Correlated bond rotations in interactions of arginine residues with ligand carboxylate groups in protein ligand complexes

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Abstract The $^{1}H/^{15}N$ HSQC NMR spectra of complexes of *Lactobacillus casei* dihydrofolate reductase containing methotrexate recorded at 1°C show four resolved signals for the four NH^{η} protons of the Arg^{57} residue. This is consistent with hindered rotation in the guanidino group resulting from interactions with the α-carboxylate of methotrexate. Increasing the temperature causes exchange line-broadening and coalescence of signals. Rotation rates for the $N^{\varepsilon}C^{\zeta}$ and $C^{\zeta}N^{\eta}$ bonds have been calculated from lineshape analysis and from zz-HSQC exchange experiments. The interactions between the methotrexate α-carboxylate group and the Arg^{57} guanidino group decrease the rotation rates for the $N^{\varepsilon}C^{\zeta}$ bond by about a factor of 10 and those for the $C^{\zeta}N^{\eta}$ bonds by more than a factor of 100 with respect to their values in free arginine. Furthermore, the relative rates of rotation about these two bonds are reversed in the protein complexes compared with their values in free arginine indicating that there are concerted rotations about the $N^{\varepsilon}C^{\zeta}$ bond of the Arg^{57} guanidino group and the $C'C^{\alpha}$ bond of the glutamate α-carboxylate group of methotrexate.

Key words: Dihydrofolate reductase; ^{15}N-NMR; Arginine guanidino group; Bond rotation rate; zz-HSQC

1. Introduction

The sidechains of arginine residues are frequently involved in hydrogen bonding and electrostatic interactions in protein-ligand complexes and NMR is proving to be a very effective method for characterising such interactions [1–5]. For example, in complexes of SH2 domains formed with bound phosphopeptides several important arginine-ligand interactions between arginine NH^{η} hydrogens and the phosphorylated tyrosines have been characterised [2–4]. Similar interactions in complexes of *Lactobacillus casei* dihydrofolate reductase between a conserved arginine residue and a carboxylate group on the antifolate drug methotrexate (see Fig. 1) have also been reported [5]. Previous studies have been aimed at identifying the NH hydrogens specifically involved in the interactions and characterising the structures of the interacting groups. We have now extended these investigations to include studies of dynamic processes within the interacting groups and have uncovered interesting findings about correlated bond rotations in these systems. The success of such studies relies on the relative ease of detection of ^{1}H- and ^{15}N-NMR signals from NH groups in ^{15}N-labelled proteins using gradient-enhanced two dimensional $^{1}H/^{15}N$ HSQC NMR experiments. The methods have proved particularly useful for detecting the guanidino NH^{ε} and NH^{η} nuclei in arginine residues (see Fig. 1). Most of the arginine residues in proteins show only a single broad coalesced resonance in their $^{1}H/^{15}N$ HSQC spectrum corresponding to the four NH^{η} protons and two N^{η} nitrogens in the guanidino group (see Fig. 2). The coalesced signals arise from exchange between the NH^{η} nuclei caused by rotations about the $N^{\varepsilon}C^{\zeta}$ and $C^{\zeta}N^{\eta}$ bonds. Several workers [3,6,7] have shown that this is the situation in free arginine at ~25°C. In experiments at lower temperatures (−40°C) in water/methanol mixtures, the bond rotations can be slowed down sufficiently to freeze out the rotamers so that separate signals from the individual NH^{η} protons and nitrogens are observed [3,7]. Rates of rotation have been estimated from analysis of the line shapes at different temperatures; the rate of rotation about the $N^{\varepsilon}C^{\zeta}$ bond is 400–640 s^{-1} at 15°C for free arginine [3] and the rates of rotation about the two $C^{\zeta}N^{\eta}$ bonds are 7800 and 17 500 s^{-1} at 25°C for N^{α}-acetylarginine isopropyl ester [6]. In a study of complexes of *L. casei* dihydrofolate reductase containing methotrexate, four separate NH^{η}signals were observed for the Arg^{57} residue indicating hindered rotation in its guanidino group even at 20°C [5]. From a consideration of the ^{1}H and ^{15}N chemical shifts it was possible to deduce that the guanidino group of Arg^{57} interacts with the α-carboxylate group of the glutamic acid moiety of methotrexate in an end-on symmetrical fashion (see Fig. 1) [5,8]. In the present study, we have characterised the rates of rotation about the $N^{\varepsilon}C^{\zeta}$ and $C^{\zeta}N^{\eta}$ bonds in binary and ternary complexes of *L. casei* DHFR with methotrexate and NADPH and used these to provide information about correlated motions in the guanidino group of Arg^{57} and the interacting carboxylate group of methotrexate.

