Dynamics of Tryptophan Binding to *Escherichia coli Trp* Repressor Wild Type and AV77 Mutant: An NMR Study†

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ABSTRACT: Binding of L-tryptophan to *Escherichia coli trp* repressor wild type (WT) and AV77 mutant was studied by ^{1}H NMR spectroscopy. Ligand binding to the proteins resulted in changes in line widths and chemical shifts of ligand resonances, but no changes in the coupling constant were observed. Line width and chemical shift changes of the H_4 L-tryptophan proton were monitored as a function of temperature and ligand and protein concentrations. For the WT repressor, the H_4 proton displays slow exchange at low temperatures (20−35 °C), while fast exchange occurs in the range from 45 to 65 °C. From 35 to 40 °C, the range of intermediate exchange, lines are broadened beyond detection. For the AV77 mutant, the intermediate and fast exchange regions are shifted at least 5 °C to higher temperatures. Line shapes of L-tryptophan H_4 proton resonances were simulated using a general expression based on McConnell's modified Bloch equations for a two-site exchange. From the simulations, an exchange frequency (ν_{exch}) of about 3000 Hz was obtained at 45 °C for WT and about 1000 Hz for AV77 mutant. The activation energy for the process is 32.7 kcal $K^{-1}mol^{-1}$ for the WT and 29.1 kcal K^{-1} mol^{-1} for AV77. At 45 °C, the dissociation and association rate constants (k_{-1} and k_{+1}, respectively) were calculated to be 2.0×10^3 s^{-1} and 9.9×10^6 M^{-1} s^{-1}, for the WT. For the AV77 mutant, k_{-1} and k_{+1} were 6.4×10^2 s^{-1} and 2.7×10^6 M^{-1} s^{-1}, respectively. The association rates are in the lower border of those values expected for diffusion-controlled processes. Taken together, the data indicate that a sizable barrier of energy is involved in the binding of L-tryptophan to *trp* repressor WT and AV77 mutant. The biological implications of the binding of L-tryptophan to AV77 mutant as compared to the wild type are discussed.

The *trp* repressor of *Escherichia coli* is a protein responsible primarily for the regulation of L-tryptophan biosynthesis. As shown by crystallographic (Schevitz et al., 1985; Zhang et al., 1987) and NMR studies (Arrowsmith et al., 1991; Zhao et al., 1993), the protein is composed of six helices, labeled A−F. Helices A, B, C, and F form a hydrophobic core, and helices D and E form a helix−turn−helix motif that contains the DNA binding site. The symmetric dimeric protein binds two molecules of the corepressor L-tryptophan to form a holorepressor with high affinity for specific DNA operons in *E. coli.* The tryptophan binding site is formed by residues of helices B, C, and E.

The mutation Ala^{77}→Val transforms the protein into a superrepressor (Kelley & Yanofsky, 1985) with enhanced activity *in vivo* compared to the wild type in the presence of low concentrations of L-tryptophan (Arvidson et al., 1993; Shapiro et al., 1993). However, the mechanism by which this is accomplished is not clear, especially taking into consideration the results obtained *in vitro.* The affinity of the superrepressor apo form to the operator is increased, being comparable to that of the holo form of the wild type, but, on the other hand, the affinity of the holo form of AV77 mutant to DNA is similar to that of the wild type (Hurlburt & Yanofsky, 1990). The binding of L-tryptophan to the protein also seems not to be affected (Hurlburt & Yanofsky, 1990). It was argued that the enhanced activity of AV77 *in vivo* is due to a decrease in the binding to nonspecific DNA rather than to an increased affinity to the specific operator (Arvidson et al., 1993).

Recent studies on the structure and dynamics of AV77 mutant, both in the apo and in the holo forms (Gryk & Jardetzky, 1995), provide an interesting observation. It was shown that although there are no substantial differences between the WT and AV77 repressor structures, most of the hydrogen-bonded amide protons in the DNA binding region are stabilized by at least 1 order of magnitude for both forms of the mutant repressors (apo and holo). It was postulated that the enhanced activity of AV77 *in vivo* can be ascribed to a decrease in the instability of the DNA binding region. In support to this idea is the fact that the binding of the corepressor to the wild-type *trp* repressor also increases the stability of the DNA binding region (Gryk et al., 1994; Finucane & Jardetzky, 1995).

Here we report on the dynamics of binding of L-tryptophan to *trp* repressor, comparing the behavior of the WT with the mutant AV77. The process of exchange between L-tryptophan and both repressors was studied by analyzing ^{1}H NMR spectra of tryptophan in the presence of both repressors, at different ligand:protein ratios, at different temperatures. Full line shape analysis of L-tryptophan H_4 proton resonances in the free and bound states was performed by simulating the experimental lines. The simulations yielded the frequency of exchange between L-tryptophan and the repressors as well as the binding constant. Other parameters could also be obtained, as the dissociation and association rate constants (k_{-1} and k_{+1}, respectively).

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MATERIALS AND METHODS

Sample Preparation. *Trp* repressor wild type and AV77 mutant were isolated from *Escherichia coli* strains CY15070 and CY15071, respectively, both carrying the pJPR2 expression plasmid (Paluh & Yanofsky, 1986). Both strains were grown in minimal medium (Vogel & Bonner, 1956). For strain CY15070, ampicillin (200 mg L^{-1}) was added to the medium, and for strain CY15071, ampicillin (200 mg L^{-1}) and threonine (100 mg L^{-1}) were added. The procedures for protein extraction and purification were essentially as described by Paluh and Yanofsky (1986).

