Eur Biophys J (1985) 11 : 211–218

European Biophysics Journal

^{1}H and ^{15}N NMR studies of protonation and hydrogen-bonding in the binding of trimethoprim to dihydrofolate reductase

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Received August 6, 1984/Accepted October 8, 1984

Abstract. The binding of trimethoprim and [1,3,2-amino-$^{15}N_3$]-trimethoprim to *Lactobacillus casei* dihydrofolate reductase has been studied by ^{15}N and ^{1}H NMR spectroscopy. ^{15} N NMR spectra of the bound drug were obtained by using polarisation transfer pulse sequences. The ^{15}N chemical shifts and ^{1}H-^{15}N spin-coupling constants show unambiguously that the drug is protonated on N1 when bound to the enzyme.

The N1-proton resonance in the complex has been assigned using the ^{15}N-enriched molecule. The temperature-dependence of the linewidth of this resonance has been used to estimate the rate of exchange of this proton with the solvent: $160 \pm 10\ s^{-1}$ at 313 K, with an activation energy of 75 ($\pm$ 9) $kJ \cdot mole^{-1}$. This is considerably faster than the dissociation rate of the drug from this complex, demonstrating that there are local fluctuations in the structure of the complex.

Key words: Dihydrofolate reductase, trimethoprim, NMR (^{15}N, ^{1}H), hydrogen bonding, protonation

Introduction

Dihydrofolate reductase (EC 1.5.1.3) is the target for the widely used 'anti-folate' drugs, such as methotrexate, trimethoprim, and pyrimethamine. All the most potent inhibitors of the enzyme contain a 2,4-diamino substituted pteridine, pyrimidine or related ring, and, as originally suggested by Baker (1967), protonation of this ring appears to play an important role in their binding to the reductase. Experimental evidence that inhibitors are protonated when bound to the enzyme has come from ultraviolet absorption (e.g., Erickson and Mathews 1972; Poe et al. 1974, 1975; Hood and Roberts 1978) and ^{13}C NMR spectroscopy (Cocco et al. 1981, 1983; Roberts et al. 1981; Blakley et al. 1983). X-ray crystallographic studies (Matthews et al. 1977, 1983; Baker et al. 1981, 1983; Volz et al. 1982; Bolin et al. 1982) have shown that, when 2,4-diamino-substituted pteridines, pyrimidines, and triazines are bound to dihydrofolate reductase from *Escherichia coli, Lactobacillus casei* or chicken liver, N1 and the 2-amino group are within hydrogen-bonding distance of a carboxylate group (aspartate in bacterial reductase, glutamate in the enzyme from vertebrates). The charge-charge and hydrogen-bonding interaction between this carboxylate and the protonated inhibitors clearly makes a major contribution to the overall binding energy, and is probably the principal determinant of the mode of binding of these inhibitors, which is quite different from that of the substrates (Roberts 1983).

In the ^{13}C NMR studies cited above, the conclusion regarding the protonation state of the bound inhibitor was based on the chemical shift of the $^{13}C2$ resonance. In view of the crucial importance of the carboxylate-inhibitor interaction, we sought to study it by more direct NMR methods. ^{15}N NMR has been shown to provide clear-cut information on ionization states and hydrogen-bonding in proteins (e.g., Bachovchin and Roberts 1978; Kanamori and Roberts 1983a, b; Franken et al. 1984, and references therein), while ^{1}H NMR can be used to study hydrogen-bonded protons directly (as, notably, in nucleic acid double helices; see, e.g., Schimmel and Redfield 1981; Reid 1981; Kearns 1984). In the present paper we report ^{15}N and ^{1}H studies of N1 and the 2-amino group of trimethoprim bound to *L. casei* dihydrofolate reductase.

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Materials and methods

Dihydrofolate reductase was isolated and purified from *Lactobacillus casei* MTX/R as described by Dann et al. (1976). [1,3,2-amino-$^{15}N_3$]-trimethoprim was prepared by condensation of [$^{15}N_3$]-guanidine (95% enrichment; U.S. Services, Inc.) with β-anilino-α-(3,4,5-trimethoxybenzyl)acrylonitrile (Cresswell et al. 1972; Cresswell and Mentha 1975). $NADP^+$, NADPH, and trimethoprim were obtained from Sigma Chemical Co.

