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Title:

RecBCD possesses strong coupling between DNA and nucleotides binding that may propel stepping mechanism during translocation.

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Key words: RecBCD, Helicase, Nucleotide-Binding Linkage, Thermodynamic Coupling

Specific contributions: VG, RZ, AK & AH designed, performed research, analyzed data and wrote the paper. OD performed research.

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Abstract

Double strand breaks are the severest genomic damage requiring rapid repair response. In prokaryotes, members of the RecBCD family initiate DNA unwinding essential for double strand break repair mechanism by homologous recombination. RecBCD is a highly processive DNA helicase with an unwinding rate approaching ~1,600 $bp \cdot s^{-1}$. The ATPase reaction mechanism enabling RecBCD to achieve this fast unwinding rate and its enzymatic adaptation are not fully understood. Here, we present thermodynamic investigation of DNA and nucleotide binding to RecBCD to reveal the linkage binding and the degree of coupling between its nucleotides cofactor and DNA substrates binding. We find that RecBCD exhibits weak binding state in the presence of ADP towards double overhang DNA substrate (dohDNA), and the same degree of coupling is observed for RecBCD affinity toward ADP, only in the presence of dohDNA. In the absence of nucleotide cofactor (APO state) or in the presence of AMPpNp, much weaker coupling is observed between the binding of DNA and the nucleotides state towards RecBCD. Other DNA substrates that do not fully engaged with RecBCD optimally do not exhibits similar degree of coupling. This may be the first evidence for strong and weak binding states that can in principle regulate 'stepping mechanism' during processive translocation of RecBCD.

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Introduction

Double Strand Breaks (DSBs) in the genome are the severest damage in DNA of all kingdoms of life. Helicases play an essential role in the repair mechanisms for DSBs in every living organism. In prokaryotes, members of the RecBCD family initiate unwinding of DSBs in preparation for strand invasion essential for repair by homologous recombination [1]. RecBCD is a highly processive DNA helicase exhibiting an exceptionally high unwinding rate of ~ 1,600 base pairs (bp) per second (s^{-1}) [2]. RecBCD is a heterotrimer of one copy of each of the DNA translocases and helicases namely, RecB, RecD [3, 4]. RecC subunit "staples" RecB and RecD subunits [5], and plays a crucial role in destabilizing the duplex DNA ahead of the translocases activities of RecB and RecD unwinding [5] and recognizes the Chi sequence [6, 7]. RecBCD utilizes ssDNA translocation on opposite DNA polarities with RecB moving on the 3' - 5' strand and RecD moving on the 5' - 3' strand with overall net translocation of RecBCD complex along the duplex DNA [4, 8-10]. Although the repair mechanisms of DSBs are found in all living organism, there is no known eukaryotic homologous to RecBCD in terms of its structural organization or rapid and processive unwinding.

Specifically, for RecBCD to transverse processively with the two motors translocating along the single strands of the DNA, a stepping-like mechanism is most likely required. For net vectorial translocation, a simple alternation between strong and weak DNA binding states can provide such mechanism of stepping [11-13]. Threading through ssDNA channels still must be via alteration between attached (strong binding state) to detached states (weak binding states). In this sense RecBCD can be viewed as a double headed ATPase molecular machine, i.e., a molecular motor that has two motors that transverse across the DNA lattice. This is highly analogues to a molecular motor such as myosin V whereas its processive stepping along the actin

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filament can be describe by walking hand over hand [14]. Threading ssDNA through semi open DNA tunnel forms in RecB and partially in RecD will increase processivity but will still be requiring cycles of coordinated association-dissociation transitions to permit net vectorial translocation. The basis for this long-distance stepping motility lies in the specific nucleotide state/s that modulate the affinity towards the lattice between strong and weak affinities, allowing the motor its dissociation and re-association with respect to the lattice [15]. However, the thermodynamics basis for the strong and weak nucleotides states during translocation has not yet been resolved for RecBCD. Therefore, identifying the existence of such intermediates during the mechanochemical ATPase cycle is important to understand how RecBCD may perform its processive and rapid translocation. The nucleotide ligand bound state (ATP, ADP·P_i, or ADP) modulates the motor affinities towards the lattice it transverse on, as have been shown for all myosins, kinesin as well as for some helicases [16-20]. For RecBCD this has not yet been determined systematically with DNA substrate with respect to the nucleotide state.

In this work, we characterized RecBCD's binding to diverse DNA substrates and nucleotides mimicking intermediate states along its reaction cycle to reveal nucleotide binding linkage within RecBCD. We show that RecBCD and RecBCD·DNA·nucleotides complex exhibit in specific state weak coupling and in other strong coupling between the nucleotide and the DNA bound to RecBCD. Specifically, we found that RecBCD·ADP complex exhibits weak affinity towards dohDNA (~40-fold reduction) unlike any other state or DNA substrate. The opposite is also true in which RecBCD· dohDNA exhibits somewhat weaker affinity towards ADP. This may present one state in RecBCD of strong coupling between dohDNA and ADP binding. The AMPpNp state weakens the affinity towards the 3ohDNA by 2- fold while the ADP strengthen the affinity of 3ohDNA substrate towards RecBCD by ~5-fold (Fig. 1D & 1E). Since dohDNA

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shows strongest impact on affinities, we propose that such strong coupling must be mediated via multiple DNA contact sites as partial DNA overhang DNA substrates don't show such impact. Long range communication may exists between the subunits during translocations to facilitate strong to weak binding transitions.

Results

RecBCD oligomeric state and DNA stoichiometric binding

We examine nucleotide binding linkage to RecBCD by performing equilibrium binding assays of DNA substrates in the absence and presence of AMPpNp and ADP. For this we have devised a set of DNA substrates that can mimic three major states engaged with RecBCD, initiation (hpDNA), translocation (ssDNA), and unwinding and translocation with overhang on both 5' and 3' (dohDNA), and two additional substrates with either 5' or 3' ohDNA engaged with RecD or RecB, respectively [5, 21] (Fig. 1, Table 1). To measure DNA and nucleotide binding linkage with respect to RecBCD complex, the oligomeric state in solution of RecBCD under our experimental conditions must be determined, similarly to what have been performed ruling out the existence of a dimer of RecBCD during catalysis [7]. Figure 2 shows the stoichiometric binding of ssDNA and hpDNA (Fig. 1, Table 1) under the condition in which [DNA] >> K_D and RecBCD is titrated well above the K_D (Fig. 3, Table 2) measured by Fluorescence Anisotropy (FA) (Fig. 2A) and fitted according to Equation 1:

Under stoichiometric conditions $[DNA]_T >> K_D$, R is the plotted (experimental) mole/mole ratio

$$[RecBCD \cdot DNA] = \frac{\left(R + n + \frac{K_D}{[DNA]_T}\right) + \sqrt{\left(R + n + \frac{K_D}{[DNA]_T}\right)^2 - 4R \cdot n}}{2n} \qquad \text{(Eq. 1)}$$

of RecBCD/DNA and n is the ratio of the fit of RecBCD to DNA, and the DNA_T is the DNA (ssDNA or hpDNA) concentration. This analysis show that RecBCD:hpDNA and RecBCd:ssDNA bind with 1:1 and 1:2 stoichiometric ratio. Previously the stoichiometry binding

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of fluorescently labeled single stranded overhang (similar to substrate in Fig. 1C) had similar stoichiometry ratio to RecBCD heterotrimer [22].

