# $^{13}C$ NMR Study of the Effects of Mutation on the Tryptophan Dynamics in Chymotrypsin Inhibitor 2: Correlations with Structure and Stability†

Stephen J. Matthews, Sati K. Jandu,‡ and Robin J. Leatherbarrow*

*Department of Chemistry, Imperial College of Science, Technology and Medicine, South Kensington, London SW7 2AY, U.K.*

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ABSTRACT: Recombinant chymotrypsin inhibitor 2 (CI-2) and the three mutants Ile39 → Val, Ile39 → Leu, and Arg67 → Ala were successfully enriched with [2-$^{13}C$]tryptophan at position 24 within the hydrophobic core of the protein. Carbon-13 NMR relaxation measurements were then used to investigate the effect of these mutations on the dynamics of the tryptophan residue. In addition, the stability of wild-type and mutant CI-2s was measured by their susceptibility to unfolding by guanidine hydrochloride. The mutant proteins were all found to be less stable, giving $\Delta\Delta G_U$ values relative to wild-type of 1.17, 1.96, and 1.21 kcal mol$^{-1}$, respectively. The indole moiety of the tryptophan residue was found to be more mobile in all the mutants studied than in wild-type CI-2. Order parameters of 0.69, 0.60, 0.56, and 0.44 were derived for wild-type, Ile39 → Val, Ile39 → Leu, and Arg67 → Ala CI-2, respectively. It is concluded that there is a correlation between the protein stability and the picosecond dynamics within the hydrophobic core and that mutations can influence the dynamic behavior of the residues that are relatively distant in the three-dimensional structure.

There have been numerous studies that have attempted to correlate conformational processes within proteins with their stability (Wagner et al., 1979; Wagner & Wüthrich, 1978, 1979; Richarz et al., 1980; Wüthrich et al., 1980; Alber et al., 1987; Nagai et al., 1987; Pielak et al., 1988; Jandu et al., 1990). The most comprehensive studies have been on basic pancreatic trypsin inhibitor (BPTI), where correlations between amide proton exchange, aromatic ring rotation, and thermal stability of chemically modified analogues of BPTI were probed using $^1H$ nuclear magnetic resonance (NMR) (Wagner et al., 1979; Wagner & Wüthrich, 1978, 1979; Wüthrich et al., 1980). Amide proton exchange was found to correlate closely with stability; however, ring rotation remained unaffected. It was therefore concluded that amide proton exchange is promoted by translational and rotational motions of hydrophobic clusters, i.e., "global fluctuations", and ring rotation by internal motion within an individual cluster.

Within proteins, aromatic ring rotation of buried residues is generally rare, and $^{13}C$ relaxation has been used to investigate motions on a faster time scale. An early study showed a few differences in methyl group relaxation rates between native and a selectively reduced BPTI, although these were within the intrinsically large experimental error of the experiments (Richarz et al., 1980; Wüthrich et al., 1980). With the recent availability of sensitive high-field spectrometers and the use of isotopic enrichment, highly accurate $^{13}C$ relaxation data on protein systems can be obtained with relative ease. However, no recent investigations concerning the effects of mutations on the fast internal dynamics within proteins have been reported. This paper describes the use of $^{13}C$ relaxation, isotopic enrichment, and site-specific mutagenesis for studying the internal dynamics of the single tryptophan ring within four subtly altered analogues of chymotrypsin inhibitor 2 (CI-2).

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* Author to whom correspondence should be addressed.

‡ Present address: Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Herts HP2 7TD.

CI-2 is a small globular protein from barley (9200 Da) that strongly inhibits serine proteases such as subtilisin and chymotrypsin and is a member of the potato-inhibitor 1 family of inhibitors (Boisen et al., 1981; Laskowski et al., 1980; Svendsen et al., 1980a,b; Longstaff et al., 1990). The three-dimensional structure of CI-2 has been well characterized in both crystalline and solution states by X-ray crystallography and NMR (McPhalen et al., 1987; Clore et al., 1987a,b). This makes CI-2 particularly attractive for protein engineering studies. The mutations studied in this paper fall into two categories. First, hydrogen bond capability is removed by the replacement of the arginine at position 67 by alanine (Figure 1). The resulting reduction in stability and changes to the dynamics of a neighboring phenylalanine ring have already been characterized for this mutant (Jandu et al., 1990; Leatherbarrow & Matthews, 1992). Secondly, modification of the side chain of isoleucine 39 residue within the hydrophobic core of the molecule. The isoleucine has been successively replaced by the amino acids leucine and valine. The side chain of isoleucine 39 is located in van der Waals contact with the indole ring of tryptophan 24 (Figure 1). In contrast, residue 67 is far from the tryptophan side chain.

