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Probing the Rate-Limiting Step for Intramolecular Transfer of a Transcription Factor between Specific Sites on the Same DNA Molecule by $^{15}N_z$-Exchange NMR Spectroscopy

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Supporting Information

ABSTRACT: The kinetics of translocation of the homeodomain transcription factor HoxD9 between specific sites of the same or opposite polarities on the same DNA molecule have been studied by $^{15}N_z$-exchange NMR spectroscopy. We show that exchange occurs by two facilitated diffusion mechanisms: a second-order intermolecular exchange reaction between specific sites located on different DNA molecules without the protein dissociating into free solution that predominates at high concentrations of free DNA, and a first-order intramolecular process involving direct transfer between specific sites located on the same DNA molecule. Control experiments using a mixture of two DNA molecules, each possessing only a single specific site, indicate that transfer between specific sites by full dissociation of HoxD9 into solution followed by reassociation is too slow to measure by z-exchange spectroscopy. Intramolecular transfer with comparable rate constants occurs between sites of the same and opposing polarity, indicating that both rotation-coupled sliding and hopping/flipping (analogous to geminate recombination) occur. The half-life for intramolecular transfer (0.5−1 s) is many orders of magnitude larger than the calculated transfer time (1−100 μs) by sliding, leading us to conclude that the intramolecular transfer rates measured by z-exchange spectroscopy represent the rate-limiting step for a one-base-pair shift from the specific site to the immediately adjacent nonspecific site. At zero concentration of added salt, the intramolecular transfer rate constants between sites of opposing polarity are smaller than those between sites of the same polarity, suggesting that hopping/flipping may become rate-limiting at very low salt concentrations.

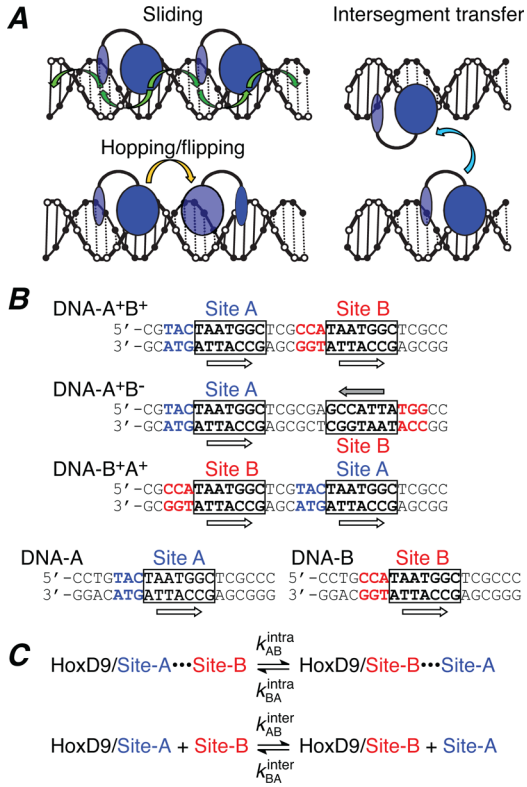

Figure 1. Facilitated diffusion in specific protein−DNA binding. (A) Schematic depiction of intramolecular sliding and hopping/flipping, and intermolecular intersegment transfer. (B) Summary of DNA duplexes used. The locations of HoxD9 specific binding sites are indicated by the boxes. The A and B sites differ by three base pair mutations immediately 5′ of the specific site, colored in blue and red, respectively. The polarity of each specific site is indicated by an arrow. (C) Intramolecular (top) and direct intermolecular transfer of HoxD9 between the A and B sites.

Transcription factors need to locate their specific target site(s) within an overwhelming sea of nonspecific DNA. A conventional three-dimensional diffusion search is not the most efficient way to locate a specific DNA binding site since a protein, once bound to a nonspecific DNA site, must first fully dissociate into free solution, diffuse in three-dimensions by Brownian motion, and subsequently reassociate at a distant site on either the same DNA molecule or another DNA molecule. This process, referred to as "jumping", must occur many times before the correct specific DNA site is located. Three facilitated diffusion mechanisms can be employed to speed up the search process:[1] (i) one-dimensional diffusion along the DNA, otherwise known as rotation-coupled sliding with the protein

