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Rapid corepressor exchange from the *trp*-repressor/operator complex: An NMR study of [ul-^{13}C/^{15}N]-L-tryptophan

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Summary

[ul-^{13}C/^{15}N]-L-tryptophan was prepared biosynthetically and its dynamic properties and intermolecular interaction with a complex of *Escherichia coli trp*-repressor and a 20 base-pair operator DNA were studied by heteronuclear isotope-edited NMR experiments. The resonances of the free and bound corepressor (L-Trp) were unambiguously identified from gradient-enhanced ^{15}N-^{1}H HSQC, ^{13}C-^{1}H HSQC, ^{13}C- and ^{15}N-edited 2D NOESY spectra. The exchange off-rate of the corepressor between the bound and free states was determined to be 3.4 ± 0.52 s^{-1} at 45 °C, almost three orders of magnitude faster than the dissociation of the protein–DNA complex. Examination of the experimental NOE buildup curves indicates that it may be desirable to use longer mixing times than would normally be used for a large molecule, in order to detect weak intermolecular NOEs in the presence of exchange. Intermolecular NOEs from bound corepressor to *trp*-repressor *and* DNA were analyzed with respect to the mechanism of ligand exchange. This analysis suggests that, in order for the ligand to diffuse out of the complex, there must be significant movement or 'breathing' of the protein and/or DNA.

Introduction

Escherichia coli trp apo-repressor is a homodimer of two 107-residue polypeptides. The noncooperative binding of two corepressor (L-tryptophan) molecules to this homodimer initiates an allosteric transition, causing the repressor to bind to operator sites in five operons involved in tryptophan metabolism and transport (*Trp*EDCBA, *aro*H, *aro*L, *Trp*R and *mtr*; Squires et al., 1975; Gunsalus and Yanofsky, 1980; Zurawski et al., 1981; Klig et al., 1988; Heatwole and Somerville, 1991; Sarsero et al., 1991). Both genetic (Bennett and Yanofsky, 1978) and biochemical (Klig et al., 1987; Carey, 1988; Carey et al., 1991; Haran et al., 1992) studies have shown that the *trp*-repressor binds to an 18 base-pair consensus operator sequence with 1:1 stoichiometry. To operator sequences of ~33 base pairs or longer, *trp*-repressor can bind in a tandem fashion with a 2:1 repressor:operator stoichiometry (Kumamoto et al., 1987; Liu and Matthews, 1993; Sutton et al., 1993).

Structures have been determined for *trp* apo- and holorepressor, both in solution by NMR spectroscopy (Arrowsmith et al., 1991; Zhao et al., 1993) and by X-ray crystallography (Schevitz et al., 1985; Zhang et al., 1987). The results of both methods are in good agreement with regard to the global structural features. However, the NMR data suggested that the D and E helices, forming a helix-turn-helix DNA binding motif, are unstable in aqueous solution (Arrowsmith et al., 1991; Czaplicki et al., 1991). The major 'activating' effect of the corepressor both in solution and in the crystalline state is to shift the helix-turn-helix motifs into better position for interactions

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Abbreviations: NOESY, nuclear Overhauser enhancement spectroscopy; HSQC, heteronuclear single-quantum coherence; PFG, pulsed field gradient; L-Trp, L-tryptophan.

with successive major grooves of B-DNA. In solution, an additional effect of the ligand is to stabilize the helix-turn-helix motif, especially the second helix which comprises part of the ligand binding pocket (Zhao et al., 1993).

Three-dimensional structures of *trp* holo-repressor/operator DNA complexes have also been determined by X-ray crystallography (Otwinowski et al., 1988; Lawson and Carey, 1993) and recently in solution by NMR (Zhang et al., 1994). The NMR structures agree with the X-ray results, both in the overall orientation of the two molecules as well as in specific amino acid side chain-DNA interactions, although the presence of intervening water molecules could not be confirmed in solution. In addition to structural information, NMR spectroscopy provides valuable information on the dynamics of the protein–DNA interaction, since NMR parameters can be strongly influenced by motions on the nano- to millisecond time scale. In NMR studies of the holo-repressor/DNA complex (Zhang et al., 1994), three dynamic processes were identified as being important to the stability and structure of the complex. These processes, in order of decreasing time scale, are (1) protein–DNA association/dissociation; (2) fluctuation or 'breathing' of the helices of the helix-turn-helix motif; and (3) exchange of the corepressor in and out of the complex.

The corepressor, L-tryptophan, is a key player in all three of these processes. Equilibrium binding constants have been determined for tryptophan binding to apo-repressor (K_D = 15–18 μM at 25 °C; Joachimiak et al., 1983; Lane et al., 1986; Marmorstein et al., 1987) and for holorepressor binding to operator DNA (K_D = 0.2–6 nM; Klig et al., 1987; Carey, 1988; Marmorstein and Sigler, 1989; Hurlburt and Yanofsky, 1992). Hurlburt and Yanofsky (1990,1992) have used filter binding to determine the half-life of the holorepressor–operator complex to be ~3 min at 37 °C. They also showed that the tryptophan rapidly dissociates from the complex, too fast for a dissociation constant to be determined using their technique. The affinity of tryptophan for the protein–DNA complex is greater than for the protein itself, probably due to additional favorable interactions with operator DNA. A putative hydrogen bond between the corepressor and DNA has been proposed from both X-ray and NMR data, based on the close contact of the heterocyclic amino nitrogen of L-tryptophan with a phosphate oxygen of the operator backbone (Otwinowski et al., 1988). The N-H bond of the indole ring is at an acceptable angle to form a hydrogen bond; however, neither X-ray nor NMR is capable of observing the hydrogen bond directly.