Further analysis by analytic size exclusion chromatography (SEC) of RecBCD·hpDNA complex in comparison to RecBCD (Fig. 2B) show clearly a monodisperse peak of approximate MW of ~330 kDa (determined by the linear range of known MW protein marker) in complex with the major peak of the hpDNA with a ratio of 1:1, 280:260 nm absorbance (in comparison to only RecBCD of 3:1, 280:260 ratio). This further confirming the stoichiometry of RecBCD: hpDNA of 1:1.

DNA binding affinities to RecBCD and RecBCD·nucleotides complex

We analyzed different DNA substrates binding to RecBCD and RecBCD·nucleotide complexes by FA (Fig. 3A-E). All DNA binding isotherms exhibited hyperbolic dependence curves which enabled us to determine the equilibrium binding constants for the different DNA substrates (Table 2). Our binding model for the DNA substrates to RecBCD is described by the following stoichiometric reaction scheme (Scheme 1):

$$\text{RecBCD} + \textit{hp}\text{DNA}/\textit{doh}\text{DNA}/\textit{oh}\text{DNA} \underset{}{\overset{K_D}{\rightleftharpoons}} \text{RecBCD}\square\textit{x}\text{DNA} \quad \text{(Scheme 1)}$$

Then the general solution for this equilibrium binding scheme is in the form of the following quadratic Equation 2:

$$[RecBCD \cdot DNA] = \frac{([RecBCD]_T + [DNA]_T + K_D) + \sqrt{([RecBCD]_T + [DNA]_T + K_D)^2 - 4[RecBCD]_T \cdot [DNA]_T}}{2} \quad \text{(Eq. 2)}$$

Where, $[DNA]_T$ is the monitored species; $[RecBCD]_T$ is titrated species and $[RecBCD \cdot DNA]$ is the bound species. For FA measurements, the conditions to hold is the total fluorescence intensity remains constant [23, 24] throughout the titration, and under our measurements conditions the changes in the FTI was small enough to be neglected, therefore allowing direct

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fitting of the FA binding isotherm curve using Eq. 2 to determine the equilibrium binding constants.

Initially binding of hpDNA to RecBCD in the absence and presence of nucleotide cofactors already revealed modulation in the affinities where ADP induced somewhat (3-fold) weaker affinity in comparison the APO state. In somewhat contrast, nucleotide cofactors did not show any dramatic effect on the affinity of RecBCD towards ssDNA binding.

When we tested the binding of dohDNA (Table 1C) to RecBCD·ADP (Fig. 3C, Table 2), we observed a dramatic change in the affinity suggesting strong coupling between the binding sites. In the presence of ADP, RecBCD exhibited a strongest reduction in the affinity towards dohDNA substrates (40-fold and 25-fold, in comparison to APO and AMPpNp states, respectively, Table 2). This strong coupling between ADP and dohDNA binding may be key to reveal modulation in the affinity of RecBCD towards DNA to allow strong and weak binding states during ATPase cycling [25, 26]. To further dissect the source of the weak binding in the presence of ADP, we characterized the binding of two additionally DNA substrates each containing either a 5ohDNA or 3ohDNA supposingly engaging with RecD or RecB, respectively (Fig. 1, Table 1). For both single stranded overhang substrates in the presence of ADP, RecBCD exhibits the strongest affinity in comparison when AMPpNp is bound or in the absence of nucleotide cofactor.

Nonetheless, the strong coupling observed when both ssDNA overhangs 5’ and 3’ are engaged with RecB and RecD in the presence of ADP argues in favor of the existence of binding linkage. In contrast, when RecB or RecD are DNA free the nucleotide state exhibited much weaker coupling among the three states, APO, AMPpNp or ADP bound. This suggest that there is some allosteric communication between RecB and RecD under the premise that both subunits

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are engaged with ssDNA via an element that may be outside the DNA binding sites within RecB and RecD. One explanation maybe that a full contact encompassing, RecB-Arm, RecB and RecD account for the observed strong coupling in the dohDNA.

Analysis of ssDNA binding to RecBCD with the Hill Equation reveal cooperativity in the presence of ADP

RecBCD binds two ssDNA, therefore we can utilize a binding model such as Hill to account for multiple binding sites according to Scheme 2:

$$\text{RecBCD} + ss\text{DNA} \xrightleftharpoons{K_D} \text{RecBCD}\square(ss\text{DNA})_2 \qquad \text{(Scheme 2)}$$

to extract the two parameters K_H and n_H as shown in Equation 3:

$$\theta = \frac{[RecBCD]^n}{[RecBCD]^n + K^n} \qquad \text{(Eq. 3)}$$

Where, is θ the fraction bound K is the apparent dissociation constant, n is the Hill coefficient, while assuming that the ssDNA binding sites are similar, not identical. The results obtained with fitting the data to the Hill equation provided further insights into the coupling between nucleotides and ssDNA binding. While the trend of the order of affinities towards ssDNA remains the same as with Eq. 2, the Hill coefficient in this case shows that while ADP weakens the affinity towards ssDNA, the cooperativity n_H is increased to ~ 2. ssDNA binding to RecBCD has intermediate cooperativity whereas the presence of AMPpNp the cooperativity is slightly negative (n_H ~ 0.7). This is very exciting results as this suggests that when the two DNA binding sites in RecB and RecD are bound with ssDNA, nucleotides cofactor impose different degree of cooperativity. Moreover, these was not revealed using the simple binding model.

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Nucleotides binding to RecBCD in the presence of DNA substrates exhibit biphasic binding patterns

We have utilized FRET to measure the binding equilibrium constant for mant-Nucleotides derivatives to RecBCD (Zananiri, Gaydar, *et al.* 2017). Unique to RecBCD, the binding isotherm curves of mant-Nucleotides to RecBCD and RecBCD·DNA complexes all exhibited a biphasic binding pattern. Such binding is best described by the sum of two Hill plot (Zananiri, Gaydar, *et al.* 2017). Thus, mant-Nucleotide binding curves of the fluorescence change as a function of the free ligand concentration are best fitted to Equation 4:

$$y = p \cdot \frac{1}{1 + \left(\frac{K_s}{[mN]}\right)^{n_s}} + (1 - p) \cdot \frac{1}{1 + \left(\frac{K_w}{[mN]}\right)^{n_w}} \qquad \text{(Eq. 4)}$$

Where y is, the fraction bound, mN is the ligand concentration, K_s (strong nucleotide state) and K_w are the equilibrium constants of the first and second phase, respectively. n_s and n_w are the Hills coefficients of the first and second phase, respectively, and p is the proportionality constant ($0 \leq p \leq 1$). Previously, we show that the first phase of the binding titration curve reflect binding to the canonical ATP binding sites residing within RecBCD and RecD and the second phase describe binding to additional weak binding sites both are given by macroscopic equilibrium constant's K_s and K_w (Zananiri, Gaydar, *et al.* 2017).

RecBCD and RecBCD·DNA complexes show overall K_s values, ranging from ~13 to 140 μM and n_s 0.8 to 3.0 (Table 3). The second binding phase exhibited K_w values in the range of ~140 to 420 μM, an order of magnitude larger than K_s, indicating significantly weaker nucleotides' binding affinity sites (Table 3). In addition, those sites exhibit a degree of cooperativity, n_w, ranging from 2.5 to 12.0, but mostly with significant errors, indicating that their may be some cooperative binding (Table 3) according to the Hill coefficient. However, we previously determined that there are at least two additional nucleotide binding sites in RecBCD

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and the high values of n_w may be a result of an overestimation due to the resolution of the fit to accurately separate the two phases.

