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NMR-based investigations into target DNA search processes of proteins

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ABSTRACT

To perform their function, transcription factors and DNA-repair/modifying enzymes must first locate their targets in the vast presence of nonspecific, but structurally similar sites on genomic DNA. Before reaching their targets, these proteins stochastically scan DNA and dynamically move from one site to another on DNA. Solution NMR spectroscopy provides unique atomic-level insights into the dynamic DNA-scanning processes, which are difficult to gain by any other experimental means. In this review, we provide an introductory overview on the NMR methods for the structural, dynamic, and kinetic investigations of target DNA search by proteins. We also discuss advantages and disadvantages of these NMR methods over other methods such as single-molecule techniques and biochemical approaches.

1. Introduction: DNA scanning by proteins

Protein-DNA interactions are vital for life. Living organisms regulate expression of genes and maintain integrity of the genome through protein-DNA interactions involving transcription factors or DNA-repair/modifying enzymes. These DNA-binding proteins must first locate their specific target sites in the vast presence of nonspecific but structurally similar sites on genomic DNA. The genome of higher eukaryotes contains billions of base pairs [1] and can potentially provide $\sim 10^9$ nonspecific sites on DNA, whereas functional target sites of each transcription factor or DNA-repair/modifying enzyme are far fewer (typically, $\sim 10^2$–10^3 sites) [2]. Functionality of these proteins should depend on their efficiency in locating targets on DNA through stochastic search processes. Therefore, it is important to understand at molecular and atomic levels how proteins scan DNA, recognize sequences, and locate the targets.

In the target search processes, the proteins nonspecifically interact with DNA. Although affinity for nonspecific DNA is weaker than that for targets, the vast quantity of nonspecific sites compensates for the weak affinity, making profound overall impacts [3–5]. It should be noted that DNA density in cell nuclei is as high as ~100 mg/ml [6]. This high density is understandable, considering that a total length of ~2 m of human genomic DNA ($=2 \times [3.2 \times 10^9$ bp$] \times [0.34 \times 10^{-9}$ m/bp]) is confined in the nucleus for which a typical diameter is ~6 μm [1]. Histones make this condensation possible, occupying ~80% of genomic DNA as nucleosomes. The average length of linker DNA segments between nucleosomes is ~56 bp [7]. The overall concentration of linker DNA segments is estimated to be as high as ~500 μM in the nuclei. Because this concentration alone is far higher than typical apparent dissociation constants of sequence-specific or structure-specific DNA-binding proteins for nonspecific DNA, the majority of these proteins must be nonspecifically bound to genomic DNA before reaching their targets.

When nonspecifically interacting with DNA, the DNA-binding proteins rapidly move from one site to another, scanning DNA to locate their targets (Fig. 1). Berg and von Hippel conceptually defined three major mechanisms for protein translocation on DNA: sliding, dissociation & re-association, and intersegment transfer [8]. Sliding corresponds to one-dimensional (1D) diffusion along DNA and involves random walks of protein being nonspecifically bound to DNA. Binding to nonspecific sites near targets can accelerate target association because sliding from nonspecific sites allows proteins to efficiently reach a target through 1D diffusion [2,9–11]. The dissociation & re-association mechanism is often called three-dimensional (3D) search. When DNA concentration is high (e.g., in the nuclei), dissociation from DNA is the rate-limiting step in this translocation mechanism. When translocation through this mechanism occurs between two sites in close proximity (e.g. within ~1–7 nm [12]), it is called hopping. Intersegment transfer (also known as direct transfer) is a unique mechanism that allows protein to directly transfer from one DNA duplex to another without going through the intermediary of free protein. This involves an intermediate where a protein molecule transiently bridges two DNA

Abbreviations: CSP, chemical shift perturbation; PRE, paramagnetic relaxation enhancement; RDC, residual dipolar coupling; ZF, zinc finger

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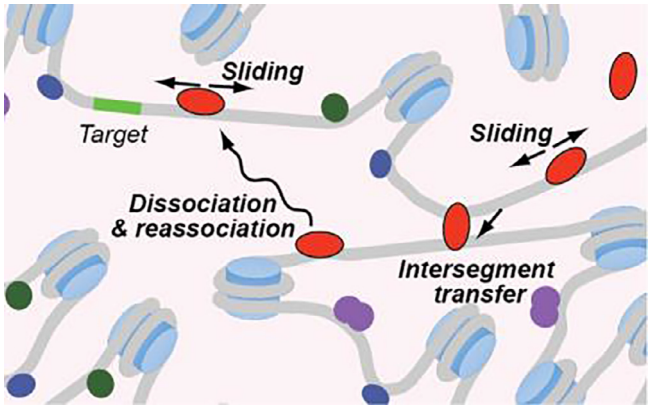

Fig. 1. Protein translocation on DNA through nonspecific interactions.

duplexes. Intersegment transfer mechanism may facilitate bypassing of nucleosome because its two ends are close in the three-dimensional structure [13]. These distinct translocation mechanisms coexist in solution, and their relative contributions to the overall efficiency of target DNA search should depend on various factors such as ionic strength, protein structure, DNA density, DNA geometry, and the presence of other proteins. However, details of the DNA scanning mechanisms are not well understood.

Our goal in this review article is to provide general readers with an introductory overview on nuclear magnetic resonance (NMR) methods for investigating the target DNA search processes of proteins. These NMR methods allow us to gain a great deal of information regarding how proteins nonspecifically interact with DNA during the search processes and how they move from one site to another on DNA. We first compare these NMR methods with biochemical and single-molecule methods for investigating target DNA search processes and explain unique strengths of these methods. Then, we will elaborate on practical details of the NMR methods that provide dynamic and kinetic information on target DNA search processes.

2. Advantages of NMR over other methods for investigating target DNA search of proteins

In the target DNA search processes, proteins dynamically move on DNA, changing their locations. This movement creates a challenge in studying these processes. Because proteins can change their locations through distinct mechanisms that are simultaneously present in the same system, characterizing a particular translocation mechanism is difficult, particularly when molecular ensembles are measured in solution. In the 21st century, there have been substantial advances in methodology for research on the target DNA search processes. Progress in single-molecule biophysics techniques is particularly remarkable, allowing direct observations of protein translocation on DNA *in vitro* and even in living cells. These single-molecule methods are recently overviewed in excellent reviews [14–21]. Elegant biochemical methods were also developed for kinetic investigations of target search and protein translocation on DNA [12,22–29]. As discussed below, NMR spectroscopy has provided unique insight into how proteins scan DNA at an atomic level. Capability and suitability of these methods for target search research are summarized in Table 1.