In this study, we have investigated the dynamic properties of L-tryptophan and the intermolecular interaction of the corepressor with both protein and DNA in the repressor/operator complex by heteronuclear ($^{13}C/^{15}N$) isotope-edited NMR experiments. For this purpose, we have isolated and purified microbially expressed L-tryptophan from media labelled with [$^{13}C_6$]-glucose and $^{15}NH_4Cl$. Isotope-edited NMR of the protein/DNA complex containing labelled corepressor has enabled the unambiguous identification of ligand resonances in both free and bound states, as well as a detailed study of the effects of ligand exchange and intermolecular interactions in this 37 kDa protein/DNA complex.

Materials and Methods

Sample preparation

Microbial fermentation of a *5-FT^r, IM^r, SG^r* mutant, *Bacillus subtilis AJ* 12099 FERM-P 7295 (Ajinomoto Co., Inc.), was used to prepare [ul-$^{13}C/^{15}N$]-L-Trp by following the procedures described previously (Ajinomoto Co., Inc., Japanese patent application 60-83594). The strain was cultured at 30 °C for 72 h in a medium containing: 98% ^{13}C [ul-^{13}C]glucose (ISOTEC Inc.), 8.0 g/dl; 99% $^{15}NH_4Cl$ (Shoko Co. Ltd.), 1.0 g/dl; KH_2PO_4, 0.1 g/dl; KCl, 0.2 g/dl; $MgSO_4 \cdot 4H_2O$, 0.04 g/dl; $FeSO_4 \cdot 7H_2O$, 0.001 g/dl; $MnSO_4 \cdot 7H_2O$, 0.001 g/dl; and casamino acids (DIFCO), 0.4 g/dl; pH 7.0. At the end of the fermentation period, L-Trp had accumulated to 730 mg/dl (9.1 w/w % to glucose). It was isolated as follows. After concentration to 1/20 of its initial volume (final L-Trp concentration ≈ 730 mg/5 ml) at pH 11 (adjusted by aqueous ammonia), 2 ml of glacial acetic acid was added to the filtered culture fluid. After several days at 4 °C, the crystallized L-Trp was collected and dried at 90 °C in order to remove crystalline water and acetic acid. Recrystallization from hot water containing a small amount of activated charcoal yielded 680 mg of [ul-$^{13}C/^{15}N$]-L-tryptophan. The ^{13}C and ^{15}N isotope concentrations were found to be very high (more than 95%) as judged by the ^{13}C NMR spectrum.

Trp apo-repressor protein was isolated from *E. coli* strain CY15070, grown on LB media and purified as previously described (Paluh and Yanofsky, 1986; Arrowsmith et al., 1990). The purified protein was dialysed against 500 mM NaCl/50 mM sodium phosphate buffer at pH 6. The concentration of the protein monomer units was measured at 280 nm using an extinction coefficient of 1.5×10^4 $M^{-1} cm^{-1}$ (Joachimiak et al., 1983). The palindromic consensus operator d(CGTACTAGAATTCTAGTACG) was synthesized on an Applied Biosystems 392 DNA/RNA synthesizer using standard phosphoramidite chemistry in 10 μM quantities and was detritylated prior to uncoupling from the resin. Residual impurities were removed by ethanol precipitation, followed by drying in a Savant Speedvac. The amount of DNA was determined at 260 nm with an extinction coefficient of 3.3×10^5 $M^{-1} cm^{-1}$ (Zhang, H. and Arrowsmith, C.H., unpublished results). The operator DNA was concentrated by lyophilization and its ^1H 1D NMR spectrum was checked against the published spectrum (Lefevre et al., 1987).

Trp holo-repressor/operator complex was formed by

mixing *trp* holo-repressor (as described above) with operator DNA in equimolar amounts, followed by repeated dilutions and concentrations via ultrafiltration (Centricon-10) into 50 mM sodium phosphate buffer at pH 6.0. The amount of L-Trp 'lost' during ultrafiltration was estimated by UV absorbance of the filtrate and an appropriate amount of fresh [ul-$^{13}C/^{15}N$]-L-tryptophan was added back into the solution on the final filtration step. The final concentration of corepressor in the protein/DNA complex was measured by the integration of free and bound indole NH resonances from the ^{15}N-decoupled (Shaka et al., 1983) ^1H 1D NMR spectrum. The concentration of the *trp* holo-repressor/operator complex for NMR experiments was ca. 1.0 mM in apo *trp*-repressor dimer, 4.0 mM $^{13}C/^{15}N$-labelled L-tryptophan and 1.0 mM double-stranded DNA. The solvent was either '100%' D_2O (Isotec Inc.) or 90% H_2O/10% D_2O with 50 mM sodium phosphate buffer at pH 6.0.

