Modeling DNA Unwinding by AddAB Helicase–Nuclease and Modulation by Chi Sequences: Comparison with AdnAB and RecBCD

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Abstract

Introduction—AddAB enzyme is a helicase–nuclease complex that initiates recombinational repair of double-stranded DNA breaks. It catalyzes processive DNA unwinding and concomitant resection of the unwound strands, which are modulated by the recognition of a recombination hotspot called Chi in the 3′-terminated strand. Despite extensive structural, biochemical and single molecule studies, the detailed molecular mechanism of DNA unwinding by the complex and modulation by Chi sequence remains unclear.

Methods—A model of DNA unwinding by the AddAB complex and modulation by Chi recognition was presented, based on which the dynamics of AddAB complex was studied analytically.

Results—The theoretical results explain well the available experimental data on effect of DNA sequence on velocity, effect of Chi recognition on velocity, static disorder peculiar to the AddAB complex, and dynamics of pausing of wild-type and mutant AddAB complexes occurring at Chi or Chi-like sequence. Predictions were provided. Comparisons of AddAB complex with other helicase–nuclease complexes such as RecBCD and AdnAB were made.

Conclusions—The study has strong implications for the molecular mechanism of DNA unwinding by the AddAB complex. The intriguing issues are addressed of why Chi recognition is an inefficient process, how AddAB complex pauses upon recognizing Chi sequence, how the paused state transits to the translocating state, why the mutant AddAB with a stronger affinity to Chi sequence has a shorter pausing lifetime, why the pausing lifetime is sensitive to the solution temperature, and so on.

Keywords—Helicase, DNA unwinding activity, DNA repair, RecBCD, AdnAB, Molecular motor.

INTRODUCTION

Double-stranded DNA breaks (DSBs) are lethal lesions that are generated frequently by a variety of exogenous and endogenous sources such as ionizing radiation and DNA replication on a chemically flawed template. Repair of DSBs by homologous recombination is initiated by the long-range resection of the DNA end to form a 3′-terminated DNA overhang suitable for RecA/Rad51-mediated strand exchange.⁹ In bacterial cells, the primary pathway for recombinational DSB repair is initiated by three structural classes of helicase–nuclease complex: *Escherichia coli* RecBCD, *Bacillus subtilis* AddAB, and *Mycobacterium smegmatis* AdnAB.^{4,5,15,16,19–21,28} A helicase–nuclease complex recognizes the broken end and then promotes its processive DNA unwinding and concomitant resection of the two unwound strands, which are modulated by the recognition of a specific sequence called Chi (crossover hotspot instigator) in the 3′-terminated strand.^{4,5,16,21,28} It is generally believed that binding of Chi sequence within the enzyme complex sequesters the 3′-terminated strand and prevents it from being digested.^{1,3} Continued unwinding and degradation of the 5′-terminated strand by the complex thus produces a long 3′-ssDNA overhang. In this work, we focus mainly on the processive DNA unwinding and modulation by Chi recognition by AddAB helicase–nuclease complex.

AddAB complex is composed of two subunits—AddA and AddB^{10,13} (see Fig. 1). AddA subunit contains a helicase domain, which is composed of the helicase core subdomains 1A and 2A and the so called accessory subdomains 1B and 2B, a nuclease domain and an ‘arm’ domain. The helicase domain translocates along 3′–5′ single strand, the nuclease domain digests 3′-terminated strand, and the arm domain contacts the downstream DNA duplex. AddB subunit contains an inactivated helicase-like domain

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FIGURE 1. Schematic diagram of structure of AddAB helicase–nuclease complexed with DNA. (a) Upon reaching Chi sequence, the Chi-binding site binds the Chi sequence. Seg I denotes the short ssDNA segment between the Chi sequence and the segment bound by AddA's subdomain 1A. (b) After recognition of the Chi sequence, an ssDNA loop can be formed between the Chi sequence and the segment bound by AddA's subdomain 1A.

and a nuclease domain. The inactivated helicase-like domain shares the same overall topology as canonical Superfamily I (SF1) helicases but lacks the characteristic motifs required for classical DNA translocation and unwinding activity, and the nuclease domain digests 5'-terminated strand. The Chi-binding site, which has a specific interaction with Chi sequence (5'-AGCGG-3'), extends across from the AddB subunit into the AddA nuclease domain.

Chi recognition by AddAB molecules is an inefficient process,²⁹ with a probability smaller than 0.1 for recognition of a single Chi sequence.² Using magnetic tweezers, Carrasco *et al.*² investigated in detail the dynamics of DNA unwinding by single AddAB molecules. It was found that individual AddAB enzymes display large variability in their velocity distributions (called static disorder). The enzymes could pause at Chi sequence and upon restart of translocation downstream of the Chi sequence the velocity decreased. AddAB was also prone to stochastic paus-

ing due to transient recognition of Chi-like sequence (a set of 5 nt with one mismatch from *bona fide* Chi sequence), with the pausing lifetime shorter than that at *bona fide* Chi sequence. Intriguingly, it was revealed that although a mutant AddAB enzyme showed higher affinity to Chi sequence than the wild type, the lifetime of the pauses induced by Chi recognition for the mutant AddAB enzyme becomes shorter.⁶ At higher temperature (37 °C) the lifetime of the pauses is decreased markedly.⁶

Despite the extensive structural, biochemical and single molecule experimental studies, the detailed molecular mechanism of modulation of DNA unwinding velocity and pausing by recognition of Chi sequence is not clear. For example, why does AddAB complex possess peculiarly large variability in velocity distributions? Why is Chi recognition by AddAB complex an inefficient process? Upon recognizing Chi sequence, how does AddAB complex pause? How does the transition from the paused state to the translocating state take place? How does DNA unwinding velocity decrease after Chi recognition? Why does the mutant AddAB with a stronger affinity to Chi sequence has a shorter pausing lifetime? Does the pausing at Chi-like sequences show a similar or different mechanism from that at *bona fide* Chi sequence? Why is the pausing lifetime sensitive to the solution temperature? In this work, we present a model of DNA unwinding by AddAB complex and modulation by Chi recognition, based on which we study analytically the dynamics of AddAB complex, explaining quantitatively the available single molecule experimental data and addressing the above-mentioned unclear issues. Moreover, to further test the model, predictions are presented. In addition, comparisons of AddAB with RecBCD and AdnAB are made.

