The effect of hydration on the dynamics of trimethoprim bound to dihydrofolate reductase

A deuterium NMR study

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ABSTRACT To determine the effect of hydration on the dynamics of a protein complex, we used deuterium nuclear magnetic resonance (NMR) techniques to examine a trimethoprim (TMP)/*E. coli* dihydrofolate reductase (DHFR) complex in its lyophilized, partially hydrated, polycrystalline, and ammonium sulfate-precipitated states. The results indicate that TMP is rigid in the lyophilized powder state. The dynamic behavior could be restored by partial rehydration. At 30 wt% hydration the deuterium spectrum of the partially hydrated sample was indistinguishable from that of the polycrystalline and ammonium sulfate-precipitated samples, suggesting that the structure of the protein/TMP complex is similar in the three physical states. Furthermore, we found that the *para*- and *meta*-methoxyl groups have very different dynamical behavior.

INTRODUCTION

Water–protein interaction is important in maintaining proteins in conformations that are vital for biological activities (1–2). The presence of solvent molecules affects the overall conformation of a protein by affecting the electrostatic interactions between charged and polar groups. At the active site crevice, solvent molecules are likely to affect the interactions between the substrate and the protein, thereby affecting the detailed mechanism of substrate binding and the enzymatic activity (3). The average solvent structure in the active site has profound influence on the electrostatic potential near the active site, thereby serving to influence the diffusional approach of charged substrates. Furthermore, the presence of water molecules can affect protein dynamics, which may in turn affect the binding of substrates and the catalytic process (4, 5). Various physical techniques have been employed to study water–protein interactions (2). Most studies have focused on the properties of water molecules (6, 7), although one would expect to observe a significant effect of solvent molecules on the structure and dynamics of the protein moiety (8–9).

Solid state NMR techniques have been employed extensively and successfully to study the structure–function relationships of biomolecules (11–13). However, application of these techniques to study macromolecular systems is hampered by the need for "solid" samples with the macromolecules in their native conformations, and yet the overall molecular rotation rate must be slow compared to the NMR time scale. Thus, protein samples employed for solid state NMR studies are often in polycrystalline form. Preparation of protein crystals is not trivial. The large quantities of protein crystals (>100 mg) needed for such studies compound the problem. We have therefore attempted to explore the possibility of using partially hydrated protein samples for such studies. Such an approach has been employed successfully to study the dynamics of glycogen and nucleic acids (8, 13). We have employed deuterium NMR techniques to monitor the onset of the dynamic process with increasing level of hydration of a drug, trimethoprim (TMP) (Fig. 1), bound to the protein dihydrofolate reductase (DHFR) from *E. coli*. To assess the validity of this approach, the results were compared with those obtained with samples in different physical states, namely the polycrystalline and ammonium sulfate-precipitated forms.

MATERIAL AND METHODS

Wild-type *E. coli* DHFR was isolated from DHFR over-producing *E. coli* strain (a generous gift from Dr. E. Howell of the University of Tennessee). DHFR was isolated according to the procedure described previously (14). Methoxyl deuterated TMP was prepared by converting 5-Bromovanillin to 5-$^2H_3$-syringaldehyde, using a modification of a published procedure (15). These deuterated materials were then converted to deuterated TMP, using the procedure of Cresswell and Mentha (16).

NMR samples were prepared by dissolving 250 mg of lyophilized protein powder in 10 ml of 10 mM phosphate buffer, pH 7.2, in deuterium-depleted water, to which dithiothreitol (DTT) and EDTA were added to 1 mM concentration. Specifically deuterated TMP was added from a stock solution to give a final ratio of 0.9 TMP:1.0 DHFR. The protein solution was allowed to stand at 4°C for 30 min prior to centrifugation and subsequent lyophilization. Hydrated samples were prepared by equilibrating the lyophilized protein complex powder with saturated sodium iodide solution (made from deuterium depleted water) for 7 to 15 days in an enclosed chamber at 4°C. To ensure uniform hydration, the protein powders were spread out on a thin teflon tape, and the weight gain was monitored regularly to determine the degree of hydration. When the desired degree of hydration was reached, the protein sample was wrapped up in the teflon tape and sealed inside a 5 mm thin-wall glass tube for NMR experiments. Poly-