NMR measurements

All experiments in H_2O solution were performed on a Varian UNITY$^+$-500 spectrometer equipped with a triple-resonance probe with an actively shielded pulsed field gradient (PFG) coil. Data were collected in quadrature detection mode at 25, 37 and 45 °C. PFG techniques were used for all H_2O experiments, resulting in good suppression of the solvent signal without saturation of the water signal. Two-dimensional ^1H-^{15}N heteronuclear correlation exchange experiments (Farrow et al., 1994) were performed yielding 80 (^{15}N) × 1024 (^1H) complex matrices with 32 transients for each t_1 increment over a range of mixing times between 27.5–550 ms. The 2D ^{15}N-^1H HSQC experiment (Bodenhausen and Ruben, 1980) was performed using the enhanced sensitivity method of Kay et al. (1992). Data were collected as a 256 (^{15}N) × 1024 (^1H) complex matrix with 128 transients for each t_1 incrementation. Gradient-enhanced 2D ^{15}N-^1H gd-HSQC-ZZ experiments (Wider et al., 1991; Kay, L.E. et al., unpublished results) with three different mixing times, i.e., 10, 50 and 75 ms, were also performed in order to estimate the exchange rates. WALTZ-16 (Shaka et al., 1983) was used for nitrogen decoupling during the detection periods of all experiments in H_2O. The ^{15}N-edited 2D gd-NOESY data were collected as a 512 × 1024 data matrix with 128 transients for each t_1 increment, using mixing times of 50–200 ms.

All ^{13}C-edited experiments were performed in D_2O solution on a Varian Unity600 spectrometer. To identify ^{13}C resonances of the free and bound corepressor, two different decoupler offset frequencies (43 ppm for $^{13}C^{\alpha}/^{13}C^{\beta}$, and 120 ppm for ring carbons) were used for ^{13}C-^1H HSQC experiments. Low-power GARP (Shaka et al., 1983) decoupling for aliphatic and aromatic carbons was used during the detection period for both ^{13}C-^1H HSQC and ^{13}C-edited 2D NOESY experiments. Mixing times of 50–800 ms were employed in collecting ^{13}C-edited 2D NOESY spectra.

All 2D data sets were processed on Sun Sparc2 or Sparc10 workstations using commercial software (NMR2, New Methods Research Inc., Syracuse, NY; VNMR, Varian Associates). Peak volumes determined by the peak-picking routine in NMR2 were used for exchange rate calculations and NOE buildup curves.

Theory and calculations

For the case of exchange between two conformations (Clore and Gronenborn, 1982), the system can be described as:

$$A \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} B$$

where k_{on} is the forward exchange rate and k_{off} is the reverse exchange rate.

For both the ^{15}N-^1H HSQC-ZZ (Wider et al., 1991) and 2D NOESY experiments, the intensities of the diagonal and exchange cross peaks for the two-site exchange case (Lee and Krishna, 1992) at small mixing times, neglecting cross-relaxation effects, can be approximated as:

$$I(\text{Diagonal}) = M_{A0}\frac{1+\frac{k_{on}}{k_{off}}\{\exp(-)(k_{on}+k_{off})T_m\}}{1+\frac{k_{on}}{k_{off}}} \quad (1)$$

$$I(\text{Exchange}) = M_{A0}\,k_{on}\frac{1-\{\exp(-)(k_{on}+k_{off})T_m\}}{k_{on}+k_{off}} \quad (2)$$

where M_{A0} is the thermal equilibrium value of magnetization A, k_{on} and k_{off} are the forward and reverse exchange rates, respectively, and T_m is the experimental mixing time.

For the present case of a ligand exchanging between free and protein-bound forms in a solution containing excess free ligand, the following equilibrium holds.

$$[E] + [L] \underset{k_-}{\overset{k_+}{\rightleftharpoons}} [EL]$$

where k_+ is the forward rate, [E] and [L] are the free protein and free ligand concentrations, respectively and [EL] is the concentration of the protein/DNA complex. In this case the pseudo-first-order rate constant (k_{on}) is given by Eq. 3:

