Journal of Biomolecular NMR, **20:** 195–202, 2001.
KLUWER/ESCOM

Mapping the ligand binding site at protein side-chains in protein-ligand complexes through NOE difference spectroscopy*

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Received 19 February 2001; Accepted 27 April 2001

Key words: binding-site mapping, difference spectroscopy, drug design, enzyme-ligand complexes, NOESY, protein NMR spectroscopy, protein-protein complexes

Abstract

This report describes a novel NMR approach for mapping the interaction surface between an unlabeled ligand and a ^{13}C,^{15}N-labeled protein. The method relies on the spin inversion properties of the dipolar relaxation pathways and records the differential relaxation of two spin modes, where ligand and protein ^{1}H magnetizations are aligned either in a parallel or anti-parallel manner. Selective inversion of protein protons is achieved in a straightforward manner by exploiting the one-bond heteronuclear scalar couplings ($^{1}J_{CH}$, $^{1}J_{NH}$). Suppression of indirect relaxation pathways mediated by bulk water or rapidly exchanging protons is achieved by selective inversion of the water signal in the middle of the NOESY mixing period. The method does not require deuteration of the protein or well separated spectral regions for the protein and the ligand, respectively. Additionally, in contrast to previous methods, the new experiment identifies side-chain enzyme ligand interactions along the intermolecular binding interface. The method is demonstrated with an application to the B_{12}-binding subunit of glutamate mutase from *Clostridium tetanomorphum* for which NMR chemical shift changes upon B_{12}-nucleotide loop binding and a high-resolution solution structure are available.

Introduction

Protein-ligand, protein-nucleic acid or protein-protein interactions are of primary interest in structural biology and often provide a clue to the physiological functioning of proteins. X-ray studies are often impaired by the difficulty to obtain well-ordered crystals suitable for diffraction. NMR spectroscopy is thus an attractive alternative to characterize both high- and low-affinity macromolecular complexes. A common approach is the uniform labeling of one of the components with ^{15}N and/or ^{13}C, while leaving the second component unlabeled. This strategy allows the identification of intermolecular NOEs across the binding interface by selectively recording the magnetization transfer between two protons, where only one is bound to a labeled heteroatom (Otting et al., 1986; Otting and Wüthrich, 1990; Sattler et al., 1999; Breeze, 2000). Isotope-filtered experiments have been tremendously improved in terms of selectivity and sensitivity and proved particularly important for the structural analyses of protein-ligand complexes and provided a wealth of structural information over the past decade (Wider et al., 1991; Ikura and Bax, 1992; Gemmecker et al., 1992; Lepre et al., 1992; Petros et al., 1992; Lee et al., 1994; Dalvit et al., 1998, 1999; Zwahlen et al., 1997; Stuart et al., 1999).

Although a high-resolution structure of the protein-ligand complex is desirable, often a map of the interaction site can already provide valuable insights. For example, in pharmaceutical research, this type of information is extremely valuable for the identification of lead compounds or to establish whether different compounds share a common binding mode. An ex-

*This paper is dedicated to Prof. Heinz Sterk on the occasion of his 60th birthday.

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tremely widely used method is based on recording chemical shift perturbation as a result of complexation (Chen et al., 1993; Gronenborn and Clore, 1993; van Nuland et al., 1995; Emerson et al., 1995; Grzesiek et al., 1996; Shuker et al., 1996; Farmer et al., 1996; Garrett et al., 1997; Ross et al., 1997). The fidelity of the method is, however, compromised by the fact that some chemical shift changes can occur also in groups distant to the binding site due to allosteric structural changes (Foster et al., 1998) or possibly as a result of altered experimental conditions.

As an alternative, Takahashi et al. proposed a new method based on NMR cross-saturation across the binding interface and nicely demonstrated the utility of the method with an application to binding site mapping in a protein-protein complex (Takahashi et al., 2000). Recently, a similar approach was devised for protein-nucleic acid complexes (Ramos et al., 2000). In this case, the spectral separation between protein ^{1}H resonances and NMR signals stemming from protons of the nucleic acids was exploited to selectively saturate the ^{1}H resonances of the nucleic acid and observe the effect of this multisite-driven NOE (Wagner and Wüthrich, 1979; Neuhaus and Williamson, 2000) on the amide resonances of the ^{15}N-labeled protein.