The greatest advantage of NMR methods over other methods is that NMR spectroscopy can provide *atomic-level* information on the highly dynamic processes whereby proteins scan DNA and locate their targets. NMR spectroscopy is particularly suited to study structural dynamics of biological macromolecules and can provide spatiotemporal information on dynamics [30]. As described in Section 4, there are several NMR methods that can provide different types of information on the DNA-scanning process. Some NMR data give structural information on proteins scanning DNA. Other NMR data give information on conformational mobility of particular domains or moieties within the proteins bound to DNA. Dynamic behavior of each basic side chain at protein-DNA interfaces can also be analyzed using NMR relaxation data [31–34]. These NMR methods can provide structural and dynamic details on the proteins moving on DNA, which are very difficult to analyze at an atomic level by any other methods.

Table 1
Comparison of the experimental approaches for investigating target DNA search of proteins.

Capability	Methods		
	NMR	Single-molecule	Biochemical
Atomic details of DNA scanning	+ + +[a]	–	–
Direct visualization of protein translocation on DNA	–	+ + +	–
Kinetic analysis of protein translocation on DNA	+ +[b]	+ +	+ +
Kinetic analysis of target DNA association	+	+ +	+ + +
Analysis of target DNA search process in cells	+	+ + +	+

Symbols are as follows: + + +, well suited; + +, feasible; +, possible, but not well explored; –, not possible.
[a] See Section 4.
[b] See Section 5.

Through NMR experiments, one can also obtain quantitative information on kinetics of protein translocation on DNA *at equilibrium*. The range of kinetic rate constants that can be determined by NMR is as wide as 0.1–10,000 s^{-1}, though suitable methods should be adopted depending on the timescale of the analyzed processes. As described in Section 5, several NMR methods are available for the kinetic investigations of protein translocation on DNA and differ in the applicable range in terms of analyzable timescale. These NMR experiments for investigating the kinetics of protein-DNA interactions can be conducted under various buffer conditions, offering flexibility in experimental design. A qualitative in-cell NMR study of protein-DNA interactions in *E. coli* cells has also been demonstrated [35]. NMR spectroscopy can thus serve as a versatile tool for investigating target DNA search processes of proteins.

3. NMR sample preparation for studying protein-DNA interactions

NMR studies on kinetics and dynamics of target DNA search by proteins require solution samples containing protein and DNA in which protein can move from one site to another. Because NMR spectroscopy is not as sensitive as many other spectroscopic techniques, high concentrations of proteins and DNA are required and high solubility of these materials is crucial for NMR experiments. Although high-field magnets and cryogenic probes have increased detection sensitivity for modern NMR spectrometers, many NMR experiments still require 0.1–1 mM proteins and DNA in a 0.25–0.5 ml solution. Milligrams of protein and DNA are necessary to prepare such a sample. Molecular size is also a limiting factor for NMR application. For typical heteronuclear multi-dimensional NMR experiments with backbone NH detection, the total molecular weight should be < 100 kDa. For methyl-TROSY-type experiments with side-chain CH_3 detection, the total molecular weight can be bigger. In fact, 200-kDa nucleosome core particles of histone octamer and 167-bp DNA were studied by methyl TROSY methods [36]. Typical buffers for NMR experiments are at pH 5–8 and an ionic strength less than 150 mM. We typically use 20–100 mM KCl (or NaCl) for NMR measurements of protein-DNA complexes. In general, sensitivity in NMR detection (particularly with cryogenic probes) is higher at a lower conductivity of solution [37,38]. When a high concentration of

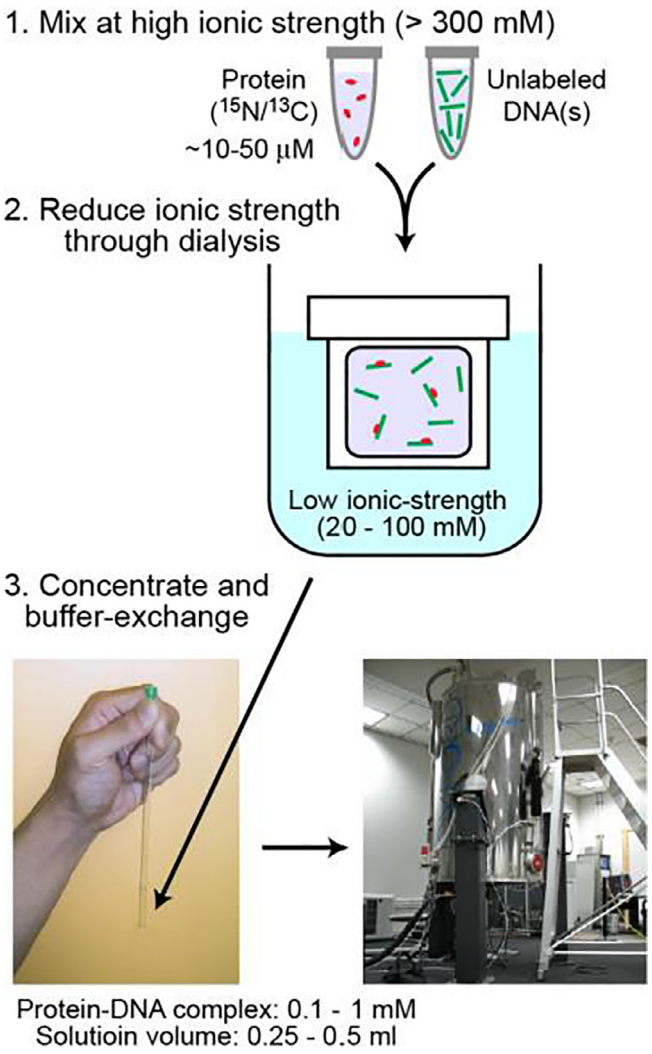

Fig. 2. Typical procedures for preparation of protein–DNA solutions for NMR investigations of target DNA search processes.

salt is required, use of sample tubes with a thinner sample diameter can mitigate the adverse effect of salt [39,40].

Due to large amounts of materials required, protein-DNA complexes might aggregate or precipitate during preparation of NMR samples, particularly when high concentrations of proteins and DNA are mixed at a low ionic strength. Fig. 2 shows our typical procedures of sample preparation for NMR studies of protein-DNA interactions. To avoid the aggregation or precipitation, we initially mix relatively low concentrations (~10–50 μM) of proteins and DNA at a relatively high ionic strength (≥300 mM KCl). In our experience, nonspecific DNA complexes of proteins exhibit a stronger tendency to precipitate at low ionic strength. After mixing the protein and DNA components at high ionic strength, we slowly reduce ionic strength. When ionic strength decreases too rapidly, some proteins precipitates (in some cases, the precipitated materials can be recovered by re-dissolving in a high ionic-strength buffer). So, we often reduce ionic strength through dialysis. A gradual decrease in salt concentration seems to generally help prevent aggregation of protein-DNA complexes, as known for nucleosome reconstitutions [41]. After reaching a desired ionic strength (typically, 20–100 mM KCl in our experiments), protein-DNA solutions are concentrated and buffer-exchanged using centrifugal concentrators such as Amicon Ultra-4 (Millipore) or Vivaspin-6 (Sartorius). A deuterated buffer, such as Tris-d_{11} or succinate-d_4, is used at this point if ^{1}H signals from buffer molecules could interfere with planned NMR experiments.

