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COMMUNICATION

A chemical approach for site-specific identification of NMR signals from protein side-chain NH_3^+ groups forming intermolecular ion pairs in protein–nucleic acid complexes

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Abstract Protein–nucleic acid interactions involve intermolecular ion pairs of protein side-chain and DNA or RNA phosphate groups. Using three protein–DNA complexes, we demonstrate that site-specific oxygen-to-sulfur substitution in phosphate groups allows for identification of NMR signals from the protein side-chain NH_3^+ groups forming the intermolecular ion pairs. A characteristic change in their ^1H and ^{15}N resonances upon this modification (i.e., substitution of phosphate to phosphorodithioate) can represent a signature of an intermolecular ion pair. Hydrogen-bond scalar coupling between protein side-chain ^{15}N and DNA phosphorodithiaote ^{31}P nuclei provides direct confirmation of the intermolecular ion pair. The same approach is likely applicable to protein–RNA complexes as well.

Keywords Protein–nucleic acid interactions · Protein side chains · NH_3^+ groups · Ion pairs · Hydrogen bonds

Ion pairing is a major stabilizer for molecular association. In protein–nucleic acid interactions, the predominant ion pairs are formed between DNA or RNA phosphate groups and protein side-chain arginine (Arg) $N\eta H_2$ groups or lysine (Lys) $N\zeta H_3^+$ groups. Although NMR spectroscopy is extremely powerful for atomic-level characterization of proteins and nucleic acids, its application to these charged moieties remains relatively rare. Typically the Arg $N\eta H_2$ groups are too difficult to analyze because of severe broadening of their NMR signals due to hindered rotations within the planar guanidino cations (Yamazaki et al. 1995). Considerable progress has been made in NMR studies of Lys side-chain NH_3^+ groups forming ion pairs with DNA (Anderson et al. 2013; Iwahara et al. 2007; Zandarashvili and Iwahara 2014), but such investigations remain non-trivial. Even after completely assigning protein backbone resonances, identifying the ^1H and ^{15}N resonances of Lys side-chain NH_3^+ groups, which are located at the fifth position from Cα atom, can be problematic. This is particularly true when low experimental temperatures are used to observe ^1H signals from NH_3^+ groups undergoing rapid hydrogen exchange (Esadze et al. 2014). For NH_3^+ assignment, additional Lys-specific triple-resonance spectra such as H3NCECD (Iwahara et al. 2007), H3NCG (Esadze et al. 2014), H2CN (Andre et al. 2007), and H2(CN)CCH-TOCSY (Esadze et al. 2014) are required in addition to general side-chain double- or triple-resonance spectra. Thus, the current methodology for Lys side-chain NH_3^+ groups requires a substantial amount of time for recording and analyzing various NMR spectra.

In this paper we present a simpler, chemical approach for identifying NMR signals from Lys side-chain NH_3^+ groups making specific intermolecular ion pairs with the phosphate backbone in protein–DNA complexes. A remarkable advantage of this approach is that it allows for site-specific identification of intermolecular ion pairs without using ^1H, ^{13}C, and ^{15}N triple-resonance experiments. This approach utilizes chemically modified DNA in which both non-bridging oxygen atoms in a phosphate group are replaced with sulfur atoms (Fig. 1a). This

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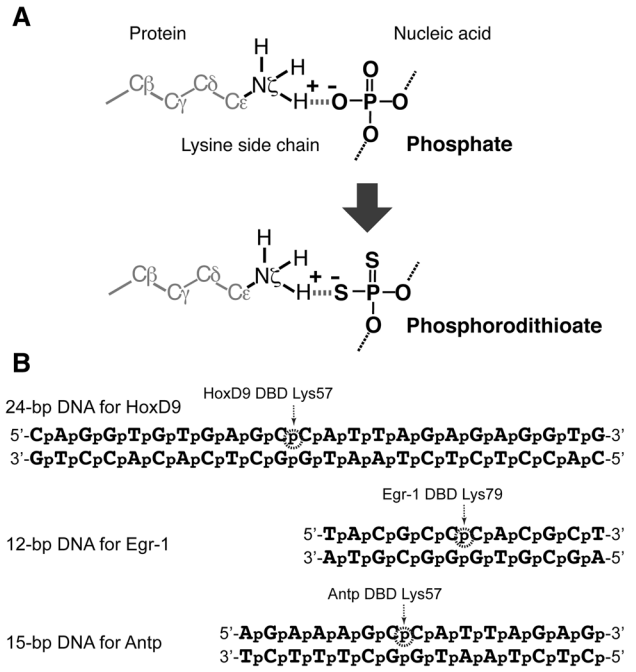