Samples were suspended in pH 6.0 or 7.6 buffer containing 50 mM NaH_2PO_4 and 500 mM NaCl, using an Amicon pressurized stir cell, and concentrated in an Amicon centrifuge concentrator. 100 μM TSP [3-(trimethylsilyl)tetradeuteriosodium propionate] was added as an internal reference for proton chemical shift, and the samples were lyophilized overnight. The dried proteins were resuspended in 98% ^2H_2O (Cambridge Isotope Laboratories) and heated to 65 °C for 10 min to allow for the labile protons to exchange. Samples were lyophilized overnight again and resuspended in 100% ^2H_2O (Cambridge Isotope Laboratories). The pH* of samples was measured before and after the NMR experiments and remained constant within 0.1 pH unit.

Protein concentrations were measured using an extinction coefficient (ϵ) of 1.48 cm^{-1} M^{-1} at 280 nm (Joachimiak et al., 1983). The same ϵ was used for both wild-type and AV77 mutant proteins, since it was calculated considering the sum of the contributions of all aromatic residues, which is not expected to differ between the two proteins.

NMR Spectroscopy. NMR spectra were acquired on a Bruker AM-500 spectrometer. The residual H_2O resonance present in samples was suppressed by a presaturation pulse of 40 L. Presaturation was used for both 1D and 2D spectra. A total of 32 scans were acquired for 1D spectra, with 10 s delay between scans. For NOESY spectra (Jeener et al., 1979), 1024 complex points were acquired in t2, with 512 slices in t1. The spectral width in both dimensions was 6024.1 Hz. NOESY spectra were performed using standard pulse sequences with a mixing time of 150 ms. All spectra were processed on a Silicon Graphics Iris Indigo workstation using the FELIX NMR data processing package developed by Hare Research. NOESY spectra were zero-filled to 2048 complex points in t2, apodized using a squared sine-bell function shifted by 45°, and base-line-corrected in both dimensions. The residual water signal was mathematically removed using a convolution algorithm to allow more accurate phasing. 1D spectra were Fourrier-transformed and base-line-corrected only, so that the natural line width was conserved.

THEORY

Trp repressor binds two molecules of tryptophan in two independent sites with identical affinities. Since no cooperativity is involved (Arvidson et al., 1986; Lane, 1986; Marmostein et al., 1986; Jin et al., 1993), the binding of tryptophan to *Trp repressor* can be represented by the equilibrium:

$$P + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} PL \qquad (1)$$

where P = *trp*R (protein), L = tryptophan (ligand), and PL is the protein−ligand complex. k_{+1} and k_{-1} are the association and dissociation rate constants, respectively.

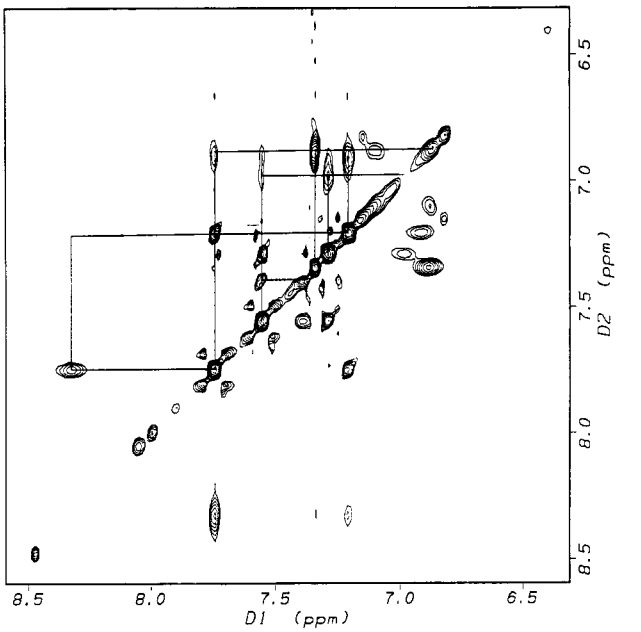

FIGURE 1: Aromatic region of the NOESY spectrum (at 20 °C) of 8.4 mM L-tryptophan in the presence of 1.1 mM *trp* repressor WT (in dimers). The sample was suspended in D_2O and contained 50 mM Na_2HPO_4 and 500 mM NaCl, pH* 7.6. Crosspeaks between bound and free resonances are indicated.

From the definition of the equilibrium constant for the process, the following relationship can be written:

$$K_D = \frac{[P][L]}{[PL]} = \frac{k_{-1}}{k_{+1}} \qquad (2)$$

For a two-site exchange, the line shape (amplitude of the signal as a function of frequency) is given by the imaginary part of $G(\nu)$ (Feeney et al., 1979):

$$G(\nu) = \frac{iC[2p_L p_{PL}\tau - \tau^2(p_L\alpha p_{PL} + p_{PL}\alpha_L)]}{p_L p_{PL} - \tau^2\alpha + \alpha_{PL}} \qquad (3)$$

where C is a scaling factor and p_L and p_{PL} are the fractional populations of free and bound ligand, respectively, and can be expressed by:

$$p_L = \frac{1}{2}\left(\frac{-(K_D + P_T - L_T) + \sqrt{(K_D + P_T - L_T)^2 + 4K_D L_T}}{L_T}\right) \qquad (4a)$$

and

$$p_{PL} = 1 - p_L \qquad (4b)$$

where P_T is the total protein concentration and L_T is the total ligand concentration.