Samples for NMR spectroscopy contained 1–1.4 mM enzyme in 50 mM potassium phosphate, 500 mM KCl, pH 6.35, with 10% (v/v) ^2H_2O for field-frequency lock. 0.9 molar equivalents of trimethoprim or [$^{15}N_3$]-trimethoprim were added to ensure that all the trimethoprim was bound to the enzyme (K_a 2×10^7 M^{-1}; Birdsall et al. 1980).

20 MHz ^{15}N NMR spectra were obtained using a Bruker WM200 spectrometer. Conventional spectra were obtained with a 20° pulse and a pulse interval of 0.4 s; the block averaging method was used to overcome the effects of pulse breakthrough, and a total of approximately 90,000 transients was averaged. INEPT spectra (insensitive nuclei enhanced by polarisation transfer; Morris and Freeman 1979; Morris 1980) were obtained using pulse timings appropriate for a triplet with $^1J_{NH}$ = 90 Hz; a relaxation delay of 1 s was used, and approximately 10,000 transients were averaged. In some experiments, noise-modulated ^1H irradiation was applied during acquisition. ^{15}N chemical shifts were referenced to $^{15}NH_4Cl$ (0.1 M in 0.1 M HCl containing 10% ^2H_2O).

500 MHz ^1H NMR spectra were obtained by using a Bruker AM500 spectrometer. Spectra in H_2O were obtained by using the 1-1 hard pulse method (Clore et al. 1983; Hore 1983), with the carrier frequency offset 4 kHz downfield from the H_2O resonance. Before Fourier transformation, the free induction decay was further processed to minimise the remaining H_2O resonance by using the DSA method of Roth et al. (1980). ^1H chemical shifts are referenced to dioxan (3.71 ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulphonate).

Results and discussion

^{15}N NMR spectroscopy

The ^{15}N chemical shifts and one-bond ^{15}N-^1H scalar coupling constants of the three enriched nitrogens in [1,3,2-amino-$^{15}N_3$]trimethoprim in aqueous and dimethylsulphoxide solution are summarised in Table 1. Comparing the chemical shifts of the monoprotonated and neutral species in DMSO solution[1], it can be seen that monoprotonation produces a very substantial (80 ppm) upfield shift of the $^{15}N1$ resonance, but much smaller shifts of the N3 and 2-NH_2 resonances. (For a discussion of ^{15}N protonation shifts in this and related systems, see Städeli et al. 1980). This large change in N1 chemical shift indicates that in trimethoprim (as in other 2,4-diamino pyrimidines; Brown and Teitei 1965) protonation occurs on N1 in free solution. This is demonstrated unambiguously by the observation of a 94-Hz ^{15}N-^1H coupling constant to N1, a value characteristic of a coupling across one bond (Levy and Lichter 1979; Martin et al. 1981).

Figure 1 shows the ^{15}N resonances of N1 and the 2-amino group of [1,3,2-amino-$^{15}N_3$]trimethoprim in its binary complex with *L. casei* dihydrofolate reductase with, for comparison, the corresponding part of the spectrum of monoprotonated trimethoprim in aqueous solution. The spectrum of the enzyme complex was obtained by using the refocused INEPT method (Morris and Freeman 1979; Morris

Table 1. ^{15}N chemical shifts and one-bond ^{15}N-^1H coupling constants of neutral and monoprotonated trimethoprim

Charge state	Solvent	N1		2-NH_2		N3
		δ^a	$^1J_{^{15}NH}{}^b$	δ^a	$^1J_{^{15}NH}{}^b$	δ^a
Monoprotonated	90% H_2O 10% ^2H_2O, pH 3.5	109.2	ND	57.6	ND	172.6
Monoprotonated	[$^2H_6$]-DMSO	112.1	94	62.4	91	180.0
Neutral[c]	[$^2H_6$]-DMSO	192.3	–	50.7	88	180.9