Overall there were four states that show strong modulation in the affinity parameter $\boldsymbol{K_s}$ as a result of the specific DNA substrates bound to RecBCD of the nucleotides state (Table 3). dohDNA bound to RecBCD induced a 2-fold weaker affinity of RecBCD towards AMPpNp in comparison to only RecBCD, and nearly no change in AMPpNp affinity towards RecBCD in complex with 5ohDNA and 3ohDNA (Figure 4A-D, Table 3). This suggest that the coupling between AMPpNp binding to RecBCD is apparent only when both ssDNA overhangs are present to fully interact with RecBCD. The presence of 3ohDNA only slightly weaken the affinity of RecBCD towards AMPpNp. Significantly, a much stronger effect of nucleotides binding affinity of RecBCD is amplified in the presence of dohDNA, specifically, in the ADP states. In this case, the affinity of RecBCD towards ADP is decreased ~7-fold in the presence of dohDNA towards ADP than in the absence of any other DNA substrates (Figure 4A-D, Table 3). Similarly, 5ohDNA and 3ohDNA weaken the affinity of RecBCD towards ADP with 7-fold and ~8-fold, respectively.

The affinity parameter $\boldsymbol{K_w}$ across all DNA substrates states appears to be changing very little in comparison to the ligated states of RecBCD without any DNA substrates (Table 3). This suggests that the weaker nucleotide binding sites (auxiliary sites) are much less prone to be affected by DNA binding. Overall, our results demonstrate that even though RecBCD cycle through an entire ATP hydrolysis states in less than a msec, still, nucleotide induced weak to strong transition and vice versa are indeed exists.

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RecBCD thermodynamic coupling between the nucleotide and DNA states

Thermodynamics detailed balance analysis allows us to quantify the degree of linkage and the thermodynamic coupling constant (TC) between the DNA and nucleotides binding states. Figure 5 presents the five by two squares per each DNA substrate with two nucleotides binding states, AMPpNp and ADP. The ratio of binding affinities of a nucleotide in the presence or absence of DNA and vice versa, sheds a light on the mechanism by which RecBCD can switch from strong and weak binding states during processive translocation. The results of TC analysis for the cycles of hpDNA, ssDNA, dohDNA, 5ohDNA, and 3ohDNA in the absence or presence of either AMPpNp or ADP are summarized in Table 4.

Thermodynamic coupling constants across the thermodynamics detailed balance square should be equal, i.e., $K_{Mp,hp}/K_{Mp} \cong K_{hp,Mp}/K_{hp}$, due to consideration of the total free energy change within a closed cycle. Thus, near equalities of TC provide an intrinsic measure of the consistency within each ligated state. Inspection of the TC in Table 4, reveals that this criterion is met to the accuracy of the measurements in most of the cycles. However, in the case of $(K_{D,doh}/K_D)/(K_{doh,D}/K_{doh})$, $(K_{D,5oh}/K_D)/(K_{5oh,D}/K_{5oh})$ and $(K_{D,3oh}/K_D)/(K_{3oh,D}/K_{3oh})$, the ratio between the TC pairs are, ~0.13, ~18, and ~30, respectively. The simplest explanation for this deviation in the TC equality could be the results of the binding model i.e., DNA or nucleotide binding isotherms are not accurately reflecting the macroscopic equilibrium constants in s these cases. This is due to the nature of the multiple binding sites of both DNA and nucleotide binding. The other is that the assumption under which equilibrium balance hold, i.e., the equilibrium is invariant under the time frame does not holds and these complexes are slowly interchanging even under steady state measurements with these DNA substrates. Currently, we cannot differentiate between these possibilities or what is the source of this inbalance. However, further

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measurements under higher stabilizing conditions may be important to resolve these differences. In summary, we show using TC that distinct states are induced to disturb the TC ratio to a degree where strong and weak coupling are observed between the strong nucleotides binding sites in the presence of dohDNA substrate with a much higher degree in ADP state.

Discussion

In this work, we have investigated the nucleotide binding linkage of RecBCD to determine quantitatively the degree of coupling between the different nucleotide states along with the different DNA substrates. Our results suggest that RecBCD TC is highly complex network partly due to the number of DNA contact sites within the different protomers, the two catalytic ATP sites and furthermore, the long range weak effect of the auxiliary nucleotide sites. We find that in order to induce linkage between DNA and nucleotide binding, the DNA substrates such as dohDNA must be fully engage with both translocase activities and with the RecB arm. The ADP states seems to perpetrate the strongest coupling in comparison to the APO or AMPpNp bound states. In this regard, all three substrates, hence dohDNA, 5ohDNA and 3ohDNA are affecting ADP binding unlike AMPpNp. When RecBCD is bound to ADP, the strongest affect is observed in the binding towards dohDNA affinity while the 5ohDNA and 3ohDNA are much less affected in comparison to the APO states. This asymmetry between the reciprocal affects that the latter two substrates have on ADP binding to RecBCD could simply be due to measuring one experiments with labeled 5' DNA end versus unlabeled DNA in the later experiments. However, this only affect the magnitude of their mutual effect on binding not the actual observed strong coupling. Therefore, we conclude that the ADP binding state is key to promote weak binding towards DNA, mostly when dohDNA is utilized but also when 5ohDNA and 3ohDNA. If ADP induces in both subunits RecB and RecD weak binding towards DNA, it

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will require that these states will never coexist in both subunits at the same time to the same extent. Furthermore, the additional parameter for the synchronized DNA binding mechanism by the two helicases subunits of RecBCD, is the Hill coefficient >1 (Table 3 & 4) upon substrates binding that is engaged by the two subunits simultaneously (ssDNA, dohDNA, 3ohDNA and 5ohDNA).

One exception exists for 3ohDNA substrate which exhibits overall weaker affinity in the absence or presence of AMPpNp in comparison to when ADP is bound. However, the TC for these states, $K_{Mp,3oh}/K_{Mp}$ and $K_{3oh,Mp}/K_{3oh}$ is very weakly coupled and the detailed balance are consistence with the thermodynamic cycle among these states suggesting that, although there may be wakening in the affinity among these states they don't trigger the strong coupling affects observed with ADP.

Structural investigation of RecBCD provided wealth of information in regards to the DNA contacts sites with RecBCD subunits, however still missing are structures with different nucleotides states to monitor conformational changes induced by the different ligates state. Nonetheless, inspection of the RecBCD structure clearly show the large surface that make direct contact with the DNA [5]. At the forefront, the RecB ARM makes extensive contacts with the dsDNA. This is followed by more contacts of the C-terminal and the junction of the dsDNA with the 'pin' domain of RecC. RecB 2A subdomain interacts with the 3' overhang ssDNA, while RecD subdomain 1A and 2A make contacts with the 5' overhang ssDNA [5]. Correlating the coupling between DNA and nucleotides binding is crucial to elucidate the mechanism of RecBCD successive translocation. Strong binding state can be viewed as the 'pulling' states while the weak binding states are the 'relieving' states allowing for productive advancements of RecBCD motor while alternating these events between RecB and RecD. Therefore, allosteric

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communication will most likely exist and will involve network of interaction across all the DNA contacts sites of all RecBCD subunits.

Thus, driven by our present work, we suggest that RecBCD has enzymatically evolved to go through these essential biochemical intermediates with minimum 'effort', with minimum energy barriers but still maintain their existence within any mechanism to allow translocation. Weather the alteration between strong and weak binding states of RecBCD is synchronized among its subunits by the nucleotide state during its processive unwinding is probably prerequisite to allow high efficiency from each ATPase hydrolysis cycle. Therefore, it will be most interesting to study how such mechanism is enabled within RecBCD ATPase subunits.

