ISWI Remodels Nucleosomes through a Random Walk

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S Supporting Information

ABSTRACT: The chromatin remodeler ISWI is capable of repositioning clusters of nucleosomes to create well-ordered arrays or moving single nucleosomes from the center of DNA fragments toward the ends without disrupting their integrity. Using standard electrophoresis assays, we have monitored the ISWI-catalyzed repositioning of different nucleosome samples each containing a different length of DNA symmetrically flanking the initially centrally positioned histone octamer. We find that ISWI moves the histone octamer between distinct and thermodynamically stable positions on the DNA according to a random walk mechanism. Through the application of a spectrophotometric assay for nucleosome repositioning, we further characterized the repositioning activity of ISWI using short nucleosome substrates and were able to determine the macroscopic rate of nucleosome repositioning by ISWI. Additionally, quantitative analysis of repositioning experiments performed at various ISWI concentrations revealed that a monomeric ISWI is sufficient to obtain the observed repositioning activity as the presence of a second ISWI bound had no effect on the rate of nucleosome repositioning. We also found that ATP hydrolysis is poorly coupled to nucleosome repositioning, suggesting that DNA translocation by ISWI is not energetically rate-limiting for the repositioning reaction. This is the first calculation of a microscopic ATPase coupling efficiency for nucleosome repositioning and also further supports our conclusion that a second bound ISWI does not contribute to the repositioning reaction.
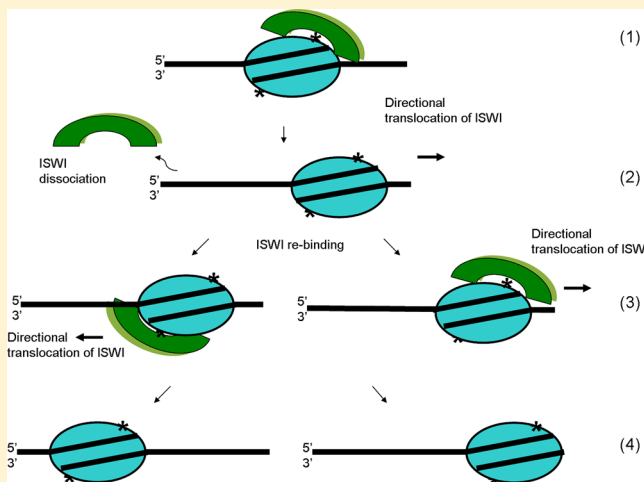
The packaging and organization of DNA into higher-order structures, termed chromatin, serves as a mechanism for the regulation of DNA repair, replication, and gene expression within the cell.[1,2] The nucleosome represents the basic packaging unit of the chromatin and consists of ~147 bp of DNA wrapped around an octamer of positively charged proteins called histones; two of each of the four core histones (H2A, H2B, H3, and H4) interact to form a stable octamer.[3,4] The wrapped DNA contacts the histone octamer at 14 different sites spaced approximately 10 bp apart, with each contact site harboring several different types of noncovalent interactions between histone proteins and DNA.[3] For DNA to be accessible to gene expression and DNA replication machinery, the chromatin must be dynamically reorganized. One mechanism of regulating this reorganization of chromatin structure involves the activity of a group of ATP-utilizing molecular motor enzymes termed chromatin remodelers.[5−7] All chromatin remodelers share a highly conserved ATPase domain and are further categorized into four subfamilies (ISWI, SWI/SNF, CHD, and INO80) based on additional domains that confer specific functional properties.[2,8,9] Because of homology of sequence and function, chromatin remodelers are classified as part of a large family of proteins called the SNF2 family, which in turn is part of helicase superfamily II (SF-II).[8] Similar to proteins belonging to the helicase families of proteins, remodelers share the ability to translocate along free or nucleosomal DNA in an ATP-dependent manner.[10−13] This ability to translocate along DNA has been shown to be critical to their nucleosome repositioning activity.[10−13] Several models have been proposed for how these remodelers reposition the histone octamer along the DNA, all of which rely on the general ability of the remodeler to translocate along the nucleosomal DNA causing at least partial distortion of histone–DNA interactions leading to the movement of the octamer to a new position on the DNA and re-establishment of DNA–histone contacts.

The 135 kDa ISWI (imitation switch) ATPase from *Xenopus laevis* is a member of the ISWI subfamily of chromatin remodeling enzymes with homologues identified in several species, including humans, *Drosophila melanogaster*, and

Saccharomyces cerevisiae.[14−17] ISWI is the molecular motor that drives the activities of several chromatin remodeling complexes. In *Xenopus*, for example, ISWI interacts with other noncatalytic protein subunits to form three additional chromatin remodeling complexes (ACF, CHRAC, and WICH[18]). Interestingly, ISWI has been shown to have basal nucleosome binding and repositioning activities independent of its association with other complexes; however, the nucleosome repositioning strategy of ISWI appears to change when it is in these different complexes[2,19−23] from creating well-spaced arrays to completely random nucleosome spacing. Additionally, the directional bias of histone repositioning, either toward or away from thermodynamically favored positions on the associated DNA, varies among these complexes.[23−28] Naturally, understanding the nucleosome repositioning activity of the fundamental ISWI motor is essential to understanding how the activities of these various chromatin remodeling complexes are differentiated and thus how the function of ISWI is regulated by the other interacting proteins in these complexes. The elucidation of these regulation mechanisms will then allow for the determination of how these different complexes are used by the cell to achieve different chromatin reorganization outcomes *in vivo*.

Many experiments have been conducted to understand the regulatory mechanisms underlying the remodeling activity of ISWI and ISWI-containing complexes. The results of recent studies have suggested that the rate of nucleosome repositioning by SNF2h, the human ISWI homologue, is dependent upon the length of flanking DNA on each side of the nucleosome core and that the interaction of SNF2h with nucleosomes is allosterically regulated by the binding of nucleotides.[11,24,25] Taken together, these data form the basis of the hypothesis that the coupling of the nucleotide regulation and flanking DNA length sensing properties modulates the repositioning activity and directionality of this remodeler.[29] However, in the preceding paper (DOI: 10.1021/bi500224t), we show that nucleosome binding by ISWI is not regulated by nucleotide binding. These results suggest that ISWI is stably anchored to the nucleosome core with high affinity and that the allosteric regulation by nucleotides may not play the dominating role in modulating the nucleosome repositioning activity of ISWI. Consistent with this conclusion are the results of two recent reports re-evaluating the proposed role of the C-terminal DNA binding domains of ISWI and the related chromatin remodeler Chd1 in the nucleosome repositioning activities of these enzymes.[30,31] In contrast to the widely accepted view, the results of these studies demonstrated that the ATPase domains of these two related chromatin remodelers are both sufficient to reposition nucleosomes. Therefore, neither energy transduction nor conformational changes between the ATPase and the DNA binding domains of these enzymes are directly required for their nucleosome repositioning activity. Instead, the DNA binding domain might affect the affinity of DNA binding and consequently the directionality and efficiency and/or processivity of nucleosome repositioning. Thus, these data demonstrate that several questions still persist regarding the mechanism of nucleosome repositioning by ISWI.