[a] ppm from external $^{15}NH_4Cl$, positive shifts downfield, ± 0.1 ppm
[b] Hz, ± 1 Hz
[c] From Städeli et al. (1980), calculated using $\delta_{^{15}NH_4Cl} = -355.3$ ppm relative to nitromethane
ND – not determined

[1] DMSO was used for this comparison, since the neutral species is insufficiently soluble in water

1980), with ^{1}H-decoupling during acquisition. Although the INEPT technique gives rise to some phase distortion (see Fig. 2), we found that for the present application, where ^{1}H and ^{15}N T_2 values are short, it gave somewhat better signal-to-noise ratios than DEPT (Doddrell et al. 1982). For free trimethoprim in aqueous solution proton exchange (particularly on N1) is too fast for efficient polarisation transfer, and both for this and for observing the unprotonated N3 in the enzyme complex (not shown in Fig. 1) conventional acquisition, using 20° pulses, was employed.

The ^{15}N chemical shifts in the binary and ternary complexes are summarised in Table 2. In all the complexes, the chemical shift is similar to that of free protonated trimethoprim and very different from that of the neutral molecule. This makes it very probable that trimethoprim is protonated on N1 when bound to the enzyme, as already suggested by ^{13}C experiments (Roberts et al. 1981).

Unequivocal evidence for this can be obtained from scalar ^{15}N-^{1}H coupling, since this is a through-bond effect (see Kanamori and Roberts 1983a). The INEPT experiment itself depends upon the existence of this coupling, which can be observed directly in spectra obtained without ^{1}H decoupling (Fig. 2). A value of about 90 Hz is measured for the coupling to N1. The precision of this value is limited

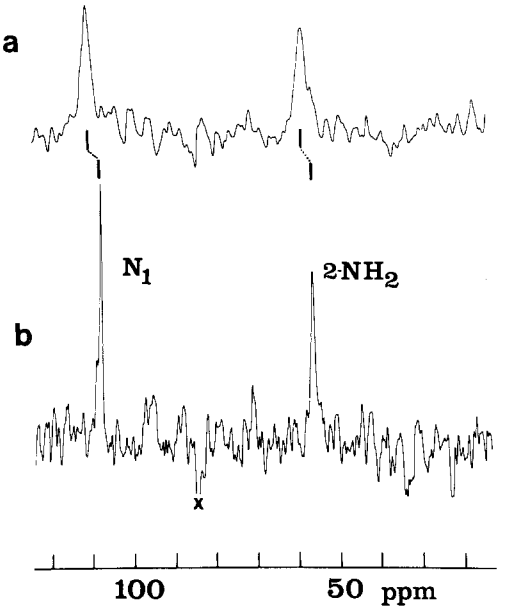

Fig. 1. Part of the ^{15}N NMR spectra of (*top*) the dihydrofolate reductase-[$^{15}N_3$]trimethoprim complex and (*bottom*) free monoprotonated [$^{15}N_3$]trimethoprim (in 0.1 M HCl). The top spectrum was obtained by using the INEPT method, and the bottom spectrum by conventional acquisition using 20° pulses; in the latter, x indicates an instrumental artefact

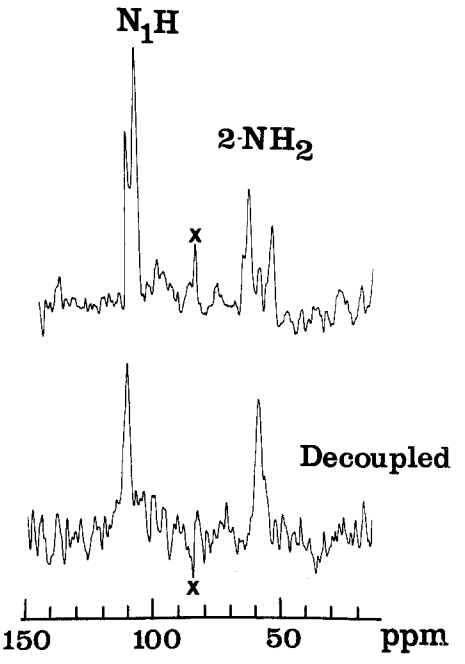

Fig. 2. Comparison of the ^{15}N NMR spectra of the dihydrofolate reductase-[$^{15}N_3$]trimethoprim complex obtained by the INEPT method with (*bottom*) and without (*top*) noise-modulated ^{1}H irradiation during acquisition. In the absence of ^{1}H irradiation, the N1 resonance appears as a doublet and the $2NH_2$ resonance as a triplet, of which only the two outer lines are detectable since only they are enhanced by polarisation transfer from the protons