For NMR studies of nonspecific DNA-protein complexes, concentration of DNA should be sufficiently higher than that of protein. We typically use molar ratios of protein to nonspecific DNA of 1:2–4. A large excess of DNA helps prevent binding of more than one protein molecule to the same DNA molecule. This is important because the multiple bindings would complicate data analysis and also enhance aggregation and precipitation. Use of mM concentrations of nonspecific DNA mimics physiological conditions because it is comparable to the concentration of linker DNA segments in the nuclei, as mentioned in Section 1. DNA length is also an important parameter that affects solubility and stability of protein-DNA complexes in NMR samples. When nonspecific DNA complexes are analyzed, DNA must not contain any high-affinity sequences that are similar to the target sequence. To confirm the absence of any high-affinity site, we use fluorescence-based competitive binding assays [42], which directly provide relative affinity of competitor with respect to that of fluorescent-probe DNA containing a high-affinity site.

4. NMR-based analysis of DNA scanning by proteins

DNA-scanning processes are highly dynamic, during which proteins rapidly and stochastically change their locations on nonspecific DNA before reaching their targets. Solution NMR spectroscopy is well suited to study such dynamic systems. Although the DNA-scanning processes represent intermediates before the proteins reach their targets, these processes can be investigated most conveniently by using solutions of nonspecific DNA complexes that do not contain any targets. NMR was used to study nonspecific protein-DNA complexes in solution for gene-regulatory proteins (e.g., the *lac* repressor [43], Egr-1 [13,44], Ets-1 [45], ETV6 [46], HMGB1 [47], HoxD9 [48], LmrR [49], Oct1 [50], Sox2 [51], and ZNF217[52]) and as well as for DNA-repair/modifying enzymes (e.g., EcoRI [53], M.HhaI [54], and UNG [55,56]). Because proteins can move on nonspecific DNA, a solution of a nonspecific complex contains many states with the protein being located at different sites. However, NMR samples of nonspecific DNA-protein complexes typically show only a single set of signals because transitions between these states are so rapid that resonances of individual states are averaged (i.e., the “fast exchange” regime in the terminology of NMR spectroscopy). An example is shown in Fig. 3. Such samples of nonspecific complexes can be used to study how proteins scan DNA before reaching their targets. The following NMR methods are particularly useful to study the DNA-scanning dynamics.

4.1. NMR relaxation

NMR relaxation data for backbone and side-chain moieties are useful to study dynamics of proteins on timescales ranging from ps – ns to μs – ms [57–60]. There are several different types of NMR relaxation parameters (e.g., longitudinal and transverse relaxation rates, cross relaxation rates, cross correlation rates, etc.), which reflect internal motions of various moieties within proteins. Relaxation parameters of ^{15}N, ^{13}C, and ^{2}H nuclei are particularly useful for investigating protein dynamics, whereas ^{1}H relaxation reflects more about structure due to strong ^{1}H–^{1}H dipole-dipole interactions. Using NMR relaxation data, one can obtain information on 1) conformational flexibility of protein backbone and side chains, 2) conformational equilibrium between different states, 3) inter-domain dynamics, and 4) kinetics of molecular interactions. Analysis of NMR relaxation for nonspecific DNA complexes of proteins allows us to learn how these proteins scan DNA.

Fig. 4A shows an example of such an analysis. This figure displays some backbone ^{15}N relaxation data for the nonspecific and specific DNA complexes of the Egr-1 zinc-finger protein. Egr-1 recognizes DNA via three zinc fingers, ZF1, ZF2, and ZF3, each of which contacts with DNA in the crystal structure of the complex with the target DNA [61–63]. Backbone ^{15}N relaxation data were compared with the nonspecific and specific complexes with 28-bp DNA. The specific DNA contains the Egr-

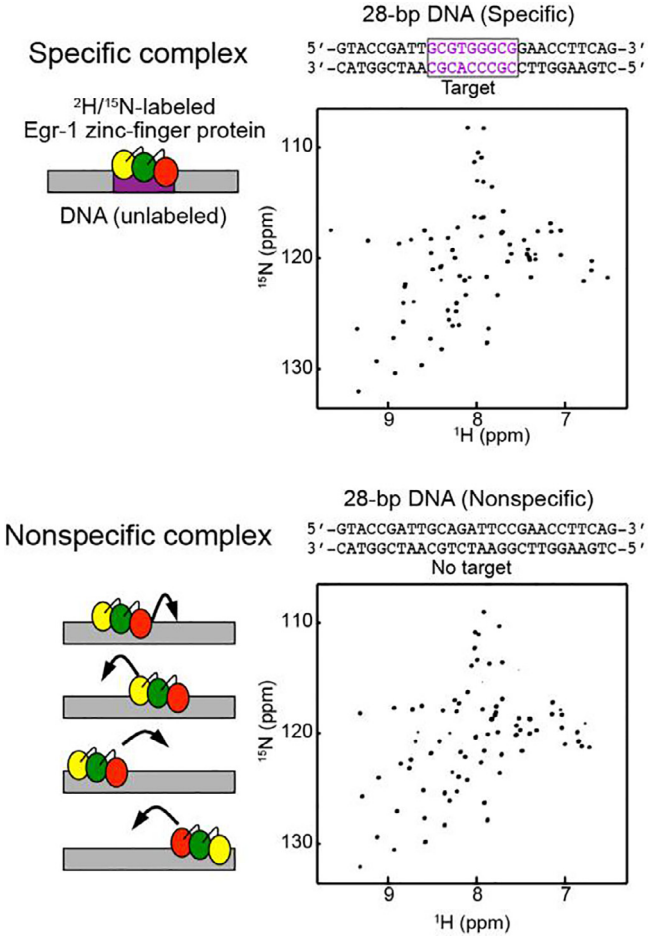

Fig. 3. ^{1}H–^{15}N heteronuclear correlation spectra recorded for the specific and nonspecific DNA complexes of the Egr-1 zinc-finger protein [13]. Although the protein can be located at various sites on nonspecific DNA with a mean residence time being ~1–10 μs at each site [23], the spectrum of the nonspecific complex shows only a single set of signals due to rapid translocation that occurs in the fast exchange regime.