## MATERIALS AND METHODS

*Mutagenesis.* The mutants Ile39 → Val, Ile39 → Leu, and Arg67 → Ala were prepared by the double-priming technique (Norris et al., 1983), using the mutagenic oligonucleotides 5′-GTCCTGCAACACCACCTTCTT-3′,5′-GTCCTGCAG-CAGCACCTTCTT-3′, and 5′-CTGGCGCAGCGGGA-GAAAC-3′, respectively. The sequences of the mutants were subsequently confirmed by DNA sequencing of the entire coding region of the gene.

*Synthesis of [2-$^{13}C$]L-Tryptophan.* [2-$^{13}C$]L-Tryptophan was synthesized from [2-$^{13}C$]indole (Cambridge Isotopes Laboratory, 99 atom %) and L-serine using the enzyme tryptophan synthetase. Recombinant enzyme was expressed in a tryptophan auxotroph mutant of *Escherichia coli*, W3110 trpAEdel2, containing the plasmid pHP3, which harbors the whole tryptophan operon. The plasmid and *E. coli* strain were a generous gift from Professor P. H. Pouwels and are

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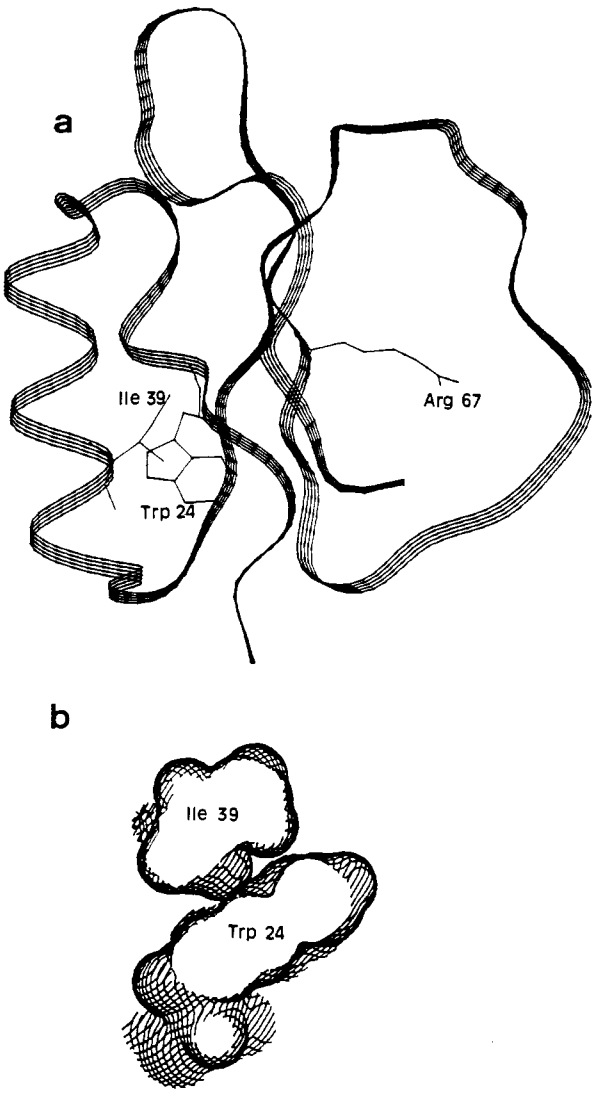


FIGURE 1: (a) Ribbon representation of the three-dimensional crystal structure of CI-2 [coordinates from McPhalen and James (1987)] showing the positions of arginine 67, isoleucine 39, and tryptophan 24. (b) Illustration of the van der Waal surfaces surrounding Ile39 and Trp24, showing their interaction.

described elsewhere (Enger-Valk et al., 1980). The cells were grown overnight at 37 °C in 2TY media containing 50 mg/L ampicillin and harvested. The cells from a 1-L fermentation were then resuspended in 100 mM sodium phosphate buffer (pH 7.8) containing 250 mg of [2-$^{13}$C]indole, 600 mg of L-serine, 3.12 g of ammonium sulfate, 250 mg of sodium sulfite, and 4 mg of the coenzyme pyridoxyl phosphate (van den Berg et al., 1988). The suspension was further incubated at 37 °C until all the indole had converted to [2-$^{13}$C]L-tryptophan which was monitored by $^1$H NMR. After approximately 6 h, when conversion was complete, the cells were removed by centrifugation, and the [2-$^{13}$C]L-tryptophan-rich supernatent (containing approximately 440 mg of trytophan) was autoclaved and refrigerated, ready for direct use in the [2-$^{13}$C]L-tryptophan labeling of CI-2.