$$k_{on} = k_+[E] \quad (3)$$

The free protein concentration can be expressed as

$$[E] \approx [E_T]K_d/(K_d + [L_T] - [E_T]) \quad (4)$$

where $[E_T]$ and $[L_T]$ are the total protein and ligand con-

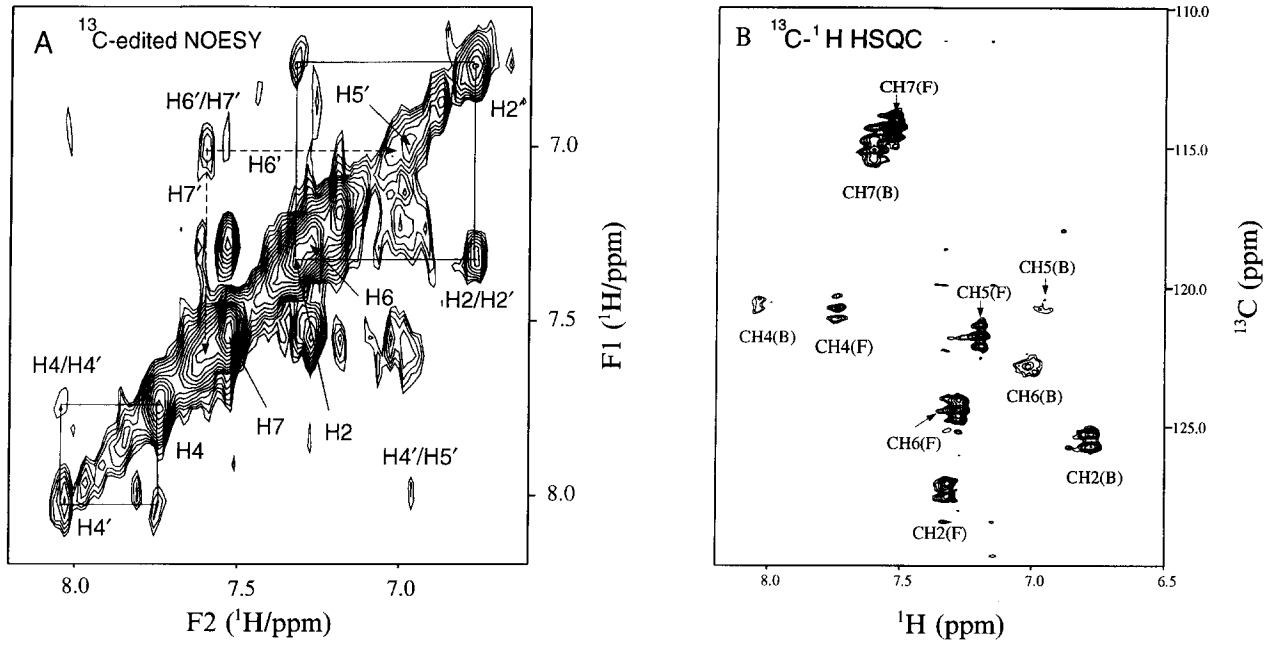

Fig. 1. (A) ^{13}C-edited 2D NOESY spectrum of *trp*-repressor/operator/[ul-^{13}C/^{15}N]-L-Trp complex with a mixing time of 150 ms at 45 °C in D_2O. The area of the indole ring proton resonances of the free and bound (primed) corepressor is shown with proton numbers. Examples of chemical exchange and sequential resonance peaks are labelled. (B) ^{13}C-^{1}H HSQC spectrum, displaying correlated peaks between covalently bonded ^{13}C and ^{1}H resonances of the indole ring of the free and bound corepressor in the protein/DNA complex. The carbon carrier frequency was set to 120 ppm. Peaks appear as multiplets in the carbon dimension due to carbon–carbon coupling. The weaker intensities of the bound ligand resonances reflect the increased T_2 relaxation of the bound species during the pulse sequence.

centrations, respectively, and K_d (k_-/k_+) is the dissociation constant. By substituting Eq. 4 and K_d into Eq. 3, k_{on} can be expressed as:

$$k_{on} = ([E_T]/[L_T])k_{off}/\{1 - ([E_T]/[L_T]) + K_d/[L_T]\} \quad (5)$$

In our case the range of the dissociation constant is small ($K_d \approx 0.01$~0.0001 mM) compared to the ligand concentration (~1 mM), and the concentration ratio between *trp*-repressor and L-tryptophan ($[E_T]/[L_T]$) is 1:2; the value of (k_{on}/k_{off}) (Eq. 5) approaches 1. If we define R as the ratio of exchange cross peak to diagonal cross peak in a NOESY spectrum (R = I(Exchange)/I(Diagonal)), the reverse exchange rate, k_{off} (pseudo-first-order rate constant) can be simply expressed from Eqs. 1, 2 and 5 as:

$$k_{off} = [(-1/2)T_m]\ln[(1 - R)/(1 + R)] \quad (6)$$

The lifetime of the corepressor in the complex can therefore be expressed as $\tau = 1/k_{off}$.

It is important to note that this method of analysis does not take into account the transfer of ^{1}H magnetization to other spins due to cross-relaxation during the mixing time. For a complex with a molecular weight as

TABLE 1
CHEMICAL SHIFTS OF THE FREE AND BOUND ^{13}C/^{15}N-LABELLED COREPRESSOR[a]

Atom	Chemical shifts (ppm)		
	^{1}H	^{13}C	^{15}N
Free			
α	4.04	57.45	
β	3.27,3.45	28.07	
Ring	7.24(H2); 7.66(H4); 7.11(H5); 7.19(H6); 7.45(H7) 10.12(R:NH)	127.0(C2); 120.7(C4); 121.8(C5); 124.6(C6); 113.5(C7)	130.20
Bound			
α	4.16	57.60	
β	2.68,3.16	28.24	
Ring	6.77(H2); 8.04(H4); 6.88(H5); 6.92(H6); 7.53(H7); 11.27(R:NH)	125.9(C2); 120.2(C4); 120.6(C5); 122.9(C6); 114.1(C7)	133.2

[a] Chemical shifts are referenced to external $^{13}CH_3CO_2Na$ (25.85 ppm) for ^{13}C and ^{15}N-labelled urea (78.98 ppm) for ^{15}N chemical shifts, respectively. Experimental conditions: 45 °C, pH 6.0.

studied here, the contribution from such effects is expected to be significant. To minimize the error due to cross-relaxation, we have used only small mixing times (50 ms) to derive rates from NOESY and HSQC-ZZ experiments. In addition, we have performed a new experiment, recently developed by Kay and co-workers (Farrow et al., 1994), which is designed to investigate exchange phenomena in the absence of cross-relaxation by measuring solely the change in ^{15}N longitudinal magnetization due to exchange.