1 recognition sequence, GCGTGGGCG, whereas the nonspecific DNA does not contain any high-affinity sequences. The backbone relaxation data for all three zinc fingers ZF1, ZF2, and ZF3 were similar for the specific DNA complex. However, the backbone ^{15}N relaxation data for ZF1 in the nonspecific complex significantly differed from those for ZF2 and ZF3 in the nonspecific complex. These and other data suggested that ZF1 is mainly dissociated from DNA when the Egr-1 zinc-finger protein scans DNA. When ZF1 is locally dissociated from DNA and ZF2 and ZF3 are bound to DNA, ZF1 undergoes almost independent domain motion, making ^{15}N longitudinal (R_1) and transverse (R_2) relaxation rates of ZF1 differ from those of ZF2 and ZF3. The linker connecting ZF1 to ZF2 shows smaller heteronuclear ^{15}N NOE values in the nonspecific complex than in the specific complex, suggesting that the linker is more flexible in the nonspecific complex. This is also consistent with the independent domain motion of ZF1 in the nonspecific complex.

4.2. Residual dipolar coupling (RDC)

Residual dipolar coupling (RDC) of nuclei in proteins weakly aligned to the magnetic field provide information about orientations of particular covalent bonds and are useful to study protein structure and dynamics [64–67]. Analysis of RDC data for proteins in the nonspecific and specific DNA complexes with DNA can allow us to assess structural dynamics of the DNA-scanning processes [13,48,50,51]. For example, RDC data for the HoxD9 homeodomain for the nonspecific and specific DNA complexes suggested that this protein interacts with nonspecific DNA in the same manner as in the specific complex [48]. In contrast, RDC data for the Egr-1 zinc-finger protein clearly showed that the three zinc fingers behave differently in the specific complex with target DNA and in the nonspecific DNA complex [13]. As mentioned above, ZF1 in the nonspecific DNA complex is locally dissociated from DNA while ZF2 and ZF3 are bound to DNA. Since ZF1 undergoes independent domain motion with respect to the other part of the complex, the overall magnitude of RDCs for ZF1 in the nonspecific complex was substantially smaller than those for ZF2 and ZF3 in the nonspecific DNA complex (Fig. 4B). Prediction of RDC for nonspecific DNA complexes is difficult because the observed samples contain many states with the protein being located at different sites. However, if structural models of individual states can be built, the overall profile of RDC for nonspecific complexes can be predicted using structure-based *de novo* RDC prediction methods [68,69]. Comparing the experimental RDC data with those predicted for the ensemble of various states in the nonspecific complex allows us to examine models on how proteins scan DNA [13,48].

4.3. Paramagnetic relaxation enhancement (PRE)

NMR paramagnetic relaxation enhancement (PRE) arising from dipole-dipole interactions between ^{1}H nuclei of a protein and unpaired electrons of an extrinsic paramagnetic group is a powerful tool for structural and dynamic studies of protein-DNA complexes [70–72]. There are several methods for site-specific, covalent attachment of a paramagnetic group to proteins or nucleic acids. For example, a paramagnetic Mn^{2+} ion can be site-specifically incorporated into the EDTA conjugated to DNA thymidine (dT-EDTA-Mn^{2+}) [73]. A paramagnetic group can also be incorporated into DNA through conjugation of a nitroxide spin label to a nucleotide base [74–76]. Intermolecular PRE rates can be readily measured using DNA containing a paramagnetic group and ^{15}N- and/or ^{13}C-labeled proteins [77,78]. In solution, proteins moving on DNA can be located near the paramagnetic group attached to a DNA base. Such a state causes strong PRE for the nuclei that are in close proximity from the Mn^{2+} ion [47,48,79]. Due to their r^{-6} dependence, PRE rates are sensitive to the presence of states in which the distances (r) between observed nuclei and the unpaired electrons of the paramagnetic group are relatively short. Even if such states are as minor as 1%, they can make a predominant contribution to observed PRE rates, allowing us to detect the low-population states [79]. When protein translocation on DNA occurs in the fast exchange regime, the PRE data can show which parts of the protein can become proximal to the paramagnetic group. This method thus provides structural information on proteins in the target DNA search processes.

4.4. Solvent PRE

Random collisions with paramagnetic co-solute molecules (e.g., gadolinium-diethylenetriamine pentaacetic acid bismethylamide [Gd-DTPA-BME] and 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl [TEMPOL]) at relatively high concentrations cause sizable PRE for ^{1}H nuclei of proteins [80–84]. This type of PRE, referred to as solvent PRE, is stronger for ^{1}H nuclei near the molecular surface accessible to solvent and can be used to identify molecular interfaces. This method is applicable to highly dynamic complexes such as nonspecific DNA-protein complexes. In the free state, the interfaces are more exposed to the solvent and therefore exhibit larger solvent PRE than in the complex with DNA even when the protein is changing its location on DNA. Although chemical shift perturbation (CSP) upon complex formation may provide similar information on the molecular interface, non-interfacial regions often exhibit significant CSP as well [85]. In principle, solvent PRE is more straightforward than CSP in identifying molecular interfaces. Relative magnitudes of solvent PRE are predictable from structure, assuming that spatial distribution of the paramagnetic co-solute