2. Materials and methods

^{15}N-labelled *L. casei* DHFR was expressed in *Escherichia coli* cells grown on a minimal medium and isolated and purified as described previously [9,10]. Methotrexate and NADPH were obtained from Sigma. All other chemicals used were of analytical grade.

The NMR experiments were performed on DHFR·methotrexate (3 mM) and DHFR·methotrexate·NADPH (4 mM) equimolar complexes examined as 0.6 ml samples in 90% H_2O/10% D_2O and 50 mM potassium phosphate and 500 mM KCl (binary) and 50 mM KCl (ternary), $pH^* = 6.5$ (the pH^* values are meter readings, unadjusted for deuterium isotope effects).

The NMR experiments were performed at 1–40°C on Varian 500 and 600 MHz spectrometers. All the NMR experiments used the

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Abbreviations: DHFR, dihydrofolate reductase; HSQC, heteronuclear single quantum coherence spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; MTX, methotrexate; zz-HSQC, zz-magnetization exchange HSQC spectroscopy.

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Watergate technique for water suppression [11] and the GARP sequence [12] for ^{15}N decoupling during the detection period. Quadrature detection in all indirectly detected dimensions was achieved using the method of States et al. [13].

The 2D HSQC sequence used in the experiments was essentially the same as that proposed by Mori et al. [14]. These experiments were performed using acquisition times of 20–40 and 128 ms in the ^{15}N and ^{1}H dimensions, respectively. The 2D zz-HSQC experiments [15,16] used for the exchange studies were carried out at 15°C using a pulse sequence described by Yamazaki and co-workers [3]. The experiment has a modified gradient enhanced HSQC pulse sequence in which a mixing time is inserted just prior to the transfer of magnetization from ^{15}N to ^{1}H. This allows observation of the transference of ^{15}N labeled heteronuclear zz-magnetization (I_zS_z) between the different sites. The experiment was carried out with a series of different mixing times (1–21 ms) and with acquisition times of 26–28 and 152 ms in the ^{15}N and ^{1}H dimensions, respectively. The data were processed with zero-filling in both dimensions using Varian software (VNMR, version 5.1) and converted to XEASY [17] for further analysis.

The rate constants were obtained from the zz-HSQC exchange experiments by measuring the normalized intensities of the cross-peak, I=[cross-peak volume/(cross-peak+autopeak) volumes] at different mixing times and analysing the data using a non-linear regression analysis. Assuming a two-site exchange and neglecting cross-relaxation during the mixing time, the normalized cross-peak intensity as a function of the mixing time (τ_m) is given by the expression $I(\tau_m)=0.5[1+\exp(-2k\tau_m)]$, where k is the rate constant of the exchange process [15].