Although we have not accessed all possible states, our analysis of the APO state, ATP mimicking state and the ADP state after P*i* release, with hpDNA, ssDNA, dohDNA, 5ohDNA, and 3ohDNA represent a wide array of biochemical intermediates along the reaction coordinates of RecBCD.

Funding

This research was supported by The Israel Science Foundation [grant No. 296/13]; and the Marie Curie Career Integration Award [grant No. 1403705/11].

Acknowledgments

We thank Dr. Stephen C. Kowalczykowski and Dr. Theetha Pavankumar for the RecBCD expression system and their assistance in RecBCD purification protocol (University of California, Davis, California).

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Materials and Methods

Reagents and Purification of RecBCD

All chemicals and reagents were the highest purity commercially available. ATP and ADP were purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). Adenosine 5′-(β,γ-imido)triphosphate (AMPpNp) was purchased from Sigma (St. Louis, MO, USA). A molar equivalent of $MgCl_2$ was added to nucleotides immediately before use. Nucleotide concentrations were determined by absorbance using an extinction coefficient ε_{259} of 15,400 M^{-1} cm^{-1}. The concentrations of N-methylanthraniloyl (mant) derivatives of ADP, 2'-deoxyADP, ATP, and 2'-deoxyATP (Jena Bioscience, Jena, Germany) were determined using ε_{255} of 23,300 M^{-1} cm^{-1}. Unless otherwise specified, all experiments were conducted in RecBCD Buffer (RB: 20 mM MOPS pH 7.4, 2 mM MgCl2, 1 mM DTT, 0.1 mM EDTA and, unless specified, 75 NaCl. Over-expression and purification of recombinant RecBCD was based on the method described by Roman et. al.[27], with additional step as indicated. All steps of purification were carried out at 4 °C, and contained 20 mM MOPS pH 7.4, 2 mM $MgCl_2$,1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, 1 mM Benzamidine and the indicated salt concentration. Four liters of *E.coli* cells expressing RecBCD were lysed using Microfluidizer, followed by centrifugation at 10,000×g. The supernatant was further clarified by centrifugation at 100,000×g and treated with Benzonase for two hrs before initial purification by DEAE chromatography (weak anion exchanger to remove nucleic acids contaminants) using a linear NaCl gradient from 75 mM to 700 mM. RecBCD-containing DEAE fractions were eluted from a Q-sepharose column (strong anion exchanger which highly selects for active RecBCD [28] using a linear NaCl gradient from 75 mM to 1 M. Fractions containing RecBCD were precipitated using $(NH_4)_2SO_4$ (45% saturation), and collected by centrifugation at 14,000×g. Precipitated RecBCD was resuspended

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and loaded onto Superdex 200 equilibrated with RB , as a final step of polishing and elution of RecBCD specifically from the monodisperse peak of the heterotrimer complex of RecBCD. Fractions containing purified RecBCD were concentrated using an Amicon concentrator (50 kDa cutoff), aliquoted and flash frozen in liquid nitrogen before storage at -80°C. The RecBCD concentration was determined using $\varepsilon_{ex,coeff.}$ of 4.2×10^5 M^{-1} cm^{-1} in Guanidine chloride. To ensure RecBCD purity from nucleic acids, only protein fractions with 280/260 nm ratio >1.3 were used (Zananiri, Gaydar, *et al.* 2017).

DNA substrates:

DNA oligonucleotides were purchased from IDT (Leuven, Belgium) and HPLC purified. The DNA substrates shown in Figure 2 were obtained by folding or hybridization of the DNA in 20 mM MOPS pH 7.4, 75 mM NaCl, 1 mM $MgCl_2$ buffer at 85°C for 3 minutes followed by slow cooling to room temperature before storage at -20°C.

DNA binding measurements by Fluorescence Anisotropy (FA):

FA measurements were performed with a PC1 spectrofluorimeter set up T-format configuration for simultaneous acquisition on two emission channels using monochromators equipped with automatic polarizers. Samples were equilibrated (60 min, RT) and then measured with λ_{ex} = 492 nm using vertical polarized light and the emitted vertical and horizontal polarized light was monitored at 90° with emission monochromators at λ_{em} =523 nm at 25 ± 0.1°C. G-factor for correction of the different gain between of vertical and horizontal PMT detectors was calculated as described by the instrument manufacturer. The buffer included 20 mM MOPS pH 7.4, 2 mM $MgCl_2$, 1 mM DTT, and 75 mM NaCl. Nucleotides concentrations in the measurements where indicated were 2 mM (MgADP, or MgAMPpNp). Fluorescent DNA substrates were held constant at 25 nM.

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mant-Nucleotide binding to RecBCD by Förster resonance energy transfer (FRET):

FRET measurements were performed with a PC1 spectrofluorimeter (ISS, Champaign, IL), utilizing excitation and emission monochromators. The observation cell was regulated with Peltier temperature controller at 25 ± 0.1°C. All equilibrium binding reactions were performed in a 10 μl Precision cell fluorescence cuvette (Farmingdale, NY, USA), which allows minimal inner filter affects [29] up to concentration of ~ 550 μM mantNucleotides. The buffer included 20 mM MOPS pH 7.4, 2 mM $MgCl_2$, 1 mM DTT, and varying concentrations of NaCl (75, 150, 200, 300 mM). mant-nucleotides were titrated with 1:1 ratio to $MgCl_2$. Equilibrium binding reactions of mantNucleotides to RecBCD were measured by FRET between RecBCD intrinsic tryptophan fluorescence (λ_{ex} = 280 nm) and bound mantNucleotide (fluorescence monitored at 90° through an emission monochromoter at λ_{em} = 436 nm) [30]. We performed subtractions of background fluorescence of free nucleotides on the observed emission peak. DNA substrates were held constant at 1 μM and RecBCD concertation was 1 μM. In the case of ADP binding to RecBCD·dohDNA, the DNA concentration was 8 μM.

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Figure Legends

Figure 1: **Schematic representation of the DNA substrates used in this work**. **A.** Hairpin DNA: hpDNA, hpDNA-F (Table 1, 1a, b) **B.** Single strand DNA: ssDNA, ssDNA-F (Table 1, 2a, b), **C.** Double strand overhang DNA: dohDNA, dohDNA-F (Table 1, 3a, b), **D.** Single strand overhang DNA: 5ohDNA, 5ohDNA-F (Table 1, 4a, b), **E.** Single strand overhang DNA: 3ohDNA, 3ohDNA-F (Table 1, 5a, b); FAM: 6-fluorescein amidi

Figure 2: Purification and Biochemical characterization of RecBCD oligomeric states without and with DNA and nucleotides. A. Stoichiometric titration of RecBCD versus ssDNA and hpDNA using FA. Concentration of ssDNA/hpDNA were fixed at 2 μM, and RecBCD was titrated up to 10 μM (5-fold DNA concentration). The fitted curve shown in black line is according to Eq. 1. This produced the following "n" values for RecBCD/ssDNA = 0.49 ± 0.1 and RecBCD/hpDNA = 1.12 ± 0.1. Data shown as mean ± s.e.m., n=2. **B.** Analytical SEC analysis by Superdex 200 of RecBCD oligomeric state in the absence and presence of hpDNA under stoichiometric conditions. The absorption spectra was normalized for its presentation. RecBCD absorption ratio 280 nm:260 nm for RecBCD in the absence of hpDNA is 3:1, in the presence hpDNA the ratio is 1:1, therefore enable the determination of hpDNA presence in RecBCD peak. Peak of RecBCD·hpDNA, corresponding to a molecular weight of ~ 330 kDa according to the MW standards.