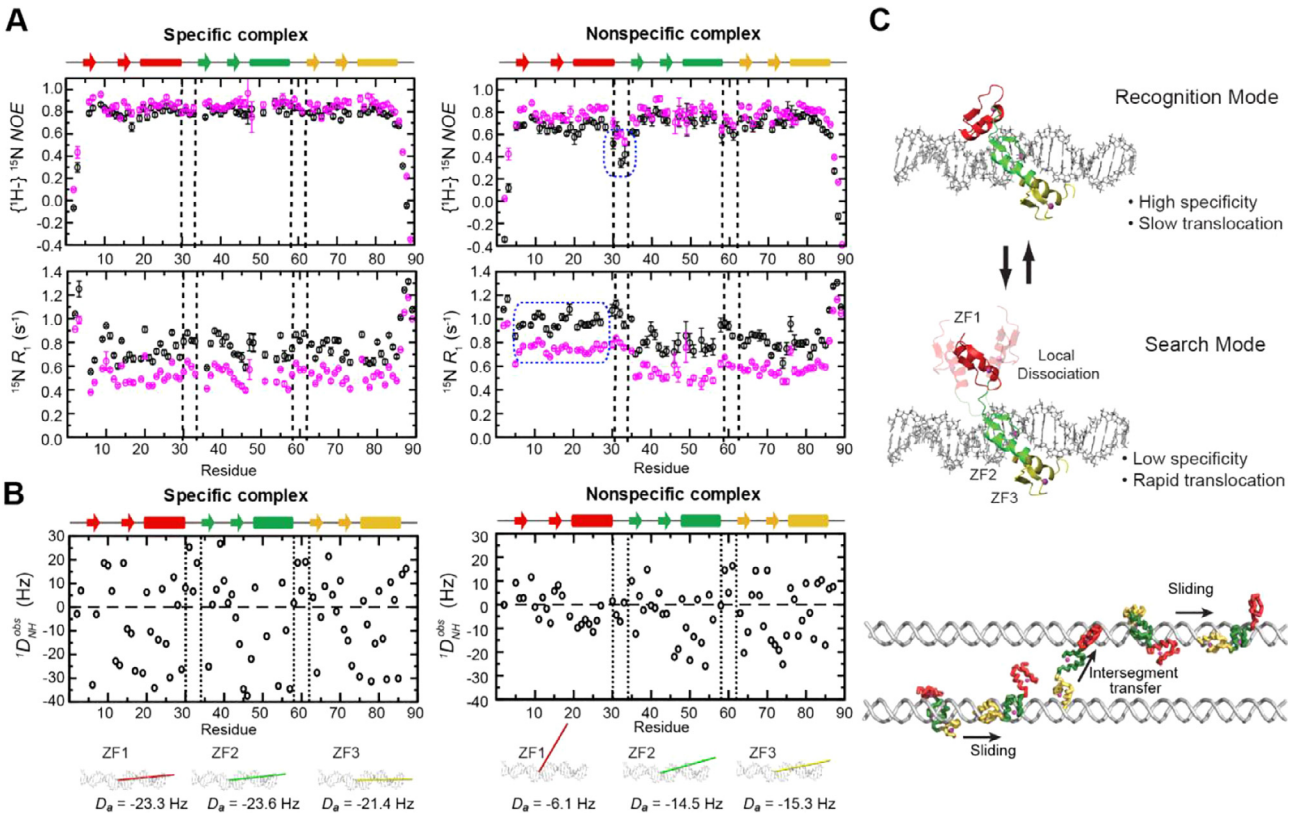

Fig. 4. DNA-scanning by the Egr-1 zinc-finger protein [13,44]. (A) Backbone heteronuclear ^{15}N NOE and ^{15}N R_1 relaxation data for nonspecific and specific DNA complexes of the Egr-1 zinc-finger protein. 28-bp DNA duplexes were used. Black and magenta data points are data obtained at the ^{1}H frequencies of 600 and 800 MHz, respectively. Blue dotted boxes show data points indicative of local dissociation of zinc finger 1 (ZF1). (B) RDC data for the nonspecific and specific DNA complexes of the Egr-1 zinc-finger protein. RDC $^{1}D_{NH}$ induced with 8 mg/ml Pf1 phage as a molecular alignment medium were analyzed. The main principal axis and the magnitude D_a of the alignment tensor for individual zinc fingers are also shown. (C) Dynamic equilibrium between the recognition and search modes. The search mode facilitates translocation of the protein on DNA. The coarse-grained molecular dynamics simulations also showed the dynamic transitions between these states [13,44]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecules is uniform [82,84]. Largely because this assumption is not necessarily valid for commonly used paramagnetic co-solute molecules, prediction of solvent PRE is only qualitatively accurate [81,84]. Nonetheless, by comparing solvent PRE data for the free protein and for the nonspecific complex, the molecular interfaces during the DNA scanning process can be mapped on the protein surface. This technique was used to analyze how the HoxD9 homeodomain and the uracil DNA glycosylate interact with nonspecific DNA [48,56].

5. NMR-based kinetic analysis of protein translocation on DNA

NMR spectroscopy can also be used to study kinetics of protein translocation on DNA. Among various spectroscopic techniques for analyzing molecular ensembles in solution, NMR is unique in that it allows investigations of fast kinetics *at equilibrium*. There are a number of NMR methods for kinetic investigations. Which one is the most appropriate depends on the timescale of the processes of interest [30]. Some are suitable for kinetic analysis of processes on a timescale of 10^{-2}–1 s (e.g., *z*-exchange spectroscopy). Others are suitable for faster processes on a timescale of 10^{-5}–10^{-3} s (e.g., line-shape analysis) or 10^{-4}–10^{-2} s (e.g., CPMG relaxation dispersion methods). Typically, sliding of protein from one nonspecific site to an adjacent site on DNA occurs on a μs timescale. Dissociation from a nonspecific site occurs on a ms timescale, whereas dissociation from a specific, high-affinity site may even require minutes to hours. Furthermore, association and intersegment transfer are second-order processes and therefore their rates depend on the concentration of free DNA.

5.1. Mixture approach

In the NMR sample, protein translocation on DNA occurs within the same DNA molecule (i.e., *intra*molecular translocation) or between sites on different DNA molecules (i.e., *inter*molecular translocation). Distinguishing these two is not trivial. A general NMR approach called the “mixture approach” has been developed to facilitate kinetic analysis of intermolecular protein translocation on DNA [13,48,86–90]. This approach makes use of two different DNA duplexes *a* and *b* of comparable binding affinity and a ^{15}N-labeled protein, from which two individual complexes of protein-DNA *a* and protein-DNA *b* and a mixture of these two complexes are prepared (Fig. 5). DNA concentrations in these samples should be high enough for the protein to completely bind to either of these DNA. When the ^{1}H–^{15}N heteronuclear spectra are recorded for these three samples, the two samples of the individual complexes with DNA *a* and DNA *b* should show different ^{1}H/^{15}N chemical shifts at least for some interfacial protein residues. For such residues, the spectrum of the mixture should exhibit one of the following features: 1) a single signal on the line between the two corresponding signals of the individual complexes, 2) two separate signals corresponding to the two complexes, or 3) very broad signal somewhere between the two signals. In the terminology of NMR spectroscopy, these three conditions are called fast, slow, and intermediate exchange regimes, respectively. If the intermolecular translocation processes occur in the fast exchange regime (this is usually the case for nonspecific DNA duplexes), NMR line-shape analysis or relaxation dispersion methods can be used to analyze the translocation kinetics (Section 5.2). If translocation of the protein occurs in the slow exchange regime (this is

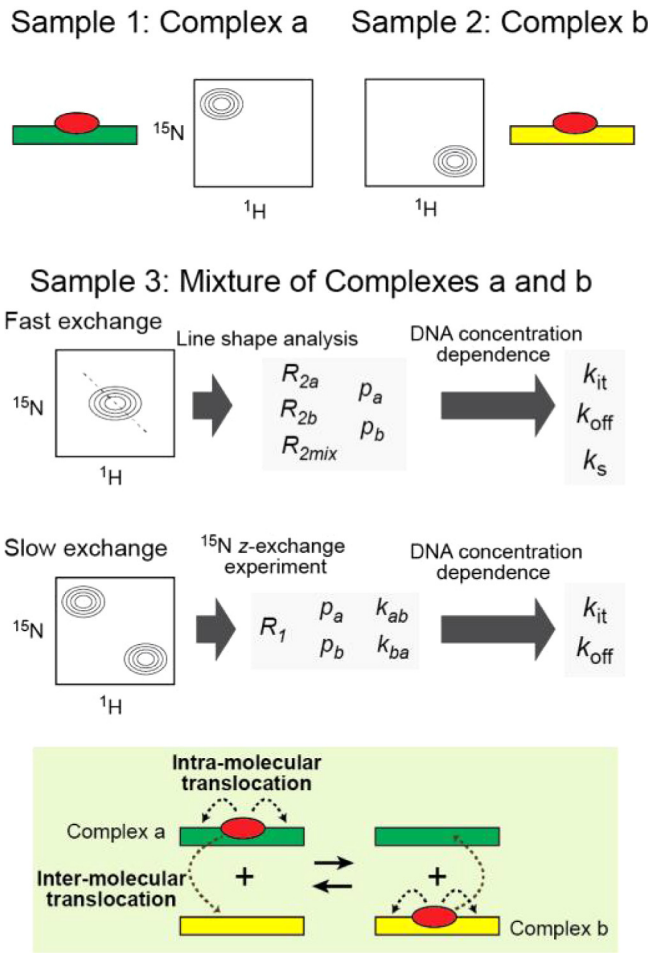

Fig. 5. The mixture approach for NMR-based investigations of kinetics of protein translocation on DNA.

typically the case when DNA duplexes containing a high-affinity site are used), the ^{15}N z-exchange method or the real-time approach can be used to analyze kinetics of intermolecular translocations between two DNA duplexes (Section 5.3).