THE MODEL

Model of ssDNA Translocation and DNA Unwinding by AddA Helicase Domain

We first present the model of ssDNA translocation and DNA unwinding by single AddA helicase domain. The structure showed that AddA helicase domain is composed of four subdomains, two of which (1A and 2A) are the helicase core subdomains found in all SF1 and SF2 helicases and the other two (1B and 2B) are the so-called accessory subdomains (see Fig. 1a). Thus, AddA helicase domain should share the same mechanism of DNA unwinding as other SF1 and SF2 helicases such as HCV NS3, PcrA, UvrD, RecQ, Dda, XPD, *etc.*, as proposed before.^{24–26}

FIGURE 2. Schematic of the model of DNA unwinding by single AddA helicase domain (see text for detailed description). (a) Subdomains 1A and 2A in nucleotide-free (ϕ) state bind with intermediate and high affinities to 3'–5' ssDNA ssDNA, respectively. (b, b') Cleft closing induced by ATP binding causes 1A to move towards 2A that is kept fixedly to ssDNA (with a probability p_{E1}) or causes 2A to move towards 1A that is kept fixedly to ssDNA (with a probability $1 - p_{E1}$). (c, c') The cleft closing changes the affinity of 2A for ssDNA from the high to low value. (d, e') Cleft opening induced by release of ATP-hydrolysis products causes 2A to move downstream away from 1A by unwinding a DNA base pair (with a probability p_{E2}). (d', e) The cleft opening can also cause 1A to move upstream away from 2A that is kept fixedly to ssDNA (with a probability $1 - p_{E2}$). (f, f') The cleft opening then changes the affinity of 2A for ssDNA from the low to high value. Shaded in gray denotes that the transition rarely occurs.

The model for AddA helicase domain is built up based on the following argument and structural evidence. (i) It is argued that subdomain 1A has an intermediate affinity for 3'–5' ssDNA, which is independent or weakly dependent on the nucleotide state, while subdomain 2A binds strongly to 3'–5' ssDNA in the absence of ATP whereas binds weakly in the presence of ATP. This would give the affinity of AddA helicase domain for ssDNA in the presence of ATP to be reduced evidently relative to that in the absence of ATP. For example, this argument is consistent with the available biochemical data for helicase HCV NS3.¹¹ The test of the similar feature for the affinity of AddA helicase domain by future experiments is desirable. (ii) The available structural data for AddAB showed that ATP binding to the open cleft between 1A and 2A results in closing of the cleft, with 1A and 2A moving

together by one DNA base, while the release of ATP-hydrolysis products opens the cleft.¹⁰

The model is schematically shown in Fig. 2. We begin a chemomechanical cycle with AddA in nucleotide-free (ϕ) state, where 2A and 1A bind ssDNA with high and intermediate affinities, respectively (Fig. 2a). The closing of cleft between the two subdomains by ATP binding either causes 1A with a high probability (p_{E1}) to move toward 2A that is fixed to ssDNA by overcoming the intermediate affinity of 1A for ssDNA (Fig. 2b) or causes 2A with a low probability ($1 - p_{E1}$) to move toward 1A that is fixed to ssDNA by overcoming the high affinity of 2A for ssDNA (Fig. 2b'). The cleft closing induces the transition of 2A from the high-affinity to low-affinity state for ssDNA, with unactivated E·ATP complex (Figs. 2b and 2b') transiting to activated E*·ATP complex (Figs. 2c or 2c'),

where E denotes AddA enzyme. After ATP hydrolysis, the opening of cleft by release of the hydrolysis products can either cause 2A with a probability (p_{E2}) to move away from 1A that is kept fixed to ssDNA by overcoming the low affinity of 2A for ssDNA plus the free energy of unwinding one base pair (Figs. 2d or 2e') or cause 1A with a probability ($1 - p_{E2}$) to move away from 2A that is kept fixed to ssDNA by overcoming the intermediate affinity of 1A for ssDNA (Figs. 2e or 2d'). The cleft opening induces the transition of 2A from the low-affinity to high-affinity state for ssDNA, with activated $E^* \cdot \phi$ complex of Figs. 2d or 2d' transiting to unactivated $E \cdot \phi$ complex of Figs. 2f or 2f', or with activated $E^* \cdot \phi$ complex of Figs. 2e or 2e' transiting to unactivated $E \cdot \phi$ complex of Fig. 2a. From Figs. 2a to 2f the AddA helicase domain has made a + 1-bp step along ssDNA, while from Figs. 2a to 2f' has made a - 1-bp step along ssDNA. As will be seen later, before and after modulation by Chi recognition the transition from Figs. 2a and 2b' occurs rarely, and at Chi sequence the transition from Figs. 2a, 2b', 2c' and 2e' to a occurs frequently but the transition from Figs. 2c', 2d' and 2f' (shaded in grey) occurs rarely. Hence, the - 1-bp steps occur rarely.

Modulation by Chi Recognition on AddAB Complex

From the structure of AddAB complex^{10,13} (see Fig. 1a), it is noted that the movement of AddA's subdomain 1A is coupled with that of AddB subunit and AddA nuclease domain, and the Chi-binding site is located in both the AddB subunit and AddA nuclease domain. In addition, it is seen that upon the Chi-binding site binding the Chi sequence, a short ssDNA segment (called ssDNA segment I, denoted by Seg I in Fig. 1a) is present in between the Chi sequence and the segment bound by AddA's subdomain 1A. As evidenced by the available biochemical data,³ the Chi-binding site has a high affinity to the Chi sequence. By contrast, it is considered here that AddB subunit has a weak affinity to ssDNA free of the Chi sequence. Thus, the AddAB complex has a weak affinity to ssDNA Seg I.

Since AddA's subdomain 1A binds ssDNA, it is expected that when Chi sequence binds strongly the Chi-binding site, the downstream movement of AddA's subdomain 1A coupled with that of AddB subunit and AddA nuclease domain can easily induce distortion of ssDNA Seg I by forming an ssDNA loop (in Fig. 1a we show the schematic of AddAB-DNA structure upon Chi recognition, where the loop has not been formed, while in Fig. 1b we show the schematic of a structure of AddAB-DNA complex after Chi recognition, where the loop has been formed). Denoting by E_{loop} (see Table 1) the energy change of

TABLE 1. Definitions of parameters used in the work.

