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Direct Observation of Enhanced Translocation of a Homeodomain between DNA Cognate Sites by NMR Exchange Spectroscopy

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NMR exchange spectroscopy provides a well-established approach for measuring rate constants under equilibrium conditions.[1] Kinetic investigation, however, of protein−DNA interactions by NMR is not straightforward. Even if exchange is slow on the chemical shift time scale, it is generally difficult to clearly observe two sets of resonances arising from free and DNA-bound protein under conditions of protein excess since, at high protein concentrations typically used in an NMR experiment, any excess protein tends to interact nonspecifically with exposed regions of the DNA in the specific complex, generally resulting in aggregation. In addition, some DNA-binding proteins may have poor solubility properties in the free state. In this contribution, we present a new, general approach for deriving kinetic information on protein−DNA interactions by NMR, which is readily applicable to many protein−DNA complexes. The results, applied to the interaction of the human Hox-D9 homeodomain with DNA, yield significant insight into the mechanism of rapid protein translocation between cognate DNA binding sites, and reconcile the long half-lives of specific protein−DNA complexes measured by biochemical analysis in vitro[2a,b] with the apparent highly dynamic behavior of such complexes observed using in vivo microscopy combined with photobleaching techniques such as FRAP.[3]

The experiment we propose makes use of two 24-bp DNA fragments *a* and *b* (Figure 1A) that differ at only a single base-pair position adjacent to the homeodomain specific target site TAATGG.[2] As a consequence, the ^{1}H−^{15}N chemical shifts of several backbone amide groups are slightly different in the two complexes, while the affinity of the two oligonucleotides for the homeodomain remains essentially unaltered. In the ^{1}H−^{15}N HSQC spectrum of a 1:1 mixture of the two complexes in 40 mM NaCl, the amide groups corresponding to complexes *a* and *b* are observed separately, indicating that any exchange process involving translocation of the homeodomain between the two oligonucleotides is slow on the chemical shift time scale (Figure 1B). Indeed, given the measured equilibrium dissociation constant K_{diss} of 1.5 ± 0.4 nM at 100 mM NaCl, determined by titration using fluorescence anisotropy spectroscopy, one would predict a lifetime of >10 s for each complex. However, exchange cross-peaks are clearly evident in a 2D ^{1}H−^{15}N correlation experiment in which exchange between the ^{15}N *z*-magnetizations of distinct species occurs during the mixing time following the t_1(^{15}N)-evolution period (Figure 1C).[4,5]

Simultaneously best-fitting the intensities of the auto- and cross-peaks as a function of mixing time (Figure 1D) using the appropriate McConnell equations[6] for the time development of magnetization in a two-site system yields apparent first-order translocation rate constants, k_{ab}^{app} and k_{ba}^{app}, whose values are dependent upon the concentration of free DNA and range from ∼5 to 20 s^{-1} (Figure 2A and E). These values are ∼3 orders of magnitude larger than the dissociation rate constant (<0.01 s^{-1}) determined by gel shift competition assays.[2a,b]