5.2. Kinetics of protein translocation on nonspecific DNA

Protein translocation on nonspecific DNA occurs through sliding, dissociation & re-association, and intersegment transfer, typically in the fast exchange regime. A discrete-state kinetics model shown in Fig. 6A can represent these translocation processes. This model assumes that a nonspecific DNA duplex contains N distinct sites for binding. For example, for 24-bp DNA and a protein that covers 9 bp at each site, N is calculated to be 16 ($=24-9+1$). Due to the structural pseudo-C_2 symmetry of each DNA site, two opposite orientations are possible for a protein to bind to each site through short-range electrostatic interactions with the DNA backbone. Each nonspecific site is assumed to exhibit the same kinetic properties with the same rate constants for sliding (k_s), dissociation (k_{off}), association (k_{on}), and intersegment transfer (k_{it}). These kinetic rate constants are microscopic rate constants defined for each site (not for the entire DNA molecule). The macroscopic association rate constant for the DNA duplex is given by $2Nk_{on}$, and thus $k_{off}/(2Nk_{on})$ corresponds to the macroscopic apparent dissociation constant ($K_{d,app}$). The sliding rate constant k_s is directly related to the macroscopic one-dimensional diffusion coefficient for sliding (D_1) by $D_1 = l^2k_s$, where l represents the distance (3.4 Å) between adjacent sites along the DNA axis [22]. D_1 given in units of bp^2 s^{-1} is equivalent to k_s. Because NMR experiments typically use DNA duplexes shorter than the persistence length (i.e., ~150 bp), intersegment transfers within the same DNA duplex are neglected, and only those between two DNA duplexes are considered. The k_{it} constant is a second-order rate constant for this type of intermolecular intersegment transfer [22].

In the mixture approach for kinetic analysis of these translocation processes on nonspecific DNA [48,89], NMR line shapes of protein backbone NH groups are analyzed for three samples: two containing individual nonspecific complexes with DNA duplexes a and b and one containing a mixture of the two nonspecific complexes. Some examples of actual experimental data are shown in Fig. 6B. This NMR approach provides accurate kinetic information on the intermolecular translocations of proteins between two DNA molecules, although this approach adopts simple two-state approximation. The validity of this approximation has been confirmed in a recent study using more rigorous McConnell equations that account for $4N + 1$ microscopic states for a system containing a protein and two nonspecific DNA duplexes [89]. The rate constants k_{off} and k_{it} can be determined through analysis of apparent transverse relaxation rates from resonance line-shapes as a function of DNA concentration. Interestingly, the same analysis also provides semi-quantitative information on the rate constant k_s and the one-dimensional diffusion coefficient D_1 for protein sliding on DNA [89]. By the mixture approach, protein translocation on nonspecific DNA was analyzed for Egr-1, HoxD9, and Sox2 proteins [13,48,51,89].

5.3. Kinetics of protein translocation between high-affinity sites on DNA

Since the residence time of protein at a high-affinity site is far longer than that at nonspecific sites, protein translocation between two high-affinity sites typically occurs in the slow exchange regime. If protein translocation between two DNA duplexes a and b occurs with an exchange rate constant k_{ex} ($= k_{ab} + k_{ba}$) being roughly ~0.5–50 s^{-1}, the ^{15}N z-exchange method [88,91,92] can be used to determine the rate constants for translocation from DNA a to b (k_{ab}) and that for translocation from DNA b to a (k_{ba}). This method has been applied to intermolecular translocation of proteins between high-affinity sites on two different DNA duplexes [79,86–88,90,93,94] and also to intramolecular translocation between two high-affinity sites on the same DNA duplex [95]. In a ^{15}N z-exchange experiment, in addition to the signals from the two complexes, signals are observed at the mixed positions with the ^{1}H resonance of the complex a and the ^{15}N resonance of the complex b (and vice versa; see Fig. 7A), which are called 'exchange cross peaks.' These additional cross peaks arise due to protein translocation between the two DNA duplexes a and b during the mixing time period in which ^{15}N nuclear magnetizations of interest remain along z, the direction of the outer magnetic field. Because the mixing time is present between the ^{15}N and ^{1}H evolution periods in the ^{15}N z-exchange experiment, some proteins interact with DNA a during the ^{15}N evolution period and interact with DNA b during the ^{1}H evolution period, causing the exchange cross peak with the ^{15}N resonance of the complex a and ^{1}H resonance of the complex b. By analyzing signal intensities of the exchange cross peaks and the auto cross peaks as a function of the mixing time, the rate constants k_{ab} and k_{ba} for protein translocation processes between the two high-affinity sites can be determined. This translocation can occur through the dissociation & re-association mechanism or through the intersegment transfer mechanisms. By measuring the k_{ab} and k_{ba} rate constants at some different concentrations of free DNA duplexes, the kinetic contributions of these two mechanisms can be determined individually [87].

