Mapping the Interfaces of Protein−Nucleic Acid Complexes Using Cross-Saturation

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Abstract: This paper reports a novel NMR method for mapping the surface of interaction between proteins and nucleic acids. The method builds upon the cross-saturation based approach recently introduced by Takahashi *et al.* (*Nature Struct. Biol.* **2000**, *7*, 220−223) and takes advantage of the presence of spectral regions which contain nucleic acid signals, but which are devoid of protein resonances, to obviate the requirement for deuteration. The data obtained are complementary to those taken from the widely used chemical shift perturbation method, and a joint analysis of the results of both methods increases the reliability with which the interface can be characterised. The approach has been assessed using a protein−RNA complex for which NMR chemical shift pertubation and X-ray crystallographic data are available.

Introduction

Protein−nucleic acid interactions play a fundamental role in regulating the steps leading from genetic information to protein synthesis, yet the structures of only a small number of protein−nucleic acid complexes have been solved to date. X-ray studies of such complexes are plagued by the difficulty of obtaining well-ordered crystals, whereas NMR studies are often hampered by unfavorable exchange processes involving the groups at the interface and by the difficulty and cost of introducing ^{13}C- and ^{15}N isotopes into nucleic acids.

In the absence of a full structure a map of the interaction site can provide valuable insight into the problem of interest. There is therefore considerable interest in methods which characterize the interaction surface and are more tractable than a full-structure determination, even if the information obtained is only partial or qualitative. The most widely used method measures the perturbations in chemical shifts that occur upon complexation—this is generally reliable, but some shift changes can occur in more remote groups as a result of allosteric structural changes[1] or as a result of small differences in the salt concentration or pH that might occur during sample preparation.

Recently, Takahashi et al. have described a new method based on NMR cross-saturation for mapping the interfaces in protein−protein complexes.[2] This method is accurate but depends on having one of the two components deuterated to a high level, which is invariably costly and is not always feasible. In the present contribution we show how the cross-saturation method can be adapted for mapping protein−nucleic acid interaction surfaces without the need for deuteration.

The cross-saturation mapping of protein−protein interfaces introduced by Takahashi et al. can be thought of as a multisite-driven NOE experiment[3,4] with saturation of the ^{1}H resonances of the nondeuterated component (the target resonances) and detection of the resulting magnetization changes on the amide protons of the deuterated component (the reporter resonances). The deuterated component is also uniformly labeled with ^{15}N, and the amide protons are detected in a 2D ^{1}H−^{15}N correlation experiment. The main requirements for deuteration are 2-fold: first to make it possible to irradiate the resonances of one component without directly affecting the other component and second to reduce the extent of spin-diffusion on the reporter side of the interface to observe larger and more specific effects.

In contrast, the ^{1}H spectra of many protein−nucleic acid complexes have a spectral window that contains nucleic acid resonances but is free of signals from the protein component—this allows signals on one side of the interface to be irradiated without requiring deuteration of the other component. One such window is centered at ∼6 ppm and contains H1′ sugar resonances and H5 base resonances of the nucleic acid, while another may be provided by the imino protons of the nucleic acid bases at shifts in excess of 12 ppm. We propose using these nucleic acid signals as target resonances.

When a nondeuterated complex is irradiated (for a period in the region of 1 s), saturation is transferred to surrounding spins by a combination of direct and relayed effects. There is a lag in the time-development of the relayed transfers, which limits their overall extent. Thus, while the relayed effects lessen the specificity of the saturation transfer, they fall far short of destroying it entirely—a significant degree of specificity can be obtained in nondeuterated systems, as demonstrated below. Indeed, to some extent the relayed transfers are an aid toward obtaining a complete picture of the interface—the target and reporter nuclei do not need to be directly involved in the interface as long as there is an efficient cross-relaxation pathway linking them. It is however still possible that some interface

