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Multiple Global Conformational States of the Hexameric RepA Helicase of Plasmid RSF1010 with Different ssDNA-Binding Capabilities Are Induced by Different Numbers of Bound Nucleotides. Analytical Ultracentrifugation and Dynamic Light Scattering Studies

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Abstract

Global conformational transitions of the hexameric RepA helicase of plasmid RSF1010, induced by the nucleoside tri and di-phosphate binding, have been examined using analytical ultracentrifugation and dynamic light scattering techniques. The global structure of the RepA hexamer in solution, modeled as an oblate ellipsoid of revolution, is very different from its crystal structure, with the axial ratio of the ellipsoid being ~4.5 as compared to only ~2.4 in the crystal structure. The large axial ratio and the experimentally determined partial specific volume strongly suggest that, in solution, the diameter of the cross-channel of the hexamer is larger than ~17 Å seen in the crystal. The global conformation of the helicase is modulated by a specific number of bound nucleotides. The enzyme exists in at least four conformational states, occurring sequentially as a function of the number of bound cofactors. These conformational states are different for ADP, as compared to β,γ -imidoadenosine 5'-triphosphate (AMP-PNP). Modulation of the global structure is separated into two phases, different for complexes with up to three bound nucleotides, from the effect observed at the saturating level of cofactors. This heterogeneity indicates different functional roles of the two modulation processes. Nucleotide control of helicase – single-stranded (ss)DNA interactions occurs through affecting the enzyme structure and the ssDNA affinity prior to DNA binding. Only one conformational state of the helicase, with two AMP-PNP molecules bound, has dramatically higher ssDNA-affinities than the complexes with ADP. Moreover the same state also has an increased site-size of the enzyme – ssDNA complexes. The implications of these findings for functional activities of a hexameric helicase are discussed.

Keywords

hexameric helicases; nucleotide binding; motor proteins; analytical ultracentrifugation; dynamic light scattering

Introduction

The RepA protein is a hexameric DNA replicative helicase that is essential for replication of RFS1010 plasmid, a broad host non-conjugative plasmid that can confer bacterial resistance to sulfonamides and streptomycin.^{1–7} The protein forms a very stable, ring-like hexameric structure in the absence of any cofactors or salt in solution.^{3,5–8} In this property, the RepA hexamer resembles the stable hexamer of the *Escherichia coli* DnaB replicative hexameric helicase, which requires only Mg^{+2} cations to maintain its structure.^{9–15} However, both RepA and DnaB helicases differ from other well known hexameric enzymes like bacteriophage T7, T4, and the *E. coli* transcription termination factor, Rho helicases, which exist in solution as a mixture of the oligomeric state and assemble into the hexamer upon interactions with nucleotide cofactors and/or the single-stranded (ss)DNA.^{16–19} The crystal structure of the RepA hexamer has been determined with 2.5 Å resolution.⁵ In the applied conditions, RepA crystallizes as a dimer of two hexamers. The diameter of a single hexamer is ~115 Å, while the diameter of the central cross-channel of the ring-like structure of the enzyme is only ~17 Å (Figure 1).^{4,5}

In the reaction of the double-stranded (ds)DNA unwinding, catalyzed by helicases, the enzymes perform a complex free energy transduction process fueled by NTP binding and/or hydrolysis.^{20–22} However, the mechanism, by which binding and/or hydrolysis of NTP regulate the enzyme activity and affinity toward the nucleic acid, is not yet completely understood for any helicase. Previous thermodynamic studies showed that the RepA hexamer binds six nucleotide molecules. Thus, all subunits of the RepA helicase can actively engage in the binding of nucleotide cofactors.^{7,8} The nucleotide macroscopic affinity decreases with the increase of the average degree of binding of the cofactors. This heterogeneity in affinity reflects the negative cooperative interactions between the binding sites. It effectively splits the cofactor binding process into two phases, with three nucleotides binding in the high-affinity phase and the remaining three nucleotides binding in the low affinity phase. Two analogous binding phases have been observed for the DnaB and Rho hexamers, indicating that it is the general property of a hexameric helicase.^{23–27}

Nevertheless, at the molecular level, little is understood about how the binding and/or hydrolysis of NTP regulate the activity of the enzyme and its affinity toward nucleic acids, for any helicase, particularly, hexameric helicases. In the presence of the saturating concentrations of the ATP non-hydrolyzable analog, β,γ -imidoadenosine 5'-triphosphate (AMP-PNP), the RepA hexamer binds the ssDNA with only moderate intrinsic affinity and, surprisingly, with strong positive cooperativity.⁶ How such a moderate affinity toward the ssDNA or positive cooperativity could be instrumental in the free energy transduction is completely unclear. The site-size of the enzyme – ssDNA complex, i.e. the number of occluded nucleotides by the protein in the complex is only 19($\pm$ 1) nucleotides, strongly suggesting that similar to other hexameric helicases the nucleic acid passes through the central cross-channel of the enzyme.^{12–15,19,28,29} However, the static picture of a single site-size of the protein – ssDNA complex does not explain how the enzyme performs the mechanical translocation, without resorting to more esoteric mechanisms, which do not possess firm experimental bases. The role of multiple nucleotide-binding sites and the observed negative cooperativity in nucleotide binding to a hexameric helicase in enzyme activities is still an unresolved problem.^{23,24}

It is clear that understanding the interactions of nucleotides with the RepA protein and their regulatory role is a key element for understanding the helicase activity of the enzyme, particularly at the molecular level. Moreover, the functional and structural homology between the RepA and DnaB hexamer makes RepA protein an excellent system for comparative studies. On the other hand, the fact that the RepA helicase is essential for the

RSF1010 plasmid replication suggests that the protein is involved in specific mechanisms during DNA replication, not yet known. The essential role of the helicase in replication of the RSF1010, a ubiquitous multiple copy plasmid, conferring resistance to antibiotics, provides an opportunity to examine the molecular aspects of such resistance.^{1,2}

Here, we present direct quantitative analysis of the global conformational transitions of the hexameric RepA helicase, induced by the nucleoside tri and di-phosphate binding. The global structure of the RepA hexamer, in solution, modeled as an oblate ellipsoid of revolution, is very different from its crystal structure, with the axial ratio of the ellipsoid being ~4.5, as compared to the axial ratio of only ~2.4 in the crystal. The global conformation is specifically modulated by a specific number of bound nucleotides and these conformational states are different for ADP, as compared to AMP-PNP. The helicase exists in at least four conformational states. Modulation of the global structure is separated into two phases, different for complexes with up to three bound nucleotides, from the effect observed at the saturating level of nucleotides. Nucleotide control of helicase interactions with the ssDNA occurs through affecting the global structure and the ssDNA affinity of the enzyme, prior to DNA binding. Only one conformational state of the helicase, with two AMP-PNP molecules bound, has orders of magnitude higher ssDNA-affinity than the enzyme complexes with ADP. Moreover the same conformational state has larger site-sizes of the enzyme – ssDNA complexes, indicating that it is the state involved in dsDNA unwinding by the helicase.

Results

Global conformation of the free RepA hexamer in solution. Analytical sedimentation velocity and dynamic light scattering studies of the RepA helicase in the absence of nucleotide cofactors

Application of the analytical sedimentation and dynamic light scattering techniques provides direct and unique information about the hydrodynamic properties of a macromolecule. In turn, the hydrodynamic properties reflect global conformational states of the macromolecule in solution.^{30–34} The sedimentation velocity profiles (monitored at 280 nm) of the RepA helicase, in buffer T5 (pH 7.6, 10 °C), are shown in Figure 2(a). The concentration of the protein is 6×10^{-6} M (hexamer) and the sedimentation run was performed at 35,000 rpm. Inspection of the profiles clearly shows that there is a single moving boundary, indicating the presence of a single molecular species.^{32–34} Independent sedimentation equilibrium studies confirm a presence of a single species with molecular weight of 185,000($\pm$ 9000) Da (data not shown). To obtain the sedimentation coefficient of the protein, $s_{20,w}$, the sedimentation velocity scans have been analyzed using the time-derivative approach.^{30,31,34,35} The advantages of the time-derivative approach over the moment method are thoroughly discussed in the literature.^{30,31} The dependence of the sedimentation coefficient of the RepA helicase upon the protein concentration is shown in Figure 2(b). The value of $s_{20,w}$ shows very little dependence upon [RepA]. Extrapolation of the plot to [RepA]=0 provides $s_{20,w}^0 = 8.36(\pm 0.08)$ S (Figure 2(b)).^{32,33}

The dependence of the normalized autocorrelation function, $g(t)$, of the scattered light from the RepA helicase solution (6×10^{-6} M (hexamer)), in buffer T5 (pH 7.6, 20 °C), upon the logarithm of time, is shown in Figure 2(c) (Materials and Methods). The autocorrelation function has been collected at the scattering angle of 90°. The continuous line is the non-linear least-squares fit of the experimental curve to a single exponential function, defined by equation (22). The fit provides an excellent description of the experimental data, indicating that a single correlation time, τ , describes the decay of the autocorrelation of the scattered light.³⁶ Moreover, virtually the same single correlation time has been obtained for autocorrelation functions collected at different scattering angles (data not shown). The

translational diffusion coefficient, $D_{20,w}$, of the RepA protein is related to the correlation time, τ , by equation (23). The dependence of $D_{20,w}$, of the RepA helicase upon the protein concentration is shown in Figure 2(d). As observed for the sedimentation coefficient, the value of $D_{20,w}$ shows little dependence upon [RepA] in the examined protein concentration range. The extrapolation of the plot in Figure 2(d) to [RepA]=0 provides

$$D_{20,w}^0 = 4.27(\pm 0.04) \times 10^{-7} \text{ cm}^2/\text{s}.$$

Determination of the sedimentation coefficient, $s_{20,w}^0$, and the diffusion coefficient, $D_{20,w}^0$, allows us to determine not only the hydrodynamic properties of the RepA protein but also to experimentally evaluate the partial specific volume of the protein, $\bar{v}$.^{32,33} Independent of any hydrodynamic model of the RepA hexamer, the sedimentation and diffusion coefficients are related to the average, translational frictional coefficient, $\bar{f}_p$, of the hexamer by:

$$s_{20,w}^0 = \frac{M(1 - \bar{v}\rho)}{N_A \bar{f}_p} \quad (1)$$

and:

$$D_{20,w}^0 = \frac{kT}{\bar{f}_p} \quad (2)$$

respectively, where M is the molecular weight of the anhydrous RepA hexamer (179,365.9), ρ is the density of the solvent in g/ml, N_A is the Avogadro number, k is the Boltzmann constant (erg/deg), and T is temperature in Kelvin. The partial specific volume, $\bar{v}$, as then described using the experimental values of $s_{20,w}^0$ and $D_{20,w}^0$, as:

$$\bar{v} = \frac{MD_{20,w}^0 - s_{20,w}^0 N_A kT}{M\rho D_{20,w}^0} \quad (3)$$

Introducing $s_{20,w}^0 = 8.36(\pm 0.08) \text{ S}$ and $D_{20,w}^0 = 4.27(\pm 0.04) \times 10^{-7} \text{ cm}^2/\text{s}$ gives $\bar{v} = 0.735(\pm 0.013) \text{ ml/g}$. Thus, the experimentally obtained value of $\bar{v}$ for the RepA hexamer is practically identical to the partial specific volume of the helicase, $\bar{v} = 0.736 \text{ ml/g}$, calculated from the amino acid composition of the protein (see Discussion).³⁷