Exchange rate constants from the ^{15}N exchange experiments were determined by fitting the decay and buildup of the auto and exchange peaks to the solution of the Bloch equations for two-site exchange, as described by Farrow et al. (1994). A least-squares fitting procedure was used to extract parameters corresponding to the best fit of the theoretical decay/buildup curves to the measured data. Errors in the fitting parameters were determined using a Monte Carlo approach (Kamath and Shriver, 1989; Palmer et al., 1991).

Results

Resonance assignments

Resonance assignments for the ring protons of the free and bound corepressor were made from characteristic connectivities of the indole ring protons from ^{13}C-edited 2D NOESY spectra. Figure 1A shows the ring proton resonances for both the free and the bound form; assignments are indicated in the figure. All of the ring proton chemical shifts of the bound corepressor are different from those of the free form. H2 (6.77 ppm) and H4 (8.04 ppm) of the bound corepressor show the largest chemical shift changes upon complexation (Table 1). The assignments of H^{α} and $H^{\beta},H^{\beta'}$ were also obtained from ^{13}C-edited 2D NOESY spectra in D_2O. The ^{13}C chemical shifts of the free and bound corepressor were readily obtained from the ^{13}C-^1H HSQC spectrum (Fig. 1B), based on the corresponding ^1H assignments.

The indole NH proton and nitrogen resonances of the free and bound corepressor were easily identified by their characteristic chemical shifts from ^{15}N-edited 1D and ^{15}N-^1H HSQC spectra in H_2O solution. The indole proton chemical shift of the free form is close to the random coil value (10.12 ppm; Wüthrich, 1986), whereas that of the bound form is significantly shifted downfield (11.27 ppm) (Fig. 2A). No significant proton chemical shift variations of either the free or the bound corepressor were observed at temperatures of 25, 37 and 45 °C (data not shown). The ^1H, ^{15}N and ^{13}C chemical shift values are listed in Table 1.

Dynamic properties of the corepressor

Exchange rates were measured for two different pairs of protons in four different types of spectra. In the ^{15}N-^1H gd-HSQC-ZZ experiment (Wider et al., 1991; Kay, L.E. et al., unpublished results), both ^{15}N-^1H correlation peaks for the bound indole ring, as well as exchange peaks were used for exchange rate measurements. The diagonal and exchange cross peaks of the indole NH were also used for rate calculations from the ^{15}N-edited 2D gd-NOESY spectrum. In the ^{13}C-edited 2D NOESY spectrum, the diagonal and exchange peak intensities of the ring H2 proton were monitored. Although exchange peaks were also detected from the H4, H5, H6 and H7 ring protons in the ^{13}C-edited 2D NOESY spectrum, these were not