Parameters	Definition
E_{loop}	Energy change of distorting ssDNA seg I to initiate ssDNA loop formation
E_{χ}	Binding energy of the Chi-binding site to the Chi sequence
ΔE_{1A}^{loop}	$E_{loop} - E_{\chi}$
$E_1^{(1A)}$	Intermediate affinity of domain 1A for ssDNA
$E_S^{(2A)}$	High affinity of domain 2A for ssDNA in unactivated State $E \cdot \phi$ and State $E \cdot ATP$
$E_W^{(2A)}$	Weak affinity of domain 2A for ssDNA in activated State $E^* \cdot ATP$ and State $E^* \cdot \phi$
ΔE_1	$E_S^{(2A)} - E_1^{(1A)}$
ΔE_2	$E_1^{(1A)} - E_W^{(2A)}$
$\Delta E_1^{(1A)}$	Reduction of $E_1^{(1A)}$ caused by strong binding of Chi-binding site to Chi sequence
E_{bp}	Free-energy change of unwinding 1 bp
ΔE_{loop}^1	$E_{loop} - \Delta E_1$
ΔE_1^{χ}	$E_{\chi} - \Delta E_1$
k	ATPase rate

distorting ssDNA Seg I to initiate loop formation, the rate of initiating loop formation is $k_{loop} P \exp(-\beta E_{loop})$, where $\beta^{-1} = k_B T$ is the thermal energy. Alternatively, the downstream movement of AddA's subdomain 1A coupled with that of AddB subunit and AddA nuclease domain can also disrupt the strong interaction of the Chi sequence with the Chi-binding site without formation of an ssDNA loop. Denoting by E_{χ} (see Table 1) the binding energy of the Chi sequence to the Chi-binding site, the rate of disrupting the interaction of the Chi sequence with the Chi-binding site is $k_{\chi} P \exp(-\beta E_{\chi})$. The probability of initiating loop formation induced by the downstream movement of AddA's subdomain 1A coupled with that of AddB subunit and AddA nuclease domain can then be approximately calculated by $p_{loop} = k_{loop} / (k_{loop} + k_{\chi})$, i.e., $p_{loop} = \exp(-\beta E_{loop}) / [\exp(-\beta E_{loop}) + \exp(-\beta E_{\chi})]$, which can be rewritten as

$$p_{loop} = \frac{\exp(-\beta \Delta E_{\chi}^{loop})}{\exp(-\beta \Delta E_{\chi}^{loop}) + 1}, \quad (1)$$

where $\Delta E_{\chi}^{loop} = E_{loop} - E_{\chi}$ (see Table 1). The probability of disrupting the interaction of the Chi sequence with the Chi-binding site is then calculated by $1 - p_{loop}$.

By contrast, in the case of RecBCD when Chi sequence binds strongly the Chi-binding site locating in RecC subunit, a much shorter ssDNA segment (also called ssDNA Seg I) is present in between the Chi sequence and the segment bound by RecB's subdomain 1A.^{14,22} In addition, since the Chi sequence in the case of RecBCD (5'-GCTGGTGG-3') has a longer length

than in the case of AddAB (5′-AGCGG-3′), the binding energy of Chi sequence to the Chi-binding site in the case of RecBCD should be larger than that in the case of AddAB. Thus, it is expected that in the case of RecBCD a much larger energy change is required to distort ssDNA Seg I or to disrupt the interaction of the Chi sequence with the Chi-binding site than the binding energy between RecC and RecB’s subdomain 1A. Consequently, the downstream movement of RecB’s subdomain 1A would result in the disruption of the relatively weak interaction between RecB’s subdomain 1A and RecC with a nearly 100% probability, as proposed before.²⁷ As RecB moves downstream an ssDNA loop between RecB and RecC forms gradually.

In addition, we argue here that upon Chi recognition the strong interaction of Chi sequence with AddB subunit induces a conformational change of AddB subunit, as experimental data indicated,⁶ which in turn induces a slight conformational change of AddA’s subdomain 1A *via* the strong interaction with AddB subunit. The slight conformational change of AddA’s subdomain 1A affects the interaction of 1A with ssDNA by reducing slightly the affinity ($E_1^{(1A)}$) between them (see Table 1). We denote this reduction of $E_1^{(1A)}$ by $\Delta E_1^{(1A)}$ (see Table 1).

RESULTS

Based on the model presented in the above section, we will study the dynamics of AddAB complex in this section. In “DNA Unwinding Velocity” and “Dynamics of Pauses Induced by Chi Recognition” sections we will focus on constant temperature $T = 293$ K (20 °C) to explain the single molecule experimental data of Carrasco *et al.*² In “Temperature Dependence of the Dynamics” section we will study the temperature dependence of the dynamics.

DNA Unwinding Velocity

Based on the model (Fig. 2), for the single AddA helicase domain its dynamics of DNA unwinding is determined by three parameters for the enzyme: probability p_{E1} (defined in Fig. 2), probability p_{E2} (defined in Fig. 2), and ATPase rate of the AddA helicase domain, k (see Table 1). Here, we focus on saturating ATP concentration and thus k corresponds to the rate constant of ATPase activity at saturating ATP. Probabilities p_{E1} and p_{E2} can be calculated as follows. Denoting by $E_1^{(1A)}$ the intermediate affinity of subdomain 1A for ssDNA and by $E_S^{(2A)}$ the high affinity of subdomain 2A for ssDNA in unactivated State $E \cdot \phi$ and State $E \cdot \text{ATP}$ (see Table 1), the rate for subdomain 1A to move forward (i.e., the rate of transition from Figs. 2a and 2b) is

$k_F^{(1A)} \propto \exp(-\beta E_1^{(1A)})$ and the rate for subdomain 2A to move backward (i.e., the rate of transition from Figs. 2a and 2b) is $k_B^{(2A)} \propto \exp(-\beta E_S^{(2A)})$. The probability p_{E1} can then be approximately calculated by $p_{E1} = k_F^{(1A)} / (k_F^{(1A)} + k_B^{(2A)})$, i.e., $p_{E1} = \exp(-\beta E_1^{(1A)}) / [\exp(-\beta E_1^{(1A)}) + (-\beta E_S^{(2A)})]$, which is rewritten as

$$p_{E1} = \frac{1}{1 + \exp(-\beta \Delta E_1)}, \quad (2)$$

where $\Delta E_1 = E_S^{(2A)} + E_1^{(1A)}$ (see Table 1). Similarly, denoting by $E_W^{(2A)}$ the weak affinity of subdomain 2A for ssDNA in activated State $E^* \cdot \text{ATP}$ and State $E^* \cdot \phi$ and by E_{bp} the base-pairing energy of a DNA base pair (see Table 1), the probability p_{E2} can then be approximately calculated by $p_{E2} = \exp[-\beta(E_W^{(2A)} + E_{bp})] / \{\exp[-\beta(E_W^{(2A)} + E_{bp})] + \exp(-\beta E_1^{(1A)})\}$, which is rewritten as

$$p_{E2} = \frac{\exp(-\beta E_{bp})}{\exp(-\beta E_{bp}) + \exp(-\beta \Delta E_2)}, \quad (3)$$

where $\Delta E_2 = E_1^{(1A)} - E_W^{(2A)}$ (see Table 1). With p_{E1} , p_{E2} and k , the DNA unwinding velocity can be calculated by

$$v = [p_{E1}p_{E2} - (1 - p_{E1})(1 - p_{E2})]kd, \quad (4)$$

where $d = 1$ bp is the step size.