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tracking the DNA grooves, is thought to be efficient over a range of about 50 base pairs; (ii) intramolecular hopping, which in effect is equivalent to geminate recombination, albeit at a different site in close proximity (within 10 bp) to the first site, circumvents full dissociation into free solution, which can be very slow; and (iii) direct intersegment transfer, whereby a transcription factor can be transferred from one site to another, either on different DNA molecules between sites very far apart in sequence on the same DNA molecule but close in space (as a consequence looping), without dissociating into free solution, using what has been described as a "monkey bar" mechanism[2] (Figure 1A). We have previously used z-exchange NMR spectroscopy to directly demonstrate the existence of intersegment transfer between slightly different specific sites located on separate DNA molecules.[3] We have also used paramagnetic relaxation enhancement and residual dipolar coupling NMR measurements to directly demonstrate the existence of rotation-coupled sliding.[4] In this paper we make use of z-exchange spectroscopy to probe the mechanism and rate-limiting steps involved in intramolecular transfer of a transcription factor, namely the homeodomain HoxD9, from one specific site to another specific site located on the same DNA molecule.

The specific binding site for HoxD9 is 7 base pairs in length, but minor groove contacts to the phosphate backbone involving the N-terminal tail likely extend an additional 2 base pairs at the 5′-end.[5] The experimental design involves mutating 3 base pairs immediately 5′ of the specific binding site to create two sites, A and B (Figure 1B). These mutations have only a small effect on affinity but result in measurable differences in $^{1}H_N/^{15}N$ chemical shifts within the N-terminal tail of bound HoxD9 (Figure 2A) that can be exploited to study the transfer of HoxD9 between sites A and B by $^{15}N_z$-exchange spectroscopy (Figure 2B). Five DNA duplexes were employed (Figure 1B). In DNA-A^+B^+ and DNA-B^+A^+, the polarity of the two sites is the same (reading the sequence of the top strand in the 5′-to-3′ direction) such that the orientation of HoxD9 is maintained at the two sites, but the order of the sites is reversed; in DNA-A^+B^-, the polarity of site B is reversed so that the orientation of HoxD9 bound at the B site is rotated by 180° relative to that at the A site, about an axis perpendicular to the long axis of the DNA; last, DNA-A and DNA-B contain only a single site (A and B, respectively) and serve as controls since exchange of HoxD9 between the A and B sites can only occur via either jumping or intersegment transfer, in contrast to the three duplexes containing both A and B sites, where exchange can occur via both intra- and intermolecular processes. For DNA-A^+B^+ and DNA-B^+A^+, intramolecular transfer of HoxD9 between the A and B sites can occur by sliding alone; for DNA-A^+B^-, however, intramolecular exchange between the A and B sites can only occur by a combination of hopping/flipping and sliding.

Exchange cross-peaks are apparent in the 2D TROSY-based $^{15}N_z$-exchange experiment[6] in which exchange of ^{15}N z-magnetization between distinct species occurs during the mixing time following the ^{15}N chemical shift evolution period (inset Figure 2B). The apparent first-order rate constants for the transfer of HoxD9 from A to B and vice versa (k_{AB}^{app} and k_{BA}^{app}, respectively) are obtained by simultaneously best-fitting the time dependence of the intensities of the auto and exchange cross-peaks in the $^{15}N_z$-exchange experiment by solving the coupled McConnell differential equations for the time evolution of magnetization in a two-site exchange system as described previously.[3a]