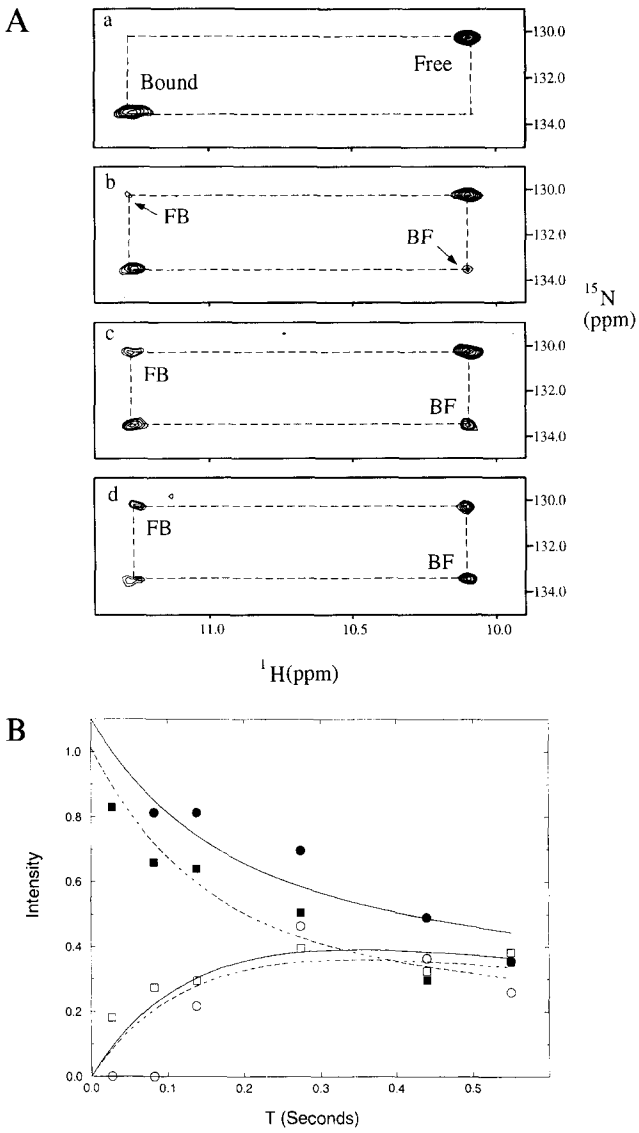

Fig. 2. (A) ^{15}N-^1H heteronuclear correlated exchange spectrum, showing both auto (B, bound; F, free) and exchange peaks (BF and FB) of the indole ring NH of L-[ul-^{13}C/^{15}N]trp in the protein/DNA complex. Mixing times of (a) 27.5, (b) 137.5, (c) 275 and (d) 550 ms were used. (B) Experimental data and fitted curves for the indole NH of L-Trp. Filled and open symbols are used to indicate experimental data points for the auto and exchange peaks, respectively. Circles indicate that magnetization originates from the free form, whereas squares indicate that magnetization originates from the bound form.

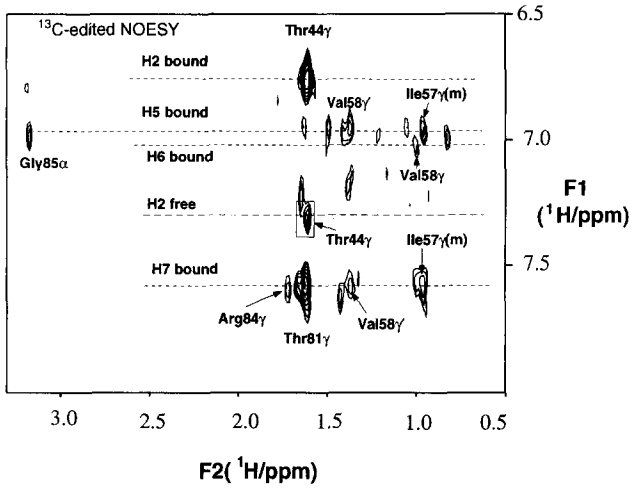

Fig. 3. ^{13}C-edited 2D NOESY spectrum of [ul-^{13}C/^{15}N]-L-Trp recorded with a mixing time of 150 ms at 45 °C in D_2O solution. Direct and exchange-mediated (boxed) intermolecular NOEs between corepressor and *trp*-repressor are shown.

used because the peak volumes were inaccurate due to resonance overlap.

To determine an exchange rate (k_{off}) of the corepressor free of cross-relaxation effects, experiments were carried out for the simultaneous measurement of longitudinal ^{15}N decay rates and chemical exchange rates (Farrow et al., 1994). Because this ^{15}N exchange experiment measures changes in ^{15}N magnetization, it is free from cross-relaxation effects that can complicate measurements involving ^{1}H longitudinal magnetization such as those described above (Lippens et al., 1992). Figure 2A shows both auto and exchange peaks of the indole NH proton and nitrogen with mixing times of 27.5, 137.5, 275.0 and 550 ms. It is clearly seen that the intensities of exchange peaks increase and those of auto peaks decrease with increasing mixing times. Figure 2B shows the measured data points and curves obtained from a least-squares fitting procedure (Farrow et al., 1994). The calculated longitudinal decay rate of the indole ^{15}N in the bound state and the exchange off-rate are 1.25 ± 0.39 s^{-1} and 3.40 ± 0.52 s^{-1}, respectively.

Table 2 summarizes the exchange rates and lifetimes calculated from the four independent experimental data sets described above. The experimental error in each measurement is approximately 10–15%, due to the poor signal-to-noise ratio associated with the shorter mixing times and the broad line widths of the bound ligand. Taking this experimental error into account, all values are similar, giving an off-rate for corepressor exchange of approximately 3 s^{-1}. Comparison of the absolute values of the off-rate measured from the ^{15}N exchange experiment with the other three values suggests that errors in the latter, due to cross-relaxation in the bound state, may be on the order of ~20%. Taking into account the experimental error in each value puts an upper limit of ~50% on the effects of cross-relaxation.

NOE measurements

In the ^{15}N- and ^{13}C-edited 2D NOESY spectra collected here, five types of cross peaks were observed: (1) chemical exchange peaks between free and bound corepressor; (2) intermolecular NOEs between bound corepressor and *trp*-repressor; (3) intermolecular NOEs between bound corepressor and operator DNA; (4) intramolecular NOEs within bound and free corepressor; and (5) exchange-mediated NOEs from free ligand to *trp*-repressor. Most of the NOEs to the *trp*-repressor are in a crowded region between 0.9 and 2.0 ppm, characteristic of the hydrophobic binding pocket (Fig. 3). The corepressor has close contacts with both *trp*-repressor and operator, although the contact surface with the protein is much greater than with the operator, mostly involving hydrophobic interactions with side chains of the protein (methyl groups of Thr^{81}, Thr^{44}, Ile^{57}, Val^{58} and methylene groups of Arg^{54} and Arg^{84}). The indole H2 proton has weak NOE contacts to the sugar protons (H3' and H4') of A(12) of the DNA, indicating interaction of the corepressor with the operator. The observed intermolecular NOE intensities from the present study are summarized qualitatively in Table 3.