Fig. 1 Oxygen-to-sulfur substitution as a tool to identify intermolecular ion pairs at protein–DNA interfaces. **a** Intermolecular ion pairs of lysine side-chain NH_3^+ and DNA phosphate/phosphrodithioate groups. **b** DNA duplexes used in this study. *Circles* indicate the modification sites, which form intermolecular ion pairs with protein NH_3^+ groups. For each duplex, two samples with or without the oxygen-to-sulfur substitution were prepared

phosphorodithioate (–O–PS_2^-–O–) is a relatively minor chemical modification of DNA because it has similar charge characteristics and tetrahedral geometry compared to the normal phosphate (–O–PO_2^-–O–). Phosphorodithioates can be easily incorporated into DNA during chemical synthesis using phosphoramidite chemistry on standard oligonucleotide synthesizers (Farschtschi and Gorenstein 1988; Nielsen et al. 1988; Wiesler et al. 1993), and the thiophosphoramidites required are readily available commercially (AM Biotech or Glen Research). Dithioated DNA is chemically stable, and interestingly, exhibits even higher affinities for some DNA-binding proteins compared to unmodified DNA (Marshall et al. 1992; Marshall and Caruthers 1993; Yang et al. 1999, 2002). In our recent work on the HoxD9 DNA-binding domain (DBD)–DNA complex (Anderson et al. 2013), we found that ^{1}H and ^{15}N resonances of a Lys NH_3^+ group forming an intermolecular ion pair were significantly perturbed upon dithioation of the phosphate group. However, in this system only 3 Lys NH_3^+ groups were observed, so it was not clear whether oxygen-to-sulfur substitution could be used as a general tool to site-specifically identify intermolecular ion pairs at protein–DNA interfaces.

In the current work, we examine the possibility of a generalized dithioation effect on ^{1}H and ^{15}N resonances by investigating three different protein–DNA complexes: 22-kDa complex of HoxD9 DBD (62 residues) and 24-bp DNA; 19-kDa complex of the Egr-1 DBD (90 residues) and 12-bp DNA; and 17-kDa complex of Antp DBD (61 residues) and 15-bp DNA. HoxD9 and Antp are homeodomain proteins, whereas Egr-1 (also known as Zif268) is a zinc-finger protein. We prepared HoxD9, Egr-1, and Antp DBDs as described previously (Fraenkel and Pabo 1998; Iwahara et al. 2006; Zandarashvili et al. 2012). Using various triple-resonance spectra, we initially assigned ^{1}H–^{15}N resonances of Lys NH_3^+ groups of these complexes through previously established procedures (Esadze et al. 2014; Zandarashvili et al. 2013). For these complexes, we incorporated a phosphorodithioate group at a position of a phosphate group that forms a putative intermolecular ion pair with a Lys side-chain NH_3^+ group. Based on available structural information, we chose the modification of the DNA phosphate groups that interact with HoxD9 Lys57, Egr-1 Lys79, and Antp Lys57 (Fig. 1b).

We recorded Lys side-chain NH_3^+-selective ^{1}H–^{15}N heteronuclear in-phase single-quantum coherence (HISQC) (Iwahara et al. 2007) spectra for the protein–DNA complexes with or without the oxygen-to-sulfur substitution (Fig. 2a). For each sample, a solution of 0.8 mM complex at pH 5.8 was sealed into a co-axial tube, in which D_2O for NMR lock was separately sealed to avoid NDH_2^+ and ND_2H^+ species of NH_3^+ groups (Iwahara et al. 2007). The HISQC spectra of the protein–DNA complexes of HoxD9, Egr-1, and Antp DBDs were recorded at 35, 10, and 25 °C, respectively. Upon the oxygen-to-sulfur substitution, the NH_3^+ groups interacting with the phosphate groups at the modification sites exhibited considerable changes in ^{1}H and ^{15}N chemical shifts, while the other Lys NH_3^+ groups showed only minor changes. An obvious perturbation pattern for dithioates on the NH_3^+ group became evident, as all the three different protein–DNA complexes showed shifts of the ^{1}H–^{15}N cross-peak in the same direction: ^{15}N chemical shift increased by $\sim$1 ppm and ^{1}H chemical shift decreased by $\sim$0.2 ppm for the NH_3^+ groups (Fig. 2b). Their changes in ^{15}N chemical shifts were particularly characteristic, and we suggest that this large ^{15}N chemical shift perturbation upon dithioation be exploited as a chemical approach for identifying NMR signals from NH_3^+ groups that form intermolecular ion pairs with DNA phosphates.