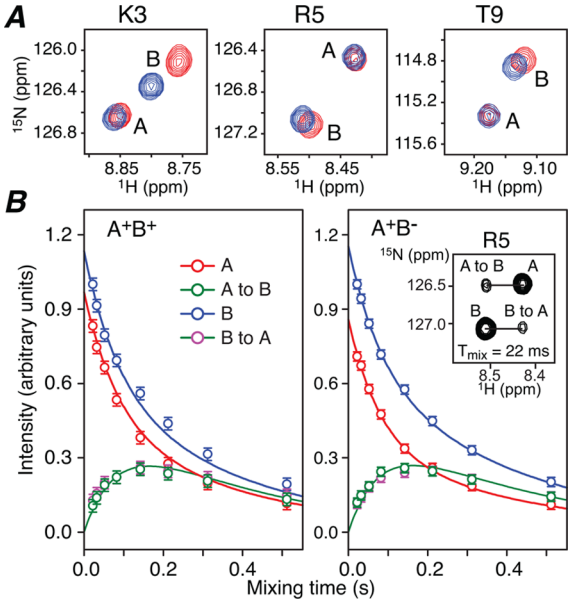

Figure 2. TROSY-based $^{15}N_z$-exchange spectroscopy. (A) Examples of cross-peaks with different $^{15}N/^{1}H_N$ chemical shifts when bound to the A and B sites of DNA-A^+B^+ (blue) and DNA-A^+B^- (red). (B) Dependence of the intensities of auto and exchange cross-peaks on mixing time (T_{mix}) in the TROSY-based $^{15}N_z$-exchange spectra (open circles) together with the best-fit curves (solid lines) obtained using a simple model for a phenomenological pseudo-first-order exchange reaction (see SI). The experimental intensities, for display purposes (see SI), represent the averages for Lys3, Arg5, and Thr9. The inset in the right panel shows the auto and exchange cross-peaks for Arg5 at T_{mix} = 22 ms. Experiments were performed at 600 MHz and 25 °C on samples of 0.45 mM uniformly $^{2}H/^{15}N$-labeled HoxD9 and 0.40 mM DNA duplex (DNA-A^+B^+ or DNA-A^+B^-) at natural isotopic abundance in buffer containing 20 mM HEPES/5 mM Na^+-HEPES, pH 7.0, 30 mM NaCl, and 95% H_2O/5% D_2O (see SI).

Intersegment transfer is a second-order process involving a bimolecular exchange reaction (Figures 1A,C).[3] k_{AB}^{app} and k_{BA}^{app} are linearly dependent upon the concentration of free DNA binding sites, respectively (Figure 3). Thus, the bimolecular rate constants for intermolecular transfer from A to B and vice versa, (k_{AB}^{inter} and k_{BA}^{inter}, respectively) are obtained directly from the slope of a plot of the apparent first-order rate constants versus the concentration of free B and A sites (which are easily determined since the equilibrium dissociation constant, K_D, is less than 1 nM under the experimental conditions employed,[3a,4a] and hence binding is stoichiometric); the intercept of this plot yields a concentration-independent first-order rate constant which can arise from dissociation of bound HoxD9 into free solution (when $k_{off} \ll k_{on}[DNA_{free}] = k_{off}[DNA_{free}]/K_D$, where k_{off} and k_{on} are the dissociation and association rate constants, respectively)[3a] and/or intramolecular transfer between sites A and B. The contribution of the former is negligible since z-exchange experiments with DNA-A and DNA-B, where intramolecular transfer between sites A and B cannot occur, yield an intercept of 0 s^{-1} (Figure 3A). Thus, dissociation of HoxD9 from sites A and B into free solution is too slow to be measured by z-exchange spectroscopy, in agreement with our previous z-exchange measurements,[3a] as well as with biochemical measurements which yield estimates for the dissociation rate constant of <0.01 s^{-1}.[7] One can therefore conclude that the measurable intercepts for the three DNA duplexes containing both A and B sites (Figure 3B–D)

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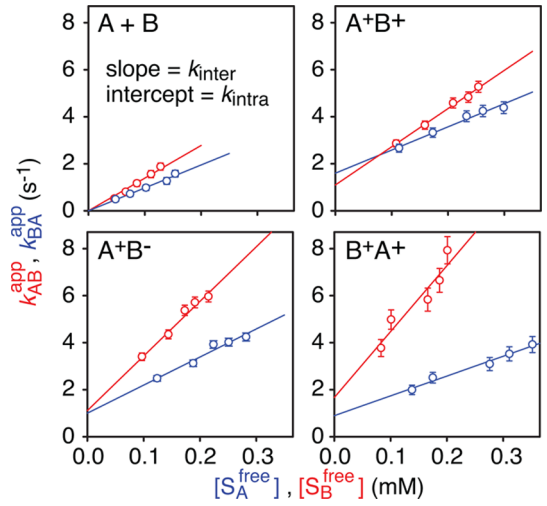

Figure 3. Dependence of k_{AB}^{app} and k_{BA}^{app} on the concentration of free DNA specific sites (S_B^{free} and S_A^{free}, respectively). Experimental conditions as in Table 1 and Figure 2. For experiments with DNA-A^+B^+, DNA-A^+B^-, and DNA-B^+A^+, the ratio of DNA to protein was kept constant at 1.12:1 so that 80% of the DNA molecules have HoxD9 bound to one of the two specific sites and 20% have both specific sites occupied. The ratio of DNA-A and DNA-B to HoxD9 was kept constant at 1:1:1.

are entirely attributable to intramolecular exchange between the A and B sites and yield the first-order rate constants k_{AB}^{intra} and k_{BA}^{intra}.