In order to overcome the limitation that the ^{1}H spectral windows of the two constituents of the macromolecular complex of interest have to be well separated, we propose another method which makes use of the spin inversion properties of the longitudinal relaxation pathways in a network of dipolarly coupled spins as exemplified by the Solomon equations. Identification of residues involved in ligand binding is achieved by recording the differential decay of longitudinal protein ^{1}H magnetization after preparation of the sum or difference of ligand and ^{13}C,^{15}N-labeled protein ^{1}H Zeeman polarizations, respectively.

Materials and methods

All NMR experiments were performed on a Varian UNITYPlus 500 MHz spectrometer equipped with a pulsed field gradient unit and triple resonance probes with actively shielded z gradients. All spectra were recorded at 26 °C. ^{13}C,^{15}N-labeled MutS was overexpressed in *E. coli* and purified as described previously (Tollinger et al., 1998). The NMR sample contained 2.0 mM ^{13}C,^{15}N-labeled MutS, 5 mM DTT, 1 mM EDTA, 0.03% NaN_3, and 10 mM phosphate buffer at pH 6.9, dissolved in 90% H_2O/10% D_2O. In the binary complex, the ^{13}C,^{15}N MutS:B_{12}-nucleotide ratio was 1.0:12.0. The B_{12}-nucleotide ([5,6-dimethyl-1-(α-D-ribofuranosyl)-benzimidazole]-3′-[R-1-aminopropyl-2-diphosphate]) was prepared from hydrazinolysis of cyano-cobalamin (vitamin B_{12}) following a procedure described by Eschenmoser and co-workers (Eschenmoser, 1988; Bartels et al., 1991). The dissociation constant K_d of the complex was determined from a titration of the protein with the B_{12}-nucleotide by a non-linear least squares fitting of the chemical shift changes as a function of ligand concentration. In a titration experiment, a sample of ^{15}N-labeled MutS (0.8 mM) and a 66.1 mM solution of B_{12}-nucleotide dissolved in the same buffer as the protein were used. The B_{12}-nucleotide solution was added stepwise to the MutS solution, and at each step a sensitivity-enhanced 2D ^{15}N HSQC experiment was performed. Protein:ligand ratios were between 1:0.5 and 1:32. All spectra were processed using NMRPipe/NMRDraw software (Delaglio et al., 1995) and analyzed using the program NMRView (Johnson and Blevins, 1994).

The proposed relaxation experiment for mapping enzyme-ligand binding sites in macromolecular complexes is similar to a 2D CT ^{13}C-^{1}H HSQC experiment in terms of the flow of magnetization and is outlined in Figure 1. The main difference is a scalar coupling (e.g., $^1J_{CH}$ and $^1J_{NH}$) evolution period ξ, which is followed by a NOESY period, τ_m. In the middle of the NOESY mixing period τ_m, a water selective inversion pulse is incorporated to suppress relaxation pathways (Zwahlen et al., 1994) which are mediated by transiently bound water molecules or other exchangeable protons (e.g., hydroxyl protons of serines, threonines or tyrosines). Two data sets are recorded (Figure 1A, 1B). In one experiment (Figure 1A), the heteronuclear inversion pulses are omitted and the one-bond $^1J_{CH}$ and $^1J_{NH}$ scalar couplings are refocused. The 90° ^{1}H radio-frequency (rf) pulse (before the NOESY period) converts both ligand and protein ^{1}H transverse magnetization to longitudinal magnetizations and creates a parallel alignment of both Zeeman reservoirs (Σ mode). In contrast, in the second experiment (Figure 1B) the heteronuclear inversion pulses are applied and both $^1J_{CH}$ and $^1J_{NH}$ couplings evolve during an evolution delay optimized for the scalar coupling constants. For ^{13}C-^{1}H spin systems, the scalar couplings evolve during the time $\tau_C = 2\xi - 2\delta = 1/^1J_{CH}$, whereas the evolution time for the $^1J_{NH}$ coupling is $\tau_N = 2\xi = 1/^1J_{NH}$. In this case, the two Zeeman polarizations of the ligand and protein protons are