To confirm the direct interactions between the DNA phosphate/phosphorodithioate and protein NH_3^+ groups, one can use three-bond scalar coupling $^{h3}J_{NP}$ between protein ^{15}N and DNA ^{31}P nuclei across an ionic hydrogen bond (Anderson et al. 2013). Owing to intrinsically slow ^{15}N relaxation of in-phase single-quantum transverse coherence of the AX_3 spin system (Esadze et al. 2011), measurements of scalar coupling constants significantly smaller than 1 Hz

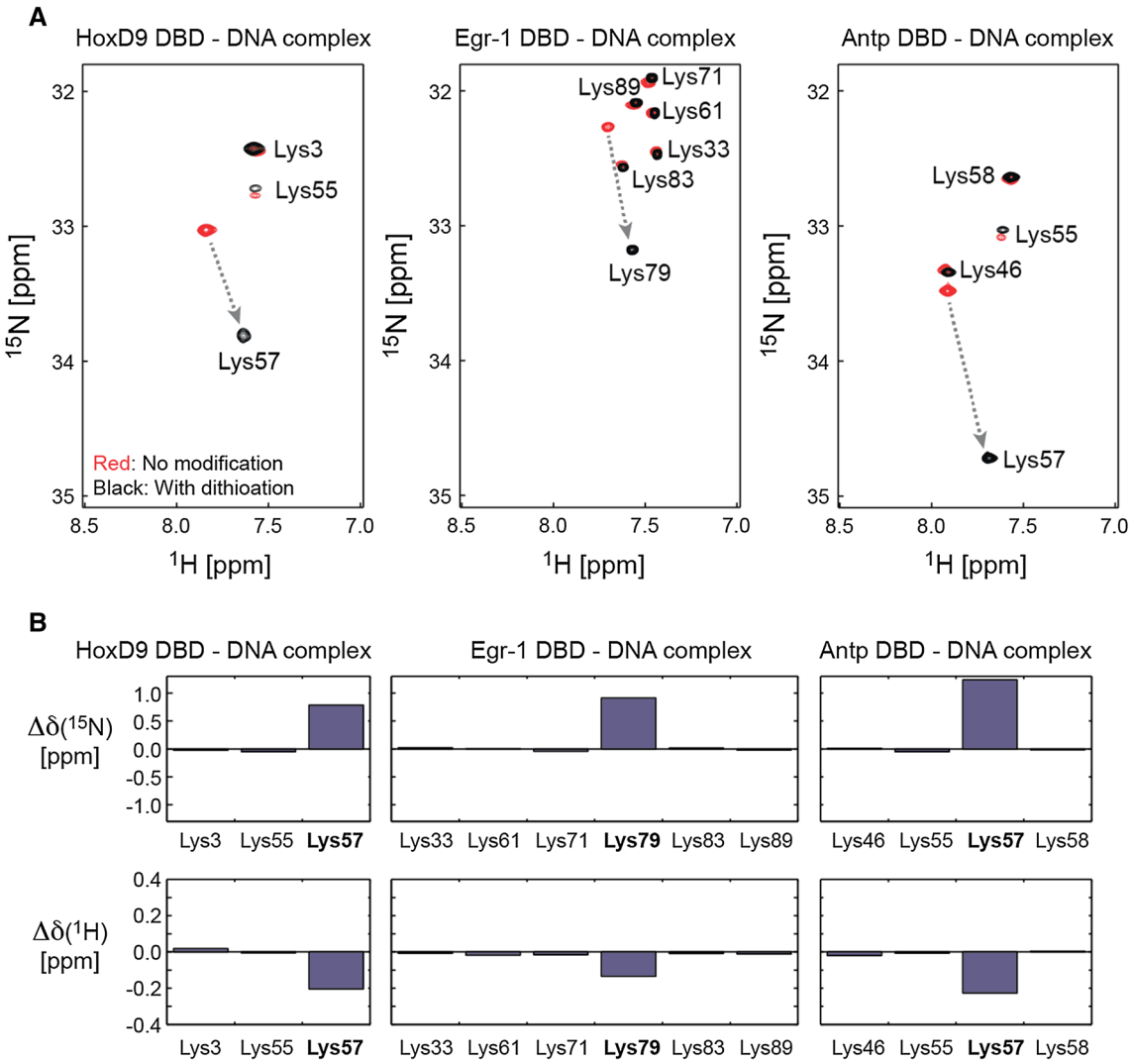

Fig. 2 Chemical shift perturbation of NH_3^+ groups upon the oxygen-to-sulfur substitution in DNA phosphate groups (see Fig. 1b). **a** Overlays of Lys NH_3^+-selective ^1H–^{15}N HISQC spectra recorded for the protein–DNA complexes at pH 5.8 with (*black*) and without (*red*) the oxygen-to-sulfur substitution. The complexes of ^{15}N-labeled proteins (HoxD9 and Egr-1 were also perdeuterated) and isotopically unlabeled DNA were used for these spectra. **b** Changes in NH_3^+ ^1H and ^{15}N chemical shifts upon the site-specific oxygen-to-sulfur substitution in the DNA phosphate groups interacting with Lys57 of HoxD9, Lys79 of Egr-1, or Lys57 of Antp DBD. Plotted values are $\Delta\delta = \delta$ (with dithioation) $- \delta$ (no modification)