Having the experimental values of $s_{20,w}^0$, $D_{20,w}^0$, and $\bar{v}$, one can address the shape of the free RepA hexamer in solution. The crystal structure of the hexamer indicates that the simplest model of its global shape is that of the oblate ellipsoid of revolution, characterized by major and minor axes, a and b , respectively (Figure 1).^{32,33,35} The average translational frictional coefficient, $\bar{f}_p$, of an oblate ellipsoid of revolution is defined, in terms of the axial ratio of the ellipsoid, by the Perrin equation as:^{32,33,35}

$$\bar{f}_p = \left\{ \frac{\sqrt{\frac{p^2-1}{p^2}}}{p^{1/3} \left\{ \arctan \left[p + \sqrt{\frac{p^2-1}{p^2}} \right] \right\}} \right\} 6\pi\eta R_h \quad (4)$$

where R_h is the hydrodynamic radius of the corresponding hydrated sphere, defined as:

$$R_h = \left[\frac{3M(\bar{v} + h\bar{v}_s)}{N_A 4\pi} \right]^{\frac{1}{3}} \quad (5)$$

p is the axial ratio of the major ellipsoid axis to the minor one, i.e. $p=a/b$ (Figure 1), M is the molecular weight of the anhydrous RepA hexamer, η is the viscosity of the solvent (poise), h is the degree of the protein hydration expressed as $gH_2O/g_{protein}$, and $\bar{v}_s$ is the partial specific volume of the solvent equal to the inverse of ρ . The degree of hydration can be estimated by the Kuntz method.³⁸ For the RepA hexamer, it provides $h=0.4$ $gH_2O/g_{protein}$. However, this value of h is calculated for the mixture of amino acids completely exposed to the solvent and represents a maximum possible value of the degree of hydration. Part of the amino acid residues will not be accessible to the solvent in the native structure of the protein. Thus, the value of h should be corrected for the part of residues in the native structure that is not accessible to the solvent. The correction factor can be obtained in a systematic way by comparing Kuntz's values for a series of proteins with the degree of hydration of the folded structure of the same protein.^{10,35,39,40} The correction factor amounts to ~ 0.75 , thus, only $\sim 75\%$ of the maximum values of h is associated with the folded protein molecule. The corrected value of the degree of hydration for the RepA hexamer is then $h=0.3$ $gH_2O/g_{protein}$.^{39,40}

The computer simulation of the sedimentation and diffusion coefficients, $s_{20,w}^0$ and $D_{20,w}^0$ of the RepA hexamer as a function of the axial ratio, p , of the oblate ellipsoid, using the corrected value of the degree of hydration, h , is shown in Figure 3(a) and (b), respectively. The plots have been obtained using equations (1). For comparison, the dependence of $s_{20,w}^0$ and $D_{20,w}^0$ as a function of the axial ratio of the oblate ellipsoid for the completely non-hydrated hexamer ($h=0$) and the hexamer with the maximum degree of hydration ($h=0.4$), are also included. For the corrected degree of hydration, the values of $s_{20,w}^0=8.36(\pm 0.08)$ S and $D_{20,w}^0=4.27(\pm 0.04) \times 10^{-7}$ cm^2/s indicate that the RepA hexamer in solution, has a non-spherical, asymmetric structure and, when modeled as an oblate ellipsoid of revolution, has an apparent axial ratio of $p=4.5\pm 0.5$. For the completely non-hydrated and maximum hydrated hexamer, the same axial ratio is $p=7.3\pm 0.6$ and $p=3.9\pm 0.4$, respectively (Figure 3(a) and (b)). An estimate of the same axial ratio determined for the crystal structure of the RepA hexamer (Figure 1) provides $p=2.4\pm 0.2$. Therefore, the determined axial ratio, $p=4.5\pm 0.5$, of the free RepA hexamer, in solution, indicates that the global structure of the hexamer in solution is significantly different from the global structure of the same hexamer determined in the crystallographic studies (see Discussion).

The effect of ADP and ATP non-hydrolyzable analog, AMP-PNP, on the global conformation of the RepA hexamer. Model-independent studies

To address the effect of the nucleotide cofactors on the global conformation of the RepA hexamer, we examined the sedimentation and diffusion coefficients, $s_{20,w}$ and $D_{20,w}$, of the enzyme as a function of concentrations of ADP or the ATP non-hydrolyzable analog, AMP-PNP. The use of AMP-PNP is dictated by the fact that the RepA helicase hydrolyzes ATP in the presence of the ssDNA in the time scale of the hydrodynamic measurements (data not shown; see below). Moreover, binding of AMP-PNP to the helicase is very similar to the binding of ATP.^{7,8} It is characterized by strong negative cooperativity and significantly affects the affinity of the helicase for the ssDNA.^{7,8} In the case of the sedimentation velocity experiments, the reference cell contains the same concentration of the nucleotide cofactor,

thus, allowing us to exclusively monitor the absorbance of the enzyme.^{34,35,41} The dependence of $s_{20,w}$ of the RepA hexamer upon the logarithm of the ADP concentration is shown in Figure 4(a). An analogous plot for $D_{20,w}$ is shown in Figure 4(c). The behavior of the system is very complex. The initial values of $s_{20,w} \sim 8.4$ and $D_{20,w} \sim 4.3 \times 10^{-7} \text{ cm}^2/\text{s}$ do not change until [ADP] approaches the value of $\sim 10^{-5} \text{ M}$. As [ADP] further increases, $s_{20,w}$ reaches the minimum value of $\sim 7.8 \text{ S}$ at [ADP] $\sim 10^{-4} \text{ M}$ and then increases to the plateau, $s_{20,w} \sim 8.8 \text{ S}$, at high ADP concentrations. At the same $\sim 10^{-4} \text{ M}$ [ADP], $D_{20,w}$ reaches the maximum value of $\sim 4.7 \times 10^{-7} \text{ cm}^2/\text{s}$ and, subsequently, sharply decreases to the value of $\sim 4 \times 10^{-7} \text{ cm}^2/\text{s}$.

The dependence of $s_{20,w}$ and $D_{20,w}$ upon the total average degree of binding, $\Sigma\Theta_i$, of ADP on the RepA hexamer is shown in Figure 4(c) and (d), respectively. The values of $\Sigma\Theta_i$, at a given total concentrations of the RepA hexamer and ADP, have been obtained using previously determined thermodynamic binding isotherms of the ADP association with the RepA hexamer, under the same solution conditions.^{7,8} It is evident that association of the first ADP molecule barely causes detectable changes in the $s_{20,w}$ and $D_{20,w}$ values. However, a well-defined minimum of sedimentation coefficient ($\sim 7.8 \text{ S}$) occurs when the total average degree of binding, $\Sigma\Theta_i$, reaches the value of ~ 2 , while when $\Sigma\Theta_i$ reaches the value of $\sim 3-4$, $s_{20,w}$ strongly increases reaching the plateau ($\sim 8.8 \text{ S}$) at $\Sigma\Theta_i \sim 4.5$. Analogously, $D_{20,w}$ only slightly changes for $\Sigma\Theta_i$ below ~ 1 . On the other hand, the value of $D_{20,w}$ reaches a maximum at $\Sigma\Theta_i \sim 2$ and then sharply decreases to the plateau value of $\sim 4 \times 10^{-7} \text{ cm}^2/\text{s}$, for the total average degree of binding exceeding about four ADP molecules bound to the RepA hexamer.^{7,8}

We have also performed fits of the sedimentation velocity data, with both the sedimentation and diffusion coefficients as fitting parameters, for the RepA hexamer in the absence of the nucleotide cofactors and in the presence of ADP ($5 \times 10^{-5} \text{ M}$), using the determined partial specific volumes of the protein. This has been accomplished using the program, sed-fit.⁴² For the RepA hexamer the fit provided $s_{20,w} \sim 8.25 \text{ S}$ and $D_{20,w} \sim 4.38 \times 10^{-7} \text{ cm}^2/\text{s}$. For the RepA hexamer in the presence of ADP, the sedimentation and diffusion coefficients, obtained from the fit, are $s_{20,w} \sim 8.05 \text{ S}$ and $D_{20,w} \sim 4.63 \times 10^{-7} \text{ cm}^2/\text{s}$, respectively. These values are, within experimental error, identical to the values obtained using independent measurements of $s_{20,w}$ and $D_{20,w}$, as described above. This is expected because, once the partial specific volume is known, both, $s_{20,w}$ and $D_{20,w}$, are related through model-independent equations (1), (2), and (3). Nevertheless, such fits of a set of data, obtained using a single method, although a valuable test of the consistency of the data, are not independent determinations of $\bar{v}$, $s_{20,w}$, and $D_{20,w}$, as described here.

Corresponding studies, as described above for ADP, have been performed for the complex of the RepA hexamer with the ATP non-hydrolyzable analog, AMP-PNP. The dependence of $s_{20,w}$ and $D_{20,w}$ of the RepA hexamer in buffer T5 (pH 7.6, 20 °C) upon the logarithm of the AMP-PNP concentration is shown in Figure 5(a) and (c), respectively. Although the qualitative character of the changes of the two hydrodynamic quantities examined is similar to the behavior observed for ADP, there are profound differences between the effects exerted by the nucleoside tri-phosphate *versus* the nucleoside di-phosphate. The decrease of $s_{20,w}$ and the increase of $D_{20,w}$ occur at [AMP-PNP] substantially lower than observed in the case of ADP (Figures 4(a) and (c) and 5(a) and (c)). The minimum value of $s_{20,w}$ ($\sim 7.5 \text{ S}$) and the maximum value of $D_{20,w}$ ($\sim 5.1 \times 10^{-7} \text{ cm}^2/\text{s}$) are significantly lower and higher, respectively, than the corresponding values observed for ADP. Moreover, there is an intermediate plateau at $s_{20,w} \sim 8.3 \text{ S}$ at the high nucleotide concentration range, not observed for ADP. Furthermore, the maximum of the plot for the diffusion constant is much less pronounced than the equivalent plot obtained for the nucleoside di-phosphate (see below).

The dependence of $s_{20,w}$ and $D_{20,w}$ upon the total average degree of binding, $\Sigma\Theta_i$, of AMP-PNP on the RepA hexamer is shown in Figure 5(b) and (d). The values of $\Sigma\Theta_i$ for AMP-PNP, at given total concentrations of ADP and the RepA hexamer, have been obtained using previously determined thermodynamic binding isotherms of the AMP-PNP association with the RepA hexamer under the same solution conditions.^{7,8} It is evident that the minimum value of $s_{20,w}$ and the maximum value of $D_{20,w}$ occurs at the total average degree of binding of AMP-PNP, $\Sigma\Theta_i$, of ~ 1 , as compared to $\Sigma\Theta_i \sim 2$ for ADP. The value of $s_{20,w}$ strongly increases with the increase of $\Sigma\Theta_i$ and the observed intermediate plateau at $s_{20,w} \sim 8.3$ S is reached when $\Sigma\Theta_i \sim 3$. We could not examine the observed further increase of $s_{20,w}$ with the increase of $\Sigma\Theta_i$, in the higher concentration range of the nucleotide cofactor because of the tendency of the RepA – AMP-PNP complex to precipitate at [AMP-PNP] exceeding ~ 10 mM at the applied protein concentration. On the other hand, unlike the behavior observed for ADP, the values of the diffusion coefficient only very gradually decrease, as the total average degree of the binding of AMP-PNP on the RepA hexamer increases above the value of ~ 3 (Figure 5(d)) (see Discussion).

Statistical thermodynamic model of the global conformational changes of the RepA hexamer induced by different numbers of the bound nucleoside di- and tri-phosphates

Recall, the RepA hexamer binds six nucleotide cofactors and the binding is characterized by negative cooperativity between the binding sites.^{7,8} The binding process is well described by a hexagon model with only two parameters that characterize the binding, the intrinsic binding constant, K , and the parameter, σ , describing cooperative interactions.^{7,8,23,24,43} The partition function for the RepA – nucleotide system, Z_H , is then:

$$Z_H = 1 + 6x + 3(3 + 2\sigma)x^2 + 2(1 + 6\sigma + 3\sigma^2)x^3 + 3(3\sigma^2 + 2\sigma^3)x^4 + 6\sigma^4x^5 + \sigma^6x^6 \quad (6)$$

where $x = K [N_F]$, and $[N_F]$ is the free nucleotide concentration. The total average degree of binding, $\Sigma\Theta_i$, is defined by the standard statistical thermodynamic formula, $\partial \ln Z_H / \partial \ln [N_F]$ as:⁴³

$$\Sigma \Theta_i = \frac{6x + 6(3 + 2\sigma)x^2 + 6(1 + 6\sigma + 3\sigma^2)x^3 + 12(3\sigma^2 + 2\sigma^3)x^4 + 30\sigma^4x^5 + 6\sigma^6x^6}{Z_H} \quad (7)$$

Inspection of the plots in Figures 4 and 5 indicates that there are at least four different and discrete global conformational states of the RepA hexamer induced by nucleotide cofactor binding (see below). Moreover, these states are different for complexes with ADP, as compared to the complexes with the ATP non-hydrolyzable analog, AMP-PNP. Thus, the simplest statistical thermodynamic model that can account for the observed dependence of $s_{20,w}$ and $D_{20,w}$ of the RepA hexamer upon the number of the bound nucleotide cofactor molecules, must include four different global conformational states of the hexamer. Furthermore, for a given nucleotide cofactor, the model must consistently describe the behavior of two independently determined hydrodynamic parameters, the sedimentation and the diffusion coefficient, as functions of the cofactor concentration or as functions of the total average degree of binding of the cofactor.