Table 2. ^{15}N chemical shifts of trimethoprim bound to *L. casei* dihydrofolate reductase

complex	Chemical shift[a]		
	N1	2-NH_2	N3
Enzyme-trimethoprim	111.3 (+ 2.1)	59.6 (+ 2.0)	179.4 (+ 6.8)
Enzyme-trimethoprim-NADP^{+}[b]	113.0 (+ 4.0)	62.0 (+ 4.3)	ND
	111.0 (+ 1.8)	58.3 (+ 0.6)	ND
Enzyme-trimethoprim-NADPH	109.8 (+ 0.6)	59.1 (+ 1.5)	ND

[a] ppm from external $^{15}NH_4Cl$; in parentheses, relative to free monoprotonated trimethoprim
[b] From Birdsall et al. (1984); this complex exists in two conformational states
ND – not determined

by the phase distortions in the INEPT spectrum, and a more precise value of 93.5 Hz is obtained from the ^1H spectrum (see below). This is very similar to the value for monoprotonated trimethoprim in DMSO (Table 1), and clearly within the range of values characteristic of ^{15}N-^1H coupling across a single bond. This is therefore unambiguous evidence that N1 of trimethoprim is protonated when the drug is bound to dihydrofolate reductase.

The observation of this doublet splitting on the $^{15}N1$ resonance also implies that the rate of exchange of the N1-proton with the solvent is less than about $^1J_{^{15}N1H} \sim 90\ s^{-1}$. By contrast, no INEPT enhancement of the $^{15}N1$ resonance is observed for free protonated trimethoprim in aqueous solution, indicating that N1-proton exchange is significantly faster in the free molecule. In the enzyme-trimethoprim complex, increasing temperature leads to a marked decrease in intensity of the $^{15}N1$ but not the 2-$^{15}NH_2$ resonance in spectra obtained by the INEPT method; by 313 K the $^{15}N1$ signal has less than 10% of the intensity of the 2-$^{15}NH_2$ signal. This can most readily be explained by postulating that the increased rate of N1-proton exchange at higher temperatures leads to a partial collapse of the doublet splitting and hence to a decrease in the efficiency of polarisation transfer. This would imply that at 313 K the rate of exchange is of the order of $90\ s^{-1}$; quantitative estimates of the exchange rates by ^1H NMR (see below) confirm this.

The differences in ^{15}N chemical shift between trimethoprim bound to the enzyme and the free monoprotonated molecule in aqueous solution are shown in parentheses in Table 2. They are in the same direction as the solvent shifts on going from water to DMSO (Table 1), and are of a similar magnitude. ^{15}N chemical shifts are well known to be very sensitive to intermolecular effects, such as hydrogen-bonding and ion-pair formation (Duthaler and Roberts 1978; Schuster and Roberts 1979; Levy and Lichter 1979; Martin et al. 1981; Witanowski et al. 1981), but these effects are very hard to interpret in structural terms. The largest shift, 6.8 ppm, is seen for N3. This nitrogen presumably acts as a (relatively weak) hydrogen bond acceptor in aqueous solution, but appears to form no hydrogen bonds in the complex of trimethoprim with the *E. coli* reductase (Baker et al. 1981). Its ^{15}N chemical shift when bound to the enzyme is virtually identical to that in DMSO, where it will not be hydrogen-bonded, and the change in chemical shift on binding may thus largely reflect the absence of hydrogen-bonding to this nitrogen in the complex.