Here we report the characterization of the ability of ISWI to reposition various nucleosomal substrates using both a gel-based assay and a new, fluorescence anisotropy-based assay to monitor repositioning. We found that nucleosome repositioning by ISWI generated a distinct distribution of histone octamer translational positions. Furthermore, analysis of time courses of

ISWI repositioning nucleosome substrates with limited lengths of DNA, and hence limited translational positions, was consistent with ISWI remodeling the nucleosomes through a random walk mechanism. Our characterization of nucleosome binding in the preceding paper (DOI: 10.1021/bi500224t) was utilized in additional analysis of repositioning time courses observed with nucleosomes containing shorter lengths of flanking DNA. This analysis revealed that even though two ISWI can bind to a nucleosome, the presence of a second ISWI monomer bound to the nucleosome did not affect the rate at which the nucleosome was repositioned, suggesting that a monomeric ISWI is sufficient to obtain the observed repositioning activity.

■ EXPERIMENTAL PROCEDURES

Recombinant ISWI Expression and Purification. The pPIC3.5-CBP-Xpress-zz expression construct encoding *Xenopus laevis* ISWI was used to express and purify the recombinant protein from the GS115 strain of *Pichia pastoris* as described in the preceding paper (DOI: 10.1021/bi500224t).

Nucleosome Reconstitution Reactions. pET28 plasmids containing untagged yeast H2A, H2B, H3, and H4 were used to express and purify the histone proteins as described previously.[32,33] DNA fragments containing the 148 bp 601 high-affinity nucleosome positioning sequence[34] and an additional length of flanking DNA were amplified using large scale polymerase chain reaction followed by purification of the amplified fragment. Either nonlabeled primers or Alexa488 end-labeled primers (IDT) were used to reconstitute the mononucleosome substrates with the desired fluorophore label and flanking DNA length using salt gradient dialysis as described previously.[32,33] Reconstituted mononucleosomes were evaluated using a 5% native polyacrylamide−bisacrylamide gel (60:1) run at 100 V in 0.25× Tris-Borate-EDTA (TBE) buffer followed by staining using SYBR gold or exposed for fluorescence imaging using a Typhoon imager (GE Healthcare).

ISWI ATPase Activity Assay. Nucleosome substrates were incubated with ISWI (see tables and figure legends for specific concentrations) in reaction buffer [10 mM HEPES (pH 7.0), 20 mM KCl, 10 mM $MgCl_2$, 4% glycerol, 0.1 mg/mL BSA, and 0.5 mM DTT] at 25 °C. The reactions were initiated by addition of 1 mM cold ATP containing 7.5 μCi of [α-^{32}P]ATP. Aliquots were withdrawn at specific time points and mixed with an equal volume of 0.5 M EDTA to stop the reaction. To separate ADP from ATP species, reaction mixtures were analyzed using thin liquid chromatography PEI-cellulose plates (EMD chemicals) in 0.6 M potassium phosphate (pH 3.4) buffer and quantified using a Typhoon Phosphor imager. The ATPase rate for each nucleosome substrate was determined from a linear fit of the data.

Gel-Based Repositioning Assays. ISWI (10 nM) was incubated with 50 nM nucleosome substrates in reaction buffer [10 mM HEPES (pH 7.0), 20 mM KCl, 10 mM $MgCl_2$, 4% glycerol, 0.1 mg/mL BSA, and 0.5 mM DTT] at 25 °C. Repositioning reactions were initiated by the addition of 1 mM ATP and allowed to proceed for specific time points before being stopped by the addition of a quenching solution containing EDTA and competitor plasmid DNA. The reaction mixtures were then analyzed using a 5% native polyacrylamide−bisacrylamide gel (60:1) run at 100 V in 0.25× TBE buffer followed by staining using SYBR gold and visualized using a Typhoon imager (GE Healthcare).

Anisotropy-Based Repositioning Assays. Varying ISWI concentrations ranging from 5 to 20 nM (specific concentrations listed in figure legends) were incubated with 10 nM Alexa488-labeled nucleosome substrates in reaction buffer [10 mM HEPES (pH 7.0), 20 mM KCl, 10 mM $MgCl_2$, 4% glycerol, 0.1 mg/mL BSA, and 0.5 mM DTT] at 25 °C. Reactions were initiated by the addition of 1 mM ATP, and the movement of the octamer was detected by monitoring changes in the fluorescence anisotropy of the fluorophore using a Syergy2 fluorescence spectrophotometer (BioTek) set at 485 nm excitation and monitoring emission at 520 nm.

Data Analysis. The simplest model consistent with our three-state random walk model for the ISWI-catalyzed repositioning of an 18N18 nucleosome is shown in Scheme 2. In Scheme 2, R_i denotes the population of nucleosomes bound with ISWI with histone octamers in the ith translational position; $i = 0$ denotes the central position, defined by the NPS, and $i = \pm1$ denotes positions one translational step from the central position. NR_i denotes the population of nucleosomes with histone octamers in the ith translational position, but without ISWI bound. The rate constant for the dissociation of ISWI from nucleosomes is denoted by k_d, and the rate constant for ISWI binding nucleosomes is denoted by k_b. The macroscopic rate constant for octamer movement between translational positions is denoted by k_r. As shown in the Appendix, the equation for the time dependence of the population of octamers at the $i = \pm1$ positions is given by eq 1.

$$\frac{[R_{+1}](t) + [R_{-1}](t) + [NR_{+1}](t) + [NR_{-1}](t)}{\sum_i [[R_i](t) + [NR_i](t)]}$$
$$= \frac{1}{2}(1 - e^{-2Kk_r t}) \tag{1}$$

The variable K in eq 1 denotes the fraction of ISWI bound initially at R_0. Similarly, for repositioning of 24N24 nucleosomes in which there are two positions for the histone octamer on either side of the central position, defined by the NPS, the equation for the time dependence of the population of octamers at the $i = \pm2$ positions is given by eq 2.

$$\frac{[R_{+2}](t) + [R_{-2}](t) + [NR_{+2}](t) + [NR_{-2}](t)}{\sum_i [[R_i](t) + [NR_i](t)]}$$
$$= \left(\frac{e^{-2Kk_r t}}{4}\right)(1 - e^{-Kk_r t})^2 \tag{2}$$

All ATPase time courses were simultaneously globally analyzed using eq 3.

$$[ADP](t) = (k_{ATP,PN}[PN] + k_{ATP,P_2N}[P_2N])t \tag{3}$$

where $k_{ATP,PN}$ and k_{ATP,P_2N} are the steady state rates of ATP hydrolysis for the PN and P_2N states, respectively. The PN and P_2N states denote nucleosomes with one or two ISWI monomers bound, respectively.

Analysis of repositioning time courses using eqs 1 and 2 and ATPase time courses using eq 3 was performed using Conlin.[35] Unless otherwise noted, all traces presented in the figures have been normalized to the final asymptotic value of the anisotropy change as determined from this analysis. Finally, unless otherwise noted, all uncertainties represent 68% confidence intervals (±1 standard deviation) as determined by Monte Carlo analysis.