Figure 3: Fluorescence Anisotropy equilibrium binding measurements of RecBCD and RecBCD·nucleotides complex to fluorescently labeled DNA. DNA substrates were labaled with 6-fluorescein amidi. Fluroesecne DNA was held at 25 nM and RecBCD was titrated to reach saturation of the binding isotherm. **A.** hpDNA binding to RecBCD (●), RecBCD·AMPpNp (●) RecBCD·ADP (●). **B.** ssDNA binding to RecBCD (●), RecBCD·AMPpNp (●)

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RecBCD·ADP (●). **C.** dohDNA binding to RecBCD (●), **D. E.** RecBCD·AMPpNp (●) RecBCD·ADP (●) Error bars report the s.d of three independent measurements, n = 3. Lines through the data points are the best fit to Eq.2. Panel C was fitted to Hill equation. λ_{ex} = 492 nm and λem. = 523 nm. Nucleotides concentrations where indicated were 2 mM (MgADP, or MgAMPpNp). **F.** Data in B fitted to Hill equation model

Figure 4: Equilibrium binding of RecBCD and RecBCD·DNA to mantNucleotides. **A. and B.** Titration curves of mantAMPpNp (A) and mantADP (B) binding to RecBCD and RecBCD·DNA substrates exhibit biphasic pattern. Data points are normalized as fraction bound. (A) and (B) RecBCD (●), RecBCD·hpDNA (●), RecBCD·ssDNA (●), **C. and D.** titration curves of mantAMPpNp (C) and mantADP (D) binding to RecBCD and RecBCD·DNA substrates exhibit biphasic pattern. RecBCD·dohDNA (●), RecBCD·5ohDNA (●), and RecBCD·3ohDNA (●). Lines show the best fit to Eq. 3. Data shown as mean ± s.e.m., n = 3.

Figure 5: Detailed thermodynamic balance schemes of RecBCD ligated states. The schemes describe the ligated states of the initiation complex (first row, hairpin DNA, stoichiometry 1:1), translocation complex (second row, ssDNA, stoichiometry 1:2), unwinding complex (third row, dohDNA, stoichiometry 1:1). The boxes in the forth and fifth rows are asymmetric substrates engaged either with RecD (5ohDNA) or RecB (3ohDNA) only. The equilibrium constants, and hence the nucleotides binding occupancy in the model are relating only to the strong nucleotides binding sties. The equilibrium constants and the free energy calculated are shown in Tables 2 and 3 and are according to the detailed balanced shown in the reaction schemes. H - RecBCD, hp - hpDNA, ss - ssDNA, doh - dohDNA, 5oh - 5ohDNA, 3oh - 3ohDNA, D - ADP, Mp -

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AMPpNp, RecBCD enzyme is represented by orange, green, blue ovals for RecB, RecC and RecD, respectively. DNA is drawn as black solid lines.

Tables

Table 1: Sequences of the DNA substrates used for DNA and nucleotides binding experiments

#	Sequence Description	Sequence 5’ - 3’	DNA Length
1a	21 bp hairpin DNA	CATGTGACTCGTTACCTGAGTTTTTACTCAGGTAACGAGTCACATG	46
1b	21 bp hairpin DNA 3’ FAM	GATGTGACTCGTTACCTGAGTTTTTACTCAGGTAACGAGTCACATC-FAM	46
2a	24 nt ssDNA	AGAGAGAGAGAGAGAGAGAGAGAG	24
2b	24 nt ssDNA 5’ FAM	FAM-AGAGAGAGAGAGAGAGAGAGAGAG	24
3a	10-6 nt 5’-3’ overhang	CGCGCGCATGTGACTCGTTACCTGAGTTTTTACTCAGGTAACGAGTCACATGATATATATAT	62
3b	10-6 nt 5’-3’ overhang FAM	FAM-CGCGCGCATGTGACTCGTTACCTGAGTTTTTACTCAGGTAACGAGTCACATGATATATATAT	62
4a	6 nt 5’ overhang	CGCGCGCATGTGACTCGTTACCTGAGTTTTTACTCAGGTAACGAGTCACATG	52
4b	6 nt 5’ overhang FAM	FAM-CGCGCGCATGTGACTCGTTACCTGAGTTTTTACTCAGGTAACGAGTCACATG	52
5a	10 nt 3’ overhang	CATGTGACTCGTTACCTGAGTTTTTACTCAGGTAACGAGTCACATGATATATATAT	56
5b	10 nt 3’ overhang FAM	FAM-CATGTGACTCGTTACCTGAGTTTTTACTCAGGTAACGAGTCACATGATATATATAT	56

1. FAM: 6-fluorescein amidi

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Table 2: Equilibrium constants for DNA substrates binding to RecBCD and RecBCD·nucleotides complex.

Annotation	Complex	[1]K_D (nM)		$\Delta G°'_{association}$ (kJ·mol^{-1})
K_{hp}	RecBCD·hpDNA-F	42 ± 9		- 42.1 ± 0.5
$K_{hp,Mp}$	RecBCD·AMPpNp·hpDNA-F	85 ± 19		- 40.3 ± 0.6
$K_{hp,D}$	RecBCD·ADP·hpDNA-F	127 ± 31		- 39.3 ± 0.6
K_{ss}	RecBCD·ssDNA-F	7 ± 2		- 46.6 ± 0.5
$K_{ss,Mp}$	RecBCD·AMPpNp·ssDNA-F	4.5 ± 2		- 47.6 ± 1.8
$K_{ss,D}$	RecBCD·ADP·ssDNA-F	13 ± 5		- 45.0 ± 0.2
K_{doh}	RecBCD·dohDNA-F	19.2 ± 5		- 44.0 ± 0.6
$K_{doh,Mp}$	RecBCD·AMPpNp·dohDNA	34.0 ± 7.0		- 42.6 ± 0.5
$K_{doh,D}$	RecBCD·ADP·dohDNA	823.0 ± 261.0		- 34.7 ± 0.8
K_{5oh}	RecBCD·5ohDNA-F	29.6 ± 7		- 43.0 ± 0.6
$K_{5oh,Mp}$	RecBCD·AMPpNp·5ohDNA-F	31 ± 8		- 43.0 ± 0.6
$K_{5oh,D}$	RecBCD·ADP·5ohDNA-F	8 ± 2		- 46.2 ± 0.6
K_{3oh}	RecBCD·3ohDNA-F	92.6 ± 15.4		- 40.1 ± 0.4
$K_{3oh,Mp}$	RecBCD·AMPpNp·3ohDNA-F	166.3 ± 41		- 38.7 ± 0.6
$K_{3oh,D}$	RecBCD·ADP·3ohDNA-F	16.3 ± 7		- 44.4 ± 1.1
		K_H (nM)	n_H	
K_{ss}	RecBCD·ssDNA-F	16.5 ± 3.1	1.4 ± 0.32	- 44.4 ± 0.5
$K_{ss,Mp}$	RecBCD·AMPpNp·ssDNA-F	7.0 ± 5.0	0.7 ± 0.20	- 46.5 ± 1.8
$K_{ss,D}$	RecBCD·ADP·ssDNA-F	23.1 ± 2.2	2.1 ± 0.34	- 43.6 ± 0.2

[1]Equilibrium constants reflect the macroscopic binding affinity of DNA binding to RecBCD complex

Table 3: Equilibrium constants for nucleotides binding to RecBCD and RecBCD·DNA complex