For the AddAB complex, the movement of AddA’s subdomain 1A is coupled with that of AddB subunit and AddA nuclease domain. Thus, before and after Chi recognition the DNA unwinding velocity v can still be calculated by Eqs. (2)–(4), but with $E_1^{(1A)}$ being replaced with $E_1^{(1AB)} = E_1^{(1A)} + E_B$, where $E_1^{(1A)}$ is the intermediate affinity of subdomain 1A for ssDNA, as defined above, and E_B is the weak affinity of AddB subunit and AddA nuclease domain for ssDNA free of Chi and Chi-like sequences. For an approximation, we consider $E_B \approx 0$.

To calculate the DNA unwinding velocity by the AddAB complex in regions free of Chi and Chi-like sequences using Eqs. (2)–(4), we require values of parameters ΔE_1 , ΔE_2 , k and E_{bp} (see Table 1). The single molecule data showed that at saturating ATP the mean DNA unwinding velocity by AddAB is about 375 bp/s at 20 °C temperature.² Considering that the ssDNA translocation velocity should be slightly larger than DNA unwinding velocity and nearly each ATP hydrolysis produces a forward step of 1 nt during ssDNA translocation, we take rate constant of ATPase activity to be slightly larger than 375 s^{−1}. Thus, we take ATPase rate $k = 410$ s^{−1} at $T = 20$ °C in our calculations (see Table 2), which is independent of Chi

recognition. From the nearest-neighboring thermodynamic model for DNA duplex stability,²³ the average value of a DNA base-pairing energy is calculated to be $E_{bp} = 2.5k_B T$ (see Table 2). Since $E_S^{(2A)}$ has a large value, we take $\Delta E_1 \gg 0$ (see Table 2). The average value of ΔE_2 is taken to be $4k_B T$ (see Table 2).

First, we study the effect of the change in DNA base-pairing energy on DNA unwinding velocity without Chi recognition. From the nearest-neighboring thermodynamic model for DNA duplex stability,²³ it is estimated that for an A:T base pair the base-pairing energy is about $E_{bp} = 1.5k_B T$, whereas for a G:C base pair the base-pairing energy is about $E_{bp} = 4k_B T$. Thus, we calculate DNA unwinding velocity v as a function E_{bp} in the range of $E_{bp} = 1.5k_B T - 4k_B T$ (Fig. 3). It is seen that DNA unwinding velocity v in GC-rich regions (with larger values of E_{bp}) is evidently smaller than in AT-rich regions (with smaller values of E_{bp}), which is consistent with the single molecule experimental data.²

Second, we study the effect of Chi recognition on DNA unwinding velocity. Based on our model, when the Chi-binding site binds Chi sequence the affinity of AddA's subdomain 1A for ssDNA, $E_1^{(1A)}$, is reduced by $\Delta E_1^{(1A)} > 0$, giving ΔE_2 to be replaced with $\Delta E_2 -$

TABLE 2. Parameter values at temperature $T = 293$ K (20 °C).

Parameters	Values
k	410 s^{-1}
E_{bp} (average value)	$2.5k_B T$ (10.1 pN nm)
ΔE_1	$\gg 0$
ΔE_2 (average value before Chi recognition)	$4k_B T$ (16.2 pN nm)
ΔE_1^{loop}	$6k_B T$ (24.3 pN nm)
ΔE_1^z (Chi sequence)	$6.9k_B T$ (27.9 pN nm)
ΔE_1^z (Chi-like sequences)	$4.9k_B T$ (19.8 pN nm)

Values of E_{bp} , ΔE_2 , ΔE_1^{loop} and ΔE_1^z outside brackets are in units of $k_B T$ with $T = 293$ K, while values inside brackets are in units of pN nm.

FIGURE 3. DNA unwinding velocity of AddAB complex vs. DNA base-pairing energy.

$\Delta E_1^{(1A)}$, and ΔE_1 to be replaced with $\Delta E_1 + \Delta E_1^{(1A)} \gg 0$. Here, we consider only the case that after Chi recognition the Chi-binding site is still bound to the Chi sequence and ssDNA loop is formed. In Fig. 4 we show the calculated results of the relative decrease of velocity, $(v_{\text{before}} - v_{\text{after}})/v_{\text{before}}$, as a function of $\Delta E_1^{(1A)}$, where v_{before} and v_{after} are values of v before and after Chi sequence bound to the Chi-binding site, respectively. It is seen that the velocity decreases evidently with the increase of $\Delta E_1^{(1A)}$. In particular, at $\Delta E_1^{(1A)} = 0.7k_B T$, the velocity decreases by about 16%, which is identical to the single molecule data,² implying that the strong interaction of Chi sequence with AddB induces the affinity of AddA's subdomain 1A for ssDNA to decrease by about $0.7k_B T$.