RESULTS

ISWI Distributes the Nucleosomes into Distinct Translational Positions. We began our characterization of the nucleosome repositioning activity of ISWI using central or asymmetric nucleosome substrates reconstituted with the 601 high-affinity positioning sequence[31,36−39] in a native gel electrophoresis assay. In this assay, the length of DNA flanking either side of the histone octamer or, equivalently, the position of the octamer on the DNA affects the mobility of the nucleosome on the gel, with centrally positioned nucleosomes displaying the slowest gel mobility.[23,40,41] The ISWI-catalyzed repositioning of three nucleosomes with symmetric lengths of flanking DNA (51, 71, and 91 bp) extending on both sides of the nucleosome core (N) is shown in Figure 1. As shown in
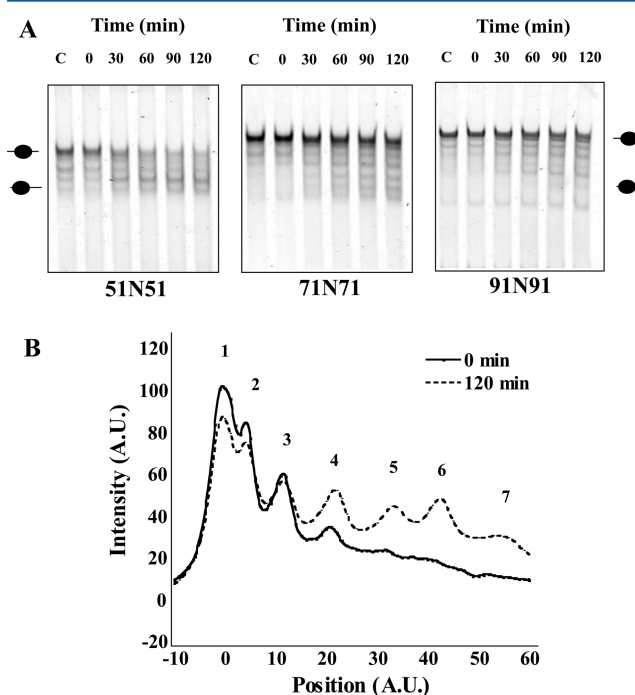
Figure 1. Native gel-based repositioning of various nucleosome substrates by ISWI. (A) Repositioning of 51N51, 71N71, and 91N91 nucleosomes (50 nM) by ISWI (25 nM). ISWI and nucleosomes were incubated together at 25 °C, and repositioning reactions were initiated by addition of 1 mM ATP. Reactions were stopped at the indicated time points by the addition of stopping buffer and resolved using a 5% TBE−acrylamide native gel. The first lane in each gel (C) shows a control reaction without ISWI that was allowed to proceed for 120 min before being stopped. Gels were stained for DNA and imaged as indicated in Experimental Procedures. (B) Analysis of changes in translational positions over time for the 91N91 nucleosome substrate.

Figure 1, we found that ISWI changed the distribution of octamer locations on the DNA from initially being primarily centered on the DNA to being spread over a series of translational positions. Furthermore, the number of apparent translational positions was dependent on the total length of the flanking DNA. From a linear analysis of the number of apparent translational positions as a function of the length of the flanking DNA, we determined that a new position was associated with each ~12 bp of additional flanking DNA (data not shown). Similarly, ISWI was able to reposition asymmetrical nucleosome substrates away from their original position into a similar distribution of distinct translational positions (Figure 1A of the

Supporting Information). Additionally, repositioning reactions with different asymmetrical substrates demonstrated the ability of ISWI to move the octamer in both directions along the DNA (Figure 1A,B of the Supporting Information).

The ATPase activity of ISWI was linear over the entire repositioning reaction time (Figure 2 of the Supporting Information). Altering the repositioning assay conditions by increasing the concentration of ISWI or the continuous titration of additional ISWI and ATP into the reactions also did not affect the final distribution of octamer positions (data not shown). Thus, this distribution appears to be a stable dynamic equilibrium of the possible translational positions for the histone octamer on the DNA.

ISWI Remodels the Nucleosomes through a Random Walk. The final dynamic equilibrium of histone octamer positions on the DNA is consistent with ISWI moving the octamers between these defined positions through a random walk mechanism.[42] Specifically, the processivity with which ISWI moves the octamers is so low that the location of an octamer is shifted, on average, only to the nearest translational position before ISWI dissociation. In subsequent ISWI binding, there is no "memory" of the previous direction of translocation, so there is equal probability of the octamer moving in either direction.[42] To simplify the determination of the microscopic rate constants associated with this mechanism, we sought to analyze the ISWI-catalyzed repositioning of nucleosome substrates with only one or two possible translational positions for the histone octamer on the flanking DNA.

We also developed a new assay for monitoring nucleosome repositioning in which the effect of histone octamer position on the motion of the flanking DNA is measured. In this assay, the movement of the histone octamer toward the end of the DNA constricts the motion of the DNA and thus increases the anisotropy of a fluorophore attached there, similar to how the movement of the counterweight along the pendulum of a metronome changes the frequency of the metronome's oscillation.