In the case of ADP, the plots in Figure 4(a) and (c) indicate that the global conformation of the hexamer, with one ADP molecule bound to the Rep hexamer, has the same sedimentation and diffusion coefficient as the free helicase. We designate these parameters as s_{F1} and D_{F1} . The global conformational state with two ADP molecules bound to the hexamer must have different hydrodynamic properties than the free RepA hexamer or the

hexamer with only one ADP molecule bound, as the values of $s_{20,w}$ and $D_{20,w}$ decrease and increase in the [ADP] concentration range dominated by $\Sigma\Theta_i = 1$ and $\Sigma\Theta_i = 2$, and reach the corresponding minimum and maximum at $\Sigma\Theta_i = 2$ (Figure 4(a) and (c)).^{7,8} Therefore, we assign the values, s_{D2} and D_{D2} , for the conformational state with two ADP molecules bound. Next, the state with three ADP molecules bound must have its global conformation different from the state with two ADP molecules bound. This is because both $s_{20,w}$ and $D_{20,w}$ strongly change around $\Sigma\Theta_i = 3$, yet, their values are still far from the values of both hydrodynamic parameters observed at the ADP concentration range dominated by $\Sigma\Theta_i \geq 4$. We assign the values s_{D3} and D_{D3} to this state. Finally, the states with four, five and six molecules of ADP bound to the RepA hexamer have the same value of s_{D4} and D_{D4} assigned to them, i.e. the values of $s_{20,w}$ and $D_{20,w}$ observed at the plateau, which is reached at $\Sigma\Theta_i \geq 4$. The observed dependence of $s_{20,w}$ and $D_{20,w}$ of the RepA hexamer upon the concentration of ADP is then:

$$s_{20,w} = \frac{s_{F1}(1+6x) + s_{D2}[3(3+2\sigma)x^2] + s_{D3}[2(1+6\sigma+3\sigma^2)x^3] + s_{D4}[3(3\sigma^2+2\sigma^3)x^4 + 6\sigma^4x^5 + \sigma^6x^6]}{Z_H} \quad (8)$$

and:

$$D_{20,w} = \frac{D_{F1}(1+6x) + D_{D2}[3(3+2\sigma)x^2] + D_{D3}[2(1+6\sigma+3\sigma^2)x^3] + D_{D4}[3(3\sigma^2+2\sigma^3)x^4 + 6\sigma^4x^5 + \sigma^6x^6]}{Z_H} \quad (9)$$

There are six parameters, K , σ , s_{F1} , s_{D2} , s_{D3} , and s_{D4} in equation (8), and six parameters, K , σ , D_{F1} , D_{D2} , D_{D3} , and D_{D4} in equation (9). However, several of them can be independently obtained. $K=1.4 \times 10^4 \text{ M}^{-1}$ and $\sigma=0.17$, for the ADP binding to the RepA helicase, have been determined in our previous thermodynamic studies of nucleotide cofactor binding to the enzyme.^{7,8} The values of $s_{F1} = 8.3(\pm 0.08) \text{ S}$ and $s_{D4} = 8.85(\pm 0.1) \text{ S}$ are available from the plots in Figures 2(b), 4(a), and (b). Thus, there are only two unknown parameters, s_{D2} and s_{D3} , remaining to be determined. The continuous line in Figure 4(a) and (b) are non-linear least-squares fits of the experimental curves using equations (7) and (8), with two fitting parameters s_{D2} and s_{D3} , which provides $s_{D2} = 7.6(\pm 0.1) \text{ S}$ and $s_{D3} = 8.15(\pm 0.11) \text{ S}$. For the diffusion coefficient, the value of $D_{F1} = 4.33(\pm 0.05) \times 10^{-7} \text{ cm}^2/\text{s}$ is available from the plots in Figures 2(d), 4(c) and (d). The remaining unknown parameters are D_{D2} , D_{D3} , and D_{D4} . The continuous line in Figure 4(c) and (d) are non-linear least-squares fits of the experimental curves using equations (7) and (9), with three fitting parameters D_{D2} , D_{D3} , and D_{D4} , which provides $D_{D2} = 5.05(\pm 0.1) \times 10^{-7} \text{ cm}^2/\text{s}$, $D_{D3} = 4.03(\pm 0.1) \times 10^{-7} \text{ cm}^2/\text{s}$, and $D_{D4} = 4.0(\pm 0.1) \times 10^{-7} \text{ cm}^2/\text{s}$. All hydrodynamic parameters are included in Table 1. Both fits provide excellent descriptions of the observed experimental behavior of the sedimentation and diffusion coefficients of the RepA – ADP complex as a function of the number of the ADP molecules bound to the RepA hexamer (see Discussion).

In the case of AMP-PNP, the plots in Figure 5(a)–(d) indicate that the global conformation of the hexamer, with one AMP-PNP molecule bound, has a very different sedimentation and diffusion coefficient, as compared to the same parameter of the free RepA helicase. We designate these parameters as s_F , D_F , s_{T1} , and D_{T1} , for the free hexamer and the hexamer with one AMP-PNP molecule bound. The conformational state with two AMP-PNP molecules bound to the hexamer must have different hydrodynamic properties than the free RepA hexamer, or the hexamer with one AMP-PNP molecule bound, as the values of $s_{20,w}$ and $D_{20,w}$ decrease and increase in the [AMP-PNP] concentration range dominated by $\Sigma\Theta_i = 1$ and $\Sigma\Theta_i = 2$ (Figure 5(a) and (c)). We assign the values s_{T2} and D_{T2} for the conformational state with two AMP-PNP molecules bound. Next, the plot in Figure 5(a) and

(b) indicate that the state with three AMP-PNP molecules bound has its global conformation different from the state with two AMP-PNP molecules bound, because at the AMP-PNP concentration range, dominated by $\Sigma\Theta_i = 3$, the value of $s_{20,w}$ reaches an intermediate plateau. We assign the value s_{T3} to this state. Further increases of [AMP-PNP] and correspondingly $\Sigma\Theta_i$ indicate that the sedimentation coefficient is higher for the RepA hexamer with four, five, and six nucleotide molecules bound than for the state with the three bound AMP-PNP molecules, although we could not pursue examination of the RepA – AMP-PNP complex at these values of $\Sigma\Theta_i$ because of the tendency of the complex to precipitate (see above). We assign a single value of s_{T4} to these states. On the other hand, the value of $D_{20,w}$ shows only a slight and very gradual change, once the value of $\Sigma\Theta_i$ exceeds 3, indicating that complexes with three, four, five, and six nucleotides bound have very similar values of the diffusion coefficient. We assign a single value of D_{T3} to these complexes. The observed dependence of $s_{20,w}$ and $D_{20,w}$ of the RepA hexamer upon the concentration of AMP-PNP is then:

$$s_{20,w} = \frac{s_F + s_{T1} 6x + s_{T2} [3(3+2\sigma)x^2] + s_{T3} [2(1+6\sigma+3\sigma^2)x^3] + s_{T4} [3(3\sigma^2+2\sigma^3)x^4 + 6\sigma^4x^5 + \sigma^6x^6]}{Z_H} \quad (10)$$

and:

$$D_{20,w} = \frac{D_F + D_{T1} 6x + D_{T2} [3(3+2\sigma)x^2] + D_{T3} [2(1+6\sigma+3\sigma^2)x^3] + 3(3\sigma^2+2\sigma^3)x^4 + 6\sigma^4x^5 + \sigma^6x^6]}{Z_H} \quad (11)$$

There are seven parameters, K , σ , s_F , s_{T1} , s_{T2} , s_{T3} , and s_{T4} in equation (10), and six parameters, K , σ , D_F , D_{T1} , D_{T2} , and D_{T3} , in equation (11). As discussed above in the case of ADP, several of these parameters can be independently obtained or estimated. The values of s_F is simply the value of $s_{20,w}$ of the free RepA hexamer under the same solution conditions (Figure 2(b)). The intrinsic binding constant, $K=2.2 \times 10^4 \text{ M}^{-1}$ and $\sigma=0.013$, for the AMP-PNP binding to the RepA helicase have been determined in previous thermodynamic studies of the nucleotide cofactor binding to the enzyme.^{7,8} The values of $s_{T3} = 8.3(\pm 0.1) \text{ S}$ can be obtained from the intermediate plateau in the plots in Figure 5(a) and (b). Thus, there are three unknown parameters, s_{T1} , s_{T2} , and s_{T4} , which remain to be determined. The continuous line in Figure 5(a) and (b) are non-linear least-squares fits of the experimental curves using equations (7) and (10), which provide $s_{T1} = 6.5(\pm 0.1) \text{ S}$, $s_{T2} = 8.29(\pm 0.08) \text{ S}$, and $s_{T4} = 8.83(\pm 0.25) \text{ S}$. In the case of the diffusion coefficient, D_F , corresponds to $D_{20,w}$ of free RepA hexamer, which has been determined independently (Figure 2(d)). The remaining unknown parameters are D_{D1} , D_{D2} , and D_{D3} . The continuous lines in Figure 5(c) and (d) are non-linear least-squares fits of the experimental curves using equations (7) and (11), with three fitting parameters D_{D1} , D_{D2} , and D_{D3} , which provides $D_{D1} = 4.9 (\pm 0.1) \times 10^{-7} \text{ cm}^2/\text{s}$, $D_{D2} = 5.1(\pm 0.1) \times 10^{-7} \text{ cm}^2/\text{s}$, and $D_{D3} = 4.9(\pm 0.1) \times 10^{-7} \text{ cm}^2/\text{s}$ (see Discussion). All hydrodynamic parameters are included in Table 2. As previously obtained for the RepA – ADP complex, the fits provide an excellent description of the observed experimental behavior of $s_{20,w}$ and $D_{20,w}$ as a function of the AMP-PNP concentration (see Discussion).