The center-to-center distance between sites A and B is 13 base pairs (=44 Å for B-type DNA). One-dimensional diffusion coefficients (D_1) for rotation-coupled sliding of proteins of varying size on DNA measured by single-molecule spectroscopy range from 0.0001 to 2 $\mu m^2\ s^{-1}$ (ref 8) and are approximately dependent on the inverse cube of the protein radius.[8c] Given the small size of HoxD9 (radius ~8 Å), the expected D_1 value is ~10 $\mu m^2\ s^{-1}$. The predicted rate constant for transfer between sites A and B by rotation-coupled sliding along nonspecific DNA stretches with D_1 = 0.1−10 $\mu m^2\ s^{-1}$ is given by $k_{sliding} = 2D_1/\langle L^2 \rangle$ (where L is the distance between the two sites) and lies between 10^4 and $10^6\ s^{-1}$, which is many orders of magnitude larger than the values of k_{AB}^{intra} and k_{BA}^{intra} measured by z-exchange spectroscopy (cf. Table 1). This leads

Table 1. Kinetic Rate Constants Obtained from $^{15}N_z$-Exchange Spectroscopy[a]

	intramolecular		intermolecular	
DNA	k_{AB}^{intra} (s^{-1})	k_{BA}^{intra} (s^{-1})	k_{AB}^{inter} ($mM^{-1}\ s^{-1}$)	k_{BA}^{inter} ($mM^{-1}\ s^{-1}$)
A + B	0.0	0.0	13.9 ± 0.5	9.7 ± 0.2
A^+B^+	1.1 ± 0.4	1.6 ± 0.3	16.2 ± 2.0	9.8 ± 1.4
A^+B^-	1.1 ± 0.3	1.0 ± 0.2	23.2 ± 2.1	11.9 ± 1.2
B^+A^+	1.7 ± 0.6	0.9 ± 0.3	28.0 ± 4.3	8.4 ± 1.4

[a] 25 °C in buffer containing 20 mM HEPES/5 mM Na^+-HEPES, pH 7.0, 30 mM NaCl, 95% H_2O/5% D_2O.

us to conclude that the latter rate constants represent the rate-limiting step for intramolecular transfer from one specific site to another. The simplest process that would result in such a rate-limiting step is the one-base-pair shift required for the protein to move from its specific site to the immediately adjacent nonspecific site.

In the case of DNA-A^+B^-, intramolecular transfer between the A and B sites cannot occur by sliding alone, since the protein has to undergo a 180° reorientation on the DNA (i.e., hopping/flipping). Given that the rate constants for intramolecular transfer are comparable for all three DNA duplexes bearing A and B sites (Table 1), one can also conclude that a 180° flip of HoxD9, when bound nonspecifically to DNA, is also a very rapid process. Mechanistically this would occur by a first-order process analogous to geminate recombination: that is, dissociation from the DNA without diffusion into free solution followed by rapid rotation and reassociation. Since the orientation of HoxD9 relative to the long axis of the DNA is the same for specific and nonspecific binding,[4b] a 180° flip on any given nonspecific site would entail minimal energetic cost.

The values of the intramolecular transfer rate constants reported in Table 1 strongly support the hypothesis that the rate-limiting step involves a single one-base-pair shift of the protein from a specific site to an immediately adjacent nonspecific site. The values of k_{AB}^{intra} for DNA-A^+B^+ and DNA-A^+B^- and k_{BA}^{intra} for DNA-B^+A^+ are the same (~1 s^{-1}), as expected since a single-base-pair shift in the 3′ direction from the specific site located at the 5′-end (top strand) of the three duplexes results in occupancy of an identical nonspecific site (5′-AATGGCT). The values of k_{BA}^{intra} and k_{AB}^{intra} for DNA-A^+B^+ and DNA-B^+A^+, however, are about 50% larger (~1.6 s^{-1}): in both cases, the single-base-pair shift in the 5′ direction from the specific site located at the 3′-end (top strand) of the two duplexes results in occupancy of a different nonspecific site (5′-ATAATGG and 5′-CTAATGG, respectively, that differ only at the position of the first base pair).