If the residence time of a protein on a high-affinity site on DNA is on the order of minutes or longer, the kinetics of translocation can be analyzed using a real-time NMR approach [96]. In this approach, a sample of protein-DNA complex is initially prepared. Then, another DNA of comparable affinity is added to the solution and the series of 2D NMR spectra are recorded to monitor translocation of the protein from the original DNA to the added DNA. By analyzing intensities of signals from the two complexes as a function of time, one can determine the

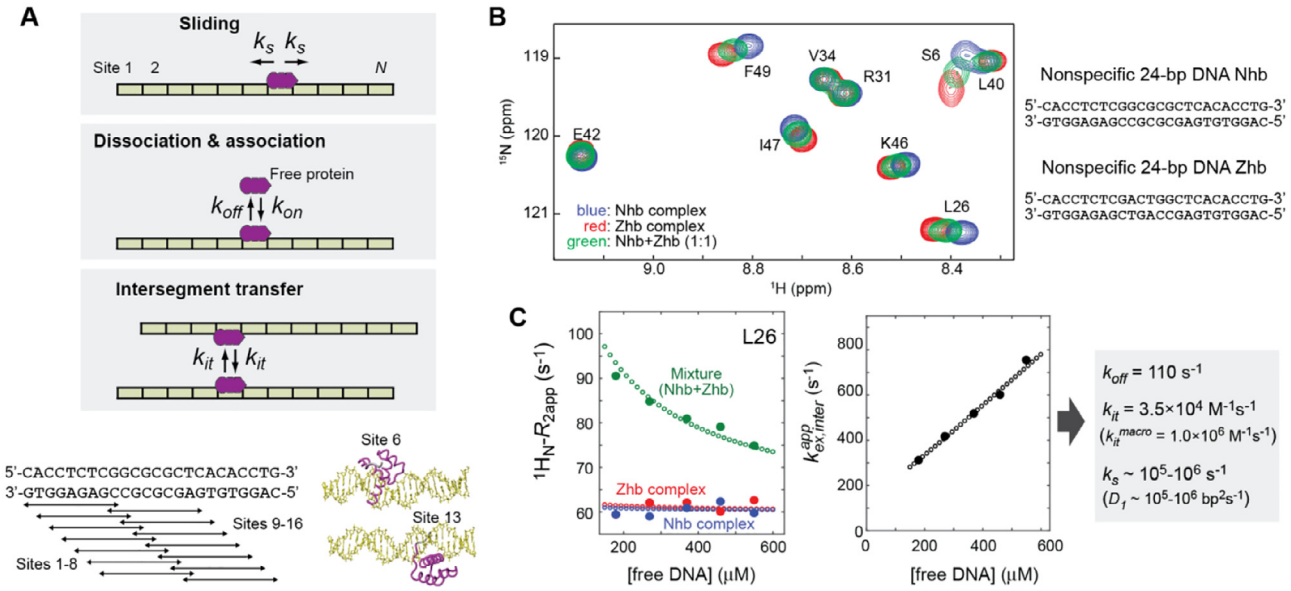

Fig. 6. NMR-based analysis of kinetics of protein translocation on nonspecific DNA. (A) A discrete-state kinetic model for protein translocation on nonspecific DNA [89]. Note that the rate constants k_{on} and k_{it} are defined as microscopic rate constants for each site. The corresponding macroscopic rate constants are $2Nk_{on}$ and $2Nk_{it}$. (B, C) Mixture approach data on HoxD9 translocation on nonspecific DNA. Panel B shows overlaid spectra recorded for the individual complexes and their mixture. Panel C shows determination of the rate constants k_{off}, k_{it}, and k_s from the apparent transverse relaxation rates of the three samples as a function of the concentration of free DNA [89].

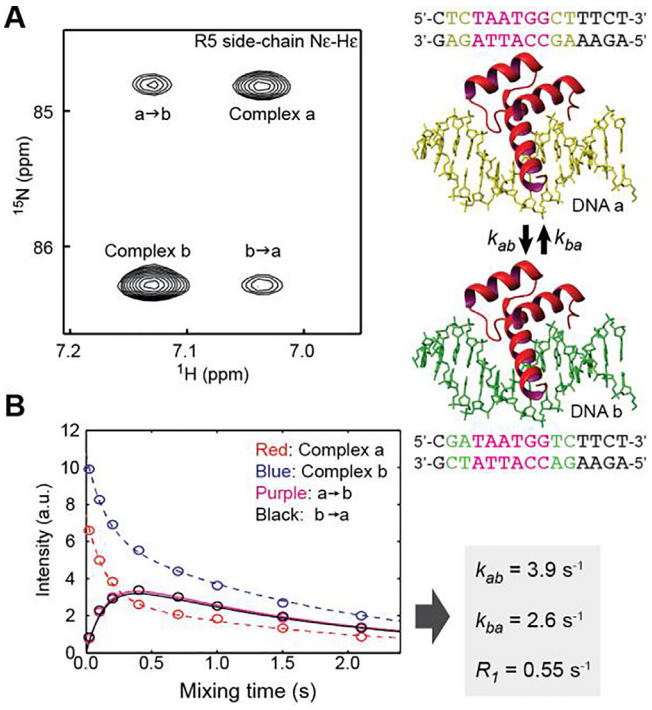

Fig. 7. ^{15}N z-exchange data on kinetics of translocation of the Antp homeodomain between the two high-affinity sites on the DNA duplexes *a* and *b* [94]. (A) A ^{1}H–^{15}N spectrum recorded in ^{15}N z-exchange experiment. (B) Intensities of the auto and exchange cross peaks as a function of the z-exchange mixing time. The rate constants determined from this dataset are also shown.

kinetic rate constant for translocation between the two DNA duplexes. Fast acquisition methods (e.g. SOFAST-HMQC) [97–99], which allow recording of each 2D ^{1}H–^{15}N heteronuclear correlation spectrum within a minute, are useful to shorten the time interval for the real-time kinetics measurements. Translocation of the Egr-1 zinc-finger protein between two target sites was analyzed using NMR in this manner [96].

It should be noted that slow translocation processes over minutes-hours could readily be measured by biochemical assays requiring far less amounts of proteins and DNA. Nonetheless, when the translocation process turned out to be too slow to analyze with the ^{15}N z-exchange method, NMR-based real-time kinetics approach could be convenient because the same set of materials can be readily tested.

5.4. Self-decoupling of intermolecular hydrogen-bond scalar couplings

The above-mentioned NMR methods for kinetic analysis of protein translocation on DNA require use of two DNA duplexes. Recently, a unique NMR-based kinetic approach that does not require two DNA duplexes has been proposed [94]. This NMR method utilizes intermolecular hydrogen-bond scalar couplings ($^{h3}J_{NP}$) between protein ^{15}N and DNA ^{31}P nuclei. Ionic-strength dependence of $^{h3}J_{NP}$ could provide information on the residence time of protein at a high-affinity site on DNA [94]. This method does not require a mixture of two DNA duplexes. The observation of intermolecular hydrogen-bond scalar couplings is possible only if the residence time is sufficiently long ($> \sim 10^{-2}$ s); otherwise, this coupling disappears through the process called self-decoupling (Fig. 8A). Qualitatively, when an intermolecular hydrogen-bond scalar coupling is observed with a magnitude comparable to that of the intrinsic coupling constant ^{h}J, the residence time of the complex should be longer than $(2\pi|^{h}J|)^{-1}$. The intrinsic values of hydrogen-bond scalar couplings can be calculated from structural information by quantum chemical calculations or from the empirical relationship between the coupling constants and the hydrogen-bond geometry [100–102]. Detailed analysis of the self-decoupling of intermolecular hydrogen-bond scalar couplings as a function of ionic strength can provide more quantitative information about the residence time of the complex. For the Antp homeodomain, the exchange rates measured with the self-decoupling-based method were in good agreement with those measured with the ^{15}N z-exchange methods (Fig. 8B) [94]. This self-decoupling-based method is unique in that it does not require different signatures for the states involved in the exchange, although such conditions are typically crucial for other methods.