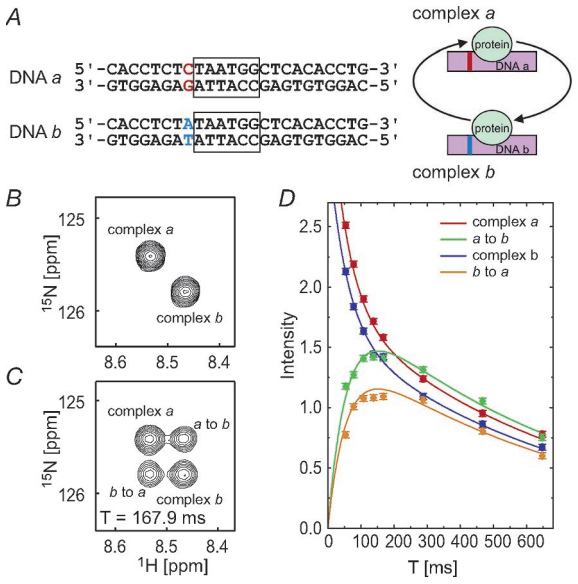

Figure 1. Exchange between two homeodomain−DNA complexes. (*A*) 24-bp DNA fragments, DNA *a* and DNA *b*, differing at a single bp (red and blue, respectively). The protein−DNA complexes involving DNA *a* and DNA *b* are referred to as complexes *a* and *b*, respectively. Experiments were carried out using ^{2}H/^{15}N-labeled Hox-D9 homeodomain and unlabeled DNA. (*B*) Cross-peaks for the backbone amide group of Arg5 in the ^{1}H−^{15}N HSQC spectrum recorded on a 1:1 mixture of complexes *a* and *b*. (*C*) Same spectral region in the ^{15}N$_z$-exchange experiment[4a] recorded with a mixing time of 167.9 ms. (*D*) Measured intensities of auto- and exchange cross-peaks and best-fit curves using a simple model for the phenomenological first-order exchange reaction. Best-fitting was carried out using the program FACSIMILE.[9] Experiments were carried out at 35 °C in 10 mM Tris·HCl (pH 6.8), 40 mM NaCl, 7% D_2O with 0.68 mM protein, 0.44 mM DNA *a* and 0.44 mM DNA *b* (under these conditions, all the protein is bound to the two oligonucleotides, and the concentrations of complexes *a* and *b* are ∼0.34 mM each).

If protein exchange between complexes *a* and *b* occurred through spontaneous dissociation of the protein followed by reassociation of free protein and DNA (Scheme 1 in Figure 2B), the rate-limiting process would be governed by the dissociation rate constants k_a^{off} and k_b^{off} under conditions where the total DNA (i.e. [DNA *a*] + [DNA *b*]) is in excess over protein and $k^{off} \ll k^{on}$ [DNA$_{free}$] (= k^{off} [DNA$_{free}$]/K_{diss}, where k^{on} is the association rate constant). k_{ab}^{app} and k_{ba}^{app} would be approximately equal to $k_a^{off}/2$ and $k_b^{off}/2$, respectively (the statistical factor of 2 arising from transitions between the same species), and should be independent of the free DNA concentration (cf. the simulations shown in Figure 2C). Thus, Scheme 1 clearly cannot account for the NMR observations. An alternative mechanism, represented by Scheme 2 (in Figure 2D) and akin to the "intersegment transfer process" postulated as a potential means of accelerating the rate of target recognition in protein−DNA interactions,[7] accounts quantitatively for the NMR data. Since the transfer

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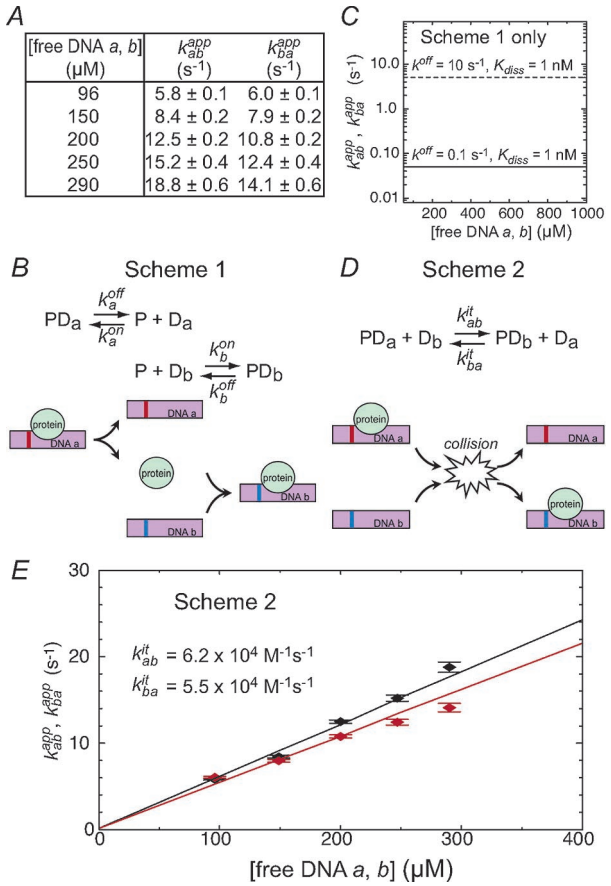