$1/\tau$ is the exchange frequency (ν_{ex}), and can be written as

$$\frac{1}{\tau} = \nu_{ex} = \frac{1}{\tau_{PL}} + \frac{1}{\tau_L} = k_{-1}\left(1 + \frac{p_{PL}}{p_L}\right) \qquad (5)$$

where $1/\tau_{PL}$ and $1/\tau_L$ are the lifetimes in the bound and free state, respectively, and

$$\alpha_L = -(2\pi i[\nu_L - \nu] + [1/T_{2L}^*] + p_{PL}/\tau) \quad (6a)$$

$$\alpha_{PL} = -(2\pi i[\nu_{PL} - \nu] + [1/T_{2PL}^*] + p_L/\tau) \quad (6b)$$

where $1/T_{2L}^*$ and $1/T_{2PL}^*$ are the apparent transversal relaxation times in the absence of chemical exchange of free and bound ligand, respectively. ν_P and ν_{PL} are the resonance frequencies of the free and bound ligand, respectively. ν is the variable frequency. Combining eqs 2–6, $G(\nu)$ will be a function of

$$G(\nu) = f(\nu_L, \nu_{PL}, T_{2L}^*, T_{2PL}^*, K_D, \nu_{ex}, L_T, P_T, \nu) \quad (7)$$

To include the J coupling constant in the simulation of line shape of the L-tryptophan H_4 proton, eq 7 was rewritten as

$$G_f(\nu) = \frac{1}{2}[G_A(\nu) + G_B(\nu)] \quad (8)$$

where

$$G_A(\nu) = f\left(\nu_L + \frac{1}{2}J, \nu_{PL} + \frac{1}{2}J, T_{2L}^*, T_{2PL}^*, K_D, \nu_{ex}, L_T, P_T, \nu\right) \quad (9a)$$

$$G_B(\nu) = f\left(\nu_L - \frac{1}{2}J, \nu_{PL} - \frac{1}{2}J, T_{2L}^*, T_{2PL}^*, K_D, \nu_{ex}, L_T, P_T, \nu\right) \quad (9b)$$

are modified forms of eq 7, including the terms $\nu_L \pm {}^1/_2J$ and $\nu_{LP} \pm {}^1/_2J$.

More precise values for the exchange frequency ν_{ex} and dissociation constant K_D were obtained by minimizing the following merit function χ^2:

$$\chi^2(\nu_{ex}, K_D, a, b) = \frac{1}{N}\sum_{i=1}^{N}\left\{\frac{[aG(\nu_{ex}, K_D, \nu_i)_{calc} + b] - G(\nu_i)_{exp}}{\sigma}\right\}^2 \quad (10)$$

where the index i refers to the ith experimental point and a and b are scaling and shift factors, respectively. The subscript "exp" refers to the experimental intensities. The subscript "calc" refers to the calculated values obtained from eq 3, with the known concentrations P_T and L_T kept constant. σ is the standard deviation of the peak intensity and was estimated from the noise level of the spectrum.

The chemical shifts ν_L and ν_{PL}, obtained from NOESY spectra at 20 °C were assumed to be invariant with temperature. The four parameters (ν_{ex}, K_D, a, b) for data at higher temperature range (45–65 °C) were optimized using the minimizing function χ^2 (eq 10) using a Marquardt nonlinear least-squared routine (Press et al., 1989). For data at the lower temperature range (20–35 °C), K_D was extrapolated from high-temperature values, and only three parameters (ν_{ex}, a, b) were optimized.

RESULTS

L-Tryptophan experiences a magnetically different environment when it binds to *trp* repressor, resulting in different chemical shift values for the free and the bound states. Precise values for the chemical shifts of both states were obtained from the NOESY spectrum of the ligand in the presence of the protein, at 20 °C. Figure 1 shows the aromatic region of the NOESY spectrum of L-tryptophan in D_2O, at pH* 7.6, in the presence of *trp* repressor WT. The connectivities between the bound and the free peaks are indicated. Tryptophan chemical shifts in the free and bound states are listed in Table 1. The values obtained agree with those reported at 22 °C by Hyde et al. (1991), within 0.01 ppm.

Table 1: Chemical Shifts (ppm) at 20 °C of the Aromatic Protons of L-Tryptophan Free and Bound to *Trp* Repressor Wild Type[a]

	chemical shift (ppm)	
proton	free[b]	bound[c]
H_4	7.74	8.32
H_7	7.55	7.39
H_2	7.33	6.88
H_6	7.29	6.98
H_5	7.21	6.91

[a] The sample was prepared in D_2O and contained 50 mM Na_2HPO_4 and 500 mM NaCl, pH* 7.6. [b] 6.87 mM L-tryptophan. [c] *Trp* repressor WT 1.1 mM (in subunits) + 8.4 mM L-tryptophan.

In order to determine the exchange frequency of tryptophan with *trp* repressor WT and AV77, a series of 1D ^1H NMR spectra were obtained at different ligand:protein ratios and temperatures. The H_4 proton resonance of tryptophan was chosen to be monitored because it presents the smallest degree of overlap to protein peaks, both in the free and in the bound states. Figure 2 shows part of the aromatic region of the ^1H NMR spectra of L-tryptophan containing the H_4 proton resonances in the free and bound states, at different temperatures, in the presence of WT and AV77, at similar ligand:protein ratios. Between 7.65 and 8.40 ppm, protein peaks are observed in addition to the H_4 resonance peaks of L-tryptophan: H_2 of His35, H_2 of His16, and H_4 of Trp19 (assigned in the figure). The spectra are similar for both WT repressor and the AV77 mutant, the latter showing slightly broader line widths in general. As the temperature increases, all protein resonances sharpen, and the chemical shifts move toward higher field, but at different extents. In the temperature range of 20–65 °C, while H_4 of Trp19 and H_2 of His35 change about 0.07 and 0.02 ppm, respectively, H_2 of His16 presents a strong temperature dependence, moving upfield by more than 0.35 ppm. This effect is due to the influence of the H_3 proton of His16 whose pK_a has a strong dependence on temperature. His16 is more exposed to the solvent while His35 is protected by protein packing (Zhao et al., 1993), and therefore does not present the same effect.