Third, we give an explanation of the single molecule experimental data showing large variability of velocity distributions or static disorder peculiar to the helicase–nuclease complexes AddAB² and RecBCD.¹² As mentioned just above (Fig. 4), we argue that the affinity of AddA's subdomain 1A for ssDNA, $E_1^{(1A)}$, is sensitively influenced by the conformational change of AddB subunit induced by Chi recognition. This implies that $E_1^{(1A)}$ is affected sensitively by the change of the environment, i.e., a slight variation of the environment can affect value of $E_1^{(1A)}$ and thus affect value of ΔE_2 . In other words, due to the slight variations of the environment ΔE_2 can have different values. In Fig. 5 we show the calculated results of DNA unwinding velocity as a function of ΔE_2 . It is seen that varying value of ΔE_2 gives a large variation of DNA unwinding velocity. In summary, we explain that the large vari-

FIGURE 4. Relative decrease of DNA unwinding velocity of AddAB complex vs. the reduction of the intermediate affinity of AddA's subdomain 1A for ssDNA ($\Delta E_1^{(1A)}$) induced by the interaction of the Chi-binding site with Chi sequence. The relative decrease of velocity is defined as $(v_{\text{before}} - v_{\text{after}})/v_{\text{before}}$, where v_{before} and v_{after} are values of velocity before and after Chi sequence bound to the Chi-binding site, respectively. The vertical dotted line indicates the value of $\Delta E_1^{(1A)}$ at which the relative decrease of velocity, $(v_{\text{before}} - v_{\text{after}})/v_{\text{before}} = 16\%$.

FIGURE 5. DNA unwinding velocity of AddAB complex vs. ΔE_2 . $\Delta E_2 = E_1^{(1A)} - E_W^{(2A)}$ is the difference between the intermediate binding energy of subdomain 1A to ssDNA ($E_1^{(1A)}$) and the weak binding energy of subdomain 2A to ssDNA in activated State E*ATP and State E* ϕ ($E_W^{(2A)}$).

ability of velocity distributions or static disorder results from the variation of value of ΔE_2 or $E_1^{(1A)}$, in turn arising from the slight variation of the environment. Different values of $E_1^{(1A)}$ giving different velocities proposed here could correspond to different conformational sub-states giving different velocities proposed before by Liu *et al.*¹²

Dynamics of Pauses Induced by Chi Recognition

At Chi sequence, the effect of its strong interaction with the Chi-binding site on the movement of AddA helicase domain is equivalent to replacing $E_1^{(1A)}$ with $E_1^{(1A)} + E_{\text{loop}}$ or $E_1^{(1A)} + E_\chi$ (see “[Modulation by Chi Recognition on AddAB Complex](#)” section). Thus, ΔE_1 is replaced with $\Delta E_1 - E_{\text{loop}}$ or $\Delta E_1 - E_\chi$. Since both E_{loop} and E_χ have large values, it is expected that both $\Delta E_1 - E_{\text{loop}}$ and $\Delta E_1 - E_\chi$ have negative values of large magnitudes, giving p_{E1} to be very small, as noted from Eq. (2). This implies that during the closing of the cleft between subdomains 1A and 2A induced by ATP binding, 1A moves with a very low probability toward 2A. Instead, the cleft closing causes 2A to move toward 1A by overcoming the strong interaction of 2A with ssDNA.¹ Then, the cleft opening induced by the release of ATP-hydrolysis products causes 2A to

move away from 1A by overcoming the weak interaction of 2A with ssDNA. As a result, multiple ATPase cycles take place with no movement of 1A along ssDNA, implying the occurrence of a pause. The termination of the pause corresponds to the case when 1A moves along ssDNA toward 2A during cleft closing induced by ATP binding, either with the Chi-binding site not leaving the Chi sequence but with formation of ssDNA loop or with the Chi-binding site leaving the Chi sequence without formation of ssDNA loop. Then, the AddAB complex translocates along DNA unhindered, because after the ssDNA loop has formed the increase of the loop size has little resistance to the forward movement of AddA’s subdomain 1A coupled with that of AddB subunit and AddA nuclease domain or after the Chi-binding site has left the Chi sequence the AddB subunit and AddA nuclease domain have a very weak affinity ($E_B \approx 0$) to ssDNA.

Upon Chi recognition, the probability P_{E1} that 1A moves toward 2A during cleft closing induced by ATP binding can be approximately calculated by

$$p_{E1} = \frac{\exp[-\beta(E_1^{(1A)} + E_{\text{loop}})]}{\exp[-\beta(E_1^{(1A)} + E_{\text{loop}})] + \exp(-\beta E_S^{(2A)})} P_{\text{loop}} + \frac{\exp[-\beta(E_1^{(1A)} + E_\chi)]}{\exp[-\beta(E_1^{(1A)} + E_\chi)] + \exp(-\beta E_S^{(2A)})} (1 - P_{\text{loop}}). \quad (5)$$

Equation (5) can be rewritten as

$$p_{E1} = \frac{\exp(-\beta \Delta E_1^{\text{loop}})}{\exp(-\beta \Delta E_1^{\text{loop}}) + 1} P_{\text{loop}} + \frac{\exp(-\beta \Delta E_1^\chi)}{\exp(-\beta \Delta E_1^\chi) + 1} (1 - P_{\text{loop}}), \quad (6)$$

where $\Delta E_1^{\text{loop}} = E_{\text{loop}} - \Delta E_1$ and $\Delta E_1^\chi = E_\chi - \Delta E_1$ (see Table 1). Then, the mean lifetime of the pause is calculated by

$$\tau_{\text{pause}} = \frac{1}{k p_{E1}}. \quad (7)$$

First, we consider the pause that occurs at Chi sequence for mutant enzyme (AddAB^{E129A}) with E129A mutation in AddB domain 1B. The experimental data showed that the E129A mutation increases greatly (by about 16-fold relative to the wild-type case) the binding affinity to Chi sequence.⁶ Thus, it is expected that for the case of AddAB^{E129A}, $E_\chi \gg E_{\text{loop}}$, i.e., $\Delta E_\chi^{\text{loop}} = E_{\text{loop}} - E_\chi \ll 0$. From Eq. (1) we have $p_{\text{loop}} \approx 1$, implying that after the pause the Chi sequence still binds the Chi-binding site. Thus, it is expected that if there are many Chi sequences in a DNA substrate, only one pause can occur in one trace of DNA unwinding. With $p_{\text{loop}} = 1$ and by taking

¹It is also possible that the force arising from the cleft closing induced by ATP binding cannot overcome the strong binding force of 2A to ssDNA. Thus, it is expected that after ATP binding the cleft is still kept in the open conformation or 2A is moved in a position between that in the open cleft and that in the closed cleft by distorting the ssDNA, both causing appearance of the internal tension. These are consistent with the structural data for AddAB structures bound with DNA containing Chi sequence.¹⁰ Then, the release of ATP-hydrolysis products makes 2A in the position in the open cleft with the disappearance of the internal tension. Thus, multiple ATPase cycles take place with no movement of 1A along DNA.