are feasible for ^{15}N nuclei of Lys NH_3^+ groups (Anderson et al. 2013; Zandarashvili et al. 2011). For the Antp DBD–DNA complex containing the phosphorodithioate group, we conducted the spin-echo $^{h3}J_{NP}$-modulation constant-time HISQC experiment (Anderson et al. 2013) at 15 °C using a Bruker Avance III 600-MHz spectrometer equipped with a QCI cryogenic probe for ^1H, ^{13}C, ^{15}N, and ^{31}P nuclei. In this experiment, two sub-spectra for Lys NH_3^+ groups were recorded in an interleaved manner: one with $^{h3}J_{NP}$ modulation active throughout the constant-time evolution period (Fig. 3a), and the other with $^{h3}J_{NP}$ modulation cancelled (Fig. 3b). These spectra were recorded using ^{31}P pulses that selectively invert ^{31}P nuclei of DNA phosphorodithioate without affecting ^{31}P nuclei of DNA phosphate. Such selective inversion is easy because typical ^{31}P chemical shifts (relative to trimethylphosphate) are very different for DNA phosphate (~–4 ppm) and phosphorodithioate (~110 ppm) (Gorenstein 1992, 1994). The difference spectrum of the two sub-spectra should show a signal only from the NH_3^+ group that exhibits sizable $^{h3}J_{NP}$ modulation due to the ionic hydrogen bond with DNA phosphorodithioate. In fact, the signal from Lys57 was clearly observed in the difference spectrum (Fig. 3c). Using the peak intensities in the individual sub-spectra, the $^{h3}J_{NP}$ coupling constant was determined to be 0.23 Hz for the ionic hydrogen bond between Lys57 NH_3^+ and DNA