*Preparation and Purification of [2-$^{13}$C]L-Tryptophan-Labeled CI-2.* Both native CI-2 and mutants were expressed in *E. coli* TG2 (Gibson, 1984) containing the plasmid pAC4 (Longstaff et al., 1990) by overnight fermentation at 37 °C in minimal media supplemented with glucose (4 g L$^{-1}$), thiamine (100 mg L$^{-1}$), ampicillin (50 mg L$^{-1}$), isopropyl $\beta$-D-thiogalactopyranoside (50 mg L$^{-1}$), and all 20 amino acids. The recipe included the following per liter: 400 mg of alanine, 400 mg of glutamine, 400 mg of arginine, 250 mg of aspartic acid, 50 mg of cysteine, 400 mg of glycine, 100 mg of histidine, 100 mg of isoleucine, 100 mg of leucine, 100 mg of lysine, 250 mg of methionine, 100 mg of proline, 1600 mg of serine, 100 mg of threonine, 100 mg of tyrosine, 100 mg of valine, 50 mg of phenylalanine, 400 mg of glutamic acid, and 250 mg of asparagine. The appropriate volume of supernatant that contained 45 mg of the synthesized tryptophan was added just prior to induction. Purification of labeled CI-2 was essentially the same as described as Jandu et al. (1990). Approximately 0.44 g of [2-$^{13}$C]L-tryptophan was used to label successfully all the proteins studied; between 1- and 3-L fermentations were used for wild-type, Ile39 → Val, and Arg67 → Ala CI-2. However, for the modestly expressing Ile39 → Leu CI-2 mutant, a 6-L fermentation was necessary to provide 20 mg of pure protein. The purity of all the proteins was assessed by SDS–polyacrylamide gel electrophoresis and was >95% in each case. Isotopic purity was judged by analysis of $^1$H NMR spectra.

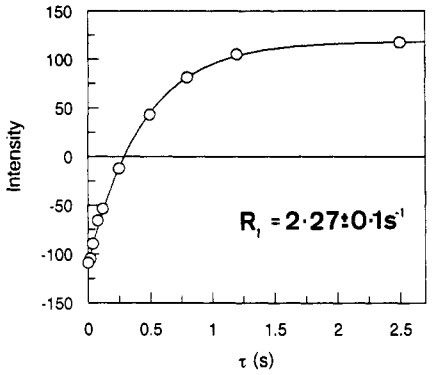


FIGURE 2: Fit of the raw intensity data from a $^{13}$C inversion–recovery experiment for [2-$^{13}$C]Trp-labeled Ile39 → Val CI-2 at 303 K.

*NMR Spectroscopy.* For all NMR experiments dry protein was dissolved in 0.6 mL of $D_2O$ (99.9 atom % D, Sigma) and the pH adjusted to 7.0. The solution was then centrifuged and transferred to a 5-mm o.d. NMR tube and degassed using the freeze–pump–thaw method. The final concentration used for all the protein samples was 3.6 mM.

Two NMR spectrometers were used, namely, a Jeol GX270 and a Bruker AM500, in order to study the frequency dependence of the relaxation data. The $^{13}$C operating frequencies are 67.8 and 125.74 MHz, respectively. The temperature of the probe was calibrated using an ethylene glycol chemical shift thermometer (Ralford et al., 1979). The temperature chosen for all experiments was 303 K (30 °C).

The $^{13}$C $T_1$ measurements were made using the fast inversion–recovery Fourier transform experiment (Canet et al., 1975) with proton decoupling. Between 10 and 15 variable delay values were chosen, and the subsequent peak intensities were fitted by using nonlinear regression to

$$M_z(\tau) = A(1) + A(2)\exp[A(3)\tau]$$

where $M_z(\tau)$ is the intensity at time $\tau$, $A(1)$ is the intensity at long time, $A(2)$ is the intensity at zero time minus $A(1)$, and $A(3)$ is the negative spin–lattice relaxation rate ($-T_1^{-1}$). Figure 2 shows a representative fit of raw intensity data from an inversion–recovery experiment for [2-$^{13}$C]Trp-labeled Ile39 → Val CI-2. The one standard deviation error of the relaxation times was better than 4% at all times. The standard gated decoupling technique (Freeman et al., 1972) was used to measure the NOE factor with a recycle time of not less than $10T_1$. With the availability of composite pulse decoupling and its associated negligible heating effect, it was possible to