Initially we characterized the repositioning activity of ISWI using a double-fluorophore-labeled F18N18F substrate. The addition of ISWI and ATP causes an increase in anisotropy as a function of time, while the addition of ISWI only or ATP only had no effect on the anisotropy (Figure 2). Furthermore, no effect on the anisotropy was observed when using ADP or
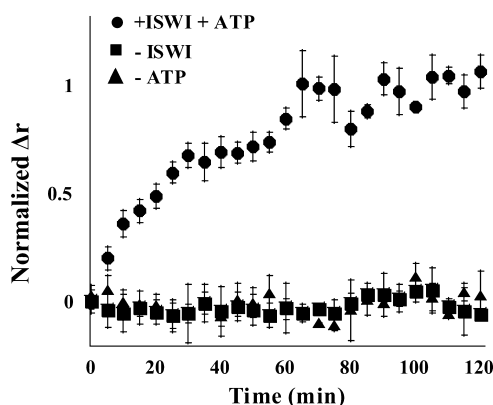
slowly hydrolyzable ATP analogues (Figure 3A of the Supporting Information). This suggests that these changes in anisotropy require the presence of ISWI and both the binding and hydrolysis of ATP. These results are therefore consistent with the change in anisotropy being associated with the movement of the histone octamer. Additionally, changes in anisotropy are not observed when a 181 bp fluorophore-labeled DNA, comparable in length to the DNA used to reconstitute the F18N18F nucleosomes, was used as the substrate in the reaction (Figure 3B of the Supporting Information). This is also consistent with movement of the octamer by ISWI being responsible for the observed time-dependent changes in anisotropy, rather than the binding or movement of ISWI. It is worth mentioning that this assay allows us to monitor changes in the total population of nucleosomes as a function of time and that the change in anisotropy we observe is an average of all species present in solution.
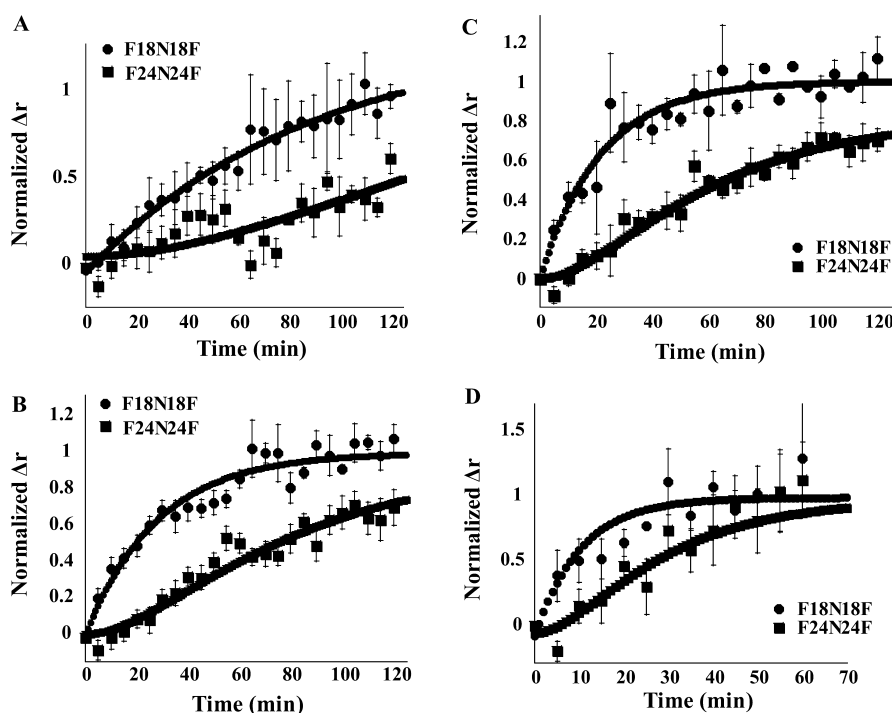
Using this molecular metronome assay, we then monitored the repositioning of nucleosome substrates with 18 or 24 bp of flanking DNA; these lengths of flanking DNA were chosen such that these substrates would be expected to have one or two, respectively, translational positions for the histone octamer on the flanking DNA. Results of experiments conducted with 10 nM ISWI and 10 nM nucleosomes are shown in Figure 3. As
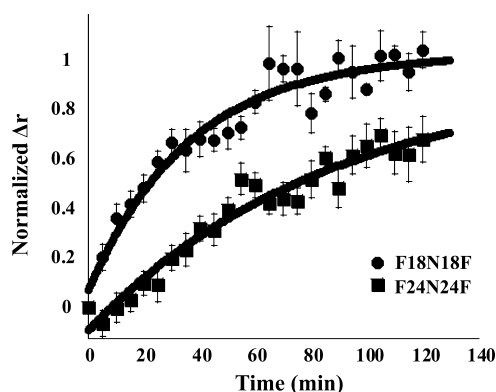
Figure 3. Fluorescence anisotropy-based repositioning of F18N18F and F24N24F. Measurements of changes in anisotropy (Δr) of 10 nM fluorophore-labeled F18N18F (●) or 24N24 (■) nucleosomes incubated with 10 nM ISWI and 1 mM ATP. The solid lines represent single-exponential fits of the data.

demonstrated in Figure 3, the apparent rate of repositioning of F24N24F nucleosomes is slower than the rate of repositioning of F18N18F nucleosomes; these rates are 0.012 ± 0.003 and $0.031 \pm 0.003 \text{ min}^{-1}$, respectively, as determined from a single-exponential fit of the time courses. This change in repositioning rate is not a function of differences in the stoichiometry or affinity with which ISWI binds these substrates as these are identical for these nucleosomes as demonstrated in the preceding paper (DOI: 10.1021/bi500224t). Rather, it is likely a simple consequence of the F24N24F substrate having more translational positions for the histone octamer than the F18N18F substrate. This is consistent with our native gel-based repositioning experiments using long nonlabeled nucleosome substrates demonstrating that the rate of repositioning for 51N51 is faster than that of 71N71 and 91N91 (Figure 1A).

To test this hypothesis further, we monitored the ISWI-catalyzed repositioning of F18N18F and F24N24F nucleo-

Figure 2. Fluorescence anisotropy-based repositioning of F18N18F by ISWI. Measurements of changes in anisotropy (Δr) of 10 nM fluorophore-labeled F18N18F nucleosome incubated with 10 nM ISWI and 1 mM ATP (●), without ISWI (■), or without ATP (▲).

Figure 4. Fluorescence anisotropy-based repositioning of F18N18F and F24N24F in the presence of various ISWI concentrations. Measurements of changes in anisotropy (Δr) of 10 nM F18N18F (●) or F24N24F (■) incubated with (A) 5, (B) 10, (C) 15, or (D) 20 nM ISWI. The reaction was started by the addition of 1 mM ATP. Isotherms were analyzed as described in Experimental Procedures. The solid lines represent fits of the data.

somes at several different concentrations of ISWI and subsequently analyzed the individual time courses for each nucleosome substrate separately to determine the number of octamer translational positions for each nucleosome substrate (see Experimental Procedures). The results of this analysis (Tables 1 and 2 of the Supporting Information) demonstrate that the best fit of the time courses is associated with F18N18F nucleosomes having one translational position on the flanking DNA and F24N24F nucleosomes having two. This is consistent with our previous estimate of ~12 bp being required for each translational position.

We next performed global analysis of the repositioning time courses, which includes both nucleosome substrates, F18N18F and F24N24F, together at each ISWI concentration. In this analysis, we assumed that the apparent rate of repositioning was constant for both substrates but that the number of translational positions was different for each substrate. In our analysis, we used the fact that the affinity and stoichiometry of ISWI–nucleosome binding are not affected by nucleotides as shown in the preceding paper (DOI: 10.1021/bi500224t) (Appendix for analysis). This analysis provided a good description of the data, as judged by the variance of the fits and visually, and furthermore demonstrated that the apparent rate of repositioning increased with an increase in ISWI concentration (Figure 4 and Table 1).

The Binding of a Second ISWI Does Not Affect the Rate of Nucleosome Repositioning. In the preceding paper (DOI: 10.1021/bi500224t), we characterized the equilibrium binding of ISWI to nucleosomes and demonstrated that two ISWI can bind to nucleosome substrates with limited lengths of flanking DNA (5−24 bp). Using the determined stoichiometric binding constants, we are able to determine the fraction of nucleosomes bound with a single ISWI and the fraction bound with two ISWIs (Table 3 of the Supporting Information); we

Table 1. Results of Global Analysis for F18N18F and F24N24F Together at Each ISWI Concentration According to eq 1 for F18N18F and eq 2 for F24N24F

[ISWI] (nM)	k_r (min^{-1})	variance of fit
5	5.65×10^{-3}	3.63×10^{-6}
10	1.78×10^{-2}	2.55×10^{-6}
15	2.31×10^{-2}	2.17×10^{-6}
20	4.87×10^{-2}	2.27×10^{-6}

denote these species as PN and P$_2$N, respectively. We then performed additional global analysis, including all of our nucleosome repositioning time courses together with these species fractions as additional constraints, to determine the repositioning activity of each species (Figure 4 of the Supporting Information). The result of this analysis is summarized in Table 2; the best fit of the data is associated with a model in which both species have the same repositioning rate. Thus, the presence of a second ISWI monomer bound did not affect the rate at which the nucleosome was repositioned, suggesting that a monomeric ISWI is sufficient to obtain the observed repositioning activity. The efficacy of eqs 1 and 2 (Experimental Procedures) in characterizing nucleosome repositioning was further verified by conducting a series of Monte Carlo simulations of nucleosome repositioning and analyzing them using eqs 1 and 2 (Appendix).