On the other hand, the chemical shift and line width patterns of the ligand as a function of temperature are quite different, due to chemical exchange between free and bound states. For the WT repressor, separated peaks for the bound and the free ligand can be distinguished from 20 to 30 °C, with line widths increasing with increasing temperature, while the chemical shifts do not change significantly. Under these conditions, the system is in slow exchange. From 35 to 40 °C, the system is in intermediate exchange, where the resonance lines of bound and free states are very broad and move toward each other until they collapse. From 45 to 65 °C, one single resonance line is observed for bound and free states whose line width decreases as the temperature increases, the chemical shifts moving upfield. Under these conditions, the system is in the fast exchange regime. The AV77 mutant has a similar behavior, showing slow, intermediate, and fast exchange regimes as well as the WT, but

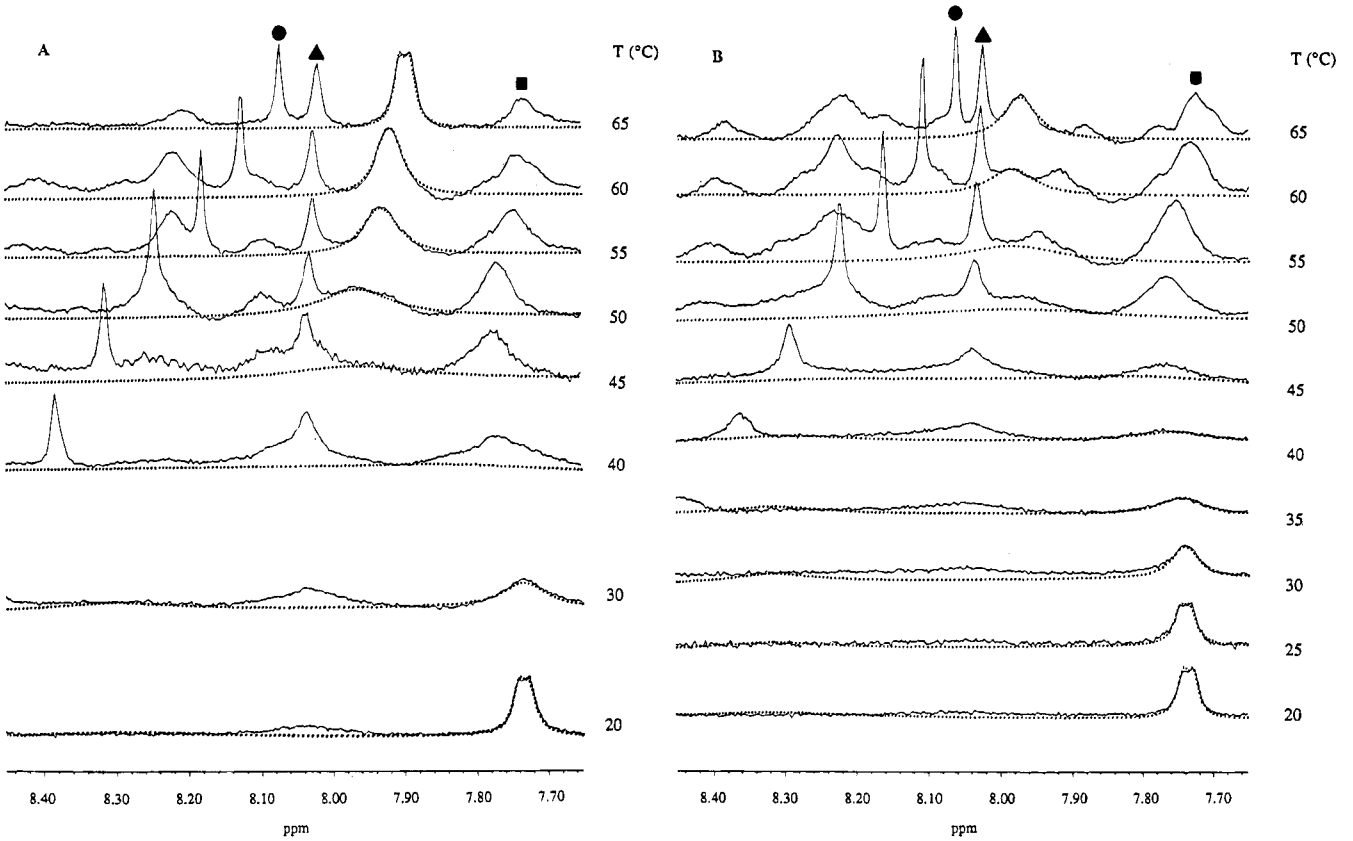

FIGURE 2: Partial aromatic region of the 1D ^{1}H NMR spectra of L-tryptophan, obtained at different temperatures. (A) 7.36 mM L-tryptophan in the presence of 3.9 mM *trp* repressor WT (in subunits). (B) 6.87 mM L-tryptophan in the presence of 3.4 mM AV77 repressor (in subunits). Samples were suspended in D_2O and contained 50 mM Na_2HPO_4 and 500 mM NaCl, pH* 6.0. The dotted lines are the simulated line shapes of the ligand H_4 proton (bound and free, in the presence of exchange) superimposed to the experimental spectra. The parameters used to simulate the spectra are described in the text. Symbols indicate protein peaks: (●) His35 (H_2); (▲) His16 (H_2); (■) Trp19 (H_4).

at temperatures slightly different. In this case, the slow exchange region extends up to 35 °C, the intermediate exchange is observed from 40 to 45 °C, and the fast exchange is observed above 50 °C.