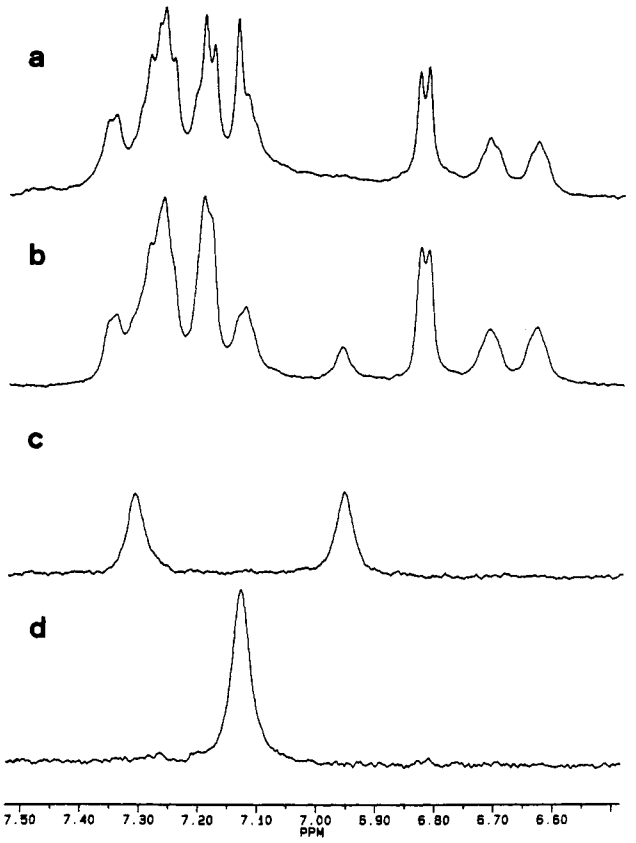


FIGURE 3: Aromatic region of 500-MHz $^1$H NMR spectra at 303 K and pH 7.0 of (a) unlabeled wild-type CI-2 and (b) wild-type CI-2 enriched with [2-$^{13}$C]tryptophan. The $^{13}$C-edited $^1$H NMR spectrum is shown (c) without and (d) with $^{13}$C decoupling.

obtain accurate NOE data, reproducible within 10% at both frequencies.

Isotope-edited $^1$H NMR spectra were obtained using a simple spin-echo difference pulse sequence (Griffey & Redfield, 1987) in which selective detection of protons coupled to $^{13}$C nuclei is achieved. The GARP1 pulse sequence (Shaka et al., 1985) was employed for $^{13}$C decoupling during acquisition.

*Unfolding Studies.* Guanidine hydrochloride (ARISTAR grade, BDH) solutions were carefully prepared from serial dilution of an 8 M stock containing 50 mM MOPS buffer (pH 7.0). For each data point of the denaturation curve, 200 $\mu$L of CI-2 solution, 50 mM MOPS, was added to 2800 $\mu$L of the appropriate concentration of denaturant solution. The samples were preequilibrated at 30 °C for 1 h, and fluorescence intensities were recorded at 30 °C with an emission wavelength of 356 nm and an excitation wavelength of 290 nm.

## RESULTS AND DISCUSSION

Figure 3 shows the aromatic region of the 500-MHz $^1$H spectrum of wild-type CI-2 (a) without and (b) with incorporation of $^{13}$C-labeled tryptophan. The 1D $^{13}$C-edited $^1$H NMR spectra of this region are also shown (c) without and (d) with $^{13}$C decoupling. The proton attached to the 2 position of the pyrrole ring is split into a doublet by the attached $^{13}$C nucleus (Figure 3c). Integration of the well-resolved high-field doublet resonance (6.96 ppm) of Figure 3b indicates the level of enrichment to be greater than 90% for all four proteins. The specificity of the labeling is illustrated by the appearance of Figure 3c and is confirmed by the proton-decoupled $^{13}$C NMR spectrum (not shown), in which a single resonance is observed at 127.2 ppm.