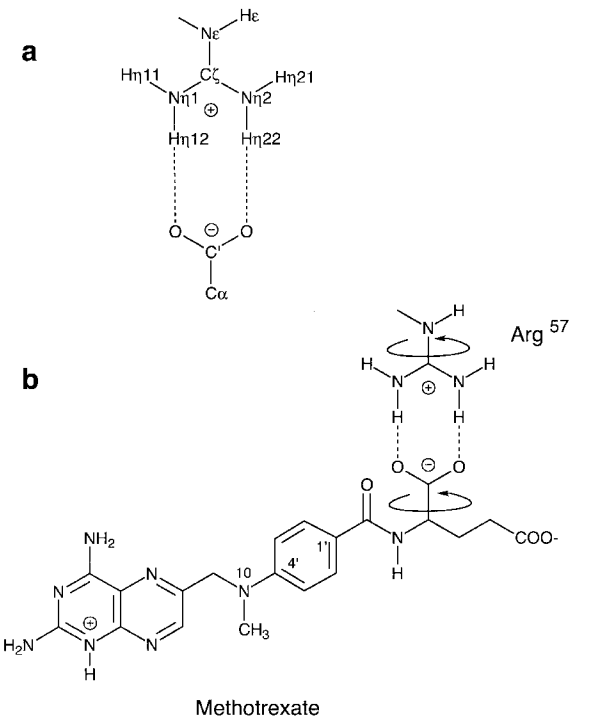
Rate constants were also determined from lineshape analysis using the standard equations for exchange processes [18]. The natural linewidths of guanidino NH signals required in these determinations were estimated by measuring the linewidths of non-exchanging amide NH and carboxyamide NH_2 signals at the appropriate temperatures.

3. Results and discussion

3.1. Rates of rotation about the $N^{\varepsilon}C^{\zeta}$ and $C^{\zeta}N^{\eta}$ bonds of Arg^{57} in complexes of dihydrofolate reductase containing methotrexate

Fig. 2a shows part of the $^{1}H/^{15}N$ HSQC spectrum of the (^{15}N-labelled)-DHFR·methotrexate·NADPH ternary complex at 1°C showing the four signals corresponding to the four NH^{η} protons and two N^{η} nitrogens in the guanidino group of the Arg^{57} residue. In earlier work [5] a 3D $^{1}H/^{15}N$ NOESY-HSQC experiment was used to assign the individual NH^{η} protons. The two lowest ^{1}H signals (9.33 and 10.00 ppm) were assigned to $NH^{\eta 12}$ and $NH^{\eta 22}$, respectively, and the large deshielding of these protons indicated that they were both interacting with the carboxylate oxygens to give an end-on symmetrical complex of the type shown in Fig. 1 [5]. A similar HSQC spectrum was observed for the Arg^{57} NH^{η} nuclei in the binary complex of DHFR with methotrexate at 1°C (see Fig. 2c).

In studies of the HSQC spectra of the DHFR·methotrexate·NADPH complex examined over the temperature range 1–40°C the four signals from the Arg^{57} guanidino group broaden as the temperature increases but remain as separate signals up to 20°C. Raising the temperature to 25°C broadens the signals in both the ^{1}H and ^{15}N dimensions to the point where the signals are no longer detected because of the exchange line broadening. At 40°C, the high field pair of signals have coalesced into a single broad signal at 6.71 and 76.89 ppm (^{1}H and ^{15}N) resulting from averaging the $H^{\eta 11}$ and $H^{\eta 21}$ and $N^{\eta 1}$ and $N^{\eta 2}$ signals. The coalescence of signals in the ^{1}H and ^{15}N dimensions over the same temperature range results from the very similar chemical shift differences of the exchanging NH nuclei for ^{1}H (170 Hz at 500 MHz) and ^{15}N (172 Hz at 50.7 MHz). The chemical shift of the coalesced signal is the averaged chemical shift expected for $H^{\eta 11}$ and $H^{\eta 21}$ in fast exchange and this indicates that there is no signal averaging between $H^{\eta 12}$ and $H^{\eta 11}$ or between $H^{\eta 22}$ and $H^{\eta 21}$. Thus, the observed pattern of signal averaging indicates fast rotation about the $N^{\varepsilon}C^{\zeta}$ bond but slow rotation about the $C^{\zeta}N^{\eta}$ bond on the NMR chemical shift time scale.



Methotrexate

Fig. 1. (a) Structure of an arginine-carboxylate complex formed with symmetrical end-on interactions and (b) structure of methotrexate showing its interaction with the guanidino group of Arg^{57} of DHFR.