Interactions of different global conformational states of the RepA hexamer, generated by the binding of different numbers of the AMP-PNP molecules to the enzyme, with the ssDNA

In the presence of high [AMP-PNP], the RepA helicase binds the ssDNA with the site-size of the protein – ssDNA complex of $19(\pm 1)$ nucleotides and a moderate intrinsic affinity.⁶ Moreover, binding to the ssDNA 41-mer, which can accommodate two RepA hexamers, is

characterized by a significant positive cooperativity between the bound helicase molecules. We address the binding of the RepA hexamer to the 41-mer in the absence and presence of nucleotide cofactors, in the context of the discovered global structural heterogeneity of the RepA hexamer, generated by the different number of the bound nucleotides, using the sedimentation velocity technique.^{10,15,34,35,41} We utilize the fact that the sedimentation coefficient of the RepA hexamer (~8.4 S) is much larger than the sedimentation coefficient of the modified ssDNA 41-mer, 5'Flu-dT(pT)₃₉ (~1.8 S), which contains fluorescein at its 5' end (Materials and Methods).^{7,8} Therefore, in our experimental design, the sedimentation of the nucleic acid can be exclusively monitored at the fluorescein absorption band (495 nm), without any interference from the protein and nucleotide cofactor absorbance. The formation of the complex between the ssDNA oligomer and the RepA hexamer is manifested by a large increase of the apparent sedimentation coefficient of the ssDNA 41-mer.^{10,15,34,35,41}

Sedimentation velocity profiles of 5'Fl-dT(pT)₃₉, in the presence of the RepA hexamer in buffer T5 (pH 7.6, 10 °C), without AMP-PNP, are shown in Figure 6(a). The profiles have been recorded at the fluorescein absorption band (495 nm). The total nucleic acid and protein concentrations are 3.5×10^{-6} M and 3.5×10^{-5} M (hexamer), respectively. Thus, the helicase is in a large excess over the nucleic acid. At this selected enzyme concentration and in the presence of high [AMP-PNP], the enzyme saturates the ssDNA oligomer.^{7,8} The fast moving boundary in Figure 6(a) corresponds to the 41-mer - RepA complex.^{6,10,15,34,35,41} The very slow moving boundary, at the applied rotational speed, corresponds to the sedimentation of the free 5'Fl-dT(pT)₃₉. There are two important aspects of these data. First, it is evident that the RepA hexamer can bind the ssDNA even in the absence of the nucleoside tri-phosphate, although with a significantly lower affinity (see below). Second, even though the enzyme binds the nucleic acid and the helicase is at concentration where, in the presence of AMP-PNP, two RepA hexamers binds to the 41-mer with substantial positive cooperativity, the sedimentation coefficient of the fast moving boundary is only ~8.3 S, indicating that only one RepA hexamer is bound to the ssDNA 41-mer.⁶ Independent sedimentation equilibrium studies confirm the 1:1 stoichiometry of the complex formed in the absence of AMP-PNP (data not shown).

From the plot in Figure 6(a) and the known total concentration of the 41-mer, [5'Fl-dT(pT)₃₉]_T, one can estimate the macroscopic binding constant, K_0 , of the RepA helicase for the ssDNA, in the absence of AMP-PNP. Using the total absorption of 5'Fl-dT(pT)₃₉, T , of the initial scans, which corresponds to the total concentration of the ssDNA 41-mer, and the absorption of the slow moving boundary, F , one can directly calculate the absorption of the 5'Fl-dT(pT)₃₉ bound to the helicase, as $C = T - F$. Thus, the concentration of the nucleic acid bound to the RepA hexamer is $(C/T) \times [5'Fl-dT(pT)_{39}]_T$. The determined total average degree of binding of the ssDNA oligomer on the RepA hexamer is then $\Sigma v_i = \{(C/T) \times [5'Fl-dT(pT)_{39}]_T\} / [\text{RepA}]_T$. This quantity is related to the macroscopic binding constant, K_0 , and the total RepA concentration, $[\text{RepA}]_T$, by:

$$K_0 = \frac{\Sigma v_i}{[\text{RepA}]_T (1 - \Sigma v_i)} \quad (12)$$

The use of $[\text{RepA}]_T$ instead of the free helicase concentration, $[\text{RepA}]_F$, in equation (12) is justified by the fact that $[\text{RepA}]_T \gg [5'Fl-dT(pT)_{39}]_T$, i.e. the free RepA concentration is practically equal to the total concentration of the enzyme. The sedimentation velocity data in Figure 6(a) provide the value of $K_0 = 3(\pm 0.5) \times 10^4 \text{ M}^{-1}$. Very similar value of $K_0 = 3.3(\pm 0.5) \times 10^4 \text{ M}^{-1}$ has been obtained independently using fluorescence emission of the 40-mer (data not shown).⁶ This value may seem high, as compared to the intrinsic binding constant of the RepA hexamer determined in the presence of AMP-PNP ($\sim 7 \times 10^4 \text{ M}^{-1}$).⁶

However, this is a macroscopic binding constant, which is related to the intrinsic binding constant, K_{int} , as $K_0 = (m-n+1)K_{\text{int}}$, where m is the length of the ssDNA oligomer in nucleotides and n is the site-size of the complex (see below).⁶ If the site-size of the hexamer in the considered complex is the same or different from the site-size of 19(±1) nucleotides, determined in the presence of AMP-PNP, by one to three nucleotides, then K_{int} is only $\sim 1 \times 10^3 \text{ M}^{-1}$ (see below).

Analogous sedimentation velocity profiles of the ssDNA 41-mer, 5'Fl-dT(pT)₃₉, in the presence of the RepA hexamer in buffer T5 (pH 7.6, 10 °C), containing $1 \times 10^{-4} \text{ M}$ AMP-PNP, are shown in Figure 6(b). At this concentration of the cofactor, complexes with one and two AMP-PNP molecules bound to the RepA hexamer dominate the distribution of the population of RepA – AMP-PNP complexes.^{7,8} There is only a single fast moving boundary in Figure 6(b) corresponding to the 41-mer - RepA complex.⁶ We cannot estimate the binding constant from these data because the concentration of the free nucleic acid is undetectably small (see below). However, the sedimentation coefficient of the boundary is only 9.8(±0.1) S, indicating that still only one RepA hexamer binds to the ssDNA 41-mer, as confirmed by independent sedimentation equilibrium experiments (data not shown). If two hexamers were bound, the expected sedimentation coefficient, $s_{20,w}$, of the complex should be $\sim 12\text{--}13 \text{ S}$, as previously determined at higher AMP-PNP concentrations and the theoretically possible value for the dimer of two RepA hexamers.⁶ The data in Figure 6(b) also provide two important results, independent of any binding models. First, the RepA hexamer, with one or two AMP-PNP molecules bound to the enzyme, binds to the ssDNA with significantly higher affinity than the RepA helicase devoid of AMP-PNP. Second, in spite of a much higher affinity, the ssDNA cannot accommodate more than one RepA hexamer in these conformational states (see below).

Sedimentation velocity studies of the RepA hexamer binding to the ssDNA 41-mer have been performed for a series of AMP-PNP concentrations. The dependence of the sedimentation coefficient of the RepA hexamer – 41-mer complex, in buffer T5 (pH 7.6, 10 °C), as a function of the AMP-PNP concentration, is shown in Figure 7. The plot shows a very intricate behavior. In the absence of AMP-PNP, the sedimentation coefficient of the complex with one RepA hexamer bound to the 41-mer is $s_{20,w} \sim 8.4 \text{ S}$ (Figure 6(a)). At very low [AMP-PNP] ($< 1 \times 10^{-5} \text{ M}$), the value of the sedimentation coefficient sharply increases, although there is still only one RepA hexamer bound to the 41-mer (see above). The plot reaches an intermediate plateau at around $6 \times 10^{-5} \text{ M}$ AMP-PNP, at $s_{20,w} \sim 9.8 \text{ S}$. A further increase of [AMP-PNP] causes further increase of $s_{20,w}$. Above [AMP-PNP] $\sim 5 \times 10^{-4} \text{ M}$, $s_{20,w}$ plateaus around $\sim 12.1 \text{ S}$, which is, as we mentioned above, the expected value for the maximum of two hexamers bound to the 5'Fl-dT(pT)₃₉.⁶ The complex behavior of the plot in Figure 7 provides the first indication that different conformational states of the RepA hexamer, induced by different numbers of bound AMP-PNP molecules, have very different ssDNA-binding capabilities.

The examined interacting system is extremely complex. One must consider several different conformational states of the RepA hexamer in solution, which compete for the nucleic acid and their contributions to the total population of the RepA hexamer change with the changes of the AMP-PNP concentration.^{6–8} The simplest statistical thermodynamic model of the competitive binding of different RepA hexamer conformational states to the ssDNA 41-mer, as a function of the AMP-PNP concentration, which takes into account these considerations, is defined by the total partition function, Z_N , as:

$$Z_N = 1 + K_0[\text{RepA}_0] + K_1[\text{RepA}_1] + K_2[\text{RepA}_2] + Z_{3-6} \quad (13)$$

where K_0 and $[\text{RepA}_0]$ are the macroscopic binding constant and concentration of the RepA hexamer without bound AMP-PNP (see above), K_1 , K_2 , $[\text{RepA}_1]$, and $[\text{RepA}_2]$ are the macroscopic binding constants and concentrations of the RepA hexamer with one and two AMP-PNP molecules bound. Above $[\text{AMP-PNP}] \geq 5 \times 10^{-4} \text{ M}$, the binding of the RepA hexamer to the 41-mer has been examined by us.⁶ The process is described by the cooperative binding of two large ligand molecules with the site-size of 19 nucleotides to the ssDNA lattice, which can accommodate only two ligand molecules, using the exact combinatorial theory for large ligand binding to a finite linear, homogeneous lattice.^{6,44–47} Thus, the term, Z_{3-6} , in the total partition function, Z_N , describes the binding of RepA hexamers with three, four, five, and six AMP-PNP molecules bound to the ssDNA 41-mer. This part of the total partition function, Z_N , is then:^{6,43}

$$Z_{3-6} = \sum_{k=1}^g \sum_{j=1}^{q-1} P_M(q, j) (K_{3-6} [\text{RepA}_{3-6}])^q \omega^j \quad (14)$$

where g is the maximum number of ligand molecules, which can bind to the nucleic lattice (in our case $g=2$), K_{3-6} is the intrinsic binding constant, ω is the cooperative interaction parameter, q is the number of ligand molecules bound, and j is the number of cooperative contacts between the q bound ligands in a particular configuration on the lattice. The combinatorial factor $P_M(q, j)$ is the number of distinct ways that q ligands bind to a lattice, with j cooperative contacts, and is defined by:

$$P_M(q, j) = \frac{(m - nq + 1)!(q - 1)!}{(m - nq + j + 1)!(q - j)!(q - j - 1)!} \quad (15)$$

where m and n are the length of the ssDNA lattice (nucleotides) and the site-size of the complex, respectively, (in our case, $m = 41$, $n = 19$). The concentration of the different conformational states of the RepA hexamer, containing a different number of bound AMP-PNP molecules, are defined using the partition function, Z_H , for the RepA hexamer – AMP-PNP binding system (equation (6)) and the total RepA concentration, $[\text{RepA}]_T$, as:

$$[\text{RepA}_0] = \frac{[\text{RepA}]_T}{Z_H} \quad (16)$$

$$[\text{RepA}_1] = \frac{6x}{Z_H} [\text{RepA}]_T \quad (17)$$

$$[\text{RepA}_2] = \frac{3(3+2\sigma)x^2}{Z_H} [\text{RepA}]_T \quad (18)$$

and

$$[\text{RepA}_{3-6}] = \left[\frac{2(1+6\sigma+3\sigma^2)x^2+3(3\sigma^2+2\sigma^3)x^4+6\sigma^4x^5+\sigma^6x^6}{Z_{\text{H}}} \right] [\text{RepA}]_{\text{T}} \quad (19)$$

Finally, the observed sedimentation coefficient of the RepA – 41-mer complex, $s_{20,w}$, as a function of the AMP-PNP concentration is defined by:

$$s_{20,w} = \frac{s_0 K_0 [\text{RepA}_0] + s_1 K_1 [\text{RepA}_1] + s_2 K_2 [\text{RepA}_2] + [s_{3-6}(m-n+1)K_{3-6} + s_{4-6}(Z_{3-6})_2][\text{RepA}_{3-6}]}{K_0 [\text{RepA}_0] + K_1 [\text{RepA}_1] + K_2 [\text{RepA}_2] + (m-n+1)K_{3-6} + (Z_{3-6})_2 [\text{RepA}_{3-6}]} \quad (20)$$

where s_1 and s_2 , are sedimentation coefficients of the RepA – 41-mer complexes with one and two AMP-PNP molecules bound to the helicase, s_{3-6} is the sedimentation coefficient of the RepA – 41-mer complexes with one RepA hexamer bound to the ssDNA and with three, four, five, and six nucleotide cofactor molecules bound to the enzyme; finally, s_{4-6} is the sedimentation coefficient of the RepA – 41-mer complexes with two RepA hexamer bound to the ssDNA and with three, four, five, and six nucleotide cofactor molecules bound to the enzyme. The term, $(Z_{3-6})_2$, is the part of the total partition function, Z_{3-6} , calculated for a single value of $g=2$.