^1H NMR spectroscopy

The resonance of the N1-proton will be directly observable in the ^1H NMR spectrum provided that exchange of this proton between N1 and water is slow on the NMR timescale. Thus the resonance is not observed for free monoprotonated trimethoprim in H_2O, but it is observed, at 9.92 ppm, in dry DMSO solution. From this chemical shift, together with the rough estimate of the exchange rate given above, it appeared that the N1-proton signal should be detectable in the spectrum of the enzyme-trimethoprim complex.

Figure 3 shows the low-field region of the ^1H NMR spectrum of the complex in H_2O solution, obtained by using the 1-1 hard pulse method (Clore et al. 1983; Hore 1983). A number of single-proton resonances are visible downfield from 6 ppm, including – close to the N1-proton chemical shift in DMSO – two at 10.31 and 11.07 ppm (14.02 and 14.78 ppm from DSS). To obtain an unambiguous assignment of the N1-proton resonance, we have compared the spectra of the enzyme-trimethoprim and en-

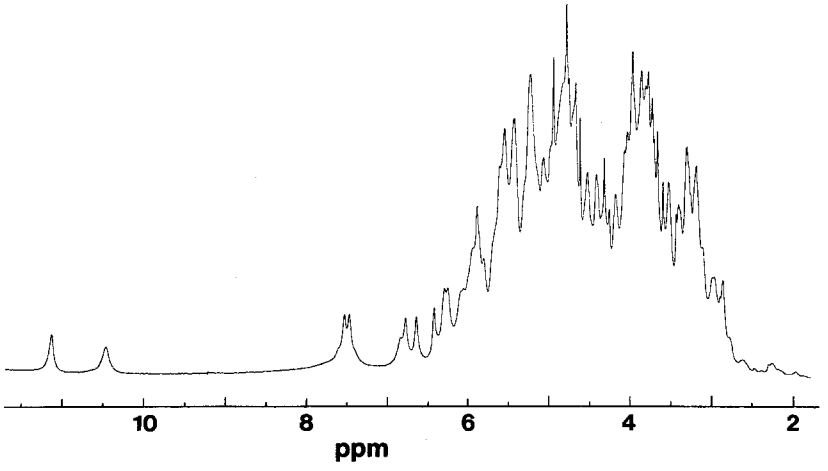

Fig. 3. The low-field region of the ^1H NMR spectrum of the dihydrofolate reductase-trimethoprim complex in 90% H_2O/10% ^2H_2O showing single proton resonances at 10.31 and 11.07 ppm

zyme-[1,3,2-amino-$^{15}N_3$]trimethoprim complexes; in the ^{15}N-enriched compound the N1-proton resonance will, of course, show a 90-Hz doublet splitting due to spin-coupling to the ^{15}N. The comparison is shown in Fig. 4. It is clear that the resonance at 11.07 ppm appears as a doublet in the spectrum of the ^{15}N-containing complex. It must thus arise either from the N1-proton or from one of the 2-amino protons of the bound trimethoprim molecule, and from its chemical shift it can be assigned to the N1-proton. The other very low-field resonance (at 10.31 ppm from dioxan) is likely to arise from the imidazolium NH of a protonated histidine residue, probably His 153 since this is the most buried histidine (Bolin et al. 1982). The difference spectrum in Fig. 4 reveals a second ^{15}N-coupled one-proton resonance, at 6.77 ppm. This can be assigned to one of the 2-amino protons. In DMSO solution, the average chemical shift of the two 2-amino protons is 5.39 ppm. The resonance of the second 2-amino proton has not been located; from Fig. 4, either the resonance must lie upfield of 6 ppm or the proton must be exchanging rapidly with the solvent. The signal at 6.77 ppm is likely to arise from that 2-amino proton which is involved in a hydrogen-bond to Asp 26 (Bolin et al. 1982). Both the N1-proton and 2-NH_2 proton resonances appear about 1 ppm further downfield when hydrogen-bonded to the carboxylate of Asp 26 in the complex than when hydrogen-bonded to DMSO in free solution.