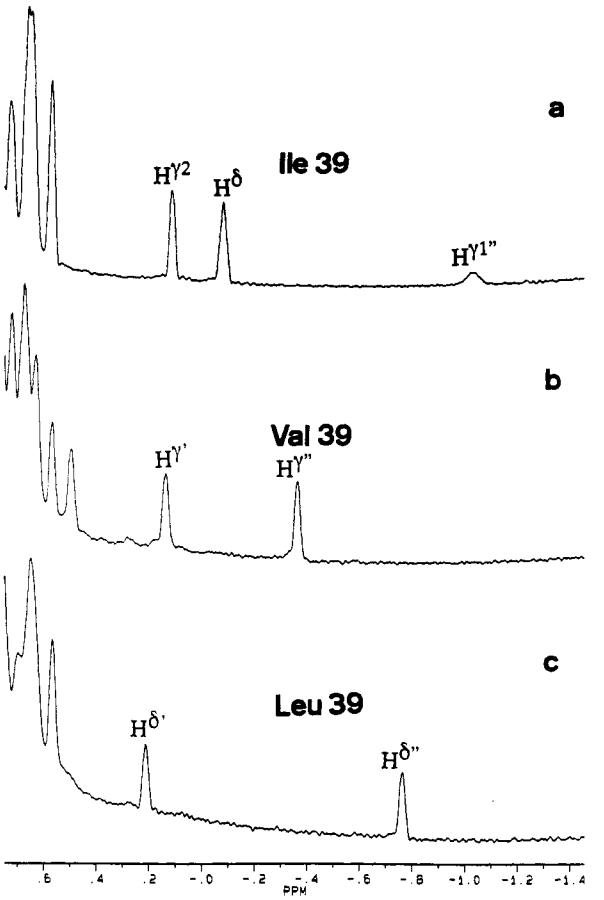


FIGURE 4: High-field methyl group region for the 500-MHz $^1$H NMR spectra of (a) wild-type, (b) Ile39 → Val, and (c) Ile39 → Leu CI-2. The region of the spectrum shown for wild-type CI-2 is essentially identical to that corresponding to the Arg67 → Ala mutant.

Figure 4 shows the high-field methyl region of the $^1$H NMR spectrum for wild-type CI-2, Ile39 → Val, and Ile39 → Leu mutants. This region of the spectrum in wild-type CI-2 is characterized by the upfield-shifted methyl and methylene resonances of Ile39 (Kjaer et al., 1987). This is a consequence of the ring current effect from the adjacent Trp24 (Figure 1). The altered high-field region of the Ile39 → Val and Ile39 → Leu CI-2 NMR spectra is presumed to arise from the methyl groups of Val39 and Leu39, which are similarly perturbed by the tryptophan aromatic system. The rest of the spectrum (not shown) for these mutants shows no major differences from that of wild-type CI-2, indicating that the overall structure of these proteins is unchanged. 2D $^1$H NMR studies on the Arg67 → Ala mutant (Ray, 1990; Jandu et al., 1990) have shown in detail that there are no significant global structural changes to the 3D structure of CI-2. These observations have been commonly found for numerous mutations in other proteins (Wagner et al. 1979; Wagner & Wüthrich, 1979; Alber et al., 1987a,b; Nagai et al., 1987; Pielak et al., 1988; Folkers et al.; 1989).

The stabilities of wild-type CI-2 and the mutants can be compared by measuring their degree of unfolding in guanidine hydrochloride solutions. The unfolding was monitored by the fluorescence arising from the single tryptophan residue at position 24. The fluorescence in denaturant solution is given by (Pace, 1986; Kellis et al., 1989)

$$F = F_N - (F_N - F_U)\frac{e^{(m[\text{denat}]-\Delta G_{H_2O})/RT}}{e^{1+(m[\text{denat}]-\Delta G_{H_2O})/RT}} \quad (1)$$

where $F_N$ and $F_U$ are the fluorescence values for the native

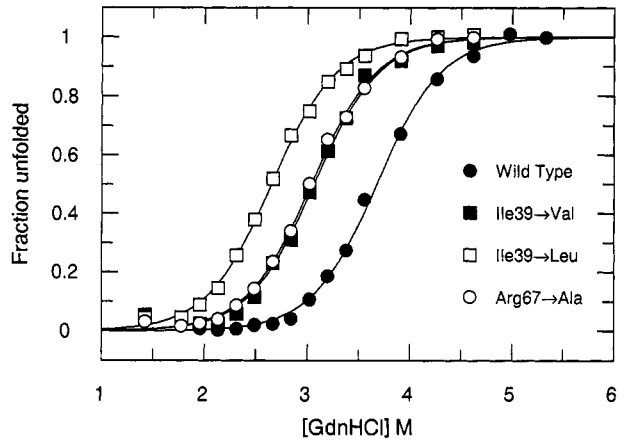


FIGURE 5: Unfolding of wild-type and mutant CI-2s in solutions of guanidine hydrochloride at 303 K and pH 7.0. The data have been normalized, and the curves represent best fits to eq 1 using nonlinear regression.