Annotation	Complex	K_s(μM)	n_s	K_w (μM)	n_w	p	$\Delta G_s°'_s$ (kJ·mol^{-1})	$\Delta G_w°'_s$ (kJ·mol^{-1})
$^4K_{Mp}$	RecBCD·mMp	52.4 ± 7.8	0.99 ± 0.1	286.9 ± 11.9	12.0 ± 4.7	0.60 ± 0.04	-24.4 ± 0.4	-20.2 ± 0.1
$K_{Mp,hp}$	RecBCD·hpDNA·mMp	73.5 ± 9.3	1.44 ± 0.3	147.0 ± 6.8	5.0 ± 1.1	0.74 ± 0.1	-23.6 ± 0.3	-21.9 ± 0.1
$K_{Mp,ss}$	RecBCD·ssDNA·mMp	20.0 ± 5.9	2.90 ± 0.9	146.9 ± 6.8	5.0 ± 1.1	0.23 ± 0.1	-26.8 ± 0.7	-21.9 ± 0.1
$K_{Mp,doh}$	RecBCD·dohDNA·mMp	106 ± 33.5	1.8 ± 0.4	343.8 ± 9.2	9.0 ± 3.2	0.50 ± 0.1	-22.7 ± 0.8	-19.6 ± 0.2
$K_{Mp,5oh}$	RecBCD·5ohDNA·mMp	47.8± 22.1	2.0 ± 1.0	285.1 ± 17.9	4.1 ± 0.8	0.30 ± 0.1	-24.6 ± 1.1	-19.8 ± 0.1
$K_{Mp,3oh}$	RecBCD·3ohDNA·mMp	71 ± 28.3	1.7 ± 0.4	295.8 ± 12.1	5.2 ± 1.5	0.50 ± 0.1	-23.7 ± 1.0	-20.2 ± 0.2
$^4K_{mD}$	RecBCD·mD	13.2 ± 3.1	.095 ± 0.1	322.5 ± 6.9	6.2 ± 0.6	.045 ± 0.03	-27.8 ± 0.6	-20.1 ± 0.1
$K_{D,hp}$	RecBCD·hpDNA·mD	29.2 ± 11.8	0.81 ± 0.2	314.9 ± 22.0	4.0 ± 0.3	0.51 ± 0.12	-25.9 ± 1.0	-20.0 ± 0.2
$K_{mD,ss}$	RecBCD·ssDNA·mD	15.7 ± 3	2.85 ± 1.7	343.0 ± 84.2	2.5 ± 0.8	0.19 ± 0.01	-27.4 ± 0.5	-19.8 ± 0.6
$K_{mD,doh}$	RecBCD·dohDNA·mD	70.9 ± 19.4	1.5 ± 0.3	338.3 ± 8.9	9.0 ± 3.0	0.51 ± 0.1	-23.7 ± 0.7	-19.8 ± 0.1
$K_{mD,5oh}$	RecBCD·5ohDNA·mD	70.5 ± 8.6	2.4 ± 0.4	354.4 ± 17.7	5.3 ± 1.1	0.38 ± .01	-23.7 ± 0.3	-19.7 ± 0.1
$K_{mD,3oh}$	RecBCD·3ohDNA·mD	80.8± 25.7	1.5 ± 0.3	342.2 ± 7.5	5.9 ± 1.2	0.44 ± 0.2	-23.3 ± 0.8	-19.8 ± 0.1

1. mantADP (mD); mantAMP-pNp (mMp);

2. Fitted parameters for the sum of two Hills equations, $\boldsymbol{K_s}$ & $\boldsymbol{K_w}$ are the equilibrium dissociation constants for nucleotides association to the strong and weak binding sites, respectively; $\boldsymbol{n_s}$ & $\boldsymbol{n_w}$ are the Hill coefficients for nucleotides' association to the strong and the weak binding sites, respectively.

3. $\Delta G°'_s = RTln(K_s)$ and $\Delta G°'_w = -RTln(K_{sw})$ are the standard binding energies at 1 M ligand, R is the gas constant (8.314 J mol^{-1} K^{-1}), T = 298.15°K for the strong and weak binding sites, respectively.

4. Previously reported (Zananiri & Gaydar *et al.* 2017).

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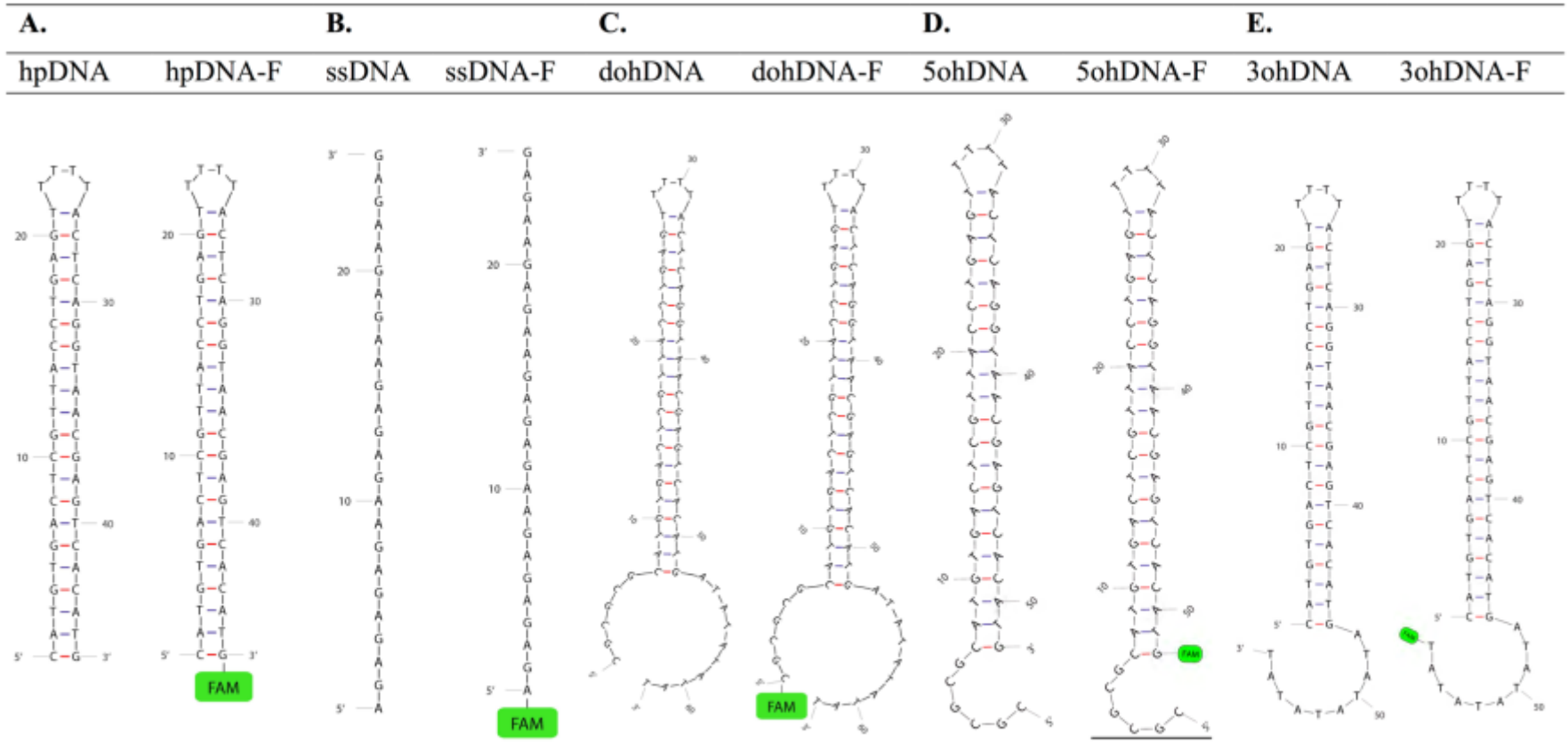
Table 4. Thermodynamics coupling parameters associated with RecBCD, RecBCD·DNA, RecBCD·DNA· nucleotides