In Fig. 4, it is apparent that the low-field component of the N1-proton doublet (in the spectrum of the ^{15}N-enriched material) is appreciably broader than the high-field component. A similar phenomenon has been observed by Rüterjans et al. (1982) in studies of the NH resonances of ^{15}N-enriched tRNA. These authors originally proposed that this differential line-broadening arose from exchange with a small population of the minor base tautomers (N-H$\cdots$N $\rightleftharpoons$ N$\cdots$H-N). We have confirmed by line-shape simulation that a model in which the proton exchanges between two sites within the complex, spending $\sim 95\%$ of the time on N1 and $\sim 5\%$ in another site where it has a chemical shift within ~ 0.6 ppm of that in the major site, can account for our results. However, Redfield (see Rüterjans et al. 1982) and Gueron et al. (1983) have pointed out that the differential relaxation rates could, alternatively, be explained by interference between dipole-dipole and chemical shift anisotropy relaxation, and this seems to be the most likely explanation for our observations. Evidence for this comes from studies of the enzyme-$NADP^+$-[$^{15}N_3$]-trimethoprim complex, which exists in two slowly interconverting conformational states (Gronenborn et al. 1981; Birdsall et al. 1984). This complex gives rise to *two* $^{15}N1$-proton doublets, from the two conformations, *each* of which shows the same differential broadening of the low-field component. This would be expected if the mechanism proposed by Redfield and Gueron is correct. By contrast, any explanation based on exchange would require one to postulate the existence of two further environments for the N1-proton (i.e., a total of four), with

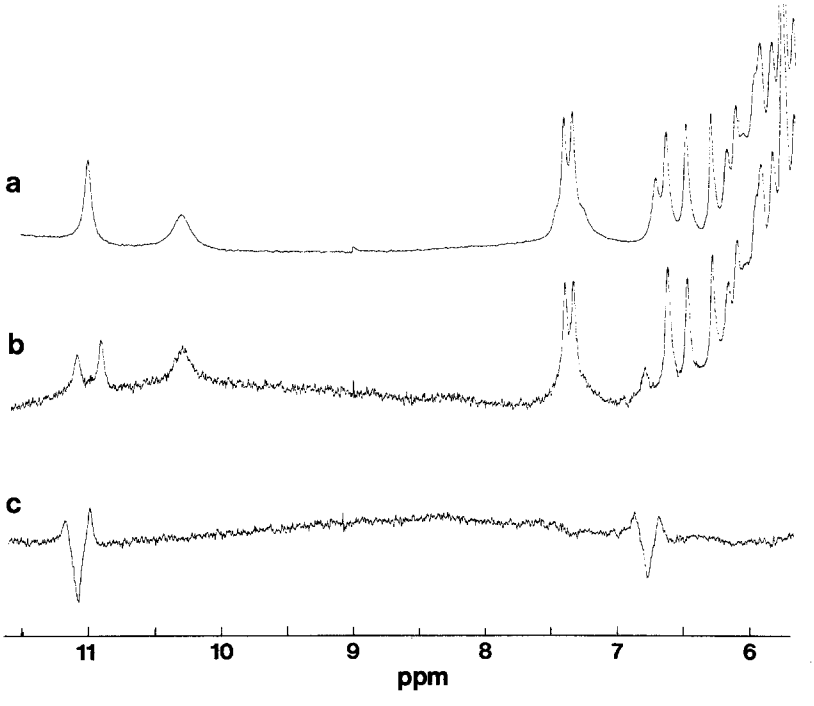

Fig. 4a–c. Comparison of the low-field region of the ^1H NMR spectra, in 90% H_2O/10% ^2H_2O, of the complexes of dihydrofolate reductase with (**a**) trimethoprim and (**b**) [$^{15}N_3$]trimethoprim. The difference (**b**)−(**a**) is shown in (**c**)

considerable constraints on the possible chemical shifts of the minor sites and on the exchange rates between the four sites. Clearly, the explanation based on interference between dipolar and chemical shift anisotropy relaxation (Gueron et al. 1983) is much the more economical.