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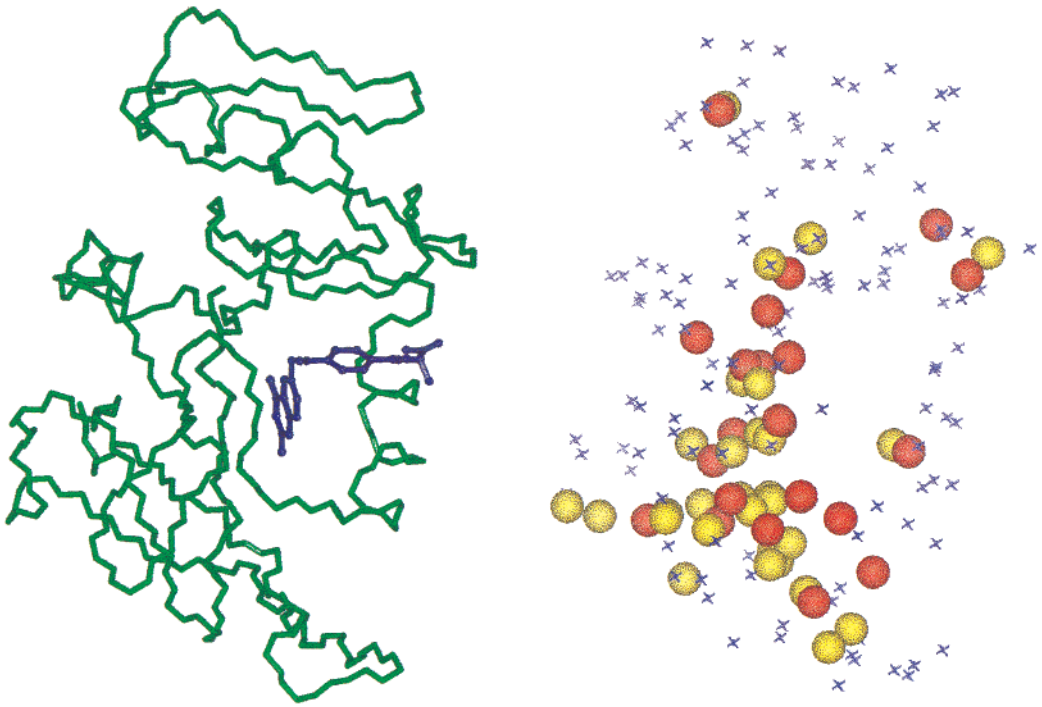

Figure 1. Left: representative NMR structure of DHFR·MTX[9] with the protein represented as backbone trace. Right: shows the position of the irradiated ^{1}H nuclei as yellow spheres, the NH resonances which are affected by cross-saturation as magenta spheres, and unaffected amide resonances as crosses.

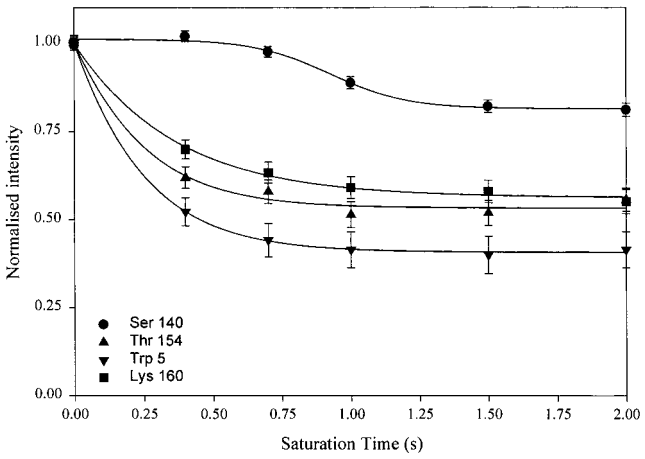

Figure 2. Intensities of selected amide resonances of the DHFR·MTX complex for different irradiation times. Experiments were recorded as described in the Experimental Procedures.

residues will remain undetected, leading to the extent of the interface being underestimated. This is in contrast to the errors which are likely to arise from chemical shift perturbation data, which tend to lead to an over-estimate of the extent of the interface.