Figure 2. Characterization of homeodomain translocation between specific target sites on two oligonucleotides. (*A*) Measured values of apparent exchange rate constants measured on a 1:1 mixture of complexes *a* and *b* (k_{ab}^{app}, translocation from *a* to *b*; k_{ba}^{app}, from *b* to *a*). Experiments were carried out at five different concentrations of free DNA. The ratio of DNA fragments *a* and *b* was kept at 1:1, and the concentration of free DNA listed is for one species. (*B*) Schematic representation of the conventional mechanism for protein translocation via dissociation and reassociation. (*C*) Results of simulations displaying the dependence of the apparent rate constants upon free DNA concentration for translocation by Scheme 1. (*D*) Schematic representation of the intersegment transfer mechanism involving direct transfer of the protein from one oligonucleotide to the other following collision of free DNA with the protein−DNA complex. (*E*) Plot of the experimentally obtained k_{ab}^{app} (black) and k_{ba}^{app} (red) rate constants versus free DNA concentration. The two solid lines represent the theoretical dependence based on the optimized values of the intersegment transfer rate constants for Scheme 2 obtained by fitting the NMR exchange data at the five DNA concentrations simultaneously.

of protein from one oligonucleotide to the other is a second-order process, both forward and backward reaction rates are proportional to the concentration of free oligonucleotides. Best-fitting all the NMR exchange data at all DNA concentrations *simultaneously* to Scheme 2, optimizing the values of the two intersegment transfer rate constants (k_{ab}^{it} and k_{ba}^{it}) and a single auto-relaxation rate constant ($R_1^{complexa} = R_1^{complexb}$) yields an excellent fit to the data (with an overall standard deviation of the fit of 1.3%) and values of 6.2(±0.1) × 10^4 and 5.5(±0.1) × 10^4 M^{-1} s^{-1} for k_{ab}^{it} and k_{ba}^{it}, respectively (see Supporting Information for details of fitting). (Note that these values indicate that the affinity of the protein for the two oligonucleotides is very similar since it is readily shown that $k_{ab}^{it}/k_{ba}^{it} = K_{diss}^{a}/K_{diss}^{b}$, where K_{diss}^{a} and K_{diss}^{b} are the equilibrium dissociation constants for complexes *a* and *b*, respectively.) Addition of Scheme 1 does not affect the intersegment transfer rate constants for values of k^{off} <0.1 s^{-1} and $k^{on} = 10^6-10^{10}$ M^{-1} s^{-1}. For $k^{off} =$ 1 s^{-1}, the intersegment transfer rate constants are reduced by 5%. From the values of k_{ab}^{it} and k_{ba}^{it} it is evident that the intersegment transfer mechanism is only significant when the free DNA concentration is higher than ∼10^{-6} M. Thus, this process would not be observable in the gel-shift mobility assays used to measure dissociation rate constants since free DNA concentrations employed in such experiments are typically in the nanomolar range.[2a,b]

In eukaryotic cells, the concentration of nuclear DNA is ∼100 mg/mL,[8] corresponding to a concentration of 150 mM on a base-pair basis. Since the recognition core for the homeodomain comprises the 4-bp sequence TAAT, the concentration of potential sites in the nucleus is quite high; even if 99% of sites are rendered inaccessible by nucleosomes, the concentration of accessible sites is still ∼10^{-5} M. Thus, intersegment transfer observed here is likely to be biologically significant. A possible physical model for the translocation process may be postulated if one considers the homeodomain as a two subdomain protein comprising the helix−turn−helix core in the major groove and the N-terminal tail in the minor groove, each of which can come on and off the DNA independent of the other.[2c] The microscopic equilibrium dissociation constants would be expected to be quite large (particularly as truncation of the N-terminal subdomain results in weak DNA binding) with rapid dissociation and reassociation of the individual subdomains, while still maintaining a global binary protein−DNA complex. At high concentrations of free DNA, the dissociated subdomain could readily attach itself to the specific site on a free DNA molecule, leading to translocation of the whole protein from one oligonucleotide to the other. The same translocation mechanism could also contribute to rapid specific target search via nonspecific protein−DNA interactions, since the concentration of nonspecific sites is enormous.

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Supporting Information Available: Details of fitting procedures, sample preparation, fluorescence and NMR spectroscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

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