect was tested with single or double alterations of both strands of the substrate, as indicated in Figure 7.

Duplex DNA oligonucleotides for fluorescence anisotropy measurements corresponded to an 18-bp consensus C'/C half-site¹⁷, with a top-strand sequence of 5'-CCC TGC CAA CTT T↓TT TGC-3'-6-FAM, where 6-FAM is 6-carboxyfluorescein; italicized nucleotides were added to enhance duplex stability. DNA duplexes were assembled by mixing equimolar concentrations of the DNA strands dissolved in buffer, heating to 95 °C for 5 min and cooling on ice for 15 min.

Single Turnover DNA Cleavage Experiments

Covalent complex formation was monitored upon incubating the enzyme with 50 nM 5' radiolabeled suicide DNA substrate in cleavage buffer at 4 °C. Single time-point and control experiments were performed with 0.1 μM DNA, 4 μM Int^{Cat}, and when present, 4 μM Int^{CB}, 4 μM Cre^{CB}, 0.1% or 1% (w/v) BSA, 0.1 or 1 mg/ml salmon sperm DNA³¹. Formation of the suicide complex was monitored after 18 hours by quenching with SDS Laemmli buffer, separating by electrophoretic mobility shift using 12% SDS-PAGE gels, and visualizing by autoradiography.

For kinetics experiments, Int^{Cat} concentrations were as indicated in the legend to Figure 6; Int^{CB} was provided at a concentration of 134 μM (~80% saturated, based on measured K_D , below). Time points for kinetic studies were obtained by quenching aliquots of the reaction with an equal volume of SDS Laemmli buffer, subjected to electrophoretic separation, visualized by autoradiography and quantitated by phosphorimager analysis (ImageQuant, GE Healthcare). Experiments were performed in triplicate and band intensities were used to calculate the mean and standard deviation of the reaction progress. Initial velocities were obtained by fitting the mean fractional progress to the integrated first order rate expression for product formation:

$$\text{Fraction cleaved} = \frac{P}{P+S} = P_0(1 - e^{-v_0 t}) - \text{background}$$

where P and S are the intensities of the product and substrate bands and v_0 is the initial velocity. The concentration dependence of the initial velocities were used to determine k_{cat} and K_M values from a nonlinear weighted least-squares fit of the initial velocity data to:

$$\text{Turnover} = \frac{v_0}{[S]_T} = \frac{V_{\text{max}}[E]_T[S]_T}{[E]_T + K_M} = \frac{k_{\text{cat}}[E]_T}{[E]_T + K_M}$$

where $[E]_T$ and $[S]_T$ correspond to the total, enzyme and substrate concentrations (see appendix for details). Reported uncertainties in k_{cat} and K_M values correspond to the 95% confidence interval obtained from the Student's T test. Nonlinear regression analysis was performed with Igor Pro v. 5.05 (Wavemetrics, Inc)

Spectroscopy

Two-dimensional FHSQC⁵¹ NMR spectra of Int^{Cat} and Int^{CB+Cat} on a 800 MHz NMR spectrometer at 25 °C, and processed with NMRPipe⁵². Fluorescence anisotropy data were recorded on a Fluorolog spectrophotometer (Jobin-Yvon, Inc.) with a 2 s integration time, 5 nm bandwidth, excitation at 495 nm and detection at 520 nm. Initial DNA concentrations were 0.015 μM (25 mM Tris pH 9.13, 100 mM NaCl, 5 mM EDTA, 5 mM DTT, 4° C).

Binding affinity

Protein-DNA dissociation constants, K_D , were determined by measuring the change in fluorescence anisotropy of 6-Fam-labeled DNA as a function of protein concentration. K_D values were obtained for Int^{CB} alone, Int^{Cat} alone, Int^{CB+Cat} and Int^{Cat} in the presence of Int^{CB} (112 μ M initial concentration), by fitting to a standard bimolecular binding quadratic⁵³:

$$A = A_{Free} + (A_{Bound} - A_{Free}) \frac{K_D + D_T + P_T - \sqrt{(K_D + D_T + P_T)^2 - 4D_T P_T}}{2D_T} + mP_T$$

where A is the measured anisotropy, A_{Free} and A_{Bound} are the anisotropy values for the free and protein-bound DNA, D_T and P_T are the total titrated protein and DNA concentrations, K_D is the dissociation constant and m is the nonspecific effect of protein concentration on the measured anisotropy. Titrations were performed at 4 °C, with initial DNA concentrations of 15 nM in 3 mL, and stock protein concentrations were 1.37, 1.8 and 0.036 mM for Int^{Cat}, Int^{CB} and Int^{CB+Cat}, respectively. Data were fit by nonlinear least squares regression (Igor Pro 5.05; Wavemetrics, Inc); for the Int^{CB} and Int^{Cat} titrations, the contribution from nonspecific effects subject to global fitting for all three titrations. Reported uncertainties correspond to the standard deviation of the fitted values. Because titration experiments were performed under conditions (4 °C) where the cleavage rate is much slower than the measurement time (~ 2 h), the effect of trace amounts of DNA cleavage on the measured anisotropy change was ignored.