The exchange of the N1-proton with the solvent will contribute to the linewidth of its resonance, and this potentially provides a method for determining the rate of this exchange. Figure 5 shows the linewidth of the N1-proton resonance as a function of temperature over the range 273–313 K. As the temperature is increased, the linewidth first decreases, reaching a minimum at about 290 K, then increases again. This behaviour is characteristic of a situation in which the linewidth is dominated by the 'natural' linewidth at low temperature and by the exchange contribution at higher temperatures – the two contributions having opposite temperature dependences. The data in Fig. 5 can be analysed quantitatively by assuming that both exchange and relaxation rates show Arrhenius behaviour as a function of temperature. The line in Fig. 5 is calculated on this assumption, with the parameters given in the caption, and can be seen to fit the data to within the experimental error. From this analysis, we estimate the exchange rate to be 160 (± 10) s^{-1} at 313 K with an activation energy of 75 (± 9) kJ · $mole^{-1}$. The temperature dependence of the 'natural' linewidth is characterised by an apparent activation energy of 13 (± 2) kJ · $mole^{-1}$; this is very similar to the activation energy of viscosity, as would be expected if the natural linewidth was determined by the overall tumbling of the protein.

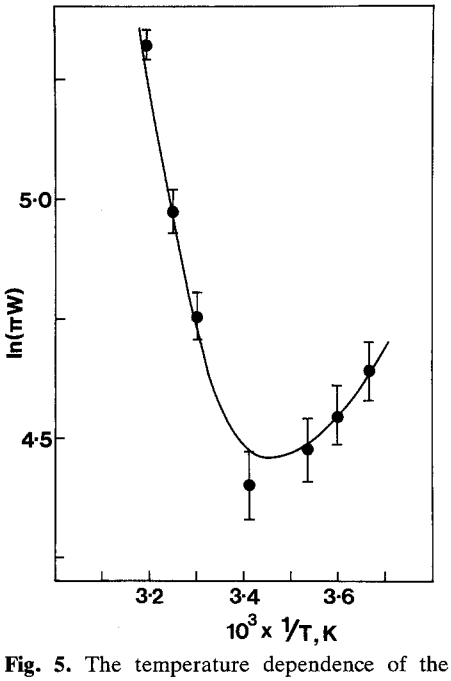

Fig. 5. The temperature dependence of the linewidth of the N1-proton resonance of trimethoprim in its complex with dihydrofolate reductase, presented as an Arrhenius plot [ln (π × linewidth) vs 1/T]. The points are experimental data, the error bars indicating the estimated maximum uncertainty in linewidth (± 2 Hz). The line was calculated using the following parameters: W_0 (273 K) = 32 Hz; E_a (W_0) = 13 kJ/mole; k_{exch} (313 K) = 160 s^{-1}; E_a (exchange) = 75 kJ/mole, where W_0 is the natural linewidth (in the absence of exchange) and E_a denotes activation energy

The N1-proton thus exchanges with the solvent at a rate of 160 s^{-1} at 313 K. This is more than 25 times faster than the rate of dissociation of the trimethoprim molecule from the enzyme (6 s^{-1} at 318 K, Cayley et al. 1979). Dissociation of the inhibitor molecule is therefore clearly not required before its N1-proton can exchange with the solvent. The exchange must result from local fluctuations in structure of the enzyme-trimethoprim complex.

The fact that no N1-proton signal can be detected for free trimethoprim in H_2O indicates that the chemical exchange process is fast in free solution. From the pK of N1, 7.1 (Roth and Strelitz 1969), one would expect the exchange to be diffusion-limited (Englander and Kallenbach 1984), having a pseudo-first order rate constant of ~ 10^3 s^{-1} at pH 7. It is thus probable that the structural fluctuation is the rate-limiting step, with a rate of 160 s^{-1}. The nature of this fluctuation is less clear – the alternative models for hydrogen exchange in proteins of solvent penetration or local unfolding have been discussed for many years (see Englander and Kallenbach 1984, and references therein). The minimum structural change required for exchange would probably be one which led to the breaking of the N1-Asp 26 hydrogen-bond. It is interesting that an interaction which is apparently so crucial to the equilibrium binding of trimethoprim is kinetically rather labile, making and breaking more than 100 times/s.

Acknowledgements. We are most grateful to Gill Ostler and John McCormick for invaluable technical assistance, to Professor Maurice Gueron for sending us a copy of his paper before publication, and to Wellcome Research Laboratories for a CASE Studentship to AWB.

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