The frequency of exchange (ν_{ex}) for the H_4 proton of L-tryptophan was obtained by fitting computationally generated line shapes to the experimental spectra, using eq 8. The chemical shift (σ), coupling constant (J), and line width (LW) of free L-tryptophan H_4 proton were measured in the absence of protein in the range of 20−65 °C. In this temperature range, σ was 7.742 ± 0.003 ppm, J was 7.35 ± 0.01 Hz, and LW was 4.14 ± 0.01 Hz. The transverse relaxation time (T_2^*) for the free ligand was calculated from the relation LW = $1/\pi T_2^*$. The parameters for L-tryptophan bound to WT repressor and the AV77 mutant were determined making the following considerations. The chemical shift value was taken from the NOESY spectra at 20 °C (Figure 1, Table 1) and was considered to remain constant with temperature. The same value was used for L-tryptophan bound to both WT and AV77 repressors, since the 1D spectra are similar and there are no significant changes in the structure of the AV77 mutant (Gryk & Jardetzky, 1995). Where the coupling constant could be measured (at very low and very high temperatures, or at very high ligand:protein ratios), it was found to be equal to the coupling constant of the free ligand, and did not change with temperature. The T_2^* values were estimated from line widths of bound peaks, but, in this case, direct measurement of line widths is difficult due to broadening. However, considering that the *trp* repressor is a rigid protein overall (Zheng *et al.*, 1995), and all protons are tumbling with the same rotational correlational time, the relaxation times of all protons are expected to be similar, except for particular protons that could present higher mobility (e.g., protons in D−E helices). The bulky side-chained tryptophan 99 does not present exceptional mobility (Zheng et al., 1995) and can be representative of the proton relaxation times of the protein protons. The protons of bound L-tryptophan are expected to have similar behavior, first because not much mobility is allowed in the binding pocket and second because the ligand tumbles with the same rotational correlational time as the protein. Therefore, it was rationalized that the H_4 proton of bound L-tryptophan should have a similar relaxation time to a protein tryptophan H_4 proton. From the relation LW = $1/\pi T_2^*$ the transverse relaxation time (T_2^*) for bound L-tryptophan at different temperatures and protein concentrations was first estimated from the line widths of the H_4 proton of tryptophan 99 of *trp* repressor WT and AV77 mutant. Further adjustments were necessary for best fitting to the experimental line shapes. The relaxation times of bond L-tryptophan used for the simulations are shown graphically in Figure 3, as a function of temperature, for different protein concentrations.

Representative resulting theoretical line shapes are shown in Figure 2 as dotted lines superimposed on the experimental spectra. Good agreement between experimental and theoretical data was obtained, with standard deviations (χ^2) for the comparison ranging from 0.6 to 0.8.

The simulations generated values for the exchange frequency (ν_{ex}) and for the dissociation constant (K_D). ν_{ex} values for both WT and AV77 mutant are shown in Figure 4 as Arrhenius plots of ln ν_{ex} against $1/T$, for different samples containing different concentrations of protein and

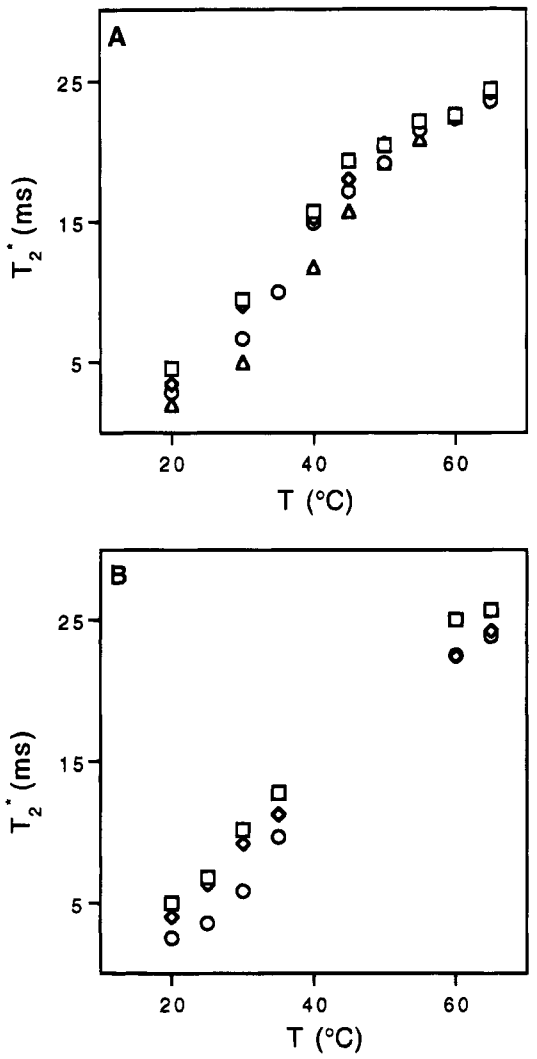

FIGURE 3: Transversal relaxation times (T_2^*) of the H_4 proton of L-tryptophan used for the simulations, when bound to (A) *trp* repressor WT: (□) 0.65 mM; (◇) 1.08 mM; (○) 1.52 mM; (△) 1.95 mM. (B) AV77 mutant: (□) 0.19 mM; (◇) 0.95 mM; (○) 1.70 mM.