ATP Hydrolysis Is Weakly Coupled to Octamer Movement. Our observation that the PN and P$_2$N species have identical repositioning rates prompted us to question the role played by the second bound ISWI in the repositioning reaction. We sought to answer this question by determining the ATPase activity associated with each species of bound ISWI during repositioning. We began by measuring the ATPase activity of ISWI at four different ISWI concentrations (50, 250, 500, and 800 nM) in the presence of 250 nM nucleosomes

Table 2. Results of Simultaneous Global Analysis for All F18N18F and F24N24F Repositioning Time Courses at Different Nucleosome Binding Affinities Using eqs 1 and 2 for the F18N18F and F24N24F Substrates, Respectively[a]

affinities	species	k_r (min^{-1})	variance of fit
$1/\beta_1 = 1.26$ nM, $1/\beta_2 = 13.92$ nM^2	PN	0.030 ± 0.002	3.34×10^{-6}
	P_2N	0.13 ± 0.01	3.37×10^{-6}
	PN + P_2N	0.0247 ± 0.0018	2.96×10^{-6}
$1/\beta_1 = 1.04$ nM, $1/\beta_2 = 11.94$ nM^2	PN	0.029 ± 0.002	3.34×10^{-6}
	P_2N	0.137 ± 0.011	3.48×10^{-6}
	PN + P_2N	0.0240 ± 0.0018	2.97×10^{-6}
$1/\beta_1 = 1.62$ nM, $1/\beta_2 = 20.32$ nM^2	PN	0.032 ± 0.002	3.30×10^{-6}
	P_2N	0.138 ± 0.011	3.30×10^{-6}
	PN + P_2N	0.0256 ± 0.0019	2.96×10^{-6}

[a]Affinities were determined using the stoichiometric binding constants reported in the preceding paper (DOI: 10.1021/bi500224t) and varied on the basis of uncertainties determined therein. P denotes ISWI, N nucleosomes, PN a nucleosome with one ISWI bound, P_2N a nucleosome with two ISWIs bound, and PN + P_2N total nucleosomes bound. As shown from the variances of the fits, the PN + P_2N species model is the best.

(10N5, 18N18, and 24N24). Although the observed ATPase rate was dependent upon the concentration of ISWI, as expected, there was no significant difference in the ATPase rates among the three nucleosome substrates at each ISWI concentration. Using the determined stoichiometric binding constants (DOI: 10.1021/bi500224t), we determined the fraction of PN, P_2N, and P species present in each of these ATPase reaction mixtures. We then used these species fractions to determine the ATPase rate associated with each species (Table 3). As shown in Table 3, the ATPase activity of the P_2N species is equal to that of the PN species within the uncertainty of the analysis.

Using the rate of repositioning determined from our global analysis of repositioning time courses measured at different ISWI concentrations, 0.0247 ± 0.0018 min^{-1}, we can calculate from Table 3 the efficiency at which ISWI couples ATP hydrolysis to octamer movement. From this calculation, we determined that 890 ± 110 ATPs are hydrolyzed for each translational step of the octamer by ISWI.

■ DISCUSSION

The mechanism of nucleosome repositioning by chromatin remodelers remains incompletely understood. Elucidating how ISWI repositions nucleosomes requires knowledge of the stoichiometry, the affinity, and the fraction of ISWI bound to the nucleosomes during the repositioning reaction. In the preceding paper (DOI: 10.1021/bi500224t), we determined these parameters and demonstrated that two ISWIs are bound at equilibrium with high affinity to nucleosome substrates with short lengths of flanking DNA and that the binding of ISWI to these substrates is not affected by nucleotides or the length of

flanking DNA. These results, together with our determination of the equilibrium constants for nucleosome binding, allow us to predict the fraction of ISWI bound to the nucleosomes during repositioning at various ISWI concentrations.

Using native gel-based repositioning assays, we observed that the chromatin remodeler ISWI is able to move histone octamers away from their initial location at the high-affinity positioning sequence and generate a distribution of octamer positions when repositioning both central and asymmetrical nucleosome substrates with long lengths of extranucleosomal DNA (51, 71, and 91 bp). These findings are consistent with previous studies that demonstrated the ability of ISWI to generate a distribution of remodeled nucleosome products.[24,25] Furthermore, movement of the octamer in both directions along the DNA was evident by the ability of ISWI to reposition a variety of asymmetrical nucleosome substrates with different initial octamer positions. Interestingly, from a linear analysis of the number of apparent translational positions as a function of the length of the flanking DNA, we determined that a new position was observed for each ~12 bp of additional flanking DNA; this spacing is consistent with the periodicity of histone–DNA contacts within the high-affinity positioning sequence (~10 bp[34,43]). The observed distribution of translational positions may therefore be influenced by both the inherent step size of the enzyme ISWI and the underlying DNA–nucleosome interactions and could very well be much larger than the individual movements of ISWI along the DNA and/or the size of potential DNA loops and/or bulges associated with repositioning. Indeed, the possibility exists that other intermediate species are created by ISWI during repositioning, but we are unable to detect these species because they are unstable and collapse into positions that are more thermodynamically favorable following ISWI dissociation.[44]

The distribution of histone octamers into a dynamic equilibrium of translational positions on the DNA is consistent with ISWI moving the octamers between these defined positions through a random walk mechanism (see Figure 5 of the Supporting Information for a model). To confirm this and more readily analyze this remodeling behavior, we sought to study the repositioning activity of ISWI by means of a new, anisotropy-based, repositioning assay using nucleosome substrates with short lengths of flanking DNA. Specifically, we designed centrally positioned nucleosomes with lengths of flanking DNA predicted by our native gel analysis to be short enough to accommodate only one or two octamer translational positions. Subsequent analysis of repositioning time courses for fluorophore-labeled F18N18F or F24N24F nucleosome substrates using a random walk model demonstrated that the best fit is associated with F18N18F nucleosomes having one translational position on the flanking DNA (one on each side of the nucleosome positioning sequence) and F24N24F nucleosomes having two translational positions on the flanking DNA (two on each side of the nucleosome positioning sequence); these results are consistent with our estimate of ~12

Table 3. Determination of Nucleosome-Stimulated ATP Hydrolysis Rates Associated with Different ISWI–Nucleosome Species (analysis performed using eq 3)

affinities	species	k_{ATP} (no. of ADPs/min)	average k_{ATP} (no. of ADPs/min)	coupling efficiency (no. of ADPs/step)	coupling efficiency (no. of ADPs/bp)
$1/\beta_1 = 1.26$ nM, $1/\beta_2 = 13.92$ nM^2	PN	17 ± 5	22 ± 2	890 ± 110	74 ± 9
	P_2N	23 ± 3			

bp being required for each observed translational position. We also globally analyzed the repositioning time courses for F18N18F and 24N24F together at several ISWI concentrations and found that the rate of repositioning increased with an increase in ISWI concentration as expected.