Influence of conformational exchange on the observed NOE intensities

For the case of conformational and chemical exchange in multiple conformations, a number of reports (Clore and Gronenborn, 1982; Keepers and James, 1984; Fejzo et al., 1991; Ni, F., 1992; Lee and Krishna, 1992; Lippens

TABLE 2
EXCHANGE RATES AND LIFETIMES OF TRYPTOPHAN IN THE REPRESSOR–OPERATOR COMPLEX AT 45 °C[a]

	^{15}N exchange	gd-HSQC-ZZ	^{13}C-edited NOESY	^{15}N-edited NOESY
I(A'A')		15.65	8.62	16.25
I(AA')		2.54	1.20	2.38
R		0.162	0.140	0.146
k_{off} (s^{-1})	3.40 ± 0.52	3.23[b]	2.81[b]	2.94[b]
Lifetime (ms)	294	310	355	340

[a] I(A'A') and I(AA') are the intensities of the diagonal and exchange peaks from the bound and free forms, respectively. R is calculated as I(AA')/I(A'A').

[b] The error was estimated as about 10%, due to inaccuracies in peak integration.

et al., 1992; London et al., 1992) have pointed out that exchange effects should be considered for an accurate interpretation of NOE data. Figure 4 shows experimental buildup curves for the intermolecular NOEs between the H2 proton of the corepressor and Thr^{44} H^{γ} of the protein (line a), H7 of the corepressor and Thr^{81} H^{γ} of the protein (line b) and an exchange-mediated NOE (line c) between H2 of the free tryptophan and Thr^{44} H^{γ} of the protein. Curve c in Fig. 4 clearly demonstrates the 'exchange-lag phase' of exchange-mediated NOEs as observed in transferred NOE measurements (Kohda et al., 1987; Rosevear and Mildvan, 1989; Lee and Krishna, 1992). Figure 4 also shows that the intermolecular NOEs reach their maximum intensities at a mixing time of 150 ms, much longer than expected for a molecular weight of 37 kDa. This is clearly the effect of ligand exchange on the NOE buildup rates, resulting in an 'effective mixing time' which differs from the real mixing time, depending on the rate of exchange (Kohda et al., 1987; Rosevear and Mildvan, 1989; Lee and Krishna, 1992; Lippens et al., 1992). These data also suggest that, in order to observe medium or weak NOEs under conditions similar to those employed here, it may be desirable to use a longer mixing time than normally used for a regular NOESY. The data also indicate that under these conditions interproton distances derived from a mixing time of 150 ms (point of maximum intensity) are likely to be longer than the true distances, due to intensity losses from the exchange effect.

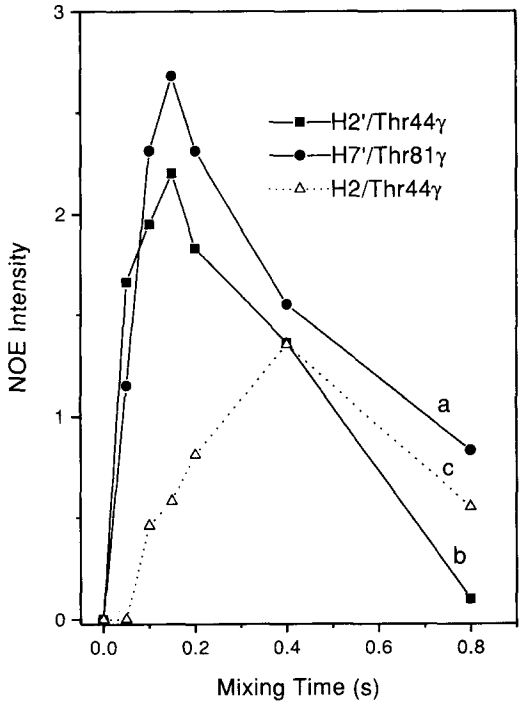

Fig. 4. Experimental intermolecular (traces a and b) and exchange-mediated (trace c) NOE buildup curves for three selected examples from ^{13}C-edited 2D NOESY data. The curves consist of simple straight lines connecting each point.

TABLE 3
INTERMOLECULAR NOEs OF COREPRESSOR TO *trp*-REPRESSOR AND OPERATOR DNA

Corepressor	Protein/DNA[a]
H^{β}	Thr^{81} H^{γ} (m)
H2	Thr^{44} H^{γ} (s), A(12) H3' (w) (DNA)
	A(12) H4' (w) (DNA)
H5	Met^{42} H^{β} (w), Arg^{54} H^{α} (w), Ile^{57} H^{γ} (w),
	Val^{58} H^{γ} (w), Val^{58} H^{γ} (m), Ile^{57} H^{γ} (Me) (m),
	Gly^{85} H^{α} (m)
H6	Val^{58} H^{γ} (w), Ile^{57} H^{γ} (w)
H7	Ile^{57} H^{γ} (Me) (m), Val^{58} H^{γ} (m),
	Thr^{81} H^{γ} (s), Arg^{84} H^{γ} (m)
NH (Ring)	Thr^{81} H^{γ}(w)

[a] NOE intensities are classified as s (strong), m (medium) and w (weak) at mixing times of 50–150 ms at 45 °C. The weak NOEs are sensitive to the processing window functions due to weaker intensities by exchange contributions (see text).