Acknowledgements

We thank A. Landy (Brown U.), S. Nunes-Duby (Brown U.), G. van Duyne (U. Penn), V. Gopalan (OSU), D. Pulukkunat (OSU), C. Amero (OSU) and P. Mehta (OSU) for reagents, protocols, guidance and suggestions, V. Gopalan, Z. Suo, and M. Ibba (OSU) for access to resources and instrumentation, the OSU CCIC, and Departments of Biochemistry and Microbiology for instrumentation support. This material is based upon work supported by the National Science Foundation under Grant No. MCB-0092962.

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Appendix

Single turnover kinetics analysis

The analytical treatment for single turnover kinetics with enzyme in excess has a similar form to the Michaelis-Menten kinetics for steady state, multiple turnover kinetics with substrate in excess. For the simple two-step enzymatic mechanism:

the rate of EP formation is given by:

$$\frac{d[EP]}{dt} = k_{cat}[ES]$$

where [ES] is the concentration of the enzyme-substrate complex. Since, for Int^{Cat}, k_{cat} is slow relative to k_1 and k_{-1} , over short time periods (i.e., little change in [S]) we can make the pre-existing equilibrium approximation for formation of the ES complex:

$$\frac{d[ES]}{dt} = k_1[E][S] - [ES](k_{-1} + k_{cat}) = 0$$

where [E] and [S] refer to the free concentrations of the enzyme and substrate, respectively. Since the enzyme is present in great excess over substrate (i.e., $[E]_T \gg [S]_T, [ES]$), we use conservation of mass to relate the free concentrations to the total concentrations of E and S:

$$[S] = [S]_T - [ES]$$

$$[E] = [E]_T - [ES] \approx [E]_T$$

Thus, the rate expression becomes:

$$\frac{d[ES]}{dt} = k_1[E]_T([S]_T - [ES]) - [ES](k_{-1} + k_{cat}) = 0$$

Solving for [ES]:

$$\begin{aligned} k_1[E]_T[S]_T - k_1[E]_T[ES] - [ES](k_{-1} + k_{cat}) &= 0 \\ [ES](k_1[E]_T + k_{-1} + k_{cat}) &= k_1[E]_T[S]_T \\ [ES] &= \frac{k_1[E]_T[S]_T}{k_1[E]_T + k_{-1} + k_{cat}} = \frac{[E]_T[S]_T}{\frac{k_{-1} + k_{cat}}{k_1} + 1}, \text{ and with } K_M = \frac{k_{-1} + k_{cat}}{k_1}, \\ [ES] &= \frac{[E]_T[S]_T}{[E]_T + K_M} \end{aligned}$$

The rate of product formation is then:

$$\frac{d[P]}{dt} = k_{cat}[ES] = \frac{k_{cat}[E]_T[S]_T}{[E]_T + K_M},$$

where V_{max} is limited by $[S]_T$ at high $[E]_T$, i.e., $V_{max} = k_{cat}[S]_T$

Equilibrium binding quadratic

The change in fluorescence polarization (anisotropy) of the fluorescently labeled DNA due to reduced molecular tumbling upon complex formation was used to quantitate the affinity of Int^{CB} and Int^{Cat} for DNA. For a binding-dependent spectroscopic parameter, the experimentally measured parameter (here anisotropy) is a sum of the contributions from the free and bound species as⁵³:

$$a = A_{Free}[D] + A_{Bound}[PD] = A_{Free}(D_T - [PD]) + A_{Bound}[PD]$$

where $[D]$ and $[PD]$ are concentrations of free and protein-bound DNA, D_T is the total DNA concentration and A_x is the anisotropy of species x . Normalizing to the total DNA concentration:

$$A = \frac{a}{D_T} = A_{Free}\left(1 - \frac{[PD]}{D_T}\right) + A_{Bound}\frac{[PD]}{D_T} = A_{Free} + (A_{Bound} - A_{Free})\frac{[PD]}{D_T}$$

The concentrations of the free and bound species are related to the equilibrium dissociation constant K_D and total protein and DNA concentrations as:

$$K_D = \frac{[P][D]}{[PD]} = \frac{(P_T - [PD])(D_T - [PD])}{[PD]}$$

where $[P]$ is the free protein concentration and P_T is the total concentration (free plus bound). Solving this equation for $[PD]$ and dividing by D_T yields the quadratic:

$$\frac{[PD]}{D_T} = \frac{K_D + D_T + P_T - \sqrt{(K_D + D_T + P_T)^2 - 4D_TP_T}}{2D_T}$$

Substituting this into the expression for the measured anisotropy (A), and adding a linear term for the effect of weak non-specific protein-DNA interactions the equation used to obtain the binding constants for Int^{CB} and Int^{Cat}:

$$A = A_{Free} + (A_{Bound} - A_{Free})\frac{K_D + D_T + P_T - \sqrt{(K_D + D_T + P_T)^2 - 4D_TP_T}}{2D_T} + mP_T$$

Figure 1.