As pointed out above, the complexity of the system is formidable. There are 12 parameters, K_0 , K_1 , K_2 , K_{3-6} , ω , s_0 , s_1 , s_2 , s_{3-6} , s_{4-6} , K , and σ that describe the observed binding process. However, as we also discussed above, several of these parameters are known from quantitative independent measurements of the same system, or can be estimated with adequate accuracy. $K=2.2 \times 10^4 \text{ M}^{-1}$, $\sigma=0.013$, $K_{3-6}=7 \times 10^4 \text{ M}^{-1}$, and $\omega=250$ have been previously determined in our thermodynamic studies of the AMP-PNP binding to the RepA helicase and the enzyme binding to the ssDNA 41-mer, at high [AMP-PNP].⁶ $K_0 = 3(\pm 0.5) \times 10^4 \text{ M}^{-1}$ and $s_0 = 8.4(\pm 0.1) \text{ S}$ have been estimated in sedimentation velocity studies described above (Figure 6(a)). The values of $s_2 = 9.8(\pm 0.3) \text{ S}$ can be estimated from the plot in Figure 7. The value of s_{4-6} , which characterizes all complexes with two RepA molecules bound to the 41-mer, can be conservatively estimated as between 12–13 S, as the largest possible complex is a dimer of two hexamers.⁶ Nevertheless, we included s_{4-6} as a fitting parameter, although we let it flow as $12.5(\pm 0.5) \text{ S}$. Thus five parameters, K_1 , K_2 , s_1 , s_{3-6} , and s_{4-6} remain to be determined. The continuous line in Figure 7 is the non-linear least-squares fit of the experimental curve, using the estimated parameters and five fitting parameters, K_1 , K_2 , s_1 , s_{3-6} , and s_{4-6} . The fit provides $K_1 = 3.9(\pm 1.3) \times 10^6 \text{ M}^{-1}$, $K_2 = 1.3(\pm 0.4) \times 10^4 \text{ M}^{-1}$, $s_1 = 8.0(\pm 0.3) \text{ S}$, $s_{3-6} = 10.3(\pm 0.4) \text{ S}$ and $s_{4-6} = 12.8(\pm 0.4) \text{ S}$. In spite of the complexity, the fit provides an excellent description of the dependence of the sedimentation coefficient of the RepA – ssDNA 41-mer – AMP-PNP tertiary complex as a function of the AMP-PNP concentration (see Discussion).

Interactions of different conformational states of the RepA hexamer, generated by the binding of different numbers of ADP molecules to the enzyme, with the ssDNA

Corresponding sedimentation velocity studies of the RepA – 41-mer complex, as described above for AMP-PNP, have been performed in the presence of ADP (data not shown). The striking difference between the effect of ADP as compared to AMP-PNP is that the presence of all ADP concentrations examined did not affect the initial distribution of the free and bound nucleic acid, observed for the solution without any nucleotide (Figure 6(a)). In other words, these data show that all different conformational states of RepA hexamer complexes with ADP have the same macroscopic binding constant as the free hexamer, i.e. $K_0 = K_1 = K_2 = K_3 = K_4 = K_5 = K_6 = 3(\pm 0.5) \times 10^4 \text{ M}^{-1}$. The dependence of the sedimentation

coefficient of the RepA hexamer – 41-mer complex, as a function of the ADP concentration is included in Figure 7. In all experiments, the total nucleic acid and protein concentrations are 3.5×10^{-6} M and 3.5×10^{-5} M (hexamer), respectively. Unlike the behavior of the system in the presence of AMP-PNP, the plot obtained in the presence of ADP has a much simpler character. As discussed above, in the absence of the nucleotide cofactor, a single RepA hexamer binds the nucleic acid and the sedimentation coefficient of the complex is $s_{20,w} \sim 8.4$ S. At low [ADP], dominated by the total average degree of ADP binding to the RepA hexamer $\Sigma \Theta_i = 1$, the value of $s_{20,w}$ drops slightly, indicating that the sedimentation coefficient of the RepA hexamer – 41-mer, with one ADP molecule bound to the hexamer, has a slightly lower value than $s_{20,w}$ of the complex with the free hexamer. However, at higher ADP concentrations, corresponding to $\Sigma \Theta_i \geq 2$, the plot levels off at $s_{20,w} = 8.35$ S, indicating the lack of any detectable changes in the hydrodynamic properties of the formed complexes with two, three, four, five, and six bound ADP molecules. Moreover, the uniform value of the sedimentation coefficient also indicates that all different conformational states of the RepA hexamer, induced by a different number of bound ADP molecules, exclusively form complexes with a 1:1 stoichiometry with the ssDNA 41-mer (see Discussion).

Analogously to the case with AMP-PNP, the concentration of the different conformational states of the RepA hexamer, containing a different number of bound ADP molecules, are defined using the partition function, Z_H , (equation (6)) and equations (16) (17) (18) (19), and the total RepA concentration, $[\text{RepA}]_T$, using the intrinsic binding constant $K = 2.2 \times 10^4$ M⁻¹ and $\sigma = 0.17$, previously determined for the RepA hexamer – ADP binding interactions.^{7,8} Taking into account the discussion above, the observed apparent sedimentation coefficient of the RepA hexamer – 41-mer - ADP tertiary complex is defined as:

$$s_{20,w} = \frac{s_0[\text{RepA}_0] + s_1[\text{RepA}_1] + s_{3-6}([\text{RepA}_2] + [\text{RepA}_{3-6}])}{[\text{RepA}_0] + [\text{RepA}_1] + [\text{RepA}_2] + [\text{RepA}_{3-6}]} \quad (21)$$

where s_0 and s_1 are the sedimentation coefficients of the RepA – 41-mer complexes without and with one ADP molecule bound to the helicase, and s_{3-6} is the sedimentation coefficient of all the remaining conformational states of the RepA hexamer with a different number of bound ADP molecules in the complex with the ssDNA 41-mer. All parameters in equation (2) 1 are known with the exception of s_1 . The broken line in Figure 7 is the non-linear least-squares fit of the experimental plot, which provides $s_1 = 7.95(\pm 0.11)$ S.

Discussion

The global structure of the free RepA hexamer in solution is different from the analogous crystal structure of the enzyme

The crystallographic studies of the RepA hexamer provided the first high-resolution as well as the global structure of a hexameric helicase.⁵ Nevertheless, the applied solvent conditions in these studies and the fact that the enzyme crystallizes only as a dimer of two hexamers rises a natural question as to what extent the determined structure of the hexamer is preserved for the free RepA hexamer in solution. Crystallographic structure of the RepA hexamer indicates that the six subunits of the hexamer form a ring-like structure with a central cross-channel with a diameter of ~ 17 Å, which can be well approximated as an oblate ellipsoid of revolution with the ratio of the long to short axis, $p = 2.4 \pm 0.2$ (Figure 1(a)). On the other hand, both analytical ultracentrifugation and dynamic light scattering techniques indicate that the axial ratio of the hexamer, in solution, is significantly larger, with $p = 4.5 \pm 0.5$. Even if one assumes the maximum degree of hydration of the protein, using Kuntz's method, the axial ratio, p , is still ~ 4 (Figure 3). These data indicate that the structure

of the free hexamer, in solution, is very different from the structure of the hexamer, which is seen in the crystal. When modeled as an oblate ellipsoid of revolution, the RepA hexamer in solution is much “flatter” than in the crystal form. The very presence of such different global structures of the RepA helicase provides the first indication that the RepA hexamer is a very dynamic entity, which flexibly responds to different environmental conditions, similar to the flexibility of the *E. coli* DnaB hexameric helicase.^{10,47,48} Results on the effect of nucleotide cofactors on the global structure of the enzyme fully confirm this conclusion (see below).

Notice, the experimentally determined partial specific volume of the hexamer, $\bar{v}=0.735(\pm 0.013)$ ml/g is virtually indistinguishable from the same quantity calculated on the basis of the amino acid composition of the protein, $\bar{v}=0.736$ ml/g. The calculated partial specific volume reflects the property of the protein matrix and its interactions with the surrounding solvent, and does not take into account any particular arrangement of the protein subunits in a complex structure such as a ring-like hexamer.^{35,37} On the other hand, the experimentally determined partial specific volume is affected by the particular arrangement of the subunits, if this arrangement affects the interactions with the solvent. The fact that the both partial specific volumes agree to such a notable extent indicates that the solvent moves freely through the cross-channel of the RepA hexamer independently of the movement of the hexamer. This also indicates that the diameter of the cross-channel of the hexamer, in solution, is indeed larger than the ~ 17 Å seen in the crystal structure. Moreover, the axial ratio, $p \sim 4.5$, also strongly suggests that the diameter of the cross-channel of the free RepA hexamer, in solution, is significantly larger than the observed in the crystal of the RepA dimer.

The number of bound nucleotide cofactor molecules dramatically modulates the global structure of the RepA hexamer

Previously, we have reported that the analogous *E. coli* DnaB hexameric helicase undergoes dramatic, global conformational transition upon saturation with ADP or ATP non-hydrolyzable analog, AMP-PNP.¹⁰ Different global conformations of the DnaB hexamer have also been observed in electron microscopic studies.^{49,50} These studies revealed the existing global conformational flexibility of the DnaB hexamer, at the saturating concentration of the nucleotide cofactors. One of the major results of this work is the discovery that the global conformation of the hexameric helicase is modulated by a specific number of bound nucleotide cofactor molecules. The applied statistical thermodynamic model of the nucleotide effect on the global conformation of the RepA hexamer provides an excellent description of the undergoing process indicating that, depending on the number of the bound nucleotide cofactors, the RepA hexamer exists in at least four different and discrete conformational states. Moreover, these discrete conformational states of the enzyme are different in the presence of ADP, as compared to the states induced by the corresponding number of the bound ATP non-hydrolyzable analog, AMP-PNP molecules.

It should be stressed that we could recognize the presence of these different conformational states only through combined application of the sedimentation velocity and dynamic light scattering measurements, which provided a significantly increased resolution of the global structure of the hexamer. For instance, if only sedimentation data were obtained, then the global structure of the RepA hexamer saturated with ADP and characterized by $s_{20,w} \sim 8.85$ S would be deemed very similar to the structure of the enzyme saturated with AMP-PNP, with $s_{20,w} \sim 8.83$ S (Table 1). On the other hand, the corresponding diffusion coefficients are $\sim 4 \times 10^{-7}$ cm²/s and $\sim 4.9 \times 10^{-7}$ cm²/s, respectively, clearly indicating profoundly different global structures (Tables 1 and 2). Moreover, with both $s_{20,w}$ and $D_{20,w}$ determined, one can address the partial specific volume of the hexamer, $\bar{v}$, and, in turn, with higher resolution,

the shape of the hexamer in solution, i.e. the axial ratio of the ellipsoid of revolution for each of the identified conformational states of the RepA hexamer.

Schematic representation of the character of the global structures of the RepA hexamer, induced by the different number of bound ADP molecules, based on the obtained hydrodynamic data, is depicted in Figure 8. Binding of the first ADP molecule to the RepA hexamer does not cause any significant changes of the hexamer global structure, as indicated by the values of $s_{20,w}$, $D_{20,w}$, and $\bar{v}$, which are very similar to the values of the same parameters obtained for the free enzyme. As a result, the global conformation of the RepA hexamer has an axial ratio, $p \sim 4.2$, calculated using the degree of hydration, $h = 0.3 \text{ gH}_2\text{O/g}_{\text{protein}}$, which is very similar to the axial ratio of the free hexamer ($p \sim 4.5$) (Table 1) (Figure 8(b)). However, binding of two ADP molecules induces a dramatic change of the global structure of the enzyme. The protein sediments slower, but it diffuses faster than the free hexamer or the enzyme with one ADP molecule bound, resulting from the large increase of the partial specific volume to $\bar{v} \sim 0.796 \text{ ml/g}$ (Table 1). Such a large increase of $\bar{v}$ suggests a significant change of the degree of hydration, h , of the hexamer, possibly, through an exposure of the polar areas of the protein.^{32,33,37–40} The axial ratio, obtained for the corrected degree of hydration ($h = 0.3$), is $p \sim 1$. Even if we assume that h changes by a factor of e.g., 2, to $h = 0.15 \text{ gH}_2\text{O/g}_{\text{protein}}$, the corresponding value of p is ~ 2.1 , still much lower than the axial ratio of the free hexamer, or the enzyme with one ADP molecule bound. Thus, the data indicate that the global structure of the RepA hexamer with two ADP molecules bound assumes a spherical shape (Figure 8(c)).