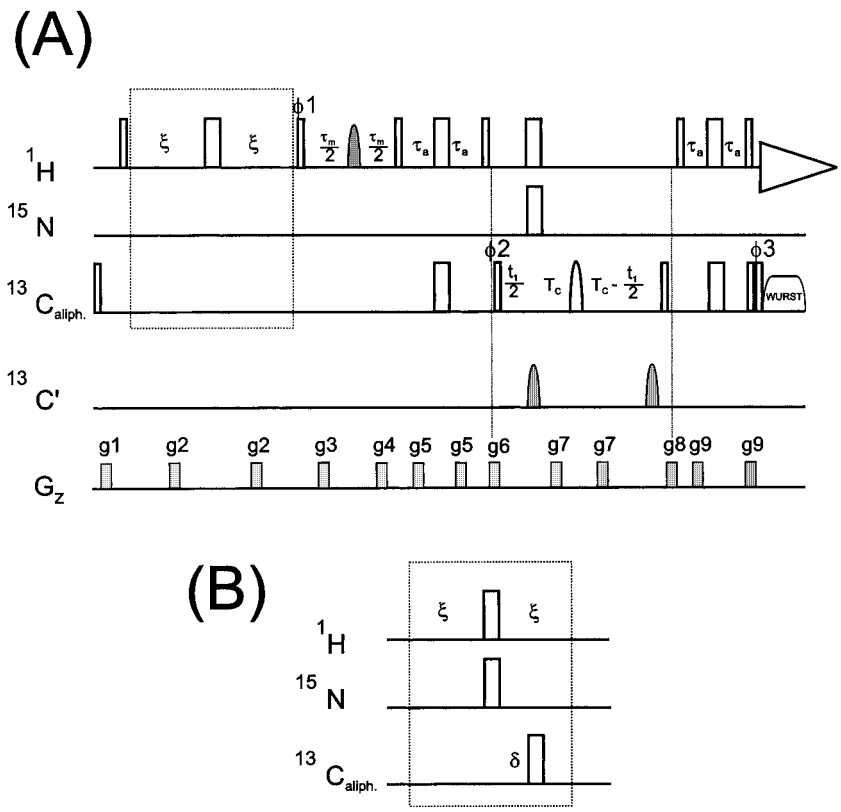

Figure 1. Pulse scheme for binding site mapping in protein-ligand complexes based on differential relaxation of longitudinal ^{1}H magnetization induced by selective ^{1}H inversion of protons attached to ^{13}C,^{15}N-labeled proteins. (A) Experiment to record the relaxation decay of the sum mode (Σ, parallel alignment of ^{13}C,^{15}N-protein and ligand ^{1}H magnetizations). Narrow and wide pulses indicate 90° and 180° pulses, respectively, and, unless indicated otherwise, all pulses are applied along the x-axis. All rectangular ^{1}H and ^{13}C′ hard pulses are applied with 28 and 20 kHz, respectively. The water selective inversion pulse applied in the middle of the NOESY period τ_m is shaped according to the IBURP2 profile (Geen and Freeman, 1991) (20 ms, 250 Hz peak rf). The ^{13}C′ shaped pulses have SEDUCE (McCoy and Mueller, 1992) profiles (240 μs, 4.55 kHz peak rf) while the $^{13}C_{aliph}$ shaped pulse (during the constant time period T_C) makes use of the REBURP (Geen and Freeman, 1991) profile (367 μs, 17.0 kHz peak rf). The second shaped ^{13}C′ rf pulse is applied to compensate for the Bloch–Siegert effects on the $^{13}C_{aliph}$ magnetization. ^{13}C decoupling during acquisition was achieved with a WURST decoupling scheme (Kupce and Freeman, 1995) ($\gamma B_1 = 3.0$ kHz). The values for ξ, δ, τ_a, and T_C were set to 5.26, 1.1, 1.7 and 29.4 ms, respectively. Gradient levels were as follows: g1 = 0.5 ms, 5 G cm^{-1}; g2 = 0.3 ms, 3 G cm^{-1}; g3 = 1.0 ms, 9 G cm^{-1}; g4 = 0.6 ms, 6 G cm^{-1}; g5 = 0.3 ms, 3 G cm^{-1}; g6 = 1.5 ms, 15 G cm^{-1}; g7 = 0.6 ms, −10 G cm^{-1}; g8 = 0.2 ms, −2.5 G cm^{-1} and g9 = 0.2 ms, 4 G cm^{-1}. The phase cycling was ϕ1 = 4(x),4(−x); ϕ2 =x,−x; ϕ3 = 2(x),2(−x); and receiver was (x),(−x),(x),(−x),(x),(−x),(x),(−x). Quadrature detection in F_1 is achieved by States-TPPI (Marion et al., 1989) of ϕ2. (B) Experimental scheme to record the decay of the difference mode (Δ, anti-parallel alignment of ^{13}C,^{15}N-protein and ligand ^{1}H magnetizations). The two data sets are recorded in an interleaved manner and separated afterwards.