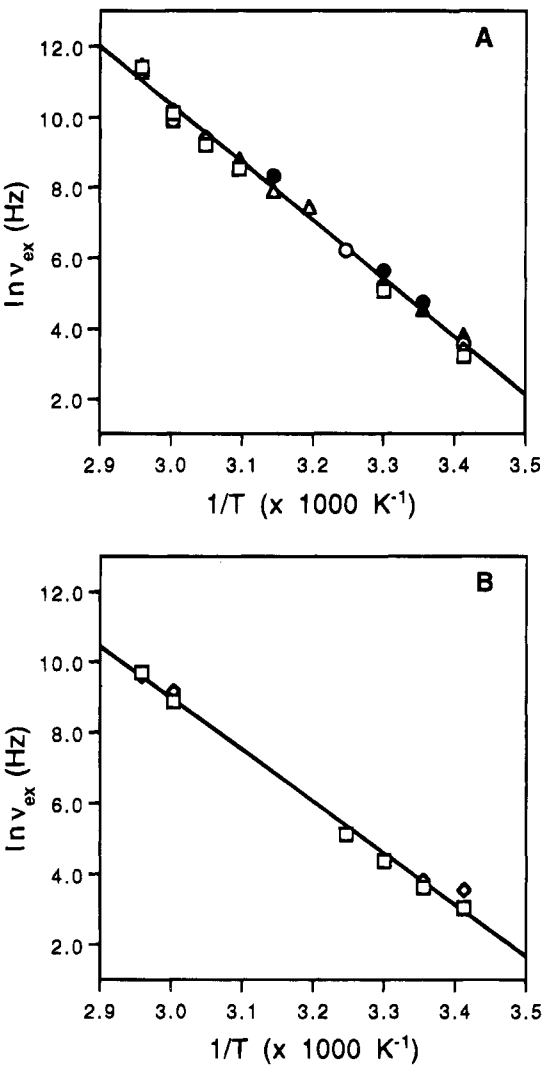

FIGURE 4: Arrhenius plots of ln ν_{exch} against $1/T$ for the exchange process of L-tryptophan with (A) L-tryptophan bound to *trp* repressor WT: (□) 0.65 mM protein + 7.36 mM ligand; (◇) 1.08 mM protein + 7.36 mM ligand; (○) 1.52 mM protein + 7.36 mM ligand; (△) 1.95 mM protein + 7.36 mM ligand; (●) 0.65 mM protein + 2.6 mM ligand; (▲) 0.65 mM protein + 3.9 mM ligand. (B) L-tryptophan bound to AV77 mutant repressor: (□) 0.19 mM protein + 6.87 mM ligand; (◇) 0.95 mM protein + 6.87 mM ligand; (○) 1.70 mM protein + 6.87 mM ligand (obscured by other symbols). The lines represent the average of data points at the same temperature.

ligand, and at different ligand:protein ratios. Similar values were obtained for all ligand:protein ratios and concentrations and at pH* 6.0 and 7.6. It was estimated that the values for ν_{ex} are subjected to an error of about 10% at most. The lines in Figure 4 represent the average between all data at the same temperature. From the slopes, the activation energies (E_a) obtained were 32.7 kcal K^{-1} mol^{-1} and 29.1 kcal K^{-1} mol^{-1} for WT and AV77 mutant, respectively (Table 2).

The K_D values obtained for WT and AV77 are shown as Arrhenius plots in Figure 5. The lines represent the average between all data at the same temperature. Considering the linearity obtained in Figures 5A,B, the enthalpy and the entropy for the binding of L-tryptophan to *trp* repressors can be assumed as being approximately constant with temperature in the range studied. ΔH and ΔS values calculated from the slope and intercept of the plots, respectively, are given in Table 2, for both WT and AV77.

Given the values for ν_{ex} and K_D, from eq 5 it is possible to calculate the dissociation rate constant (k_{-1}). From the k_{-1} values and using the definition for the dissociation constant (eq 2), k_{+1} can be calculated. The results for WT and AV77 are shown in Table 3. Arrhenius plots of the data (not shown) generate straight lines. From the slope of the plots, the activation energies were obtained, and are given in Table 2.

Table 2: Thermodynamic Parameters for the L-Tryptophan–*Trp* Repressor Exchange Process, for both WT and AV77 Mutant

	E_a (k_{ex}) [kcal/(K·mol)]	E_a (k_{-1}) [kcal/(K·mol)]	E_a (k_{+1}) [kcal/(K·mol)]	$\Delta H°$ (kcal/mol)	$\Delta S°$ [cal/(K·mol)]
WT	32.7	33.8	10.3	−5.7	−13.8
AV77	29.1	29.3	15.7	−3.2	−5.8

DISCUSSION

The line shape simulations yielded values for ν_{ex} with a high degree of accuracy. The line width of the simulated spectrum is strongly dependent on the value of ν_{ex}, and this value is limited to a very small range. In general, ν_{ex} is only weakly dependent on the relative populations of free and bound ligand and on the relaxation times. Since T_2^*