Table I: Free Energies of Unfolding for CI-2 and Mutants Derived from Guanidine Hydrochloride Unfolding Experiments at 303 K and pH 7.0

| CI-2 | $\Delta G_U$ (kcal mol$^{-1}$)[a] | $\Delta\Delta G_U$ (kcal mol$^{-1}$) |
|---|---|---|
| wild-type | 7.05 (±0.16) | 0 |
| Ile39 → Val | 5.88 (±0.16) | 1.17 |
| Ile39 → Leu | 5.09 (±0.12) | 1.96 |
| Arg67 → Ala | 5.84 (±0.13) | 1.21 |

[a] Data fitted to the same $m$ value; $m$ = 1.92 (±0.05) kcal mol$^{-1}$ M$^{-1}$.

and unfolded states, respectively. $\Delta G_{H_2O}$ is the free energy of unfolding in the absence of denaturant. All the mutations described cause the protein to unfold at lower concentrations of guanidine hydrochloride, indicating a destabilization of the protein (Figure 5). The data for native CI-2 and the three mutants were fitted, by non-linear least-squares minimization, to have a common best-fit $m$ value (Kellis et al., 1989; Jandu et al. 1990) using eq 1. The free energies of unfolding together with the differences [$\Delta\Delta G_U = \Delta G_{H_2O(\text{wild-type})} - \Delta G_{H_2O(\text{mutant})}$] are presented in Table I.

From these results, the proteins can be ranked in the order of stability as follows: wild-type > Ile39 → Val ≈ Arg67 → Ala > Ile39 → Leu CI-2. Changing arginine to alanine at position 67 removes a hydrogen-bonding capability from this position. The value obtained from this study of $\Delta\Delta G_U$ = 1.21 kcal mol$^{-1}$ at pH 7 lies in the estimated range for the deletion of a hydrogen bond, 0.5–2 kcal mol$^{-1}$ (Fersht et al., 1985). The position 39 mutants represent modifications to the hydrophobic core of the molecule. There have been numerous investigations concerning the contribution of hydrophobic interactions to protein stability (Kellis et al., 1988; Masazumi et al., 1988; Alber, 1989; Lim & Sauer, 1989; Kellis et al., 1989). In these studies, the authors have generally attempted to rationalize such conservative mutations in terms of the reduction of the surface accessible to solvent upon folding. Kellis et al. (1988) concluded that the loss of one $CH_2$ group destabilizes the protein by 1.1 kcal mol$^{-1}$ and three such groups by 4.0 kcal mol$^{-1}$. Our result for the Ile39 → Val mutation ($\Delta\Delta G_U$ = 1.17 kcal mol$^{-1}$) is entirely consistent with these conclusions. However, the Ile39 → Leu mutant, which represents little change in hydrophobicity, causes the largest decrease in stability ($\Delta\Delta G_U$ = 1.96 kcal mol$^{-1}$). As this mutation involves a negligible change in side-chain volume, other effects need to be taken into account. In this case, the structural isomerization of isoleucine to leucine must result in inefficient side-chain packing within the hydrophobic interior of the protein.

Table II: Experimental $^{13}C$ $R_1$ and $\eta$ Data for [2-$^{13}C$]Tryptophan CI-2 and Mutants Measured at 303 K and pH 7.0, Together with Theoretical Values Derived from the Simultaneous Fit to the "Model-Free" Two-Correlation Time Spectral Density

| CI-2 | experimental values[a] | theoretical values |
|---|---|---|
| wild-type | | |
| $R_1$ (s$^{-1}$) 125.74 MHz | 2.76 | 2.70 |
| $\eta$ 125.74 MHz | 0.13 | 0.19 |
| $R_1$ (s$^{-1}$) 67.8 MHz | 5.92 | 5.95 |
| $\eta$ 67.8 MHz | 0.20 | 0.22 |
| Ile39 → Val | | |
| $R_1$ (s$^{-1}$) 125.74 MHz | 2.27 | 2.31 |
| $\eta$ 125.74 MHz | 0.19 | 0.17 |
| $R_1$ (s$^{-1}$) 67.8 MHz | 5.13 | 5.11 |
| $\eta$ 67.8 MHz | 0.23 | 0.21 |
| Ile39 → Leu | | |
| $R_1$ (s$^{-1}$) 125.74 MHz | 2.08 | 2.13 |
| $\eta$ 125.74 MHz | 0.19 | 0.15 |
| $R_1$ (s$^{-1}$) 67.8 MHz | 4.78 | 4.76 |
| $\eta$ 67.8 MHz | 0.21 | 0.20 |
| Arg67 → Ala | | |
| $R_1$ (s$^{-1}$) 125.74 MHz | 1.69 | 1.68 |
| $\eta$ 125.74 MHz | 0.15 | 0.17 |
| $R_1$ (s$^{-1}$) 67.8 MHz | 3.71 | 3.71 |
| $\eta$ 67.8 MHz | 0.24 | 0.21 |