aligned in an anti-parallel manner (Δ mode). The decay of a particular longitudinal ^{1}H magnetization will be altered in the Δ mode experiment provided a dipolar coupling partner is inverted relative to the proton of interest (Neuhaus and Williamson, 2000). A differential decay of the longitudinal ^{1}H magnetization is thus observed for those protein sites which are dipolarly coupled across the binding interface to protons of the ligand. Note that the underlying principle of the proposed sequence is related to pulse sequences based on doubly-half-filtered NOESY techniques (Otting et al., 1986; Otting and Wüthrich, 1990; Sattler et al., 1999; Breeze, 2000). However, the proposed sequence probes the sum of all intermolecular dipolar interactions a particular proton of the protein exhibits to ligand protons located in the binding site. Thus protons in the binding site can be observed with good sensitivity.

To account for a variation in the one-bond heteronuclear scalar coupling constants, a reference experiment was recorded by omitting the NOESY period ($\tau_m = 0$). This gives the reference intensities for

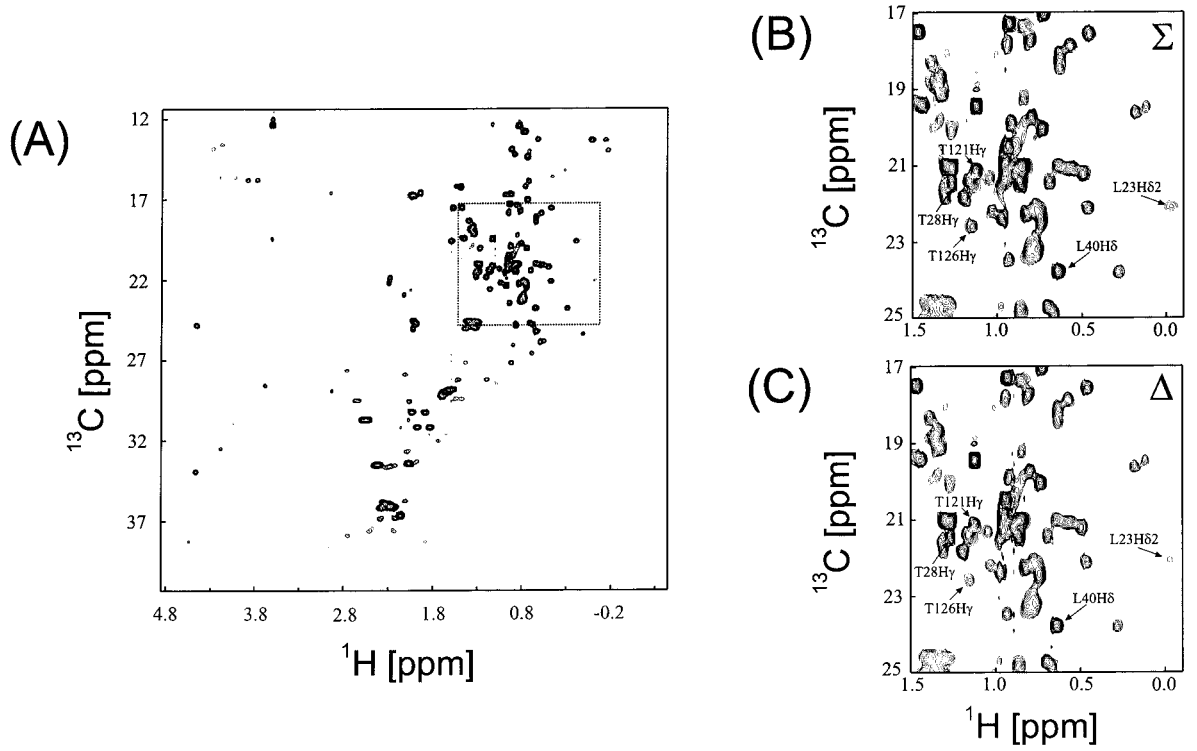

Figure 2. (A) 2D ^{13}C-^{1}H correlation map obtained with the pulse sequence of Figure 1A (Σ mode), with $\tau_m = 0$ ms. Residues located in the boxed region are also displayed in (B) and (C). (B) Σ mode with $\tau_m = 300$ ms; (C) Δ mode with $\tau_m = 300$ ms. Residues which displayed differential relaxation in the two experiments (L23δ, T121γ and T126γ) are indicated. In contrast, T28γ and L40δ did not show differential relaxation and are used as a reference (see text).

the sum mode, Σ_0, and the difference mode, Δ_0, respectively. Differential relaxation of the sum, Σ, and difference, Δ, modes, due to intermolecular NOEs between the two proton subsets (^{13}C,^{15}N-labeled protein and ligand) leads to an effective ratio of signal intensities according to

$$\Gamma = (\Delta/\Sigma)/(\Delta_0/\Sigma_0). \quad (1)$$

The NOESY period is followed by a conventional INEPT step and transfer to the heteronucleus ^{13}C. The ^{13}C chemical shift is recorded in a constant-time manner (with additional ^{13}CO decoupling).