Cartoon diagram of the tetrameric complex observed in the crystal structure of residues 75–356 of λ-Int covalently bound to a suicide DNA substrate (1Z19.pdb)¹⁵. (A) The core-binding domain (top) binds on the opposite side of the DNA substrate as the catalytic domain (bottom). An extended linker connects the domains in the intact enzyme. DNA is shown in molecular surface representation, with each continuous strand colored differently; the bright orange strand is covalently attached to the green protomer. (B) Close-up of the protein-DNA and inter-protomer interfaces observed in the crystal structure. The core-binding domain binds the DNA opposite the site of cleavage (indicated by Y342, in red). The only contacts between the two domains are the linker (indicated in the green protomer), and a contact between the sidechain

of K93 (indicated in the blue protomer) and the backbone carbonyl of S234 in a hairpin loop. The C-terminus of the green protomer, containing W350, makes contacts to the blue protomer.

Figure 2.

Trans activation of Int^{Cat} by Int^{CB}. DNA cleavage activity monitored by denaturing SDS-PAGE analysis of the formation of a covalent complex between the protein and a 5'-radiolabeled DNA suicide substrate. Lane 1, the catalytic domain alone (Int^{Cat}); lane 2, the catalytic domain in the presence of the (separately added) core-binding domain (Int^{Cat} + Int^{CB}); lane 3, the linked core and catalytic domains in the context of Int^{CB+Cat} (lane 3). The concentration of the DNA substrate was 0.1 μ M, while the Int^{Cat}, Int^{CB} and Int^{CB+Cat} concentrations were 4 μ M. Addition of Int^{CB} results in a large increase in complex formation, while linking the two domains provides an additional enhancement.

Figure 3.

In the absence of DNA, catalytic and core binding domains of λ -Int are independent. Left, overlay of two-dimensional $^1\text{H}/^{15}\text{N}$ -HSQC spectra of $\text{Int}^{\text{CB+Cat}}$ (black) and Int^{Cat} (red). Near-perfect superposition of signals from the catalytic domain in isolation (Int^{Cat}) and when linked in tandem with the core binding domain indicate the domains do not significantly interact. Right, ribbon model of $\text{Int}^{\text{CB+Cat}}$ from the crystal structure of its complex with a half-site DNA substrate ²⁰.

Figure 4.

Trans complementarity between Int^{CB} and Int^{Cat} is DNA sequence dependent. (A) Close-up of the closest contact between Int^{Cat} (blue) and Int^{CB} (cyan) in the crystal structure (1P7D) ²⁰ (B) Cleavage of DNA substrates by Int^{Cat} in the absence (odd-numbered lanes) and presence (even lanes) of Int^{CB}. The oligonucleotide substrates tested are the “wild-type” C’s and variants with adenine nucleotides at positions +5 and +6 mutated to T, G or C in pairs and individually. Trans activation is lost when both adenines are mutated to T, while variants with those positions mutated to G or C are no longer recognized and cleaved by Int^{Cat}.

Figure 5.

Time course for DNA cleavage by Int^{Cat} in the absence (left) and presence (right) of Int^{CB} . Bands in the denaturing SDS-PAGE gel indicate the migration position of uncleaved ^{32}P -labeled substrate DNA and of Int^{Cat} in covalent complex with the trapped suicide substrate. $\text{Int}^{\text{Cat}\Delta 349\text{C}197\text{S}}$ was used to obtain the kinetic data because the mutant is more active and is less prone to precipitation than wild-type Int^{Cat} ; trans activation by Int^{CB} was observed for both.

Figure 6.

Pre-steady state kinetics analysis of DNA cleavage by Int^{Cat} indicates trans activation by Int^{CB} is due to improved efficiency, not tighter substrate binding. Left, initial rates of product formation were monitored as a function of Int^{Cat} concentration in the absence (top) and presence (bottom) of Int^{CB} . Right, concentration-dependence of the initial DNA cleavage rates, in the absence (top) and presence of Int^{CB} (bottom).

Figure 7.

Int^{CB} has a negligible effect of DNA binding affinity by Int^{Cat}. (A) Binding to a fluorescently labeled DNA substrate by Int^{CB} (“+” marks), Int^{Cat} alone (open circles) and Int^{Cat} in the presence of near-saturating (70%) Int^{CB} (filled circles) was monitored by fluorescence anisotropy. Data were fit using nonlinear regression to a one-site binding model; contributions from non-specific binding were determined from global fitting of all three datasets. (B) Binding of Int^{CB+Cat} to the same fluorescently labeled DNA substrate as in A.