$\Delta E_1^{\text{loop}} = 6k_B T$ (see Table 2), from Eqs. (6) and (7) the calculated mean lifetime of the pause, $\tau_{\text{pause}} = 1$ s, which is identical to the single molecule experimental data.⁶

Then, we study unwinding of DNA duplex containing Chi sequence by mutant enzyme (AddAB^{F210A}) with a point mutation in the Chi recognition domain of AddB.² For AddAB^{F210A}, the binding energy of the Chi-binding site to the Chi sequence, $E_\chi \approx 0$. As $\Delta E_1 \gg 0$ (see Table 2), we have $\Delta E_1^\chi = E_\chi - \Delta E_1 \ll 0$. In addition, we have $E_{\text{loop}} = \Delta E_1^{\text{loop}} + \Delta E_1 \gg 0$ (see Table 2), giving $\Delta E_\chi^{\text{loop}} = E_{\text{loop}} - E_\chi \gg 0$. Thus, from Eq. (1) we have $p_{\text{loop}} \approx 0$. From Eqs. (6) and (7) we have $p_{E1} \approx 1$ and $\tau_{\text{pause}} \approx 1/k$. That is, the ‘pausing’ lifetime at Chi sequence is equal to the dwell time in each step during processive DNA unwinding in the region free of Chi or Chi-like sequence, implying that no pausing at Chi sequence can be observed for AddAB^{F210A}, which is in agreement with the single molecule experimental data.²

Third, we study the pause that occurs at Chi sequence for wild-type AddAB. It is noted that $\Delta E_\chi^{\text{loop}} = E_{\text{loop}} - E_\chi$ in Eq. (1) can be rewritten as $\Delta E_\chi^{\text{loop}} = (E_{\text{loop}} - \Delta E_1) - (E_\chi - \Delta E_1) = \Delta E_1^{\text{loop}} - \Delta E_1^\chi$. Thus, from Eqs. (1), (6) and (7) we see that to calculate probability p_{loop} and pause lifetime τ_{pause} , besides values of parameters $k = 410 \text{ s}^{-1}$ and $\Delta E_1^{\text{loop}} = 6k_B T$ (see above), we also require value of ΔE_1^χ , which is taken here as a variable parameter. In Figs. 6a and 6b we show the calculated results of probability p_{loop} and mean pause lifetime τ_{pause} , respectively, as a function of ΔE_1^χ . As expected, when ΔE_1^χ is small, the probability p_{loop} is small and the lifetime τ_{pause} is also small. Interestingly, while p_{loop} increases monotonically with the increase of ΔE_1^χ , τ_{pause} does not increase monotonically with the increase of ΔE_1^χ , and the maximum value of τ_{pause} occurs at an intermediate value of ΔE_1^χ . This implies that a larger binding energy of the Chi-site to Chi sequence does not necessarily give a longer pausing lifetime, which is in agreement with single molecule experimental data showing that the pausing lifetime for mutant AddAB^{E129A}, which has larger binding affinity to Chi sequence than the wild-type case, is shorter than that for the wild-type case.⁶ As shown just above, when ΔE_1^χ is very large, $p_{\text{loop}} = 1$ and $\tau_{\text{pause}} = 1$ s.

In particular, from Fig. 6b we see that at $\Delta E_1^\chi = 6.9k_B T$ (see Table 2), $\tau_{\text{pause}} = 1.2$ s, which is close to the single molecule experimental data of about 1.4 s for pauses that occur at position of 10× Chi loci (i.e., 10 closely spaced Chi sequences).² This implies that the interaction of the Chi sequence with the Chi-

FIGURE 6. Dynamics of pauses induced by Chi recognition. (a) Probability of the Chi-binding site not leaving the Chi sequence but with formation of ssDNA loop vs. $\Delta E_1^\chi = E_\chi - \Delta E_1$. E_χ is the binding energy of the Chi-binding site to the Chi sequence and $\Delta E_1 = E_S^{(2A)} - E_i^{(1A)}$ is the difference between the strong binding energy of subdomain 2A to ssDNA in unactivated State E- ϕ and State E-ATP ($E_S^{(2A)}$) and the intermediate binding energy of subdomain 1A to ssDNA ($E_i^{(1A)}$). (b) Lifetime of pause vs. $\Delta E_1^\chi = E_\chi - \Delta E_1$.

binding site gives $\Delta E_1^\chi = 6.9k_B T$. Moreover, from Fig. 6a we see that at $\Delta E_1^\chi = 6.9k_B T$, $p_{\text{loop}} = 0.71$. This implies that when a pause induced by Chi recognition occurs, the transition from the paused state to translocating state takes place with about 71% probability that the Chi-binding site has not left the Chi sequence, but with the formation of an ssDNA loop. In other words, there is still about $1 - p_{\text{loop}} = 29\%$ probability that the Chi-binding site has left the Chi sequence, but without formation of an ssDNA loop. The single molecule experimental data showed that about 80% of all traces paused at the position of 10× Chi loci (a set of 10 closely spaced Chi sequences).² Based on the experimental data and our above results of about 29% probability that the Chi-binding site can leave the Chi sequence, it is estimated that there are about $80\% \times 29\% = 23.2\%$ of AddAB enzymes that paused at the 10× Chi loci (called first 10× Chi loci here) and then can leave the first 10× Chi loci. Thus, there are about $23.2\% \times 80\% = 18.56\%$ of AddAB enzymes that can pause at the second 10× Chi loci in Chi10-For-For DNA substrates (two sets of 10 closely spaced Chi sequences). That is, there are about 18.56% of AddAB enzymes that can pause at both loci in Chi10-For-For DNA substrates. This is

close to the single molecule experimental data showing that about 15% of AddAB enzymes paused at both loci.²

Additionally, from Fig. 6b we see that at $\Delta E_1^X = 4.9k_B T$ (see Table 2), $\tau_{\text{pause}} = 0.4$ s, which is identical to the single molecule experimental data showing the transition from a paused state that occurs at Chi-like sequences to a translocating state with a rate constant of $2.5 \pm 0.3 \text{ s}^{-1}$.² This result implies that at Chi-like sequences, $\Delta E_1^X = 4.9k_B T$. By contrast, at *bona fide* Chi sequence, $\Delta E_1^X = 6.9k_B T$ (see just above), giving the interaction of the Chi-binding site with the Chi-like sequence to be about $2k_B T$ smaller than that with *bona fide* Chi sequence. Moreover, from Fig. 6a we see that at $\Delta E_1^X = 4.9k_B T$, $p_{\text{loop}} = 0.25$, implying that when a pause induced by Chi-like sequences occurs, there is as large as about $1 - p_{\text{loop}} = 75\%$ probability that the Chi-like sequences left the Chi-binding site, with no formation of an ssDNA loop. Thus, it is expected that if there are many Chi-like sequences in a DNA substrate, several pauses can occur in one trace of DNA unwinding, which is consistent with the single molecule experimental data.²

Temperature Dependence of the Dynamics

Up to now, we have focused on constant temperature $T = 293 \text{ K}$ (20°C). In this section we will study the temperature dependence of the dynamics.