Binding of the next ADP molecule, up to a total of three ADP molecules bound, induces a further dramatic change of the hexamer global conformation. The protein sediments faster than the enzyme with two ADP bound, but it diffuses much slower, as a result of the decreased value of the partial specific volume to $\bar{v} \sim 0.726 \text{ ml/g}$, which is slightly lower than the value obtained for the free hexamer (Table 1). The axial ratio, obtained for the corrected degree of hydration, $h = 0.3 \text{ gH}_2\text{O/g}_{\text{protein}}$, is $p = 5.9(\pm 1)$. This value of p indicates that the hexamer assumes the previous shape of an oblate ellipsoid of revolution, although flatter than the free hexamer, as schematically depicted in Figure 8(d). Binding of the additional ADP molecules to a total of four ADP bound, once again, induces a large change in the global structure of the hexamer. The protein has the highest sedimentation coefficient and also the lowest diffusion coefficient (Table 1). However, further saturation of the remaining fifth and sixth nucleotide-binding site of the helicase does not induce further changes in the global structure of the enzyme. The partial specific volume assumes the lowest value of all conformational states of the helicase with the value of $\bar{v} \sim 0.7$, which provides the axial ratio, obtained for the corrected degree of hydration ($h = 0.3$), $p \sim 6.4$. The low value of $\bar{v}$ suggests a change of the degree of hydration and exposure of hydrophobic amino acid residues in this conformational state. Still, if we assume that h changes by as much as a factor of 2, to $h = 0.6 \text{ gH}_2\text{O/g}_{\text{protein}}$, the corresponding value of p is ~ 4.4 . Thus, these data indicate that the RepA hexamer preserves its flatter global structure of the oblate ellipsoid of revolution, when saturated with the nucleoside di-phosphate (Figure 8(e)).

The response of the RepA hexamer to the different numbers of the bound ATP non-hydrolyzable analog, AMP-PNP, to the enzyme is very different from the response observed in the case of ADP. Schematic representation of the character of global structures of the RepA hexamer, induced by the different number of the bound AMP-PNP molecules, is depicted in Figure 9. Binding of the first AMP-PNP molecule to the RepA hexamer already cause a large change of the global structure of the enzyme, which is very different from the free hexamer, or the hexamer with one AMP-PNP molecule bound (Table 2). The protein sediments much slower but it diffuses faster than the free hexamer. Consequently, the partial specific volume is strongly increased to $\bar{v} \sim 0.821 \text{ ml/g}$ (Table 2). With the degree of

hydration, $h=0.3 \text{ gH}_2\text{O/g}_{\text{protein}}$, the global conformation of the RepA hexamer has an axial ratio of $p \sim 1.5$. However, as discussed above, such a large increase of $\bar{v}$ suggests a significant change of h .^{32,33,37–40} If we assume that h changes by as much as a factor of 2, to $h=0.15$, the corresponding value of p is ~ 2.4 , still much lower than the axial ratio of the free hexamer (Tables 1 and 2). Thus, unlike the hexamer with one ADP molecule bound, the global structure of the RepA hexamer, already with one nucleoside tri-phosphate molecule bound, approaches a spherical shape (Figure 9(b)). With two AMP-PNP molecules, the partial specific volume assumes the value of $\bar{v} \sim 0.782 \text{ ml/g}$ (Table 2). With the degree of hydration, $h=0.3 \text{ gH}_2\text{O/g}_{\text{protein}}$, the axial ratio of the hexamer is $p \sim 1$, while with $h=0.15 \text{ gH}_2\text{O/g}_{\text{protein}}$, the value of p is ~ 2 . Thus, the global structure of the RepA hexamer with two bound AMP-PNP molecules is very close to the spherical one and similar to the structure at the corresponding number of bound ADP molecules (Figures 8(c) and 9(c)).

However, unlike the effect of ADP, binding of three AMP-PNP molecules to the RepA hexamer induces a much less pronounced change of the hexamer global conformation (Table 2). The axial ratio, obtained for the corrected degree of hydration, $h=0.3 \text{ gH}_2\text{O/g}_{\text{protein}}$, is $p \sim 1.8$. With the assumed value of $h=0.15 \text{ gH}_2\text{O/g}_{\text{protein}}$, the value of p is ~ 2.7 . Thus, the hexamer assumes the shape of an oblate ellipsoid of revolution, although significantly less flat than the structure with the three ADP molecules bound, which has a disk-like shape (Figures 8(d) and 9(d)) (Table 2). Further saturation of the remaining fourth, fifth, and sixth nucleotide-binding sites with AMP-PNP has little effect on this global structure of the RepA hexamer, as compared to the enzyme with three ADP molecules bound. Nevertheless, the protein has the highest sedimentation coefficient and also a high diffusion coefficient. The axial ratio, obtained for the corrected degree of hydration ($h=0.3$), is $p \sim 1.9$ (Table 2). On the other hand, this value of p indicates that the global structure of the hexamer, saturated with the nucleoside tri-phosphate, is more spherical than the structure of the disk-like shape of the enzyme saturated with ADP, or the shape of oblate ellipsoid of revolution of the free hexamer in solution (Figure 9(e)).

Modulation of the global structure of the RepA hexamer occurs sequentially with all six nucleotide binding sites participating in allosteric propagation of conformational changes

There are two remarkable features of the observed effect of the nucleotide cofactor on the global structure of the RepA hexamer. Moreover, these features are independent of any binding model. First, binding of only one or two nucleotide cofactors already induces major global conformational changes of the hexamer (Figures 4 and 5). The global character of the observed changes, induced by a single cofactor molecule bound, can only occur if the nucleotide effect propagates allosterically through the entire hexamer, mediated by interacting interfaces of the neighboring subunits, i.e. with all subunits participating in the process.^{10,23,24} Second, the data in Figures 4 and 5 indicate that several very different conformational changes, in opposite directions, occur sequentially, i.e. they are characteristic for a given degree of binding of the nucleotide cofactor.

Modulation of the global structure of the RepA hexamer is separated into two phases depending on the number of bound nucleotide cofactors

Another prominent feature of the observed effect of the nucleotide cofactors on the RepA hexamer global conformation is a clear separation between the effects of binding of the first three nucleotides and binding of the subsequent three cofactor molecules, particularly, visible in the case of the ATP analog, AMP-PNP. Moreover, these features are also independent of any binding model. The binding of the first, second, and third nucleotides, each induces a specific conformational transition, different from the previous one. With the exception of the state with two cofactor molecules bound, the induced conformational changes are different for the nucleoside tri-phosphate, as compared to nucleoside di-

phosphate. Conversely, binding of the remaining three nucleotides, i.e. formation of the complex of the hexamer with four, five, and six cofactor molecules induces a much more homogeneous structure, although very different for the ATP analog, as compared to ADP. Thus, the modulation of the hexamer global structure is different for the complexes with up to three bound nucleotides, from the effect observed for the nucleotide-saturated form of the enzyme. This functional heterogeneity indicates a different role of the two modulation processes in activities of the enzyme (see below).

Only one conformational state of the RepA hexamer, induced by binding of two nucleotide tri-phosphate molecules, has dramatically higher ssDNA-affinities than the enzyme complexes with ADP

Changing the ssDNA affinity of a helicase through the binding and/or hydrolysis of the nucleoside tri-phosphates is the major element of any model of the mechanical translocation and the dsDNA unwinding by the enzyme.^{20–22} Current thinking about the effect of the nucleotide cofactors on the ssDNA-affinity of a hexameric helicase is that a single high-affinity state is achieved through saturation of the multiple nucleotide-binding sites of the enzyme with the nucleotide tri-phosphate. This is partly because the enzyme structure and binding to the ssDNA as a function of the number of bound nucleotide cofactor molecules have never been undertaken before. The data obtained for the RepA hexameric helicase in this work clearly indicate that the situation is much more intricate. The excellent agreement between the formulated statistical thermodynamic model and the observed binding of the RepA hexamer to the ssDNA as a function of the AMP-PNP or ADP concentration (Figure 7) indicates that different conformational states of the helicase, induced by the binding of a different number of nucleotide cofactors, compete for the nucleic acid. Moreover, in the case of the ATP analog, these different conformational states have profoundly different ssDNA affinities. In other words, control of interactions of the RepA hexamer with the ssDNA by the nucleotide cofactors occurs through modulating the structure and affinity of the hexamer, prior to binding to the DNA.

The data obtained here and in previous thermodynamic studies of the RepA hexamer binding to the ssDNA indicated a puzzling situation.⁶ The intrinsic affinity of the enzyme for the ssDNA, in the presence of the high nucleoside tri-phosphate concentration, is only moderately higher than the affinity observed for the enzyme in the absence of AMP-PNP (Table 2). Figure 10 shows the dependence of the natural logarithm of intrinsic affinities of the different conformational states of the RepA hexamer, induced by AMP-PNP or ADP, upon the number of AMP-PNP or ADP molecules bound to the helicase. The plot reflects the free energy difference between different ssDNA affinities without being dependent on the particular temperature of the measurement. Because of the large difference between the length of the ssDNA 41-mer and the site-size of the RepA hexamer – ssDNA complex ($19(\pm 1)$ nucleotides), the differences between site-sizes of various complexes by one to three nucleotides (see above) do not affect the plot and can only increase the differences between the intrinsic affinities. It is evident that only a complex with two nucleoside tri-phosphate molecules bound has a dramatically higher intrinsic affinity for the nucleic acid, as compared to any complexes of the enzyme with ADP or the free helicase. The affinity of the complex with two AMP-PNP molecules bound is about three orders of magnitude higher than the affinity of the complex with two ADP molecules bound, while the complex with one AMP-PNP molecule bound has an affinity higher by about two orders of magnitude than the RepA hexamer with the corresponding number of ADP molecules bound. First, the fact that there is only one complex with such an increased ssDNA affinity strongly suggests that two nucleoside tri-phosphate molecules participate in the free energy transduction during the dsDNA unwinding reaction and the mechanical translocation on the DNA lattice (see below). Second, there is not a “general” nucleoside tri-phosphate state of the hexameric

helicase, which has high ssDNA affinity, but a very specific state with well defined and a low number of the bound nucleoside tri-phosphate molecules and a very specific global structure (Figure 9).

Conformational states of the RepA hexamer with one or two bound nucleoside tri-phosphate molecules have increased site-sizes of the enzyme – ssDNA complexes

The ssDNA oligomer containing 41 nucleotides has been selected because it allows us to address three major aspects of the RepA hexamer interactions with the ssDNA: affinities, site-sizes of the formed complexes, and the presence of cooperative interactions.⁶ In the presence of high [AMP-PNP], the total DNA-binding site of the RepA hexamer has a very stringent requirement for an uninterrupted 19(±1) stretch of nucleotides, in order to form a complex with the ssDNA. For instance, already the ssDNA 37-mer cannot accept two RepA hexamers, since the length of 17 – 18 nucleotides is not enough to engage to total DNA-binding site of the enzyme. Notice that the site-sizes of the complexes with the ssDNA 41-mer, formed by the conformational states of the RepA hexamer, with one or two AMP-PNP molecules bound, must be larger than the site-size of 19(±1) nucleotides the enzyme – ssDNA complex determined at high AMP-PNP concentrations.⁶ This is because, at the applied total protein concentration (3.5×10^{-5} M (hexamer)) the affinity of the helicase in these two conformational states, particularly with two AMP-PNP molecules bound, is high enough for the second hexamer to efficiently associate with the 41-mer, even without any positive cooperative interactions (see below). Yet, binding of two hexamers, at this degree of binding of AMP-PNP, which would be manifested by large increase of $s_{20,w}$, is not observed experimentally (Figure 7). The sedimentation coefficient of two RepA hexamers bound to the 41-mer is ≥ 12 S, while only a complex characterized by $s_{20,w} \sim 9.8$ S with the highest molecular weight of $\sim 200,000$ is observed (see above) (Figure 7). The increased site-size of the helicase – ssDNA complex corroborates the spherical shape of the hexamer with one or two AMP-PNP molecules bound as compared to the flatter oblate ellipsoid of revolution of the free hexamer or the hexamer with one ADP molecule bound (Tables 1 and 2).