A two-site exchange lineshape analysis of the coalesced signal in the ^{15}N dimension indicated that the rate of rotation about the $N^{\varepsilon}C^{\zeta}$ bond is 930 ± 100 s^{-1} at 40°C. When this rate was used to simulate the lineshape of the coalesced signal in the ^{1}H dimension, the calculated linewidth (61 ± 6 Hz) accounted for most of the measured linewidth (82 ± 4 Hz). The extra linewidth contribution arises from an additional exchange process and a lineshape simulation indicated that a rate of 71 ± 22 s^{-1} would provide the additional broadening (the signals being in the slow exchange regime). This rate is an upper limit for the average rate of rotation about the $C^{\zeta}N^{\eta}$ bonds. One cannot exclude the possibility that part of the extra line-broadening arises from exchange with the water.

A simulation of the coalesced signal expected for the $H^{\eta 12}$ and $H^{\eta 22}$ protons at 40°C using the $N^{\varepsilon}C^{\zeta}$ rotation rate of 930 s^{-1} gives a very broad signal (linewidth 290 Hz) which explains why this could not be detected (see Fig. 2b).

Similar variable temperature studies over the range 1–40°C were carried out on the DHFR·methotrexate binary complex (see Fig. 2c–e). In this case, the chemical shift difference between the ^{1}H frequencies for $H^{\eta 11}$ and $H^{\eta 21}$ (52 Hz at 600 MHz) is much smaller than that between the ^{15}N frequencies $N^{\eta 1}$ and $N^{\eta 2}$ (253 Hz at 60.8 MHz) and at 30°C there is fast exchange averaging between the proton frequencies leading to coalescence while the signals from the ^{15}N nuclei remain in