Results and discussion

The utility of the method described in this paper was experimentally demonstrated with an application to the B_{12}-binding subunit of glutamate mutase (MutS) from *Clostridium tetanomorphum* in complex with the nucleotide portion of the natural cofactor coenzyme B_{12}. Glutamate mutase is an adenosylcobamide (coenzyme B_{12}) dependent enzyme that catalyzes the reversible rearrangement of (2S)-glutamate to (2S,3S)-3-methylaspartate (Barker et al., 1958). The enzyme from *Clostridium tetanomorphum* consists of two subunits (MutE, 53.7 kDa and MutS, 14.8 kDa) (Marsh and Holloway, 1993; Holloway and Marsh, 1994) and it has been shown (Tollinger et al., 1998) that the global fold of MutS in solution closely resembles that determined by X-ray crystallography for the B_{12}-binding domains of *E. coli* methionine synthase (Drennan et al., 1994a,b) and *Propionibacterium shermanii* methylmalonyl CoA mutase (Mancia et al., 1996), and *Clostridium cochlearium* glutamate mutase (Reitzer et al., 1999).

The quality of spectra obtained with the devised sequence is illustrated in Figure 2, where the ^{13}C-^{1}H correlation map obtained with the sequence of Figure 1 is shown. Although the proposed strategy is not limited to methyl groups, we focused in the present application on the detection of methyl groups and thus used a constant-time HSQC detection scheme, which compromises the observation of Cα and Cβ carbons

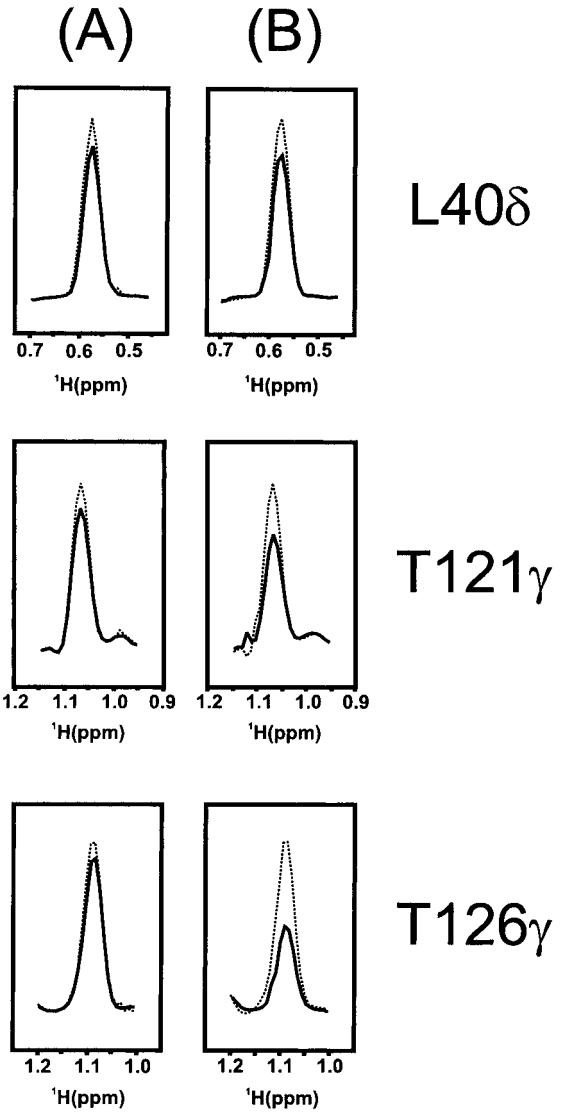

Figure 3. F_2 slices through residues L40δ, T121γ and T126γ of the ^{13}C-^{1}H correlation spectra of the MutS-B_{12}-nucleotide loop complex obtained with the sequence of Figure 1 (Σ mode: dashed line; Δ mode: solid line). (A) $\tau_m = 0$ ms; (B) $\tau_m = 300$ ms.