Functional implications. Conformational states of a hexameric helicase, with a low degree of saturation with the nucleotide cofactors, are involved in the mechanical translocation and dsDNA unwinding activities of the enzyme

The fundamental features of molecular transitions of a helicase, involved in active mechanical translocation and dsDNA unwinding reaction and fueled by the nucleoside tri-phosphate binding and hydrolysis, must include a large difference between the affinities and the difference between the site-sizes of the helicase – ssDNA complexes of the enzyme associated with the nucleoside tri-phosphate, as compared to the complex with nucleoside di-phosphate.^{20–22} The large difference in the ssDNA affinities provides free energy for the unwinding and mechanical translocation, while the difference in site-size is the mechanical requirement for the progressive movement of the enzyme, which allows the helicase to invade the duplex DNA. The discovery of a large difference in affinities and different site-sizes of the various conformational states of the RepA hexamer, discussed above, strongly suggest that the structural and functional modulations of the RepA hexamer, observed at a low degree of nucleotide binding to the hexamer, are the identified set of molecular transitions involved in the translocation and dsDNA unwinding by a hexameric helicase and, particularly, the RepA helicase. No other conformational states of the enzyme fulfill these fundamental requirements for free energy transduction, although they may differ in the site-sizes of the formed complexes with the ssDNA (Figure 10) (see below).

Figure 11 shows the plausible model of the conformational transitions involved in mechanical translocation and unwinding of the dsDNA by the RepA hexamer, based on the

data obtained. The enzyme, free of nucleotides and forming a flat ellipsoid with axial ratio $p \sim 4.5$, is bound at the opening of the DNA fork. The cross-channel is larger than in the crystal structure (see above). Binding of the first nucleoside tri-phosphate molecule induces the transformation of the hexamer into a more spherical structure with $p \sim 1.5$ – 1 , with the larger site-size and a higher affinity for the ssDNA. The increase of the sites-size can be by ~ 1 – 3 nucleotides, as the kinetic step size of the dsDNA unwinding by the analogous DnaB helicase is only ~ 1 base-pair.^{51,52} This structural transition causes the helicase to invade the duplex DNA by ~ 1 – 3 base-pairs and to weaken the stability of the duplex conformation of the nucleic acid now engulfed by the cross-channel of the helicase.^{29,51,52} Binding of the second nucleoside tri-phosphate molecule further strongly increases the affinity for the ssDNA and enables the breaking of the dsDNA structure by increasing the favorable free energy for the complex with the ssDNA conformation. Subsequent hydrolysis of two nucleoside tri-phosphate molecules weakens interactions with the DNA but preserve the site-size of the complex. The release of the two nucleoside di-phosphate molecules returns the hexamer to the shape of the oblate ellipsoid of revolution, with $p \sim 4.5$, and with the lower site-size. The binding of the nucleotide tri-phosphate or ADP to the third site on the hexamer can play an additional trigger role in changing to this lower-site size, flat conformation of the enzyme (Figures 8 and 9). The enzyme is now located ~ 1 – 3 bases from the starting point, i.e. it translocated over a distance of the unwound dsDNA. The helicase can now repeat the cycle.

Role of negative cooperative interactions in the nucleotide cofactor binding to a hexameric helicase

The proposed mechanism provides the long awaited explanation for the role of the observed negative cooperativity in the nucleotide binding to hexameric helicases.^{7,8,23–27} So far, this is an unresolved problem in understanding the mechanism of the enzymes. Recall, the binding of the nucleotide cofactors to all studied hexameric helicases is characterized by two binding phases, with first three nucleotides binding in the high-affinity phase and the next three cofactors binding in the low-affinity phase.^{7,8,23–27} In the case of both the *E. coli* DnaB and RepA hexamers, the two binding phases result from negative cooperative interactions between the six nucleotide-binding sites of the enzymes.^{7,8,23,24} Notice, AMP-PNP binds the RepA hexamer with much stronger negative cooperativity, with cooperative interaction parameter $\sigma \sim 0.013$, as compared to ~ 0.17 observed for ADP.^{7,8} The negative cooperativity of ATP binding is even stronger with $\sigma \sim 0.003$. In other words, the RepA hexamer will be saturated with nucleoside tri-phosphates beyond the first three bound nucleotides only at very high ≥ 10 mM nucleoside tri-phosphate concentrations. Therefore, the role of the negative cooperative interactions in the nucleotide binding to a hexameric helicase and splitting the binding process into two binding phases, each including the association of three cofactors, would simply serve to increase the probability for the enzyme to be in states with a low degree of nucleotide binding, which are active in dsDNA unwinding and mechanical translocation (see above; Figure 11).

Role of the conformational states of a hexameric helicase induced by the saturating number of nucleotide cofactors

Nevertheless, at very high nucleotide concentration, all six nucleotide-binding sites of the RepA hexamer will be saturated with nucleotide cofactors.^{7,8} We could not directly estimate the ssDNA site-sizes of the RepA helicase saturated with ADP, as compared to the enzyme containing three or more bound AMP-PNP molecules, because of the low affinity of both these complexes (Figures 7 and 10). However, a significantly flatter oblate ellipsoidal shape of the hexamer saturated with ADP indicates that its site-size is lower than the site-size of the enzyme with bound AMP-PNP molecules ($p \sim 1.9$) (Figures 8 and 9). Low and moderately different ssDNA affinities of the enzyme saturated with ADP or nucleoside tri-

phosphate preclude these complexes from participating in efficient free energy transduction processes.^{7,8} On the other hand, the difference in the ssDNA site-sizes between these complexes and only a moderate difference in the ssDNA affinity strongly suggest that they may exclusively play the role of an auxiliary mechanism in the mechanical translocation. This points to an important caveat in exclusively interpreting mechanical translocation data for a hexameric helicase as representing the same mechanism as the translocation mechanism involved in the dsDNA unwinding reaction.

In the case of the *E. coli* DnaB protein, it is also known that the enzyme may function as a molecular pump, which pushes the nucleic acid through the cross-channel.^{53,54} The presence of two different conformations of the enzyme, differing in the number of occluded nucleotides or base-pairs of the ss and dsDNA, respectively, which can be generated by binding and hydrolysis of the multiple nucleoside tri-phosphates, corroborates well the notion that the functional role of the conformational states of the hexamer, induced by more than three nucleotides bound to the helicase, is related to the molecular pump activity of the enzyme. Whether or not the RepA hexamer possesses a similar pump activity is, as yet, unknown. However, the unusual character of the RepA helicase interactions with the ssDNA is the fact that, in the presence of high AMP-PNP concentration, the enzyme binds the ssDNA with significant positive cooperativity with ~250. This feature could play a role in enhancing the molecular pump activity of this enzyme by overcoming only a moderate difference in the affinities between nucleoside tri and di-phosphate conformational states.

Materials and Methods

Reagents and buffers

All chemicals were reagent grade. All solutions were made with distilled and deionized 18 M Ω (Milli-Q) water. The standard buffer, T5, is 50 mM Tris adjusted to pH 7.6 at appropriate temperatures with HCl, 10% (v/w) glycerol, 10 mM NaCl, and 1 mM MgCl₂. The temperature and concentrations of salt in the buffer are indicated in the text.

RepA helicase of plasmid RSF1010—The gene of the RepA helicase has been isolated directly from plasmid RSF1010 and placed in plasmid pET30a under control of the T7 polymerase system. Isolation and purification of the protein was performed, with slight modifications, as described.¹² The protein was N99% pure as judged by SDS–polyacrylamide gel electrophoresis with Coomassie brilliant blue staining. The concentration of the protein was determined spectrophotometrically using the extinction coefficient $\epsilon_{280} = 1.66 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$, obtained with the approach based on Edelhoch's method.^{55,56}

Nucleotides and nucleic acids

ATP, ADP, and AMP-PNP, were from Sigma (Saint Louis, MO). The modified ssDNA 5'Fl-dT(pT)₃₉, was purchased from Midland Certified Reagents (Midland, TX). The modified nucleic acid contains a fluorescent label, fluorescein, attached at the 5' end through phosphoramidate chemistry. Concentrations of all ssDNA oligomers have been determined spectrophotometrically as described.^{9,12–15,29,28,34}

Analytical ultracentrifugation measurements

Sedimentation velocity experiments were performed with an Optima XL-A analytical ultracentrifuge (Beckman Inc., Palo Alto, CA) using double-sector charcoal-filled 12 mm centerpieces as described.^{9,10,15,34,35} Sedimentation velocity scans were collected at the absorption band of the RepA helicase at 280 nm. The RepA helicase – nucleotide complexes were scanned at 295 nm where the cofactor absorbance is minimal and the absorbance of the sample is dominated by the protein. Time derivative analyses of sedimentation scans were

performed with the software supplied by the manufacturer using averages of 8 to 15 scans for each concentration as p described.^{9,10,15,34,35} The values of sedimentation coefficients were corrected to $s_{20,w}$ for solvent viscosity and temperature to standard conditions.^{32,33}

Dynamic light scattering measurements

Dynamic light scattering measurements were performed using a Brookhaven instrument equipped with BI-9000AT autocorrelator with 512 channels (Brookhaven, NY). The light source was the He-Ne laser with 45 mW nominal power, emitting light at 632.8 nm. The measurements were performed with 200 μm aperture, using the borosiliconated glass cell containing 1.2 ml of the sample and a thermostated cell holder. The homodyne intensity autocorrelation functions of the scattered light from the sample were collected at three different degrees, 60°, 90°, and 120°. The translational diffusion coefficient, $D_{20,w}$, was determined from autocorrelation function, $g(t)$, defined as:^{36,57}

$$g(t) = B + A \exp\left(-\frac{t}{\tau}\right) \quad (22)$$

where B is the bases line, A is normalized amplitude, and τ , is the autocorrelation time. The autocorrelation time is defined as:

$$\tau = \frac{1}{2Dq^2} \quad (23)$$

where D is the diffusion coefficient and q is the scattering vector described by:

$$q = \frac{4\pi n}{\lambda} \sin\left(\frac{\Theta}{2}\right) \quad (24)$$

The values of diffusion coefficient, D , were corrected to $D_{20,w}$ for solvent viscosity and temperature to standard conditions.^{32,33,57} The non-linear least-squares fittings of autocorrelation functions have been performed using software provided by the manufacturer.

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Abbreviation used

AMP-PNP β,γ -imidoadenosine 5'-triphosphate

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Figure 1.

The structure of the RepA helicase hexamer determined in crystallographic studies.⁵ The structure was generated using data from RCSB Protein Data Bank, under the code 1G8Y, using ViewerPro (San Diego, CA). The six subunits form a ring-like structure with the diameter of 115 Å with the central cross-channel 17–21 Å. This global structure of the RepA hexamer can be approximated as the oblate ellipsoid of revolution with the long axis, $a=115(\pm 5)$ Å and the short axis, $b=48(\pm 3)$ Å (broken lines) (see the text for details).

Figure 2.

(a) The sedimentation velocity profiles (monitored at 280 nm) of the RepA helicase, in buffer T5 (pH 7.6, 10 °C). The concentration of the protein is 6×10^{-6} M (hexamer); 14 min. time interval; 35,000 rpm. (b) The dependence of the RepA hexamer sedimentation coefficient, $s_{20,w}$, upon the protein concentration in buffer T5 (pH 7.6, 10 °C). (c) The dependence of the normalized autocorrelation function, $g(t)$, of the scattered light from RepA helicase solution (6×10^{-6} M (hexamer)), in buffer T5 (pH 7.6, 10 °C), upon the logarithm of delay time. The autocorrelation function has been collected at 90°. The continuous line is the non-linear least-squares fit of the experimental curve to a single exponential function defined by equation (22) (Materials and Methods). (d) The dependence of the RepA hexamer diffusion coefficient, $D_{20,w}$, upon the protein concentration in buffer T5 (pH 7.6, 10 °C).