Discussion and Conclusions

A novel bacterial strain was used to isolate large quantities of uniformly ^{13}C- and ^{15}N-labelled L-tryptophan for functional studies of the regulation of the *trp*-repressor–operator interaction. With this labelled material we obtained more detailed information on the structure and dynamics of the tryptophan ligand than was possible using the natural abundance material. For example, the assignments of the α- and β-protons of the bound natural abundance ligand within the protein–DNA complex were ambiguous, due to lack of scalar connectivities in the bound state (Zhang et al., 1994). A strong exchange-mediated NOE between the H^{α} proton of the free ligand and a proton of the bound ligand at 3.16 ppm suggested that the latter was the α-proton of the bound ligand. However, the ^{13}C-^{1}H HSQC spectrum of the labelled ligand clearly shows that the proton at 3.16 ppm is covalently bound to a β-carbon. Therefore, this proton must be H^{β} of the bound ligand and the exchange-mediated NOE is from H^{α} of the free ligand to H^{β} of the bound form. The α-proton chemical shift of the bound ligand is, in fact, very close to that of the free ligand. Thus, the exchange peak between free and bound α-protons is buried in the diagonal of the NOESY spectrum and is not observable. Two-dimensional ^{13}C-edited NOESY spectra of the ligand also revealed additional NOEs from the ligand to the protein that were not identified in previous work (Zhang et al., 1994).

The use of labelled L-Trp has also provided a means for the detailed study of the exchange of the ligand from the protein–DNA complex. A semiquantitative determination of the exchange off-rate, free from effects of cross-relaxation, has allowed determination of the error on the NOE exchange peaks introduced by cross-relaxation. These errors can be as large as about 50% for the current system.

The lifetime of the ligand in the complex (~0.3 s at 45 °C) is roughly three orders of magnitude less than the half-life of the complex itself (~180 s at 37 °C; Hurlburt and Yanofsky, 1992). This relatively rapid diffusion of the corepressor in and out of the complex allows the operons involved in tryptophan biosynthesis and transport to immediately sense and respond to changes in corepressor concentration. Thus, there is a clear biological explanation for this phenomenon. However, *structurally* it is not clear how the tryptophan can get out of the complex. A detailed analysis of the NOEs from the ligand and the structure of the complex provides some clues.

The NOEs from the ligand to the protein involve mainly methyl or methylene groups in the highly hydrophobic binding pocket composed of adjacent faces of helices C (residues Arg^{54}, Ile^{57} and Val^{58}) and E (residues Thr^{81}, Arg^{84} and Gly^{85}). This same pattern of NOEs is seen for the binary corepressor–repressor complex (Arrowsmith et al., 1990), consistent with the almost identical binding mode seen in the X-ray structures of the repressor (Schevitz et al., 1985) and the DNA complex (Otwinowski et al., 1988). Since mutations in most of these residues abolish ligand binding, the interactions reflected by the NOEs listed in Table 3 are highly specific. For example, Arvidson et al. (1991) have shown that mutation of Val^{58} to any other of the 19 amino acids required higher concentrations of tryptophan for activation of DNA binding. Similarly, mutations of $Thr^{44} \rightarrow$ Met, $Arg^{54} \rightarrow$ His and $Arg^{84} \rightarrow$ His all disrupt the hydrophobic interactions reflected in the NOEs and also abolish ligand binding (Kelly and Yanofsky, 1985; Hurlburt and Yanofsky, 1990).

The NOEs from the H2 proton of the bound ligand to sugar protons of A(12) of the DNA, together with the large downfield shift of the indole NH proton resonance (Table 1), provide strong supporting evidence for the existence of a hydrogen bond between this NH and the phosphate backbone and are in agreement with X-ray crystal structures (Otwinowski et al., 1988; Lawson and Carey, 1993). Thus, the corepressor directly participates in energetically favorable interactions with the operator, and this hydrogen bond must be broken for exchange to occur.

Since the ligand is 'sandwiched' between the protein and the DNA, with contacts to both species, this would imply significant motion of the protein and/or DNA in the ligand binding region associated with exchange. Indeed, many of the residues in helix C were difficult to assign due to broader (relative to the rest of the protein) resonances and lack of observable NOEs (Zhang et al., 1994). On the other hand, helix E, the second helix of the helix-turn-helix motif, is known to be flexible in the apo- and holo-repressors (Lawson et al., 1988; Arrowsmith et al., 1991; Czaplicki et al., 1991) and fast NH exchange rates for helix E in the complex (Revington, M. and Arrowsmith, C.H., unpublished results) also suggest flexibility while bound to DNA. Helix E forms one face of the ligand binding pocket, with significant hydrophobic interactions to residues Gly^{85} and Arg^{84}. Breathing of this helix or of the helix-turn-helix motif could also be a mechanism for release of the corepressor.

Obviously, additional data are needed to further detail the mechanism of corepressor exchange in this system. Preliminary data of a complex with the corepressor analog, 5-methyl tryptophan, indicate that this ligand is in slow exchange on the NMR timescale, reflecting its 10-fold greater affinity for the protein (Marmorstein et al., 1987). A comparison of protein relaxation rates and NH exchange rates in the complexes of L-Trp and 5-methyl-L-Trp should allow a correlation to be made between flexible regions of the *trp*-repressor and the rate of ligand exchange.

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