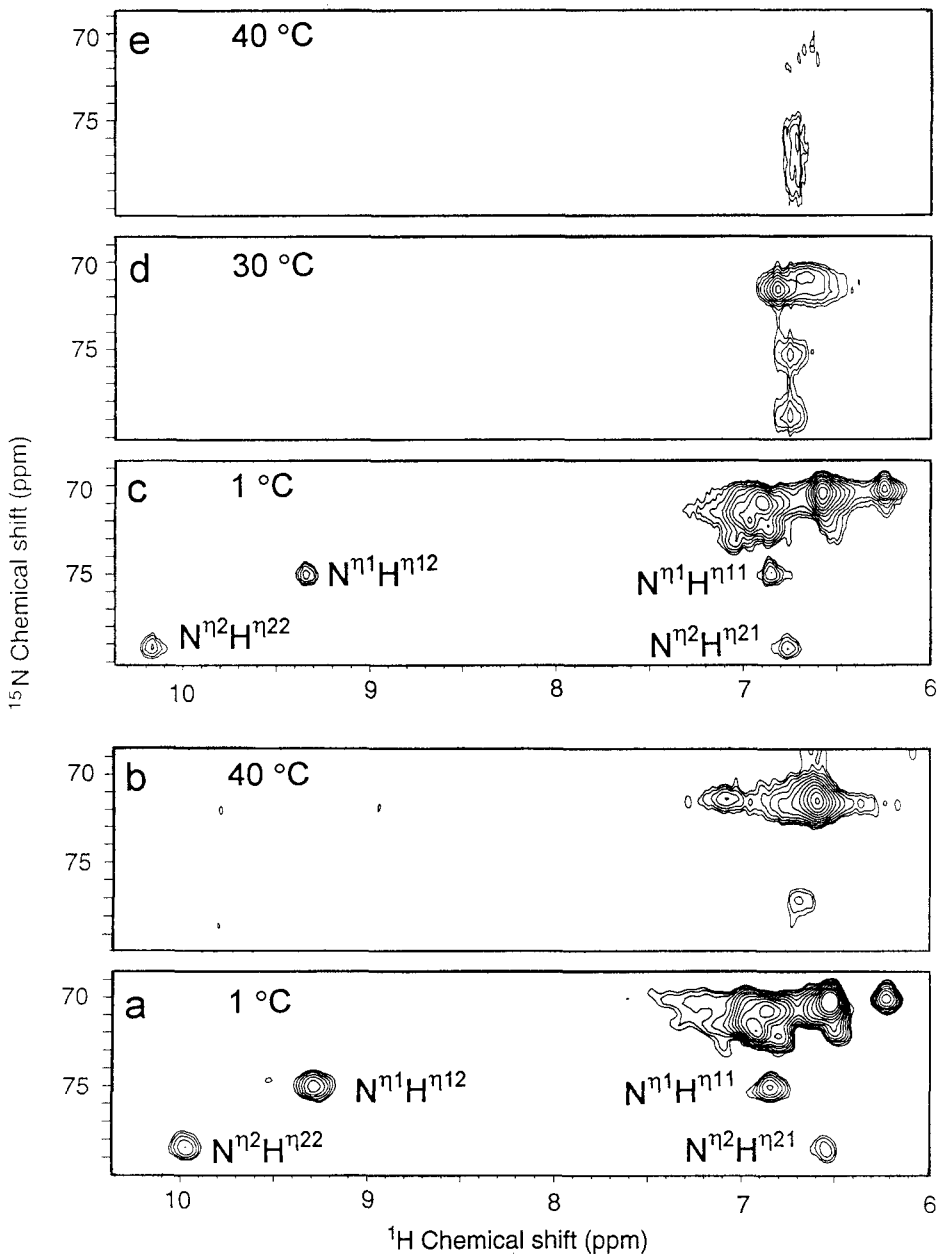


Fig. 2. Expansion of the arginine guanidino NH^{η} region of $^{1}H/^{15}N$ HSQC spectra of complexes of *L. casei* dihydrofolate reductase (DHFR) at different temperatures: DHFR·methotrexate·NADPH complex recorded at 500 MHz and at (a) 1°C: the four resolved signals detected for the NH^{η} nuclei in Arg^{57} are labelled (b) 40°C: a coalesced signal is detected at 6.71 and 76.89 ppm corresponding to exchanging nuclei from $NH^{\eta 11}$ and $NH^{\eta 21}$ of Arg^{57} in the fast exchange regime. Other Arg NH^{η} protons are detected as overlapped signals at 6.5 ± 1 and 71 ± 2 ppm. DHFR·methotrexate complex recorded at 600 MHz and at (c) 1°C, (d) 30°C, (e) 40°C.

slow exchange (Fig. 2d). At 40°C, the signals in the ^{15}N dimension are beginning to coalesce (Fig. 2e). A similar lineshape analysis to that carried out on the ternary complex gave a rate of rotation for the $N^{\varepsilon}C^{\zeta}$ bond of 565 ± 20 s^{-1} and for $C^{\zeta}N^{\eta}$ bonds an upper limit of 117 ± 17 s^{-1} at 40°C.

It was also possible to obtain a value for the rotation rates about the $N^{\varepsilon}C^{\zeta}$ of the Arg^{57} guanidino group in the DHFR complexes from exchange experiments using a zz-HSQC experiment [3,15,16]. This experiment uses a modified gradient enhanced HSQC pulse sequence [3] to detect exchange processes involving NH groups as described in Section 2. Fig. 3 shows the spectra from a series of zz-HSQC experiments on the DHFR·methotrexate·NADPH ternary complex carried out at 15°C with various mixing times. These spectra show the progressive increase in intensity of the exchange peaks with increase in mixing time. Cross peak correlations are seen between peaks which are related to corresponding protons in the two NH^{η} groups ($NH^{\eta 11}$ with $NH^{\eta 21}$ and $NH^{\eta 12}$ with $NH^{\eta 22}$). These connections are due to rotations about the $N^{\varepsilon}C^{\zeta}$ bond which result in interchange of the positions of the two NH_2 groups. The rate constant can be obtained from analysis of the changes in cross-peak intensities as a function of mixing time as indicated in Section 2. At 15°C, the rate constant for the $N^{\varepsilon}C^{\zeta}$ rotation was found to be 62 ± 20 s^{-1} for