(e.g., low signal-to-noise due to efficient carbon T_2 relaxation and/or $^{1}J_{C\beta C\gamma}$ couplings), respectively. In applications to these carbon sites it would be beneficial to omit the constant-time delay and record a conventional HSQC-type experiment. However, it should be noted that the water-selective inversion pulse in the middle of the NOESY mixing time, which is necessary to ensure that all detected NOEs stem from intermolecular dipolar interactions across the binding interface rather than indirect effects via exchanging hydroxyl protons or transiently bound water molecules (Figure 1) also inverts aliphatic Hα's (or possibly Hβ's of serines and threonines). This frequency dependence of this pulse thus limits the extension of this approach to Cα sites with Hα resonance frequencies well outside the bandwidth of the inversion pulse. Figure 3 shows F_2 slices for three methyl groups (L40δ, T121γ and T126γ). The data sets of the reference ($\tau_m = 0$) and after a NOESY mixing period of $\tau_m = 300$ ms are given. The signal intensity of the methyl group L40δ is almost unaffected by the selective inversion of the ligand proton subset, whereas the differential decays of the proton magnetizations of T121γ and T126γ are clearly observed. Figure 4 shows the differential decay (given as the ratio Γ, see Materials and methods) observed for some methyl groups in the MutS/B_{12}-nucleotide loop complex. Due to the complex relaxation network in a protein (and most likely also in the ligand) the decay will, in general, deviate from mono-exponential behavior. As we are mainly interested in a rapid and facile approach to identify protein protons interacting with ligand protons, the failure of an exact quantification of the NOE connectivities does not compromise the approach. Of course, the scalar coupling constants $^{1}J_{CH}$ are not uniform, but vary for different sites of the protein. The one-bond $^{1}J_{NH}$ couplings, on the other hand, can be safely assumed to be rather uniform. A variation of the scalar coupling constants changes the intensity of the difference mode

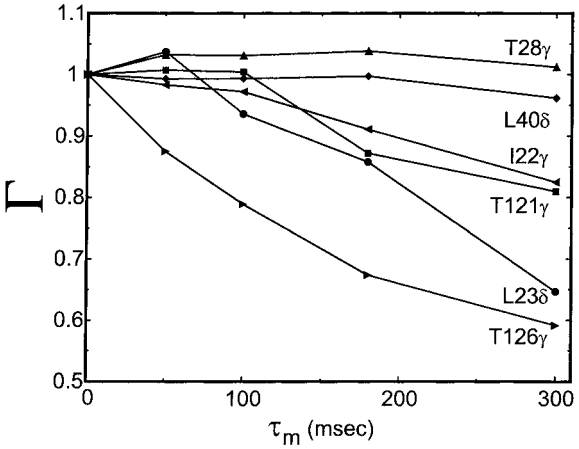

Figure 4. Representative intensity ratios Γ of selected methyl group resonances (I22γ, L23δ, T28γ, L40δ, T121γ and T126γ) in the MutS/B_{12}-nucleotide loop complex as a function of NOESY mixing time. Γ is defined as the ratio between the signal intensities obtained in the Δ and Σ mode experiment (Δ/Σ), divided by the ratio of the reference experiment (Δ_0/Σ_0) (see Materials and methods, Equation 1). Experimental parameters are given in the legend of Figure 1.

intensity because of imperfect inversion (Figure 1B). To account for this effect we recorded a reference experiment omitting the NOESY period with $\tau_m = 0$, and the observed differential decay is corrected according to Equation 1. Additionally, a non-uniform inversion of protein ^1H magnetization in the experiment recording the relaxation decay of the difference mode (Figure 1B) might also affect the differential relaxation decay in the two experiments, although no significant deviations were observed for the analyzed methyl groups in the MutS/B_{12}-nucleotide complex. This effect could be alleviated by implementation of an adiabatic inversion pulse applied during the period ξ (Figure 1B) where the sweep rate of the adiabatic inversion pulse is tuned according to an empirically observed ^{13}C chemical shift vs $^1J_{CH}$ scalar coupling profile (Zwahlen et al., 1997).

Of particular concern are unwanted NOE pathways through bulk water and rapidly exchanging hydroxyl hydrogens stemming from serine, threonine or tyrosine residues, as these might obscure the unambigious identification of residues located at the binding interface. Figure 4 convincingly demonstrates that NOESY transfer via bulk water or exchangeable hydroxyl protons is successfully suppressed by the application of the water selective inversion pulse in the middle of the NOESY period (Zwahlen et al., 1994), as exemplified by T28γ, which does not show any variation of the intensity ratio as a function of mixing time τ_m (in contrast to T121γ and T126γ, which are part of the binding site, see below).