The dependence of the ATPase rate on temperature T can be obtained by using transition state theory formula^{7,8}

$$k \approx 6 \times 10^{12} \exp\left(-\frac{E_a}{k_B T}\right), \quad (8)$$

where E_a is the activation energy of ATP hydrolysis by the enzyme, T is the absolute temperature and the rate is given in inverse seconds. From Table 2, $k = 410 \text{ s}^{-1}$ at $T = 293 \text{ K}$, with which and using Eq. (8) we obtain $E_a = 94.64 \text{ pN nm}$. Thus, the value of k at any temperature T can be calculated by Eq. (8) with $E_a = 94.64 \text{ pN nm}$. In addition, values of E_{bp} , ΔE_1 , ΔE_2 , ΔE_1^{loop} and ΔE_1^X at any temperature T are given in Table 2 (in units of pN nm).

First, we study the temperature dependence of DNA unwinding velocity in regions free of Chi and Chi-like sequences. Using Eqs. (2)–(4) and (8), the calculated results of DNA unwinding velocity vs. temperature T are shown in Fig. 7a. It is seen that as T increases the velocity increases greatly. At $T = 37^\circ \text{C}$, the velocity is about 1190 bp/s, which is close to the experimental value of about 1000 bp/s measured by Gilhooly *et al.*⁶ Then, we study the temperature dependence of pauses induced by Chi recognition. Using Eqs. (1) and (6)–(8),

FIGURE 7. Temperature dependence of the dynamics for wild-type AddAB and mutant AddAB^{E129A} complexes. (a) DNA unwinding velocity in regions free of Chi and Chi-like sequences vs. temperature T . (b) Lifetime of pause at Chi sequence vs. temperature T . The vertical dotted lines indicate the position of $T = 37^\circ \text{C}$.

the calculated results of the lifetime τ_{pause} at Chi sequence for both wild-type AddAB (with $\Delta E_1^X = 27.9 \text{ pN nm}$, see Table 2) and AddAB^{E129A} (with $\Delta E_1^X \gg 0$, see above) vs. temperature T are shown in Fig. 7b. It is seen that for both wild-type AddAB and mutant AddAB^{E129A} complexes, the pausing lifetime τ_{pause} decreases greatly with the increase of T . At $T = 37^\circ \text{C}$, $\tau_{\text{pause}} = 0.238 \text{ s}$ and 0.197 s for wild-type AddAB and AddAB^{E129A} complexes, respectively, both of which are markedly decreased relative to those at $T = 20^\circ \text{C}$. These values of τ_{pause} at $T = 37^\circ \text{C}$ are below the detection limit of the instrument ($< 0.33 \text{ s}$) used in Gilhooly *et al.*,⁶ implying that the pauses are detected less frequently in the experiments, especially for the case of AddAB^{E129A}. These calculated results are in agreement with the experimental data of Gilhooly *et al.*⁶

DISCUSSION

Explanation of Inefficient Chi Recognition in AddAB: Comparison with RecBCD

Since AddB subunit has a very weak interaction with ssDNA segment free of Chi and Chi-like sequence, with the interacting energy $E_B \approx 0$, it is expected that the ssDNA segment downstream of that

bound by AddA's subdomain 1A is flexible and thus fluctuates around the channel through AddB subunit, along which ssDNA runs. Considering the fast translocation of DNA by the AddA enzyme, it is then expected that the Chi sequence has a large probability not to fall into the potential well, where the Chi sequence has the strong interaction with the Chi-binding site that is distant away from the segment fixed by AddA's subdomain 1A. This gives an explanation of the experimental evidence showing that Chi recognition is an inefficient process.² Moreover, it is expected that the probability that the Chi sequence falls into the potential well is determined by the range of fluctuations around the potential well and is independent of the well depth. This implies that the probability of Chi recognition by a Chi-binding site is independent on its binding affinity to the Chi sequence. This is consistent with the single molecule experimental data showing that although AddAB^{E129A} has a binding affinity to the Chi sequence that is about 16-fold larger than the wild-type AddAB, they have nearly the same Chi-recognition efficiency.⁶

By contrast, in RecBCD the distance between RecB's subdomain 1A and the Chi-binding site locating in RecC subunit is shorter than the distance between AddA's subdomain 1A and the Chi-binding site locating in both AddB subunit and AddA nuclease domain.^{14,22} This implies that in RecBCD the distance between the Chi sequence and the ssDNA segment that is bound fixedly by RecB's subdomain 1A is shorter than the corresponding distance in AddAB. Thus, the Chi sequence shows a smaller range of fluctuations in RecBCD than in AddAB. Considering that DNA translocation velocity by RecB is similar to that by AddA,^{2,17} it is then expected that the Chi sequence has a larger probability to fall into the potential well where the Chi sequence has the strong interaction with the Chi-binding site in RecBCD than in AddAB. This is consistent with the available experimental data.^{2,17}

Comparisons of AddAB with AdnAB: A Model for Dynamics of AdnAB

As mentioned above, Chi recognition by AddAB enzyme is inefficient, which could be due to following reasons. AddB subunit and AddA nuclease domain have weak interaction with ssDNA free of Chi sequence, and the Chi-binding site that has a high affinity to Chi sequence is distant away from AddA's subdomain 1A bound to ssDNA. However, if we considered that AddB subunit has an intermediate or high affinity to ssDNA free of Chi sequence, it would then be expected that the Chi recognition is highly efficient. Moreover, to ensure that before Chi the 3'-terminated DNA can be digested and after Chi recognition the 3'-

terminated DNA can be protected from digestion, it is required that subunit AddB coupled with AddA nuclease domain can also translocate along 3'-5' single strand before Chi, and after Chi recognition the subunit AddB becomes stalled while subunit AddA still moves forward. As discussed as follows, AdnAB enzyme may use such mechanism to realize efficient Chi recognition.