In the preceding paper (DOI: 10.1021/bi500224t), we demonstrated that two ISWIs can bind to a nucleosome substrate with short flanking DNA. Previous studies demonstrated that two SNF2h monomers can bind to a nucleosome and that the repositioning activity of this SNF2h dimer is regulated by the effect of nucleotide binding on nucleosome binding affinity together with the flanking DNA length sensing capability of the remodeler. However, the nucleosome binding affinity of ISWI is not affected by nucleotide binding or the length of the flanking DNA as demonstrated in the preceding paper (DOI: 10.1021/bi500224t). To explore the role of the two ISWIs in the observed nucleosome repositioning activity, we used the determined stoichiometric binding constants to quantify the fraction of nucleosomes bound with a single ISWI and the fraction bound with two ISWIs. These values were used as additional constraints in a global analysis of all repositioning time courses where we found that the presence of a second ISWI monomer bound did not affect the rate at which the nucleosome was repositioned, suggesting that a monomer is sufficient for the observed repositioning activity. These findings are consistent with *in vivo* estimates of ISWI concentration suggesting that it is predominantly present as a monomer.[45] While it is not immediately clear what determines which monomer is responsible for the repositioning activity, it is possible that the binding of one ISWI monomer causes a conformational change in nucleosome structure or affects the binding of the second monomer, rendering only one of the monomers active for repositioning. The binding orientation of the active monomer may in turn determine the direction of the octamer movement (as discussed below). Further mutagenesis and structural studies will be required to resolve these questions.

We determined the efficiency at which ISWI couples ATP hydrolysis to octamer movement. Consistent with what has been observed previously, we found that ATP binding and hydrolysis are poorly coupled to octamer movement, specifically that this movement requires the consumption of hundreds of ATP molecules.[25] If we assume a size of ~12 bp for each translational movement of the octamer, then moving the octamer 1 bp requires the hydrolysis of 74 ± 9 ATPs. As the ATP coupling efficiency for DNA translocation by other DNA translocases, including chromatin remodelers, is between 0.5 ATP/bp and 3 ATPs/bp,[46−48] our result argues against the possibility that DNA translocation by ISWI is energetically rate-limiting for the repositioning reaction. Therefore, we believe that the poor coupling efficiency of ATP hydrolysis to octamer movement results either from significant hydrolysis being associated with futile repositioning or from a significant ATP consumption requirement associated with the initiation of repositioning. It is worth noting that the former might be an indication of several abortive attempts to move the octamer occurring prior to each successful repositioning event. A low probability of successful repositioning associated with ISWI binding is consistent with the poor template commitment previously reported for SNF2h.[25] Additionally, we found that the rate of ATP hydrolysis of the PN species is the same as the rate of the P_2N species; this is consistent with our global

analysis of repositioning data suggesting that a monomer is responsible for the observed nucleosome repositioning activity.

As demonstrated in the preceding paper (DOI: 10.1021/bi500224t), the presence of two ISWI binding sites on the nucleosome as evident in the ability of two ISWIs to bind to a nucleosome substrate with a very short length of extranucleosomal DNA. The random walk movement of the octamer back and forth among various translational positions requires a continuous change in the directionality of octamer movement along the DNA. Because the directionality of octamer movement is likely determined by the orientation with which ISWI is bound to the nucleosome, i.e., perhaps each binding site is associated with one direction of translocation. Such changes in the directionality of octamer movement can then potentially be achieved randomly through the dissociation and rebinding of ISWI to the nucleosome in a different orientation and in a manner independent of the previous binding. According to this model, the presence of a distribution of remodeled species then suggests that the rate at which ISWI dissociates from the nucleosomes is much faster than the rate at which it moves the octamer between translational positions. This conclusion is consistent with previous competition experiments demonstrating that SNF2h dissociates from the nucleosomes at a rate faster than the rate of repositioning taking place and is therefore not rate-limiting with respect to the repositioning process.[25]

In recent single-molecule studies of nucleosome repositioning by the ISW2 complex, it was reported that changes in the direction of nucleosome movement could occur after translocation of at least ~7 bp of DNA by ISW2. Interestingly, the fraction of nucleosomes undergoing these changes in directionality increased significantly (from 6 to 54%) in repositioning experiments with an ISW2 complex containing mutations that compromised the interaction of the C-terminal DNA binding SLIDE domain with extranucleosomal DNA. While these mutations were shown not to affect the affinity of ISW2 for nucleosomes or the interaction of its ATPase domain with the nucleosomes, DNA fingerprinting showed that the interactions of this ISW2 mutant with flanking DNA were altered and that the repositioning activity was significantly affected.[49,50] The fact that nucleotide binding has an effect on ISWI−free DNA interaction but no effect on the affinity of ISWI for nucleosomes [see the preceding paper (DOI: 10.1021/bi500224t)] may suggest a role for the ATPase cycle in regulating the interactions with flanking DNA and hence the repositioning activity of ISWI. Similar to the effects of compromising mutations in the SLIDE domain of ISW2 on repositioning efficiency and directionality of nucleosome translocation, the ATP binding and hydrolysis cycle of ISWI may contribute to the random walk behavior that we observe. Interestingly, recent studies revealed that the ATPase domain of *Drosophila* ISWI and Chd1 serves as the basic motor that is sufficient for the repositioning activity of these remodelers and that the C-terminal DNA interacting domain may serve more of a regulatory role in the nucleosome repositioning process such as determining the directionality, binding specificity, and processivity of the repositioning process.[30,31]

The ability of the catalytic subunit ISWI to reposition nucleosomes in a random walk fashion might be modified and regulated by other binding subunits as part of larger complexes. Indeed, the final outcome of repositioning activity and remodeled products is different between free ISWI and ISWI as part of a complex, and the repositioning outcome was shown

to greatly vary among different ISWI-containing complexes.[23−28] Future studies elucidating the mechanism by which these subunits regulate the repositioning activity of ISWI would be of great interest.

■ APPENDIX

We begin by considering a model in which there are three possible locations for the histone octamer on the DNA; this model is depicted in Scheme 1. In this model, these locations

Scheme 1[a]

$$R_{-1} \underset{k_1/2}{\overset{k_2}{\rightleftarrows}} R_0 \underset{k_2}{\overset{k_1/2}{\rightleftarrows}} R_{+1}$$
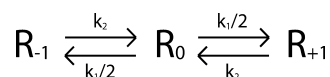
[a]R_i denotes the population of nucleosomes bound with ISWI with histone octamers in the ith translational position; $i = 0$ denotes the central position, defined by the NPS, and $i = \pm1$ denotes positions one translational step from the central position. k_1 and k_2 represent the microscopic rate constants for repositioning.