The applications presented here use the amide resonances of the ^{15}N-labeled protein as reporter resonances, as in the method of Takahashi et al. and in many chemical-shift perturbation studies. The observed effects are interpreted in conjunction with a structure (experimentally determined or modeled) of the protein component. Assignments of the nucleic acid signals are not needed, although it is necessary to know which ^{1}H signals arise from the nucleic acid and which from the protein to select the spectral window for the irradiation. This information can easily be obtained from ^{13}C- and ^{15}N isotope-edited spectra recorded from the complex formed between labeled protein and unlabeled nucleic acid.

Experimental Procedures

The 15mer RNA oligonucleotide used in this study was obtained in milligram quantities using established procedures.[5] Briefly, the oligonucleotide was prepared using the phage T7 RNA polymerase in an *in vitro* runoff transcription system. The RNA was then gel purified, electroeluted, ethanol precipitated, and dialyzed extensively against phosphate buffer with decreasing salt concentration. Residual salt and acrylamide byproducts were eliminated using a gel filtration column (Sephadex G15). The RNA 15mer was then dialyzed against the final NMR buffer.

^{15}N-labeled DHFR was overexpressed in *Escherichia coli* and purified as described previously.[6]

The KH3 domain from Nova1 protein was overexpressed as histidine-tagged fusion protein from *E. coli* BL21 cells and purified in two steps using a Nickel semi-affinity column (Ni NTA, Qiagen) and a gel filtration column (High Load 26/60 Superdex 30, Pharmacia). Electrospray mass-spectrometry analysis confirmed that the protein monomer had the expected MW.

The DHFR sample used for NMR experiments was in 90% H_2O−10% D_2O, with 50 mM KPi and 100 mM KCl at pH 6.5.

The ^{15}N-labeled Nova1-unlabeled RNA sample used for NMR experiments was 0.3 mM and was prepared in 90% H_2O−10% D_2O with 10 mM KH_2PO_4 and 150 mM NaCl at pH 6.8.

Watergate HSQC experiments[7] were recorded at 600 MHz on a Varian Inova spectrometer. Experiments were recorded at 35 °C for the DHFR·MTX and at 25 °C for the Nova1 KH3−RNA sample. Saturation of a narrow band of ^{1}H resonances was achieved by means of a windowless train of hyperbolic secant 180° pulses,[8] each 50 ms long with a peak B_1 field of 200 Hz. This pulse train was inserted immediately prior to the first pulse of an HSQC sequence, and 2D ^{1}H−^{15}N spectra were acquired with different irradiation times up to 2