Figure 3.

(a) Computer simulation of the sedimentation coefficient, $s_{20,w}^{\circ}$, as a function of the axial ratio of the oblate ellipsoid of revolution, p , for different values of the degree of hydration, h ($gH_2O/g_{protein}$); 0 (---); 0.3 (—); 0.4 (— · —). The plots have been generated using equations (1), (4), and (5). The continuous horizontal line marks the value of the sedimentation coefficient, $s_{20,w}^{\circ} = 8.36$. The arrows indicate values of the axial ratio, p , corresponding to the same $s_{20,w}^{\circ}$ at different degrees of hydration (see the text for details). (b) Computer simulation of the diffusion coefficient, $D_{20,w}^{\circ}$, as a function of the axial ratio of the oblate ellipsoid of revolution, p , for different values of the degree of hydration, h ($gH_2O/g_{protein}$); 0 (---); 0.3 (—); 0.4 (— · —). The plots have been generated using equations (2), (4), and (5). The continuous horizontal line marks the value of the sedimentation

coefficient, $s_{20,w}^0=8.36$. The arrows indicate values of the axial ratio, p , corresponding to the same $D_{20,w}^0$ at different degrees of hydration (see the text for details).

Figure 4.

(a) The dependence of the sedimentation coefficient, $s_{20,w}$, of the RepA hexamer in buffer T5 (pH 7.6, 10 °C) upon the logarithm of the ADP concentration. (b) The dependence of the sedimentation coefficient, $s_{20,w}$, of the RepA hexamer in buffer T5 (pH 7.6, 10 °C) upon the total average degree of binding, $\Sigma\Theta_i$, of ADP on the RepA hexamer. The continuous lines in (a) and (b) are non-linear least-squares fits of the experimental curves using the defined statistical thermodynamic model, described by equations (6), (7) and (8). The hydrodynamic parameters obtained are included in Table 1. (c) The dependence of the diffusion coefficient, $D_{20,w}$, of the RepA hexamer in buffer T5 (pH 7.6, 10 °C) upon the logarithm of ADP concentration. (d) The dependence of the diffusion coefficient, $D_{20,w}$, of the RepA hexamer in buffer T5 (pH 7.6, 10 °C) upon the total average degree of binding, $\Sigma\Theta_i$, of ADP on the RepA hexamer. The continuous lines in (c) and (d) are non-linear least-squares fits of the experimental curves using the defined statistical thermodynamic model, described by equations (6), (7) and (9). The obtained hydrodynamic parameters are included in Table 1 (see the text for details).

Figure 5.

(a) The dependence of the sedimentation coefficient, $s_{20,w}$, of the RepA hexamer in buffer T5 (pH 7.6, 10 °C) upon the logarithm of the AMP-PNP concentration. (b) The dependence of the sedimentation coefficient, $s_{20,w}$, of the RepA hexamer in buffer T5 (pH 7.6, 10 °C) upon the total average degree of binding, $\Sigma\Theta_i$, of AMP-PNP on the RepA hexamer. The continuous lines in (a) and (b) are non-linear least-squares fits of the experimental curves using the defined statistical thermodynamic model, described by equations (6), (7) and (10). The obtained hydrodynamic parameters are included in Table 1. (c) The dependence of the diffusion coefficient, $D_{20,w}$ of the RepA hexamer in buffer T5 (pH 7.6, 10 °C) upon the logarithm of AMP-PNP concentration. (d) The dependence of the diffusion coefficient, $D_{20,w}$, of the RepA hexamer in buffer T5 (pH 7.6, 10 °C) upon the total average degree of binding, $\Sigma\Theta_i$, of AMP-PNP on the RepA hexamer. The continuous lines in (c) and (d) are non-linear least-squares fits of the experimental curves using the defined statistical thermodynamic model, described by equations (6), (7) and (11). The hydrodynamic parameters obtained are included in Table 1 (see the text for details).

Figure 6.

(a) Absorption profiles, recorded at 495 nm, of analytical sedimentation velocity runs of the ssDNA 41-mer, 5'-Fl-dT(pT)₃₉, in the presence of the RepA helicase, in buffer T5 (pH 7.6, 10 °C) and in the absence of AMP-PNP. The total nucleic acid and protein concentrations are 3.5×10^{-6} M and 3.5×10^{-5} M (hexamer), respectively. The scans were collected at 35,000 rpm. The short initial parts of the scans correspond to the buffer-air region above the meniscus. The arrows indicate the absorption of the total concentration of the ssDNA 41-mer, (T), the concentration of the 41-mer bound in the complex with the RepA hexamer (C), and the concentration of the free nucleic acid, (F). The horizontal lines indicate the locations of the plateaus corresponding to the zero line (— - —), free 41-mer concentration (- - -), and total 41-mer concentration (——). (b) Analogous absorption profiles, recorded at 495

nm, of analytical sedimentation velocity runs of the ssDNA 41-mer, 5'-Fl-dT(pT)₃₉, in the presence of the RepA helicase, in buffer T5 (pH 7.6, 10 °C), containing 10^{-4} M AMP-PNP. The total nucleic acid and protein concentrations are 3.5×10^{-6} M and 3.5×10^{-5} M (hexamer), respectively. The scans were collected at 35,000 rpm.

Figure 7.

The sedimentation coefficient, $s_{20,w}$, of the complex of the RepA hexamer with the ssDNA 41-mer, 5'-Fl-dT(pT)₃₉, in buffer T5 (pH 7.6, 10 °C) as a function of the AMP-PNP (■) or ADP (□) concentrations. The continuous lines are non-linear least-squares fits of the experimental curves, using the defined statistical thermodynamic model for the effect of the AMP-PNP on the RepA interactions with the ssDNA 41-mer (equations (13)–(20)) and the statistical thermodynamic model for the analogous effect of ADP (equations (16)–(19), and (21)). The binding parameters obtained are included in Tables 1 and 2 (see the text for details).

Figure 8. Schematic model of the RepA hexamer global conformational transitions induced by the different numbers of bound nucleoside di-phosphate, ADP, based on the data obtained here (see the text for details).

Figure 9.

Schematic model of the RepA hexamer global conformational transitions induced by the different numbers of bound ATP non-hydrolyzable analog, AMP-PNP, based on the data obtained here (see the text for details).

Figure 10.

The dependence of the natural logarithm of intrinsic affinities of the different conformational states of the RepA hexamer, induced by AMP-PNP (■) or ADP (□), upon the number of AMP-PNP or ADP molecules bound to the helicase. The intrinsic affinities have been calculated using the same site-size of 19 nucleotides for all complexes formed (see the text for details). The continuous lines are interpolations between the points and have no theoretical basis.

Figure 11.

A plausible model of the functional role of the identified conformational states of the RepA hexamer induced by the binding of the first two nucleotide cofactors, in the free energy transduction of the dsDNA unwinding and mechanical translocation of the RepA helicase. The hexamer, free of nucleotides and forming a flat ellipsoid with axial ratio $p \sim 4.5$, is bound at the opening of the fork DNA substrate. Binding of the first ATP molecule induces the transformation of the hexamer into the spherical structure, with a larger site-size and a higher affinity for the ssDNA. This increased site-size allows the helicase to invade the duplex DNA by 1–3 base-pairs and the increased ssDNA affinity weakens the stability of the duplex conformation of the nucleic acid, which became engulfed by the cross-channel of the helicase. Binding of the second nucleoside tri-phosphate molecule further dramatically increases the affinity for the ssDNA and enables the dsDNA unwinding, by providing the large favorable free energy for the complex with the ssDNA conformation. Hydrolysis of the nucleoside tri-phosphate molecules weakens the interactions with the DNA but preserves the site-size of the complex with the two ADP molecules. The release of the two NDP molecules returns the hexamer to the shape of the oblate ellipsoid of revolution ($p \sim 4.5$), which has the lowest site-size. The binding of ATP or ADP to the third site can play an additional trigger role in changing to this lower-site size, flat conformation of the enzyme. The enzyme is now located one to three bases from the starting point, i.e. it is translocated over a distance of the unwound dsDNA and can now repeat the cycle. Notice, in the model, we assumed that the ssDNA passes through the cross-channel of the RepA hexamer. Although this assumption does not affect the major aspects of the model, it still remains to be proven.

Table 1

Hydrodynamic, structural, and binding parameters of the complexes of the RepA hexameric helicase with different numbers of bound ADP

Number of bound ADP molecules	0	1	2	3	4	5	6
$s_{20,w}$ (S)	8.29±0.08 ^a	8.29±0.08	7.6±0.1	8.15±0.11	8.85±0.15	8.85±0.11	8.85±0.11
$D_{20,w} \times 10^7$ (cm ² /s)	4.33±0.05 ^a	4.33±0.05	5.05±0.10	4.03±0.10	4.0±0.1	4.0±0.1	4.0±0.1
$\bar{v}$ (ml/g) ^b	0.736±0.013	0.741±0.008	0.796±0.08	0.726±0.008	0.7000±0.008	0.7000±0.008	0.7000±0.008
p ($h=0.3$) ^c	4.5±0.5	4.2±0.6	1±0.2	5.9±1	6.4±1	6.4±1	6.4±1
p ($h=0.15$) ^d	–	–	2.1±0.5	–	–	–	–
p ($h=0.6$) ^e	4.5±0.5	–	–	–	4.4±1.0	4.4±1.0	4.4±1.0
K_N (M ⁻¹) ^f	(3.0±0.5)×10 ⁴	(3.0±0.5)×10 ⁴	(3.0±0.5)×10 ⁴	(3.0±0.5)×10 ⁴	(3.0±0.5)×10 ⁴	(3.0±0.5)×10 ⁴	(3.0±0.5)×10 ⁴

^aError is the standard deviation using three to four experimental data.

^bCalculated using corresponding $s_{20,w}$ and $D_{20,w}$ values (see the text for details).

^cCorrected degree of hydration, h , obtained using Kuntz's method (see the text for details).

^dAssumed lowest degree of hydration.

^eAssumed highest degree of hydration.

^fMacroscopic binding constant of RepA hexamer, containing a given number, N , of bound ADP molecules, for the ssDNA 41-mer (see the text for details).

Table 2

Hydrodynamic and structural properties of the complexes of the RepA hexameric helicase with different numbers of bound molecules of the ATP non-hydrolyzable analog, AMP-PNP

Number of bound AMP-PNP molecules	0	1	2	3	4	5	6
$s_{20,w}$ (S)	8.29±0.08 ^a	6.5±0.1	8.29±0.08	8.3±0.1	8.83±0.25	8.83±0.25	8.83±0.25
$D_{20,w} \times 10^7$ (cm ² /s)	4.27±0.04 ^a	4.9±0.1	5.1±0.1	4.9±0.1	4.9±0.1	4.9±0.1	4.9±0.1
$\bar{v}$ (ml/g) ^b	0.735±0.013	0.821±0.013	0.782±0.008	0.771±0.008	0.756±0.008	0.756±0.008	0.756±0.008
p ($h=0.3$) ^c	4.5±0.5	1.6±0.4	1.0±0.2	1.8±0.4	1.9±0.4	1.9±0.4	1.9±0.4
p ($h=0.15$) ^d		2.4±0.4	2.0±0.2	2.7±0.4	2.8±0.4	2.8±0.4	2.8±0.4
K (M ⁻¹) ^e	(3±0.5)×10 ⁴	3.9×10 ⁶	1.3×10 ⁸	1.6×10 ⁶	1.6×10 ⁶	1.6×10 ⁶	7×10 ⁶
ω	—	1	1	250	250	250	250

^a Error is the standard deviation using three to four experimental data.

^b Calculated using corresponding $s_{20,w}$ and $D_{20,w}$ values (see the text for details).

^c Corrected degree of hydration, h , obtained using Kuntz's method (see the text for details).

^d Assumed lowest degree of hydration.

^e Macroscopic binding constant of RepA hexamer, containing a given number, N , of bound AMP-PNP molecules, for the ssDNA 41-mer (see text for details).