(R_i) relative to the central position are denoted by a subscript. Thus, R_0 denotes a nucleosome at the central position, and $R_{\pm1}$ denotes a nucleosome that is one translational position from the central position. Because the affinity of DNA binding by the histone octamer is likely strongest at the central position, we will assume different rate constants for the movement of the octamer away and toward this location. We define k_1 as the rate constant for the movement away from R_0 and k_2 as the rate constant for the movement toward R_0. Note that because there are two pathways for repositioning from R_0 the rate constant for each pathway is denoted as $k_1/2$ in Scheme 1.

The differential equations associated with this scheme are

$$\frac{d}{dt}[R_{-1}] = \frac{k_1}{2}[R_0] - k_2[R_{-1}] \tag{A1}$$

$$\frac{d}{dt}[R_0] = -2\left(\frac{k_1}{2}\right)[R_0] + k_2[R_{-1}] + k_2[R_{+1}] \tag{A2}$$

$$\frac{d}{dt}[R_{+1}] = \frac{k_1}{2}[R_0] - k_2[R_{+1}] \tag{A3}$$

If we assume that a fraction f of the octamers is initially at R_0 and $(1 - f)/2$ at R_{+1} and R_{-1}, then the solution of these differential equations is

$$\frac{[R_0](t)}{\sum_i [R_i](t)} = \frac{1}{k_1 + k_2}\{k_2 + [f(k_1 + k_2) - k_2]e^{-(k_1+k_2)t}\} \tag{A4}$$

$$\frac{[R_{-1}](t)}{\sum_i [R_i](t)} = \frac{[R_{+1}](t)}{\sum_i [R_i](t)} = \frac{1}{2(k_1 + k_2)}$$
$$\{k_1 + [k_2 - f(k_1 + k_2)]e^{-(k_1+k_2)t}\} \tag{A5}$$

Thus, the apparent rate constant for the approach to the steady state equilibrium is

$$k_{app} = k_1 + k_2 \tag{A6}$$

As expected, it is not possible to determine values for k_1 and k_2 from the rate at which the system approaches its steady state.

The repositioning reaction is coupled to the hydrolysis of ATP. If we assume that each movement of a histone octamer is associated with c ATP molecules being hydrolyzed (and thus c

ADP molecules being formed), the equation for the rate of change of the concentration of ADP is

$$\frac{d}{dt}[ADP] = 2c\left(\frac{k_1}{2}\right)[R_0] + ck_2([R_{+1}] + [R_{-1}]) \tag{A7}$$

The solution to this equation is given in eq A8.

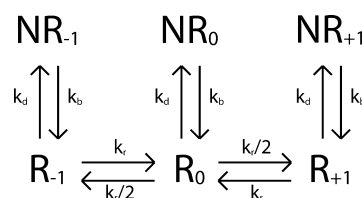$$[ADP](t) = \frac{c}{k_{obs}^2}[f(k_1^2 - k_2^2) - k_1k_2 + k_2^2]$$
$$- e^{-k_{obs}t}(k_1 - k_2)(k_{obs}f - k_2) + 2k_1k_2k_{obs}t] \tag{A8}$$

According to eq A8, the time course of ADP production will consist of a burst phase, with associated rate constant k_{obs}, followed by a steady state phase. The magnitude of the burst phase is directly proportional to the difference between k_1 and k_2. If the rates of repositioning are the same regardless of octamer position (i.e., if $k_1 = k_2 = k$), then eq A8 simplifies to eq A9.

$$[ADP](t) = ckt \tag{A9}$$

Scheme 1 can be modified to include the binding and dissociation of ISWI from the nucleosome as shown in Scheme 2. In this scheme, the histone octamer can exist in two states, R

Scheme 2[a]

$$\begin{array}{ccc}
NR_{-1} & NR_0 & NR_{+1} \\
k_d \updownarrow k_b & k_d \updownarrow k_b & k_d \updownarrow k_b \\
R_{-1} \underset{k_r/2}{\overset{k_r}{\rightleftarrows}} & R_0 \underset{k_r}{\overset{k_r/2}{\rightleftarrows}} & R_{+1}
\end{array}$$

[a]NR_i denotes the population of nucleosomes with histone octamers in the ith translational position, but without ISWI bound. The rate constant for the dissociation of ISWI from nucleosomes is denoted by k_d, and the rate constant for the binding of ISWI nucleosomes is denoted by k_b. The macroscopic rate constant for octamer movement between translational positions is denoted by k_r.

and NR. In the R state, the octamer is bound by ISWI and thus is capable of being repositioned. In the NR state, the octamer is not bound by ISWI and thus cannot be repositioned. The rate constant for the dissociation of ISWI from the nucleosome is k_d, and the rate constant for binding of ISWI to the nucleosome is k_b; in this representation, k_b is a composite rate constant that includes contributions from the concentration of ISWI present in the solution. As previously demonstrated, it is not possible to determine independent estimates of the microscopic rate constants for repositioning (k_1 and k_2 in Scheme 1), so we will assume that they are equal (denoted k_r in Scheme 2). The differential equations associated with Scheme 2 are

$$\frac{d}{dt}[R_0] = -2\left(\frac{k_r}{2}\right)[R_0] - k_d[R_0] + k_b[NR_0] + k_r[R_{+1}]$$
$$+ k_r[R_{-1}] \tag{A10}$$

$$\frac{d}{dt}[R_{+1}] = \frac{k_r}{2}[R_0] - k_r[R_{+1}] - k_d[R_{+1}] + k_b[NR_{+1}] \tag{A11}$$

$$\frac{d}{dt}[R_{-1}] = \frac{k_r}{2}[R_0] - k_r[R_{-1}] - k_d[R_{-1}] + k_b[NR_{-1}]$$
(A12)

$$\frac{d}{dt}[NR_i] = k_d[R_i] - k_b[NR_i]$$
(A13)

For the sake of simplicity, we will assume that all of the protein is bound initially at either R_0 or NR_0 and we will use the variable K to denote the fraction of the protein bound initially at R_0; $1 - K$ is thus the fraction initially bound at NR_0. Because the presence of nucleotide does not affect the affinity of ISWI for nucleosomes,[51] these fractions will remain constant throughout the repositioning reaction. Thus

$$k_b = \left(\frac{K}{1-K}\right)k_d$$
(A14)

The solutions to eqs A11−A13 are too cumbersome to reproduce here but can be simplified if we assume that the rate of dissociation of ISWI from the nucleosome is faster than the rate of repositioning (i.e., $k_d \gg k_r$). This assumption is consistent with the poor substrate commitment demonstrated by ISWI-containing chromatin remodelers for nucleosome repositioning. With this assumption, we have

$$\frac{[R_0](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{K}{2}(1 + e^{-2Kk_r t})$$
(A15)

$$\frac{[R_{+1}](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{[R_{-1}](t)}{\sum_i [R_i](t) + [NR_i](t)}$$
$$= \frac{K}{4}(1 - e^{-2Kk_r t})$$
(A16)

$$\frac{[NR_0](t)}{\sum_i [R_i](t) + [NR_i](t)} = \left(\frac{1-K}{2}\right)(1 + e^{-2Kk_r t})$$
(A17)

$$\frac{[NR_{+1}](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{[NR_{-1}](t)}{\sum_i [R_i](t) + [NR_i](t)}$$
$$= \left(\frac{1-K}{4}\right)(1 - e^{-2Kk_r t})$$
(A18)