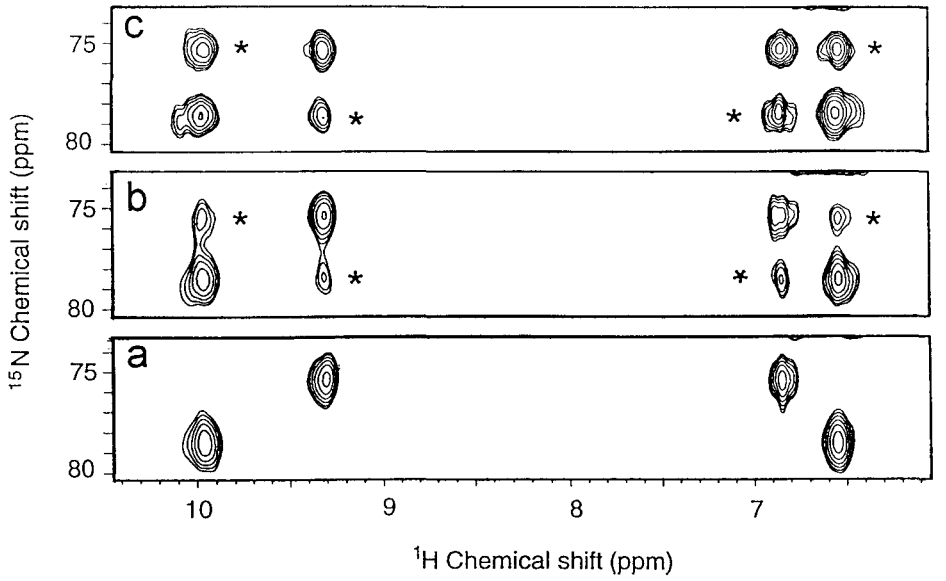


Fig. 3. Parts of the zz-HSQC spectra showing the guanidino NH^{η} signals of Arg^{57} in the complex of *L. casei* dihydrofolate reductase with methotrexate and NADPH as a function of the mixing times: (a) 16 ms, (b) 8 ms, (c) 1 ms. The exchange peaks are indicated by asterisks in the spectra.

the ternary complex. Similar experiments on the binary complex gave a rate for the $N^{\varepsilon}C^{\zeta}$ rotation of 50 ± 10 s^{-1}.

The rates of rotation about the $N^{\varepsilon}C^{\zeta}$ and $C^{\zeta}N^{\eta}$ bonds in free and complexed Arg residues are summarised in Table 1.

3.2. Comparison of the rotation rates about the $N^{\varepsilon}C^{\zeta}$ and $C^{\zeta}N^{\eta}$ bonds

It is interesting to compare the rates of $N^{\varepsilon}C^{\zeta}$ and $C^{\zeta}N^{\eta}$ bond rotations for unliganded arginine with those for an arginine complexed to a ligand carboxylate group as found for the Arg^{57} residue of DHFR in complexes containing methotrexate. For both bonds in the binary and ternary complexes, the rates of rotation are reduced compared to their values in unliganded arginine because of the interactions with the α-carboxylate group in methotrexate. The rate of $N^{\varepsilon}C^{\zeta}$ bond rotation for free arginine in aqueous solution estimated by Yamazaki and co-workers [3] using the same zz-HSQC method as used here is 636 s^{-1} at 15°C which is about 10-fold faster than the rate measured for this bond rotation in the Arg^{57} residue of both the binary (50 ± 10 s^{-1}) and ternary (62 ± 20 s^{-1}) DHFR complexes. For the $C^{\zeta}N^{\eta}$ bond rotation, the rate is reduced even more dramatically (more than 100-fold) from 7800 and 17 500 s^{-1} at 25°C for the free N^{α}-acetylarginine isopropyl ester [6] to less than 71 ± 22 s^{-1} at 40°C for Arg^{57} in the DHFR ternary complex (less than 117 ± 17 s^{-1} in the binary complex).