The application of the method to the MutS/B_{12}-nucleotide complex is particularly challenging, as the dissociation constant K_d of the complex is 5.6 $\pm$ 0.7 mM. Nevertheless, we have observed a significant decrease of the intensity ratio Γ for residues which are located in the binding site and have been identified by the conventional chemical shift perturbation method. Specifically, the following residues experienced a notable decrease in the intensity ratio Γ after a NOESY period of 300 ms: I22γ (0.82), L23δ (0.65), T121γ (0.81), and T126γ (0.59). This experimental finding nicely correlates with the crystal structure of the homologous glutamate mutase from *Clostridium cochlearium* complexed with methylcobalamine, where a similar binding interface was observed (Reitzer et al., 1999). Figure 5A shows a ribbon drawing of the structural model of MutS complexed with the B_{12}-nucleotide, based on the crystal structure of the complex between the homologous glutamate mutase from *Clostridium cochlearium* and methylcobalamine (Reitzer et al., 1999). The location and orientation of the nucleotide in the MutS/B_{12}-nucleotide complex was obtained by a superposition of the backbone nuclei of the B_{12}-binding subunit of glutamate mutase (GlmS) with corresponding residues in MutS. In Figure 5B residues are shown (dark grey) which display significant $^1H^N$ and ^{15}N chemical shift changes upon B_{12}-nucleotide binding. The residues identified by means of chemical-shift perturbation agree very well with those from the structural homology model (Figure 5A). This strongly corroborates a similar B_{12}-nucleotide binding mode for MutS. Figure 5C shows that residues I22, L23, T121 and T126 are grouped around the binding site of the B_{12}-nucleotide in MutS, thus demonstrating the specificity of the method. However, Figure 5B shows that chemical shift changes were also observed for ^1H-^{15}N-groups of residues I6, V7, L8 and G9 located in β1 of MutS (Tollinger et al., 1998), which are distant to the binding site and which are due to subtle changes of the β-strand conformation and the long-range character of shielding effects of aromatic ring systems.

Additionally, although the sequence was optimized to the observation of methyl groups, intermolecular NOEs could also be detected at some backbone Cα carbon sites. Specifically, the Hα's of V90 and G91 resonate at 5.80 and 4.30 ppm and are thus sufficiently separated from the water resonance and not perturbed by the selective inversion pulse in the middle of the NOESY period (Figure 1). For both protons, a significant differential relaxation was observed in the experiments (sum, Σ, and difference, Δ, mode). The Γ values (Equation 1) obtained for a mixing time $\tau_m = 300$ ms were 0.20 (V90) and 0.65 (G91), respectively. Again, these data are in agreement with the crystal structure of the homologous glutamate mutase from *Clostridium cochlearium* complexed with coenzyme B_{12} (Reitzer et al., 1999). However, no differential relaxation decay was found for Cβ carbon sites in the MutS/B_{12} nucleotide complex.

Although a complete description of the structure will be given elsewhere, the NOE difference data obtained with the method described in this report indicate that MutS exhibits a B_{12}-nucleotide binding mode reminiscent of other nucleotide (e.g., Rossmann fold) or B_{12}-binding proteins. As the quality of the complex structure is particularly sensitive to the number of unambiguously assigned intermolecular NOEs (Clore, 2000), the data obtained with the proposed sequence provide an important starting point for the assignment of intermolecular NOE connectivities recorded