[a] At all time the errors were better than 4% and 10% on $R_1$ and $\eta$ data, respectively.

Table II gives the raw $^{13}C$ spin–lattice relaxation rates ($R_1$) and NOE factors for the four proteins at 303 K; a significant variation in $R_1$ is observed. To quantify these differences in terms of the motional properties of the tryptophan residue, the data were fitted by nonlinear least-squares to an appropriate model. For the generally restricted motions of the indole moiety of tryptophan in proteins, the "model-free" approach of Lipari and Szabo is ideally suited. The spectral density has the general form (Lipari & Szabo, 1982a,b)

$$J(\omega) = S^2\frac{2\tau_C}{(1+\omega^2\tau_C^2)} + (1-S^2)\frac{2\tau}{(1+\omega^2\tau^2)} \quad (2)$$

where $\tau_C$ is the correlation time for overall motion and $\tau^{-1} = \tau_C^{-1} + \tau_e^{-1}$, $\tau_e$ being the effective correlation time for internal motion and $S^2$ a generalized order parameter measuring the spatial restriction of the internal motion. For dipole–dipole relaxation of a $^{13}C$ nucleus bound to a proton, which in general dominates for such nuclei, the spin–lattice relaxation time can be given as (Harris, 1983)

$$R_{1dd} = \frac{1}{T_{1dd}} = \frac{N_H}{20}\left[\frac{\mu_0\gamma_C\gamma_H\hbar}{4\pi r^3}\right]^2 [J(\omega_H-\omega_C) + 3J(\omega_C) + 6J(\omega_H+\omega_C)] \quad (3)$$

where $J(\omega)$ is the spectral density function, $r$ is the average C–H distance, and the other symbols have their usual meanings. The value for $r_{C-H}$ was taken to be 0.1098 nm. The nuclear Overhauser effect (NOE) factor is given by (Harris, 1983)

$$\eta = (\gamma_H/\gamma_C)\frac{6J(\omega_H-\omega_C) - J(\omega_H-\omega_C)}{6J(\omega_H+\omega_C) + 3J(\omega_C) + J(\omega_H-\omega_C)} \quad (4)$$

However, the $^{13}C$ nucleus within the indole ring of tryptophan can experience a small influence from the chemical shift anisotropy (CSA) mechanism (Harris, 1983)

$$T_{1CSA}^{-1} = (2/15)\gamma_C^2 B_0^2 \Delta\sigma^2 J(\omega_C) \quad (5)$$

where $\Delta\sigma$ is the chemical shift anisotropy. No precise values for the chemical shift anisotropy have been reported for

Table III: Parameters Derived from the Fit of the $^{13}C$ Relaxation Data for Wild-Type and Mutant CI-2s (Measured at 303 K and pH 7.0) to the "Model-Free" spectral Density, Together with Semiangles Calculated from the Diffusion in a Cone Model

| CI-2[a] | $\tau_e$ (ps) | $S^2$ | $\theta_0$ (deg) |
|---|---|---|---|
| wild-type | 31 (±7) | 0.69 (±0.02) | 28.1 |
| Ile39 → Val | 14 (±5) | 0.60 (±0.02) | 32.7 |
| Ile39 → Leu | 8 (±4) | 0.56 (±0.01) | 34.8 |
| Arg67 → Ala | 7 (±3) | 0.44 (±0.01) | 40.9 |

[a] Data for all four equally concentrated protein solutions were fitted simultaneously to the same overall correlation time, from which a value of $\tau_c$ = 3.9 (±0.4) ns was derived.