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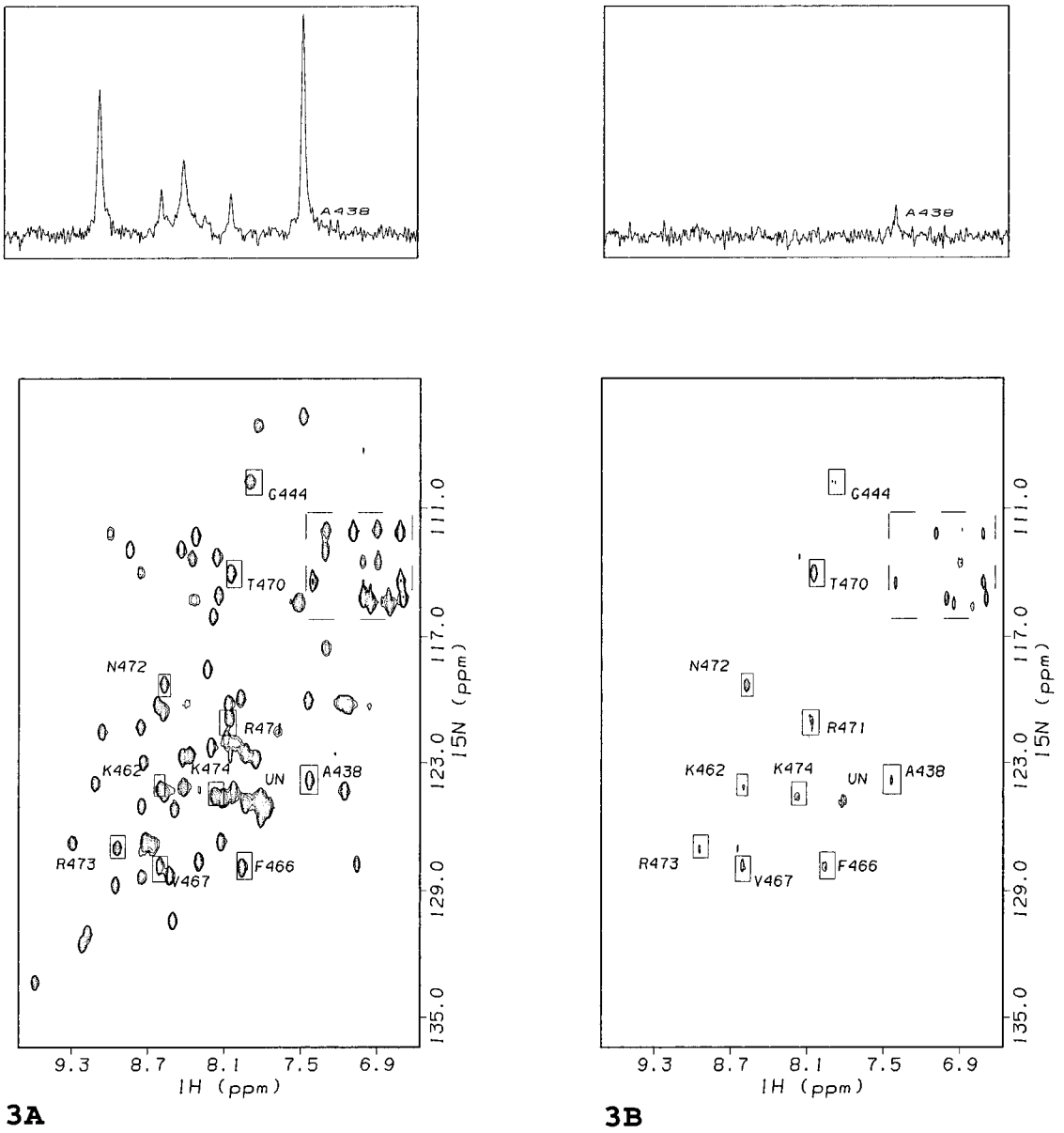

Figure 3. $^{15}N-^{1}H$ correlation spectra of the Nova1 KH3−RNA complex with pre-irradiation of a band of frequencies. (a) Control spectrum with irradiation outside the protein and RNA chemical shift range (bottom). (b) Difference spectrum between (a) and a similar experiment with adiabatic pulses centered at the frequency of the RNA anomeric protons (6.0 ppm) (bottom). Peaks attributable to RNA−protein cross-saturation are boxed and labeled. The peak marked with an asterisk is unassigned. The signals within the dashed box arise from side-chain amide protons which have been directly affected by the out-of-band irradiation to a slight extent (<5% saturation); this is too small to give rise to any of the other cross-peaks. A cross-section of each HSQC spectrum is displayed in the two top panels.

s. The direct effect of the saturation pulse-train was to achieve >80% saturation over a band of ±0.4 ppm, while affecting resonances outside a band of ±0.5 ppm by less than 5%.

Results and Discussion

The viability of method described in this paper was experimentally tested in two steps. First the degree of specificity following the selective irradiation of a small number of resonances in a macromolecule was evaluated in the 162-residue protein dihydrofolate reductase in complex with the drug methotrexate (DHFR·MTX). This system has been extensively characterized by NMR[9] and X-ray crystallography[10] and a set of resonance assignments and precise interproton distances was available. Second, the method was applied to a protein−nucleic acid complex currently under study, the complex between Nova1 KH3 and a target RNA.