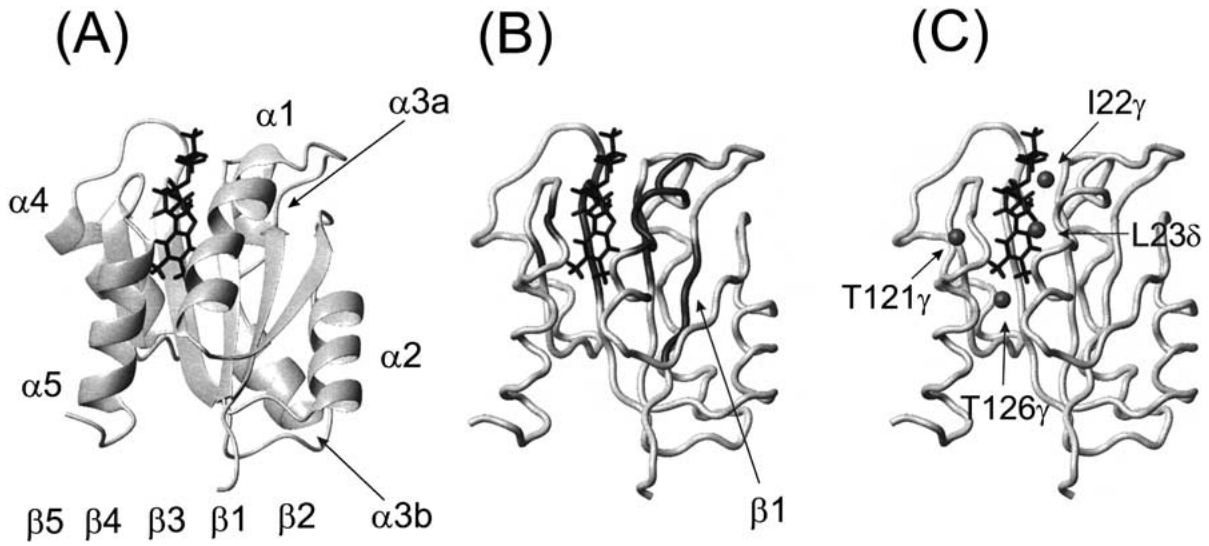

Figure 5. Ribbon drawing of MutS complexed with B_{12}-nucleotide and comparison between different methods of identifying interaction surfaces in proteins. (A) Structural homology model for the B_{12}-nucleotide binding site in MutS. The putative location of the B_{12}-nucleotide binding site in MutS was obtained from the closely related complex between glutamate mutase from *Clostridium cochlearium* and methylcobalamine (see text) (Reitzer et al., 1999). Secondary structure elements are numbered. Helix α3 is split into two parts and the two halves are numbered accordingly (α3a and α3b). Only the nucleotide moiety of bound methylcobalamine is shown. (B) The B_{12}-nucleotide binding site was identified by chemical shift perturbation (residues which exhibited significant ^{15}N and/or ^{1}H chemical shift changes are shown in dark grey) or (C) by NOE difference spectroscopy (using the pulse sequence of Figure 1). The location of residues I6, V7, L8 and G9 located in β1 of MutS (Tollinger et al., 1998) is indicated in (B) (see text). The figure was prepared using the program MOLMOL (Koradi et al., 1996).

by pulse sequences based on isotope-filter techniques (Otting et al., 1986; Otting and Wüthrich, 1990; Sattler et al., 1999; Breeze, 2000).

Conclusions

In summary, a pulse sequence has been presented for rapid identification of the ligand binding site in enzyme-ligand complexes through NOE difference spectroscopy detected at methyl group protons. The residues identified in this experiment agree very well with those determined to be part of the interface by established chemical shift perturbation methods. The particular merit of the method is the fact that indirect magnetization transfer via transiently bound bulk water molecules or rapidly exchanging hydroxyl hydrogens is effectively suppressed, no protein deuteration is required and there is no limitation in terms of spectral separation between protein and ligand signals. It also complements a recently described ^{13}C-methyl-labeling and screening strategy for the identification of lead compounds in drug research (Hajduk et al., 2000). Statistical analyses of buried surface areas in protein complexes have revealed that Leu and Val are among the three most frequently encountered residue types at molecular interfaces (Janin et al., 1988). In combination with a recently described powerful labeling procedure (Rosen et al., 1996), the proposed NOE difference method might also have broad utility in defining and characterizing functionally important protein interaction sites. Although we focused in the present application on the observation of methyl groups, the strategy is not limited to aliphatic side-chains but can be easily extended to other protein sites by a suitable modification of the detection scheme (e.g., ^{1}H-^{15}N HSQC for amide backbone sites). The data obtained on the low-affinity complex between the B_{12}-binding subunit of glutamate mutase from *Clostridium tetanomorphum* and the B_{12}-nucleotide loop indicate that the method will be also applicable to weakly interacting molecular species which are often encountered in early stages of the lead identifying process in drug design (Shuker et al., 1996).

Acknowledgements

The authors thank Prof. N.E.G. Marsh (University of Ann Arbor) for kindly supplying uniformly ^{13}C,^{15}N-labeled MutS. This research was supported by grants P 13595 (to B.K.) and P 13486 (to R.K.) from the

Austrian Science Foundation FWF, and the European Commission (TMR Project No. FMRX.CT96.0018, to B.K.).

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