Detailed thermodynamics box	Thermodynamics coupling parameter	[1]Thermodynamics coupling constants
Mp,hp	$K_{Mp,hp}/K_{Mp}$	1.4
	$K_{hp,Mp}/K_{hp}$	2
D,hp	$K_{D,hp}/K_{D}$	2.2
	$K_{hp,D}/K_{hp}$	3
Mp,ss	$K_{Mp,ss}/K_{Mp}$	0.4
	$K_{ss,Mp}/K_{ss}$	0.6
D,ss	$K_{D,ss}/K_{D}$	1.2
	$K_{ss,D}/K_{ss}$	1.9
Mp,doh	$K_{Mp,doh}/K_{Mp}$	2
	$K_{doh,Mp}/K_{doh}$	1.8
D,doh	$K_{D,doh}/K_{D}$	5.4
	$K_{doh,D}/K_{doh}$	42.9
Mp,5oh	$K_{Mp,5oh}/K_{Mp}$	0.9
	$K_{5oh,Mp}/K_{5oh}$	1
D,5oh	$K_{D,5oh}/K_{D}$	5.3
	$K_{5oh,D}/K_{5oh}$	0.3
Mp,3oh	$K_{Mp,3oh}/K_{Mp}$	1.4
	$K_{3oh,Mp}/K_{3oh}$	1.8
D,3oh	$K_{D,3oh}/K_{D}$	6.1
	$K_{3oh,D}/K_{3oh}$	0.2

1. Calculated from detailed thermodynamic squares Fig. 5.

Figure 1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B.
C.
D.
E.
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hpDNA-F
ssDNA
ssDNA-F
dohDNA
dohDNA-F
5ohDNA
5ohDNA-F
3ohDNA
3ohDNA-F
FAM
FAM
FAM
FAM

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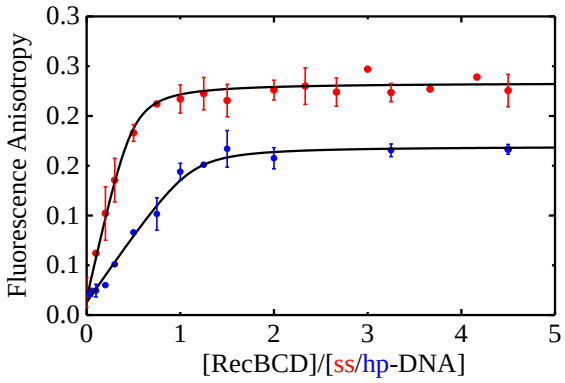
Figure 2

A.



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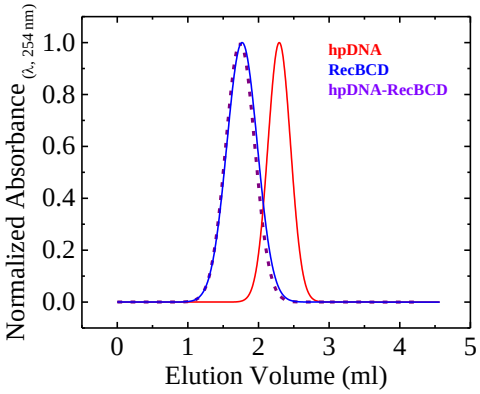