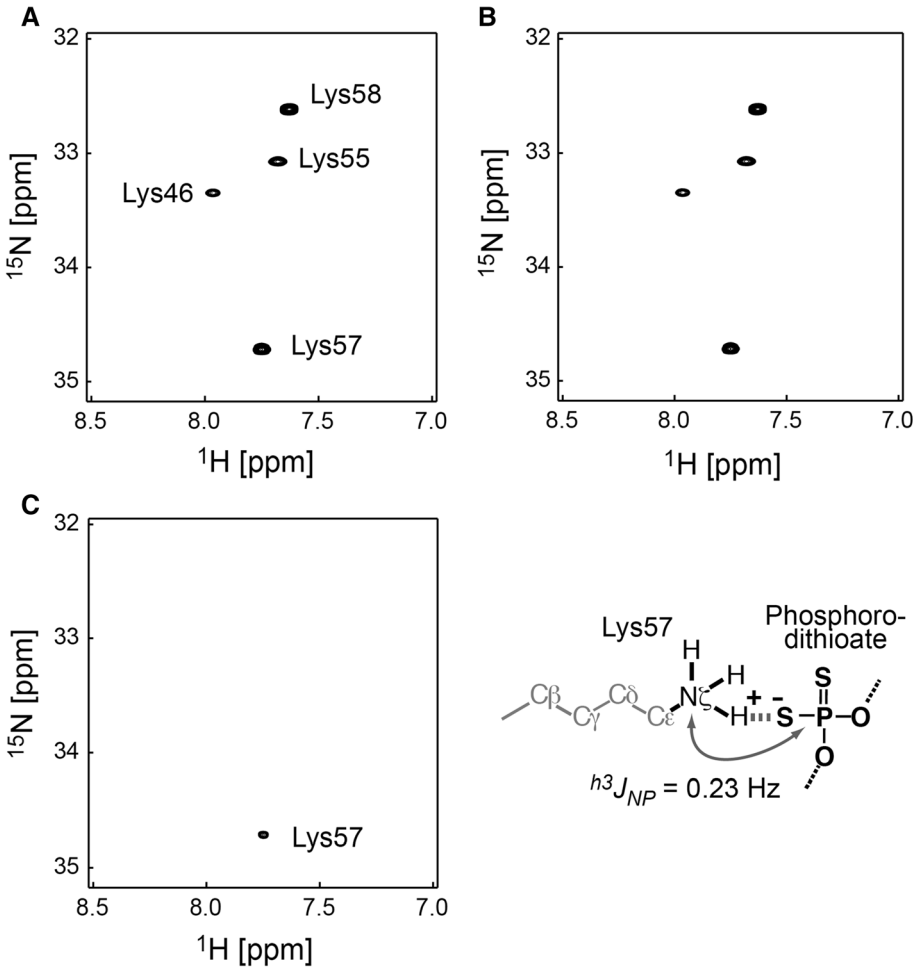

Fig. 3 Spectra of the spin-echo $^{h3}J_{NP}$ modulation constant-time HISQC experiment (Anderson et al. 2013) for the Antp DBD–DNA complex containing a phosphorodithioate at the Lys57 interaction site. ^{31}P pulses that selectively invert ^{31}P nuclei of DNA phosphodithioate (~110 ppm) were used. **a** Sub-spectrum recorded with $^{h3}J_{NP}$ modulation throughout the constant-time evolution period. **b** Sub-spectrum recorded with $^{h3}J_{NP}$ modulation cancelled. **c** Difference between the two sub-spectra. The signal in this difference spectrum represents the direct evidence for the ionic hydrogen bond between Lys57 NH_3^+ and DNA phosphorodithioate groups

phosphorodithioate groups of the Antp DBD–DNA complex.

Although this work utilized DNA in which a phosphate (–O–PO_2^-–O–) group is substituted to a phosphorodithioate (–O–PS_2^-–O–) group, one could conceivably use a phosphoromonothioate derivative (–O–PSO^-–O–) for the same purpose. In terms of chemically modified DNA synthesis the monothioates are cheaper to produce since they do not require a specialized phosphoramidite, but we opted initially to pursue dithioates because it is an isopolar chemical substitution that is achiral at the phosphorus. A monothioate synthesis produces a racemic mixture of two enantiomers for each phosphoromonothioate (–O–PSO^-–O–), which likely would complicate NMR analysis unless these species are separated. Although some success with separating stereo-chemically different monothioate derivatives of DNA (Murakami et al. 1994; Stec et al. 1985) or RNA (Frederiksen and Piccirilli 2009) by chromatography has been reported, the use of dithioate derivatives is undeniably more convenient because it does not require such separation.

In conclusion, this study demonstrates that oxygen-to-sulfur substitution in phosphate groups facilitates site-specific identification of NMR signals from NH_3^+ group forming intermolecular ion pairs at protein–DNA interfaces. The characteristic changes in NH_3^+ ^{1}H and ^{15}N resonances upon dithioation of the DNA phosphate provide an insightful indicator of intermolecular ion pair formation. Their direct ionic interactions involving hydrogen bonding can be detected by means of $^{h3}J_{NP}$ coupling. The same methods applied here to DNA should also be readily applicable to intermolecular ion pairs for protein–RNA complexes as well since monothioation during synthesis is simple and RNA thiophosphoramidites are commercially

available (AM Biotech) for dithioate RNA synthesis. Thus, our approach presented in this paper can facilitate atomic-level investigations of intermolecular ion pairs at protein–nucleic acid interfaces for many different systems. Additionally, although we did not explore in this work, the NMR information of intermolecular ion pairs could also provide intermolecular constraints for structure determination of protein–nucleic acid complexes in solution.

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