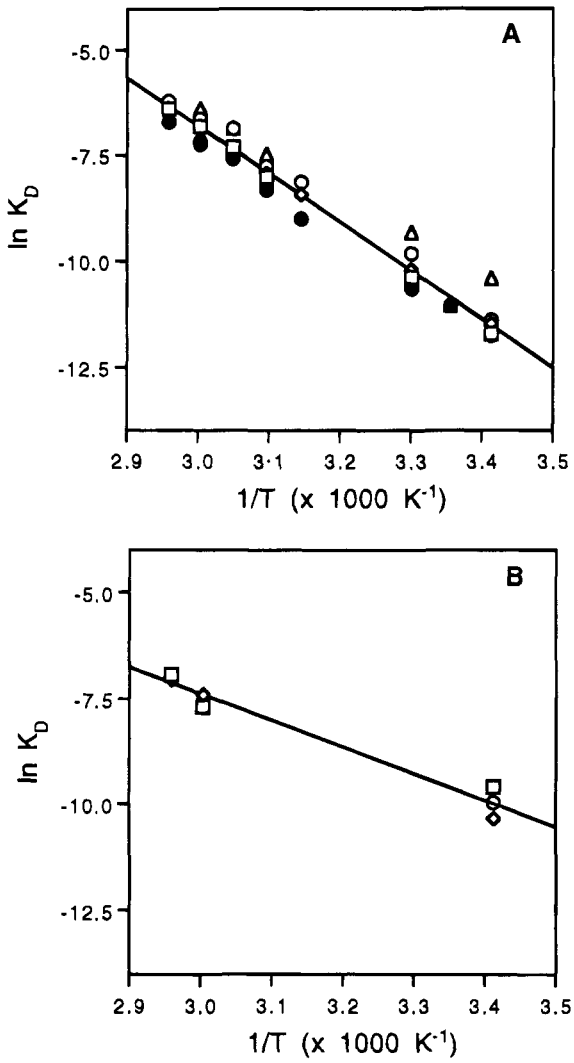

FIGURE 5: Plot of ln K_D against $1/T$ for (A) WT *trp* repressor and (B) AV77 mutant. Symbols are as in Figure 4. The lines represent the average of data points at the same temperature.

Table 3: Dissociation and Association Rate Constants for the Exchange Process between L-Tryptophan and *Trp* Repressor WT and AV77 Mutant[a]

	WT		AV77	
T (°C)	k_{-1} (s^{-1})	k_{+1} (M^{-1} s^{-1})	k_{-1} (s^{-1})	k_{+1} (M^{-1} s^{-1})
20	2.1×10^1	2.5×10^6	1.2×10^1	3.2×10^5
25	5.4×10^1	3.3×10^6	2.9×10^1	5.1×10^5
30	1.4×10^2	4.4×10^6	6.5×10^1	7.8×10^5
35	3.5×10^2	5.8×10^6	1.4×10^2	1.2×10^6
40	8.4×10^2	7.6×10^6	3.1×10^2	1.8×10^6
45	2.0×10^3	9.9×10^6	6.4×10^2	2.7×10^6
50	4.5×10^3	1.3×10^7	1.3×10^3	3.9×10^6
55	1.0×10^4	1.6×10^7	2.6×10^3	5.7×10^6
60	2.2×10^4	2.1×10^7	5.2×10^3	8.1×10^6
65	4.6×10^4	2.6×10^7	9.9×10^3	1.2×10^7

[a] Data are the average between 3 and 6 different samples.

values were either well determined (in the case of free ligand) or estimated with a reasonable degree of confidence (as described under Results for the bound ligand), small errors are contained in the values of ν_{ex}.

Three regions of exchange between tryptophan and *trp* repressor can be distinguished for the tryptophan H_4 proton, in the temperature range of 20−65 °C. For the WT repressor, slow exchange occurs from 20 to 30 °C, intermediate exchange occurs from 35 to 40 °C, and fast exchange occurs above 45 °C. For the AV77 mutant, the intermediate and fast exchange ranges are shifted 5−10 °C to higher temperatures. Studies on the dynamics of *trp* repressor were reported at 45 °C, a temperature in which the process of ligand exchange is in the interface between the intermediate and fast exchange regimes. It was shown that the amide protons of the D−E helices of the protein are in equilibrium between a "closed" and an "open" state and exchange with solvent protons on a time scale of 10^1 s^{-1}, both in the apo form and in the holo form of the WT and AV77 repressors (Gryk et al., 1995; Finucane & Jardetzky, 1995; Gryk & Jardetzky, 1995). The time scale of ν_{ex} determined in the present work at 45° C is of the order of 10^3 s^{-1}. This process is much faster than any other dynamic process reported to occur in the *trp* repressor molecule. It appears, therefore, that ligand exchange plays no role in the proton exchange mechanism, neither in the WT nor in the AV77 mutant.

In the temperature range studied, ν_{ex} is 1.3−5.6 times slower for the AV77 mutant as compared to WT. Also, k_{-1} and k_{+1} values for the AV77 mutant are slower than for WT at all temperatures studied. These findings can be rationalized as follows. It was shown that the amide protons of the DNA binding region in the AV77 mutant exchange with solvent protons at least 1 order of magnitude slower than the WT, due to stabilization of the D−E helices (Gryk & Jardetzky, 1995). The increased stability makes the molecule more rigid, and, as a result, the ligand can make better contacts with the protein, leading to a slower dissociation process (k_{-1}). On the other hand, the increased stability of the protein introduces some difficulty in the association process (k_{+1}) by restricting the number of orientations that the ligand can assume in order to reach the binding site. In summary, the general effect of increasing the stability of the protein is to slow down the overall dynamic processes that can take place in the molecule of *trp* repressor. It is worth noting that the frequency of exchange between the ligand and the repressor in the protein−operator DNA complex at 37 °C was estimated as being of the order of 2.5−5 s^{-1} (Zhang et al., 1994), values considerably smaller than those obtained in the present work in the absence of the operator, at the same temperature (766 s^{-1} and 291 s^{-1} for WT and AV77 mutant, respectively). In this case, an increased degree of motional restriction is imposed to *trp* repressor by the binding of the operator DNA and the ligand is "trapped" between the protein and the DNA, with a consequent substantial decrease in the exchange frequency.