The total population of octamers at the $i = \pm 1$ positions is given by eq A19.

$$\frac{[R_{+1}](t) + [R_{-1}](t) + [NR_{+1}](t) + [NR_{-1}](t)}{\sum_i [R_i](t) + [NR_i](t)}$$
$$= \frac{1}{2}(1 - e^{-2Kk_r t})$$
(A19)

Thus, the repositioning reaction approaches its steady state solution with an apparent rate constant of

$$k_{app} = 2Kk_r$$
(A20)

The rate of ADP production associated with repositioning is

$$[ADP](t) = ck_r Kt$$
(A21)

As expected, the rate of ADP production is linearly dependent upon the fraction of bound nucleosomes. Similarly, if there were two octamer positions available on each side of the central position, then

$$\frac{[R_0](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{K}{4}e^{-2Kk_r t}(1 + e^{-Kk_r t})^2$$
(A22)

$$\frac{[R_{+1}](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{[R_{-1}](t)}{\sum_i [R_i](t) + [NR_i](t)}$$
$$= \frac{K}{4}(1 - e^{-2Kk_r t})$$
(A23)

$$\frac{[R_{+2}](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{[R_{-2}](t)}{\sum_i [R_i](t) + [NR_i](t)}$$
$$= \frac{K}{8}e^{-2Kk_r t}(1 - e^{-Kk_r t})^2$$
(A24)

The total population of octamers at the $i = \pm 2$ positions is given by eq A25.

$$\frac{[R_{+2}](t) + [R_{-2}](t) + [NR_{+2}](t) + [NR_{-2}](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{e^{-2Kk_r t}}{4}$$
$$(1 - e^{-Kk_r t})^2$$
(A25)

The repositioning reaction is now biphasic with

$$k_{app,1} = Kk_r$$
(A26)

$$k_{app,2} = 2k_{app,1} = 2Kk_r$$
(A27)

However, the rate of ADP production is still given by eq A21.

Monte Carlo Simulations

Nucleosome repositioning time courses were simulated using a Monte Carlo program written in C++. In these simulations, the motion of an ISWI-bound histone octamer along the DNA was determined according to the probabilities that were assigned to forward motion of the octamer and dissociation of ISWI from the octamer. The corresponding kinetic rates for Scheme 2 associated with these probabilities are listed in Table A1 and

Table A1. Results of Global Analysis of Simulated Repositioning Time Courses Using eqs 1 and 2[a]

	input parameters		fit parameter	
data set	k_r (min^{-1})	k_d (min^{-1})	P	k_r (min^{-1})
1	1	2	0.33	0.882 ± 0.002
2	1	5	0.17	0.827 ± 0.003
3	1	10	0.09	0.762 ± 0.002
4	2	1	0.67	1.531 ± 0.004
5	5	1	0.83	3.77 ± 0.02
6	10	1	0.91	7.33 ± 0.05

[a]The parameter P is the processivity of the repositioning reaction defined as $k_r/(k_r + k_d)$.

Table A2 for each simulated data set. The direction of octamer movement remained constant as long as ISWI was bound. When ISWI bound to an unbound nucleosome, the direction of repositioning was assigned randomly. The probability of ISWI binding to an unbound octamer was varied to alter the fraction of bound ISWI. For all simulations, all octamers were initially centrally located on their DNA substrate and the direction of repositioning for each initially bound octamer was assigned randomly.

Two time courses were generated for each set of simulation parameters: one in which there was one additional translational position for the octamer on each side of the initial central

Table A2. Results of Global Analysis of Simulated Repositioning Time Courses Using eqs 1 and 2[a]

data set	input parameters		fit parameter	
	k_r (min^{-1})	k_d (min^{-1})	P	k_r (min^{-1})
1	1	0	1	0.912 ± 0.002
2	1	4	0.25	1.000 ± 0.003
3	1	10	0.09	0.935 ± 0.003
4	2	1	0.67	2.5 ± 0.02

[a]The parameter P is the processivity of the repositioning reaction defined as $k_r/(k_r + k_d)$.

position (corresponding to the 18N18 substrate) and one in which there were two additional translational positions for the octamer on each side of the initial central position (corresponding to the 24N24 substrate).

Data Analysis of Monte Carlo Simulations

All repositioning time courses were globally analyzed using eqs 1 and 2 as described in Experimental Procedures. This is the same procedure that was implemented for the analysis of experimental time courses. No additional experimental noise was added to these time courses, so the uncertainties associated with the parameter estimates obtained from this analysis are artificially smaller than they would be for the analysis of experimental data.

Results of Monte Carlo Simulations

In the first set of simulations, repositioning time courses at a single fraction of bound ISWI were generated and analyzed. Each set of data consists of two time courses: one for the 18N18 substrate and one for the 24N24 substrate. For all sets of data analysis, eqs 1 and 2 provided a good description of the data as judged by the variance of the fit and visually, demonstrating the general applicability of these equations for describing a variety of repositioning reactions. However, as shown in Table A1, the estimate of k_r was typically an underestimate, often significantly so.

To further investigate the efficacy of our approach in analyzing repositioning time courses, we conducted an additional set of simulations in which we generated different fractions of bound ISWI by varying the probability of binding in the simulation. For each data set, we generated time courses for two different nucleosome substrates (18N18 and 24N24) at four different fractions of bound ISWI. This more accurately mimics the conditions under which we analyzed our experimental data. For three of the four sets of data analysis, eqs 1 and 2 provided a good description of the data as judged by the variance of the fit and visually. Furthermore, as shown in Table A2, with one exception, the estimates of k_r were within 10% of the simulated value. For the one outlier data set, the quality of the fits was poor, as judged by the variance of the fit and visually, which we believe results from the presence of long-lived intermediate species in these time courses that cannot be described by eqs 1 and 2.

Taken together, we believe that these simulations not only demonstrate the efficacy of our analysis method for determining the rate constant for repositioning but also show that the generality of this approach extends beyond the restrictive assumptions used in its derivation.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Native gel-based repositioning of asymmetrical nucleosome substrates 51N5 and 10N71 (Figure 1), nucleosome-stimulated ATPase activity of ISWI (Figure 2), requirement of octamer and ATP for the observed changes in the fluorescence anisotropy-based assay (Figure 3), results of global analysis of measurements of changes in anisotropy (Figure 4), model of nucleosome repositioning by ISWI (Figure 5), results of analysis of F18N18F nucleosomes using eqs 1 and 2 (Table 1), results of analysis of F24N24F nucleosomes using eqs 1 and 2 (Table 2), and determination of the fraction of nucleosomes bound with one or two ISWIs at various ISWI concentrations using species fractions as constraints (Table 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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