tryptophan. Weaver et al. (1988) successfully adopted a value of 180 ppm, derived for benzene (Pines et al., 1972), for their analysis of $^{13}C$ relaxation in tryptophan residues. This value was therefore used in the analysis of the relaxation data presented in this paper. The complete description of the $^{13}C$ relaxation behavior for the [2-$^{13}C$]Trp-labeled proteins is

$$T_1^{-1} = T_{1dd}^{-1} + T_{1CSA}^{-1} \quad (6)$$

All proteins studied have essentially identical size and shape, and so for data measured under the same conditions the overall correlation time, $\tau_c$, should not vary. The data were fitted by nonlinear regression to eq 6, deriving a common best-fit value for $\tau_c$ but individual values for $\tau_e$ and $S^2$. Table II gives the raw $R_1$ and NOE data together with theoretical values. The table shows an excellent fit to the data, confirming the inclusion of the CSA contribution and the applicability of the model. For wild-type CI-2 (3.6 mM and 303 K) the relative contributions of the CSA and dipole–dipole mechanisms to the $^{13}C$ relaxation are 26:74 and 9:91 at 125.74 and 67.8 MHz, respectively. The parameters derived from the analysis are given in Table III. The value obtained for the correlation time for overall tumbling ($\tau_c$ = 3.9 ns) is in agreement with literature values for proteins (Richarz et al., 1980; Kay et al., 1989; Clore et al., 1990) and the results of phenylalanine and glycine relaxation studies on CI-2 (Leatherbarrow & Matthews, 1992).

Interior tryptophan and other aromatic side chains are commonly present in particularly rigid regions of proteins (Gelin & Karplus, 1975; McCammon et al., 1979; McCammon & Karplus, 1983). Small-amplitude librations and vibrations, experienced by restricted side chains in proteins, can be successfully likened to restricted diffusion within a cone of semiangle $\theta_0$, where (Lipari & Szabo, 1982b; Dellwo & Wand, 1989)

$$\theta_0 = \cos^{-1}\left[1/2((1 + 8S_{cone})^{1/2} - 1)\right] \quad (7)$$

$$0° \leq \theta_0 \leq 90°$$

in which the generalized order parameter is equated with the order parameter $S_{cone}$. The cone semiangles for tryptophan motion within CI-2 and its mutants are also given in Table III. For all the proteins studied, the values for the angle fall in the range 28–41°, which are only moderately higher than those obtained for backbone carbon positions in proteins (typically around 20°; $S^2 \sim 0.85$) (Richarz et al., 1980; Lipari & Szabo, 1982b; McCain et al., 1988; Dellwo & Wand, 1989; Kay et al., 1989; Clore et al., 1990; Palmer et al., 1991). These values indicate that the motions of the tryptophan ring are highly restricted in CI-2 and can be compared to order parameters for the more extensive tryptophan motion within peptides, for which values between 0.09 and 0.36 have been measured (Weaver et al., 1988).

The mutations studied can be summarized as follows: a reduction in side-chain volume immediately next to Trp24 (Ile39 → Val), an isomerization at this position (Ile39 → Leu), and a modification to hydrogen-bonding capability distant from the tryptophan residue (Arg67 → Ala). It might have been expected that the mutations adjacent to the ring, particularly the Ile39 → Val change, would have a greater effect on the mobility of the Trp24 side chain than would a distant mutation. However, this is clearly not the case for the mutants studied here. Instead, the motion of the tryptophan ring is less restricted in all these mutants than it is in the wild-type. Although not perfect, it seems that there is a correlation between the protein stability and the picosecond dynamics within the hydrophobic core. The effect on the dynamics is most evident for Arg67 → Ala, even though the mutation is distant from the tryptophan residue. This implies that the loss of the hydrogen bond from the external loop region of CI-2 has a cooperative effect on the motions within the hydrophobic core and suggests that the dynamic consequences of mutation are not necessarily confined to the locality of the modification.

There are several different types of internal motion within proteins which can be probed by NMR. The infrequent stochastic events of aromatic ring rotation have been shown to be independent of protein stability (Wagner et al., 1979; Wagner & Wüthrich, 1978, 1979; Wüthrich et al., 1980; Pielak et al., 1988). A similar relationship has also been observed for the fast dynamics experienced by methyl groups (Richarz et al., 1980; Wüthrich et al., 1980). In the time scale between these types of motion are the librations of highly restricted side chains, in particular aromatic systems. Until now, the correlation of these with stability has not been extensively tested. This paper has shown that the picosecond motions of Trp24, in the hydrophobic core of CI-2, are highly restricted in wild-type protein but become significantly freer with destabilizing mutations. The order parameter for such a residue describes the flexibility of its environment, implying that in this protein the rigidity of the internal hydrophobic cluster is correlated with global fluctuations of the whole molecule.

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