The values of the bimolecular intermolecular transfer rate constants (Table 1) are also of interest. The values of k_{BA}^{inter} are comparable in all four cases (8−12 $mM^{-1}\ s^{-1}$). The values of k_{AB}^{inter} for DNA-A + DNA-B and DNA-A^+B^+ are also similar (13−16 $mM^{-1}\ s^{-1}$) but significantly smaller than those for DNA-A^+B^- and DNA-B^+A^+ (23−28 $mM^{-1}\ s^{-1}$). This appears to be correlated to the proximity of the 5′-end of the B site to the end of the DNA duplex: closer for DNA-A^+B^- and DNA-B^+A^+, and farther for DNA-B and DNA-A^+B^+. Thus, end effects seem to have a larger influence on intermolecular transfer to the B site than to the A site.

Lastly, we examined the impact of added Na^+ on the intramolecular transfer rate constants by serial dilution of samples with 95% H_2O/5% D_2O, thereby simultaneously reducing the concentrations of free DNA sites and Na^+ (Figure 4). Since there is a linear relationship between log [Na^+] and

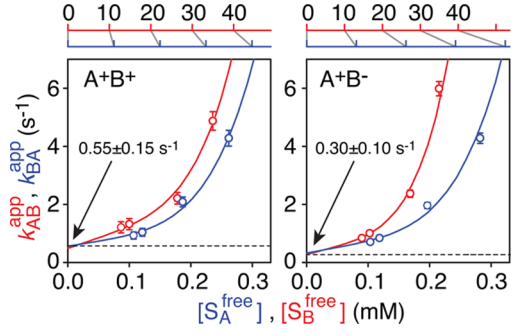

Figure 4. Dependence of apparent exchange rates on simultaneous variation of the concentrations of added Na^+ and free specific DNA sites. The data were obtained by successively diluting the original samples (cf. Figure 2) with 95% H_2O/5% D_2O.

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log k_{ex}^{app},[9] the data can be fitted using the empirical relationship $k_{ex}^{app([DNA_{free}],[Na^+])}/k_{ex}^{app([DNA_{free}],[Na^+]=35\ mM)} = a[Na^+]^b + c$. The extrapolated values of k_{ex}^{app} to zero free DNA and added salt concentration yield the values of k_{AB}^{intra} and k_{BA}^{intra} at zero apparent salt concentration (note that Na^+ cannot be eliminated completely, since it serves as a counterion for the DNA phosphate backbone). For both the DNA-A^+B^+ and DNA-A^+B^- duplexes, k_{AB}^{intra} and k_{BA}^{intra} at zero added salt converge to the same values; however, these values are about 80% larger for DNA-A^+B^+ than DNA-A^+B^- (Figure 4). Since intramolecular transfer for the former can occur by sliding alone, while both sliding and a 180° flip are required for the latter, this result suggests that, in the absence of added salt (or at very low salt), the 180° protein reorientation required for intramolecular transfer in the case of DNA-A^+B^- may also become rate-limiting. This is expected since flipping requires partial dissociation of HoxD9 from the DNA (without diffusion into free solution), which would be severely reduced at low salt, where electrostatic screening is weak.

In conclusion, we have made use of $^{15}N_z$-exchange spectroscopy to directly probe the rate-limiting steps involved in intramolecular transfer of a transcription factor between specific sites on a single DNA molecule. While the separation between the specific sites used here is small (13 base pairs center-to-center) owing to molecular weight limitations of NMR, one can calculate that, even for rotation-coupled sliding over a distance of 100 base pairs (mean passage time of 50 μs to 5 ms for D_1 values ranging from 10 to 0.1 $\mu m^2\ s^{-1}$, respectively), the initial one-base-pair shift from the specific site to the immediately adjacent nonspecific site would still be rate-limiting ($t_{1/2}$ = 0.5−1 s).

■ ASSOCIATED CONTENT

Supporting Information

Details of experimental procedures, fitting of $^{15}N_z$-exchange data, and sample preparation. 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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