In contrast with the ν_{ex} values, the K_D values generated by the simulations are subject to larger errors, mainly due to uncertainties in the determination of the chemical shifts of the bound ligand. If the chemical shift of the bound state remains constant in the range of temperatures studied, accurate values would be obtained in the region of fast exchange, where the position of the lines is strongly dependent on the ratio of bound and free populations. On the other hand, large shifts in the position of the bound resonance would lead to large errors in the determination of the binding constant. However, this seems not to be the case, for two reasons. First, the variations observed in protein proton chemical shifts as a function of temperature range from 0.02 to 0.07 ppm in the aromatic region. For methyl protons, the largest variation observed in the NOESY spectra is about 0.03−0.04 ppm in the temperature range of 45−65 °C (M. R. Gryk and O. Jardetzky, unpublished results). In spite of the fact that L-tryptophan is not covalently bound to

trp repressor, the binding pocket is very tight, and the motion of the ligand is restricted. Therefore, it is reasonable to assume that the proton chemical shifts of the bound ligand would be subject to similar variations with temperature as would protein protons. If a variation of the same magnitude occurs for the bound ligand, an error of a factor of 2 is implicit in the determined values of K_D, in the regime of fast exchange. In the region of slow exchange, bound peaks can be observed separately, and the chemical shifts do not change significantly. In this case, however, K_D is reflected mainly in the ratio between the bound and free resonance intensities, and is subject to a different source of error. It is apparent from the simulations that the sets of parameters that can be used to fit the experimental lines are limited in number. Even in the region of slow exchange, the values of K_D will not be off by more than a factor of 2. Second, the K_D values for the wild-type *trp* repressor agree well with values obtained by equilibrium dialysis and flow dialysis (Arvidson et al., 1986) when compared to the values obtained in the range of temperatures above 25 °C. The K_D values obtained in the present work for the AV77 mutant are similar to those obtained for the wild type, especially around 45 °C. The similarity of the binding constants for WT and AV77 mutant *in vitro* was previously observed by Hurlburt and Yanofsky (1990), and our results corroborate theirs.

In the temperature range of 20−65 °C, ln K_D against $1/T$ is linear (Figure 5), and the enthalpy and the entropy of L-tryptophan−*trp* repressor binding process can be considered constant. Below 20 °C, deviations from linearity have been reported, with ΔH and ΔS being no longer constant (Arvidson et al., 1986; Jin et al., 1993). In the range of temperatures studied in the present work, no deviation was observed because the lowest temperature used (20 °C) is at the upper limit of the reported deviation, observed to occur in the range of 4−20 °C. Large negative values for ΔH and ΔS were obtained in the present work for both *trp* repressor WT and AV77 mutant (Table 2). Similar values were reported for the WT repressor (Arvidson et al., 1986; Jin et al., 1993). Negative values for ΔH and ΔS are expected for a binding process involving protein conformational change and breakdown of hydrogen bonds between amino acid residues in the binding pocket and formation of new ones with the ligand in a hydrophobic environment (Zhang et al., 1987). Consistently, high values of the activation energy (E_a) for ligand binding were found (32.74 kcal mol^{-1} for the WT and 29.05 kcal mol^{-1} for AV77). It is worth noting that, for both repressors, k_{+1} is slower than the values that would be expected for diffusion-controlled processes (10^9-10^{10} M^{-1} s^{-1}), corroborating the finding that a sizable energy barrier is involved in the binding process. The similarity between the thermodynamic parameters of both WT and AV77 mutant repressors indicates that L-tryptophan binds to both proteins in a similar manner, with similar conformational changes. In fact, the comparison of NOE cross-peaks and proton chemical shifts (Gryk & Jardetzky, 1995) shows that both proteins assume virtually the same structure. It is expected, therefore, that L-tryptophan binds to the two repressors with similar energetics.

In summary, the mutation Ala^{77}→Val increases the stability of *trp* repressor, which leads to an overall decrease in the rate of dynamic processes. On the other hand, this mutation does not affect the structure of the protein in a significant way, and the mode of binding of the ligand to the protein is maintained. As a result, the affinity and energetics of binding are similar, and the same conformational changes that take place in the WT protein upon ligand binding also occur in the mutant. The effect of the mutation is to restrict the overall internal motion of the repressor, which leads to a restricted specificity of the protein to certain DNA sequences, leading to a superrepressor phenotype *in vivo*. On the other hand, increased stability is not enough to completely activate the repressor. As suggested by Arvidson et al. (1993), L-tryptophan is indeed required for proper function of the AV77 mutant *in vivo*, despite the fact that lower concentrations are required. It is apparent that the correpressor has two effects on the *trp* repressor molecule. The first is to stabilize the DNA binding helices for proper DNA recognition. The second is to position the helices in a precise orientation to allow for the binding. The mutation Ala^{77}→Val seems to promote the first effect, but lacks the conformational change necessary for full activation of the repressor.

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