AdnAB enzyme displays a similar overall primary structure to AddAB.¹⁵ However, the experimental data showed that AdnAB is distinct from AddAB in that AdnAB has a dual motor-dual nuclease organization and the relative positions among different domains in AdnAB complex are proposed to be schematically shown in Fig. 8a.^{19,20} The "leading" AdnB and "lagging" AdnA motor domains track in tandem, 3' to 5', along the same DNA single strand, and AdnB nuclease domain and AdnA nuclease domain digest 3'- and 5'-terminated strands, respectively. In contrast to AddAB where the two motor domains are bound together strongly, it is argued here that there is only a weak interaction between AdnA's subdomain 2A and AdnB's subdomain 1A, while there is a high binding energy of AdnB's subdomain 2B with AdnA's subdomain 2B and nuclease domain. Thus, from the proposed organization of different domains in AdnAB complex (see Fig. 8a), it is seen that the movement of AdnA's subdomain 1A is coupled with that of AdnB nuclease domain, and the movement of AdnB's subdomain 2B is coupled with that of AdnA nuclease domain. Based on the argument, a model of DNA unwinding by AdnAB complex and modulation by Chi recognition is described as follows (Fig. 8).

Before Chi, both AdnA and AdnB helicase domains translocate along 3'-5' single strand independently, with AdnB nuclease domain and AdnA nuclease domain digesting 3'- and 5'-terminated strands, respectively (Fig. 8a). After AdnA's subdomain 1A binding strongly to Chi sequence, the cleft closing and opening between 1A and 2A of AdnA would cause 2A to move toward and away from 1A that is kept fixedly to the Chi sequence. Thus, AdnA helicase domain becomes stalled on the ssDNA (Fig. 8b), although the ATPase activity still continues. By contrast, AdnB helicase domain still moves forward, with AdnB's subdomain 1A becoming away from AdnA's subdomain 2A (Fig. 8c). However, due to the strong coupling of AdnB's subdomain 2B with AdnA's subdomain 2B and nuclease domain, an ssDNA loop is expected to form between AdnB's subdomain 1A and AdnA's subdomain 2A, and as AdnB moves continuously the size of loop increases (Fig. 8d). Based on this model, before reaching Chi sequence the ssDNA segment binds with an intermediate affinity to AdnA's subdomain 1A and after reaching Chi sequence the AdnA's

FIGURE 8. Schematic of the model for DNA unwinding by AdnAB complex and modulation by Chi recognition. The model predicts high efficient Chi recognition. (a) Before Chi, both AdnA and AdnB helicase domains translocate along 3'–5' single strand independently, with AdnB nuclease domain and AdnA nuclease domain digesting 3'- and 5'-terminated strands, respectively. (b) Upon reaching Chi sequence, the Chi-binding site binds to the Chi sequence and AdnA helicase domain becomes stalled on the ssDNA. (c) After the Chi-binding site binds to the Chi sequence, AdnB helicase domain still moves forwards, with AdnB's subdomain 1A becoming away from AdnA's subdomain 2A. (d) Due to the strong coupling of AdnB's subdomain 2B with AdnA's subdomain 2B and nuclease domain, an ssDNA loop is expected to form between AdnB's subdomain 1A and AdnA's subdomain 2A. As AdnB moves continuously the size of loop increases.

subdomain 1A, where the Chi-binding site locates, has a high affinity to the Chi sequence. Without the fluctuation of ssDNA the Chi sequence would have a very high probability to bind the Chi-binding site. Thus, it is interesting to note that compared to the case in AddAB complex, in AdnAB complex the enhancement of Chi recognition efficiency is sacrificed by the additional consumption of ATP molecules by AdnA motor domain. Similarly, in RecBCD the enhancement of Chi recognition efficiency is realized by using the strategy with two motor domains tracking in parallel along the two separated strands instead of tracking in tandem along the same single strand.

Concluding Remarks

A model of DNA unwinding by AddAB enzymes and modulation by Chi recognition is proposed. Based on the model, the DNA unwinding velocity in the region free of Chi and Chi-like sequences and the dynamics of pausing taking place at Chi sequence or Chi-like sequence are studied analytically. Diverse experimental data on DNA unwinding velocity such as the effect of DNA sequence and Chi recognition on the DNA unwinding velocity as well as the static disorder peculiar to the AddAB enzymes are explained quantitatively. The experimental data on pausing of wild-

type and mutated AddAB enzymes taking place at Chi sequence or Chi-like sequence are also explained well.

Particularly and interestingly, our model and results show that when a pause induced by Chi recognition occurs, the transition from the paused state to the translocating state takes place with about 2/3 probability that the Chi sequence has not left the Chi-binding site, with the formation of an ssDNA loop. Thus, there is still about 1/3 probability that the Chi sequence has left the Chi-binding site, with no formation of an ssDNA loop. This implies that after an AddAB enzyme pauses at the first Chi sequence, it still has a small probability to pause at the second Chi sequence. This is contrast to case for a RecBCD enzyme, which cannot pause at the second Chi sequence after the pausing induced by the recognition of the first Chi sequence.¹⁸

In order to precisely test our results, we propose to use a DNA substrate containing only A:T base pairs but with a Chi sequence intervened at many places. In the region free of the Chi sequence, the presence of only A:T base pairs ensures that no Chi-like sequence is present and thus no short pause can occur. Based on our model and results, it is predicted that there is a probability albeit small that more than one pause can be observed in one trace of the unwinding of the designed DNA substrate by an AddAB enzyme. By contrast, if it is considered that after the Chi-binding site binds the Chi sequence the Chi-binding site cannot leave the Chi sequence, at most one pause can be observed in one trace of the unwinding of the designed DNA substrate, as in the case of RecBCD. Moreover, it is proposed to use a DNA substrate containing only A:T base pairs but with a Chi-like sequence intervened at many places. Based on our model and results, it is predicted that there is a large probability that more than one short pause can be observed in one trace of the unwinding of the designed DNA substrate by an AddAB enzyme.

Finally, we mention that the model only provides a simplistic picture of the complex AddAB molecular machine. The more detailed and full mechanistic description of the machine remains to be studied in the future.

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CONFLICT OF INTERESTS

Ping Xie declares that he has no conflicts of interest.

ETHICAL STANDARDS

No human studies were carried out by the author for this article. No animal studies were carried out by the author for this article.

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