3.3. Correlated bond rotations

The most interesting observation is that the rates of rotation about the $N^{\varepsilon}C^{\zeta}$ bonds at 40°C (930 s^{-1} (ternary) and 565 s^{-1} (binary)) are greater than those about the $C^{\zeta}N^{\eta}$ bonds (less than 71 s^{-1} (ternary) and 117 s^{-1} (binary)) reversing the pattern seen for these rotation rates in free arginine. This surprising result needs some explanation because the hydrogen bonding interactions involving the NH^{η} protons with groups on the ligand or protein might have been expected to influence the two bond rotations similarly or proportionally reflecting the rate of hydrogen bond breaking. Consideration of Fig. 1a indicates that a faster rate for the rotation about the $N^{\varepsilon}C^{\zeta}$ bond could only be achieved if there are simultaneous motions in the guanidino and carboxylate groups such that concerted rotations occur about the $N^{\varepsilon}C^{\zeta}$ bond and the $C'C^{\alpha}$ bond of the α-carboxylate group. This model of the rotational dynamics is fully consistent with the symmetrical end-on interaction involving stable hydrogen bonds to both NH^{η} groups (Fig. 1) which was proposed earlier based on chemical shift information [5]. In this model, rotation can take place about the $N^{\varepsilon}C^{\zeta}$ bond even when the guanidino-

Table 1
Rates of rotation about the $N^{\varepsilon}C^{\zeta}$ and $C^{\zeta}N^{\eta}$ bonds in free and complexed arginine residues

Residue	Bond	Rate constants (s^{-1})		
		15°C	25°C	40°C
Arg^{57} in DHFR·MTX	$N^{\varepsilon}C^{\zeta}$	50 ± 10^{a}		565 ± 20^{b}
Arg^{57} in DHFR·MTX·NADPH	$N^{\varepsilon}C^{\zeta}$	62 ± 20^{a}		930 ± 100^{b}
Free arginine[c]	$N^{\varepsilon}C^{\zeta}$	400–640	900–1500 [a,b]	3000–4000
Arg^{57} in DHFR·MTX	$C^{\zeta}N^{\eta}$			$< 117 \pm 17^{b}$
Arg^{57} in DHFR·MTX·NADPH	$C^{\zeta}N^{\eta}$			$< 71 \pm 22^{b}$
Free N^{α}-Ac-Arg-O^{i}Pr[d]	$C^{\zeta}N^{\eta}$		7800–17500[b]	

[a]From zz-HSQC exchange analysis.
[b]From line shape analysis.
[c]Data reported in [3,7].
[d]Data reported for N^{α}-acetylarginine isopropyl ester in [6].

carboxylate interactions remain intact whereas rotation about a $C^{\zeta}N^{\eta}$ bond can only occur when all interactions with each NH_2 group are broken. Thus the two types of rotation can be influenced differently by the rates of hydrogen bond breaking, with the rate for the $C^{\zeta}N^{\eta}$ bonds being slower than for the $N^{\varepsilon}C^{\zeta}$ bond because the former rotation can only take place when the guanidino-carboxylate hydrogen bonds have been broken. The correlated bond rotations resemble those observed previously for the flipping of the benzoyl aromatic ring of a methotrexate analogue bound to DHFR [19]. In this case, the flipping of the aromatic ring occurs while the other parts of the methotrexate remain tightly bound in the complex. The ring flipping requires correlated rotations, within the bound methotrexate, about the two bonds (C1′CO and C4′N10) connecting the *para*-positions of its benzoyl ring to the other parts of methotrexate (see Fig. 1). Of course, in the case of the interaction between the arginine and carboxylate groups (Fig. 1) the correlated rotations involve two bonds which are from different molecules.

The observation of two different examples of correlated bond motions within methotrexate-protein complexes suggests that such correlated motions probably occur frequently in proteins. NMR provides a direct method for detecting and characterising these correlated motions.

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