DHFR. Dihydrofolate reductase (DHFR) catalyses the NADPH-linked reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. The irradiation of a narrow window in the spectrum of a DHFR·MTX sample (see Experimental Procedures) resulted in the saturation of 22 protein resonances, and the effect on the HSQC was a decrease in the intensity of only a small proportion of the amide resonances. The results obtained with an irradiation period of one second are shown in Figure 1, in which the highlighted residues are those for which the NH intensity changed by 15% or more; this value reflects the smallest effect that could be identified with confidence as dictated by the signal-to-noise ratio of the spectra. The amides which have been affected by cross-saturation are grouped around the irradiated

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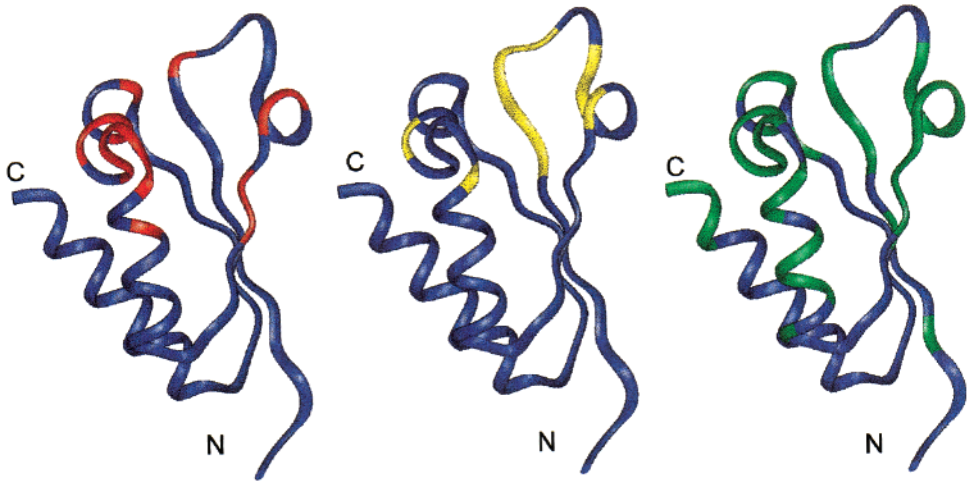

Figure 4. Structural homology model of Nova1 KH3 protein (Swissprot) showing the protein backbone as a ribbon. Three different methods of identifying surface residues are compared. Left: residues reported to make direct contact in the crystal structure of a closely related complex[15] (red). Center: residues identified using the spectrum in Figure 2b (yellow). Right: chemical shift perturbation (green).

atoms and 26 (out of a total of 28) are within 5.4 Å of an irradiation site, demonstrating that selectivity can be achieved without deuteration. Further insight into the magnetization transfer processes involved can be gleaned from the build-up curves for the affected NH groups (Figure 2)—the response of S140 shows a pronounced lag typical of a multistep process, while others are dominated by single-step transfers.

Nova1 KH3−RNA Complex. Nova1 is a multidomain RNA-binding protein responsible for the neurological disorder POMA.[11] The protein construct used is 80 amino acids long, has a mass of 9.4 kDa, and is thought to dimerize in solution.[12] The 15 nucleotide RNA target has a mass of 6.3 kDa and includes two copies of a four-nucleotide motif specific for Noval KH3 binding. Essentially complete assignments of the backbone resonances of the free protein are available,[13] as are chemical-shift perturbation data for this complex[13] and for a different KH domain in complex with a single stranded DNA oligomer.[14] No KH3 resonance is observable in the 5.6−6.4 ppm region. Also, the crystal structure of a related complex, the Nova2 KH3−RNA has been published recently[15] providing information on the interaction surface. HSQC experiments as described in the Experimental Procedures were carried out on a sample of the Nova1 KH3−RNA complex. The results of a difference experiment obtained with an irradiation time of 1 s are shown in Figure 3; intensity changes were detected on only a small number of protein amide cross-peaks.

The residues identified in this experiment agree very well with those found to be involved in the interface of the closely related complex by X-ray crystallography,[15] as shown in Figure 4. A rather larger number of candidate interface residues are identified by means of the chemical-shift perturbation method. The combined use of the cross-saturation data and the chemical shift perturbation data allows the interaction surface to be identified with greater confidence.

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