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Figure 3

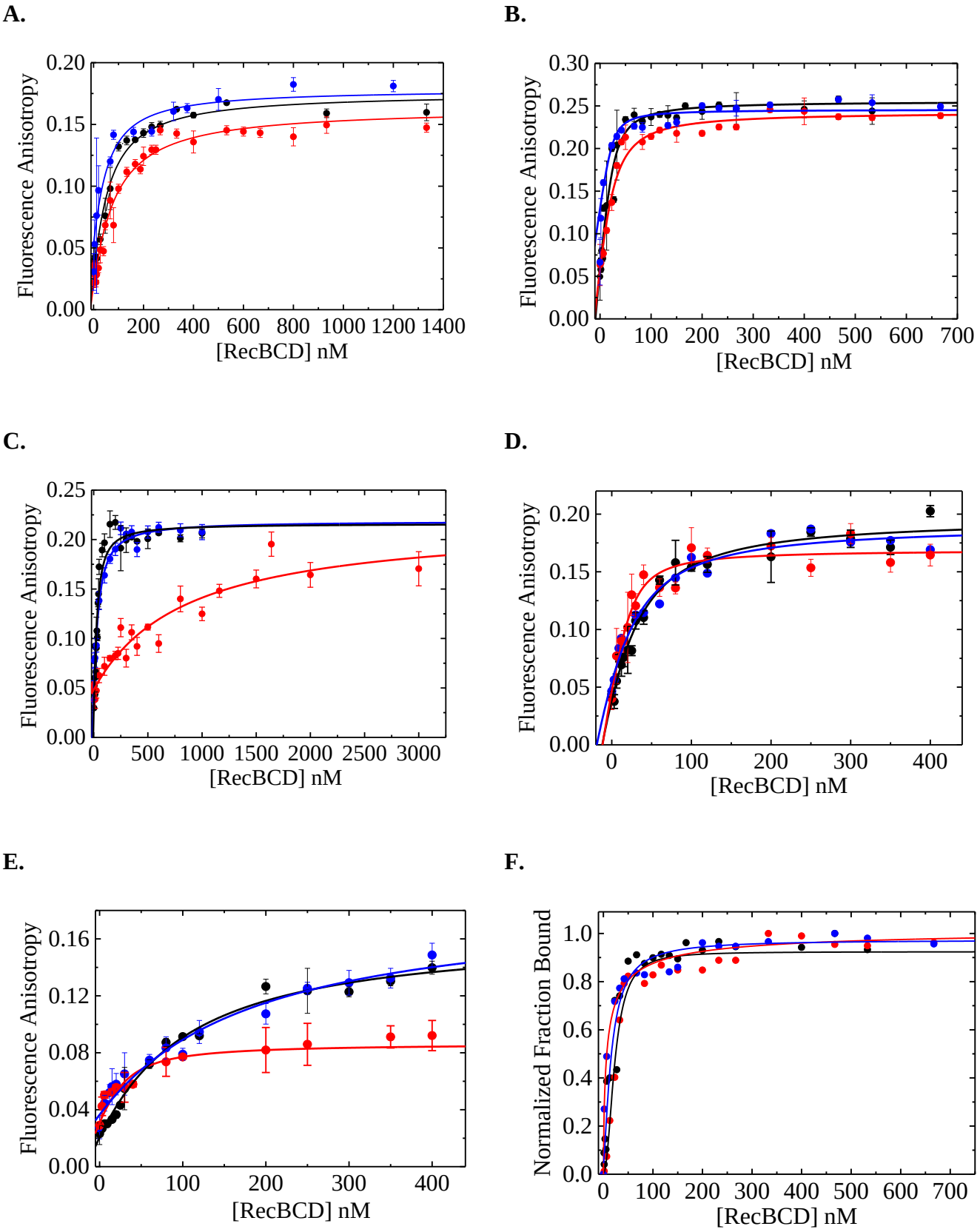

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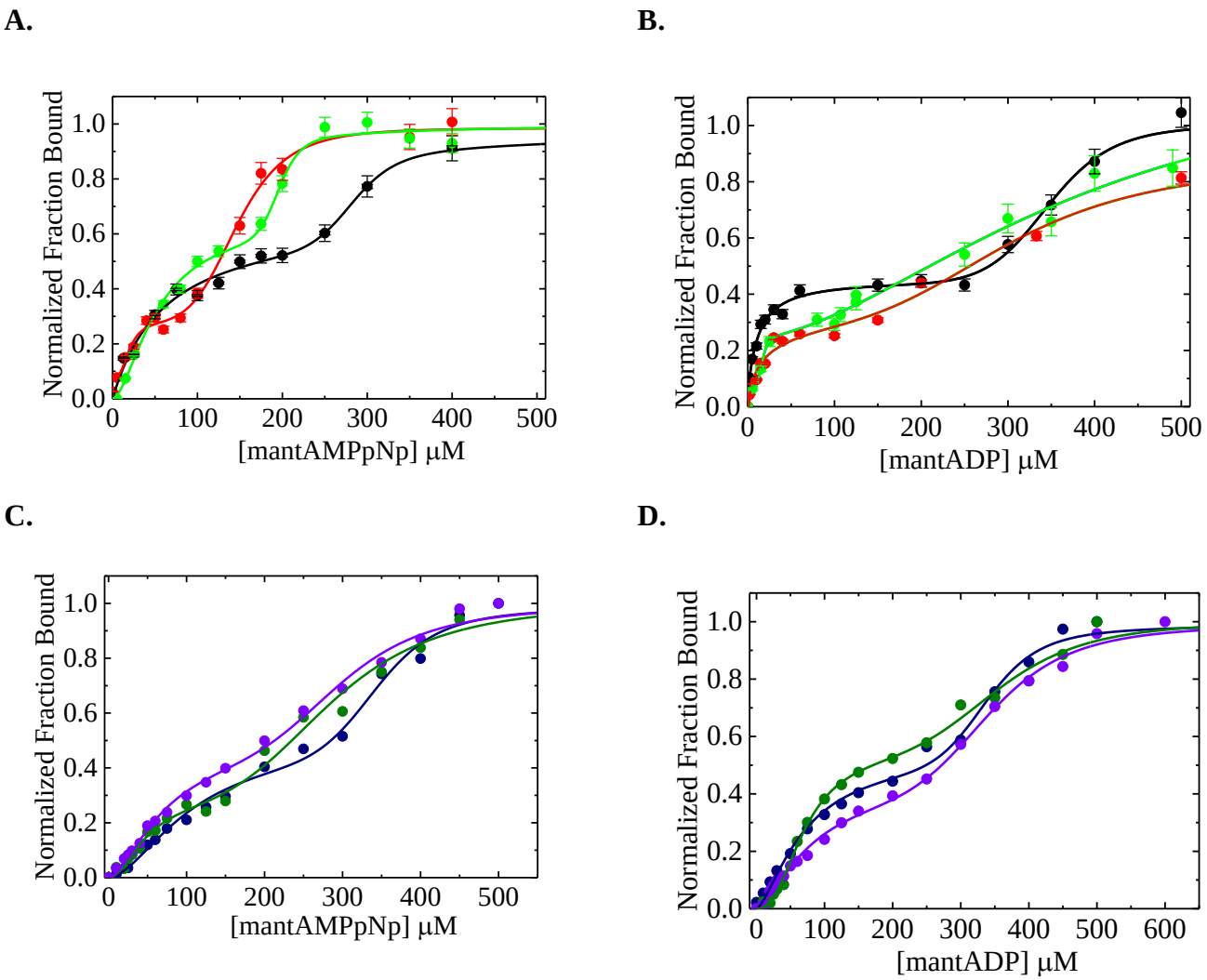
Figure 4



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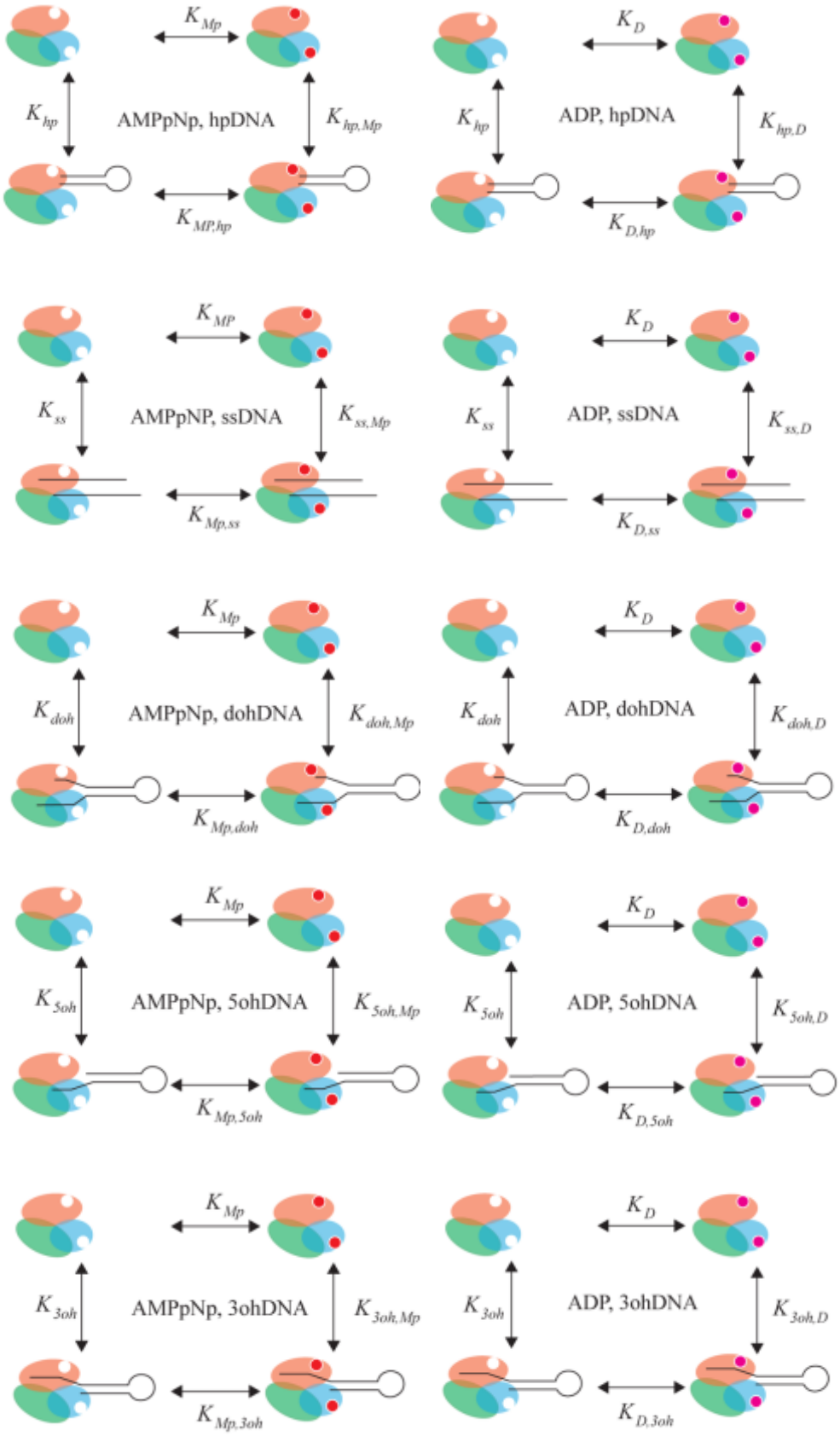
Figure 5