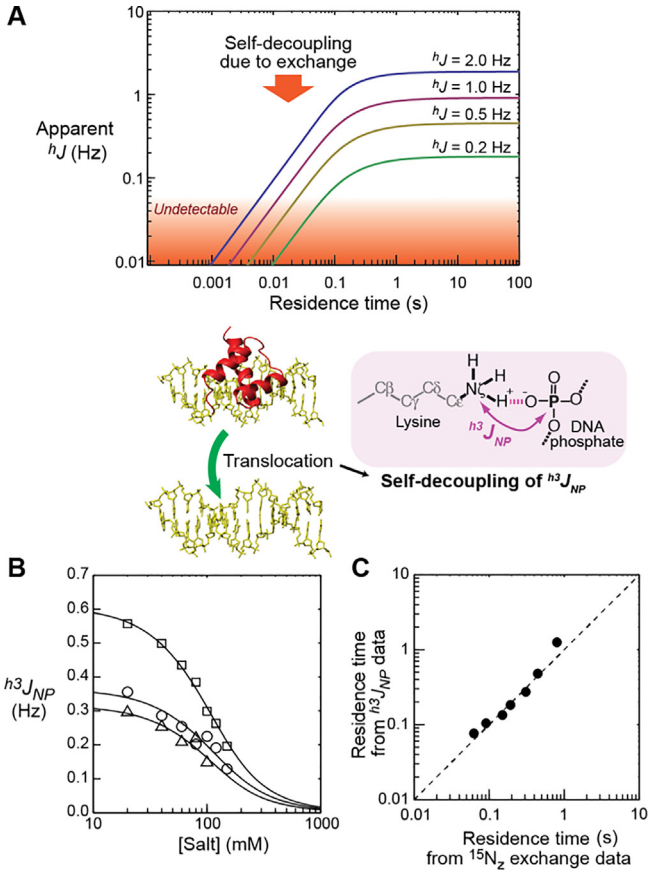

Fig. 8. Kinetic investigation of protein translocation on DNA through analysis of self-decoupling of intermolecular hydrogen-bond scalar coupling between protein ^{15}N and DNA ^{31}P nuclei [94]. (A) Theoretical relationship between the apparent value of intermolecular hydrogen-bond scalar coupling and the residence time of a molecular complex. Four cases simulated with different values of the intrinsic coupling constants are shown. (B) $^{h3}J_{NP}$ coupling constants measured for the hydrogen bonds between Lys side-chain ^{15}N and DNA phosphate ^{31}P nuclei in the Antp homeodomain-DNA complex at some different salt concentrations. The $^{h3}J_{NP}$ coupling constants were measured as described by Anderson et al. [31] (C) Comparison of the residence times of the Antp homeodomain at the recognition sequence measured with the $^{h3}J_{NP}$ method and those measured with ^{15}N z-exchange method.

6. NMR instruments

The above-mentioned NMR methods require a high-field NMR spectrometer (a magnetic field higher than 11 Tesla; ^{1}H frequency ≥500 MH) equipped with a multi-channel probe capable of heteronuclear multidimensional experiments. Some quantitative NMR methods require data collection at multiple magnetic fields (e.g., ^{1}H frequencies at 600 and 800 MHz). We use Bruker Avance III NMR spectrometers operated at ^{1}H frequencies of 800, 750, and 600 MHz. Cryogenic ^{1}H, ^{13}C, ^{15}N triple-resonance TCI (800 and 750 MHz) or ^{1}H, ^{13}C, ^{15}N, ^{31}P quadruple-resonance QCI (600 MHz) probes are typically used for these spectrometers. Cryogenic probes achieve ~3-fold higher signal-to-noise ratio in ^{1}H or ^{13}C NMR detection by cooling the detector to a cryogenic temperature for reduction in thermal noise, while retaining a physiological temperature (typically, 2–40 °C) for measured samples. This high sensitivity is helpful for quantitative NMR methods for investigating target DNA search processes of proteins. The ^{1}H, ^{13}C, ^{15}N, ^{31}P QCI cryogenic probe is particularly useful for studying protein-DNA interactions because it allows for precise measurements of intermolecular hydrogen-bond scalar couplings between DNA ^{31}P and protein ^{15}N nuclei [31,32,42,44,94,103,104]. Although this cryogenic probe has an additional coil for ^{31}P nuclei, its sensitivity is almost as good as that of a corresponding TCI cryogenic probe (lower only by 11%, which can readily be compensated by a slightly larger number of scans).

7. Combining with other methods

Interpretation of NMR data on the target DNA search process can be greatly facilitated by integrating with other methods. In our lab, we routinely combine NMR and stopped-flow fluorescence data on the target DNA search processes. Our stopped-flow fluorescence kinetic methods allow us to obtain kinetic information on protein translocation on DNA via sliding, dissociation & re-association, and intersegment transfer mechanisms [22,23]. The kinetic results from the NMR and fluorescence studies were consistent for the Egr-1 zinc-finger protein [13,23]. The fluorescence data suggested that the Egr-1 zinc-finger protein spends ~1–10 μs at each nonspecific site and then slides to an adjacent site. This was also consistent with NMR observation that protein translocation on nonspecific DNA occurs in the fast exchange regime. The NMR and fluorescence methods can be applicable under the same buffer conditions and data obtained with these methods are complementary. Computational studies are also complementary. The coarse-grained molecular dynamics simulations showed that the search mode of Egr-1 facilitates intersegment transfer between nonspecific DNA duplexes (see Fig. 4C) [13]. Applying these experimental and computational methods to the Egr-1 zinc-finger protein, we were able to validate the theoretical model involving the dynamic conformational equilibrium between the recognition and search modes during the target DNA search process [13,44]. This model was originally proposed by some theoretical researchers to explain how transcription factors can simultaneously achieve two opposing factors: highly specific binding and sufficiently rapid search [105–107]. Through mutagenesis, we modulated the dynamic conformational equilibrium between the search and recognition modes and directly assessed the conformational shifts using NMR spectroscopy [44]. Using fluorescence and biochemical assays, we analyzed how the shifts of the conformational equilibrium influence binding affinity, target search kinetics, and efficiency in displacing other proteins from the target sites. A shift toward the recognition mode caused an increase in affinity for DNA and a decrease in search efficiency. In contrast, a shift toward the search mode caused a decrease in affinity and an increase in search efficiency. This demonstrated that target search by these proteins can be accelerated via engineering based on structural dynamic knowledge of the DNA-scanning process. Thus, NMR spectroscopy can help us deepen our knowledge of target DNA search by proteins and apply the knowledge to engineer proteins that can find targets more efficiently for artificial gene regulation or genome editing.

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