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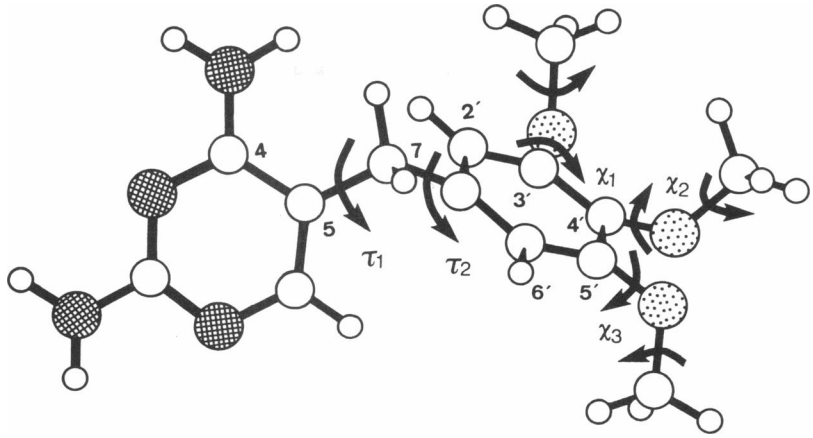

FIGURE 1 The structure of trimethoprim (TMP).

crystals of TMP/DHFR complex were grown according to the method of Champness et al. (17).

Deuterium NMR spectra were obtained on a home-built spectrometer operating at deuterium frequency of 46.1 MHz, using an acoustic ringing suppression–quadruple echo technique with a $\pi/2$ pulse of 2 μs Echo delay time t = 40 μs. and recycle time of 0.5 s (18). Quadruple echo signals were digitized at 0.5 or 1.0 μs at 1 k point resolution using a Nicolet transient recorder. Signals were zero-filled to 4,096 points prior to Fourier transformation. Special attention was given to ensure that digitization began at the top of the echo. Cold nitrogen gas was used to cool down the sample and the temperature was regulated by a home-built temperature controller to within ±1°C.

RESULTS AND DISCUSSION

Fig. 2 shows a series of deuterium powder spectra of polycrystalline (3′,4′-OMe-$^2H_6$)TMP and (3′,4′-OMe-$^2H_6$)TMP/DHFR complex (D-6 sample) in partially hydrated and ammonium sulfate precipitated forms. The residual splitting, i.e., the separation between the two most prominent "horns," of the polycrystalline TMP sample is 34 kHz. This splitting does not change with temperature between −60 and 70°C (19), which is characteristic of a rotating methyl group with no additional motion (10, 12). Thus, the methoxyl groups of polycrystalline TMP exert no motion other than the methyl group rotation. The spectrum of the lyophilized protein/TMP complex resembles that of the "rigid" spectrum of polycrystalline TMP, suggesting that in the anhydrous, lyophilized powder of the protein binary complex the methoxyl groups of TMP exert only methyl group reorientation. Upon increasing the hydration level to 30 wt%, the spectral lineshape gradually transforms to a round-top shape. The separation between the two sharp edges reduces to 21 kHz. A further increase in hydration level above 30 wt% caused no additional change in the deuterium spectrum (see also reference 19). Thus, the transformation appears to be complete at 30 wt% of hydration. Furthermore, the spectrum of the (3′,4′-OMe-$^2H_6$)TMP/DHFR complex precipitated from 95% ammonium sulfate solution is nearly identical to that of the 30 wt% sample.

Fig. 3 shows a set of deuterium spectra of polycrystalline and 43 wt% hydrated (4′-OMe-$^2H_3$)TMP/DHFR complex (D-3 sample) taken at 20°C. The spectra also show that the hydration induces molecular motion, as revealed by the reduced splitting from 34 kHz for the anhydrous sample to 21 kHz for the 43 wt% hydrated sample. The most interesting feature of these two spectra is the remarkable resemblance of them, suggesting that the protein conformation at the TMP binding site is similar in the two physical states. Furthermore, the lineshape of the deuterium spectrum of the D-3 sample is very different from that of the D-6 sample, suggesting that the motional behaviors of the *meta*- and *para*-methoxyl groups are different. Fig. 4 shows a set of ^2H spectra of (4′-D_3)TMP/DHFR complex, obtained at various temperatures. Lineshape simulation shows that the 4′-methoxyl group is undergoing a vibrational motion with respect to the C′4-O bond of amplitude ±27° and rates as indicated on Fig. 4 (19). The detailed motional mechanism of the TMP molecule will be presented elsewhere.

In summary, we can reach four major conclusions from this study:

(*a*) In lyophilized powder the dynamics of TMP bound to the active site of DHFR is totally suppressed. This could be due to the presence of a more compact active site in the absence of hydration water so that there is not enough room for the methoxyl group to move around. Hydration induces conformational changes in such a way that the active site become enlarged so that there is enough space for the TMP molecule to move around. Alternatively, the binding site of DHFR may still be crowded even in the presence of sufficient hydration water. However, the presence of water may serve as a "lubricant" to allow the side chains surrounding the methoxyl groups of TMP to move around so that the

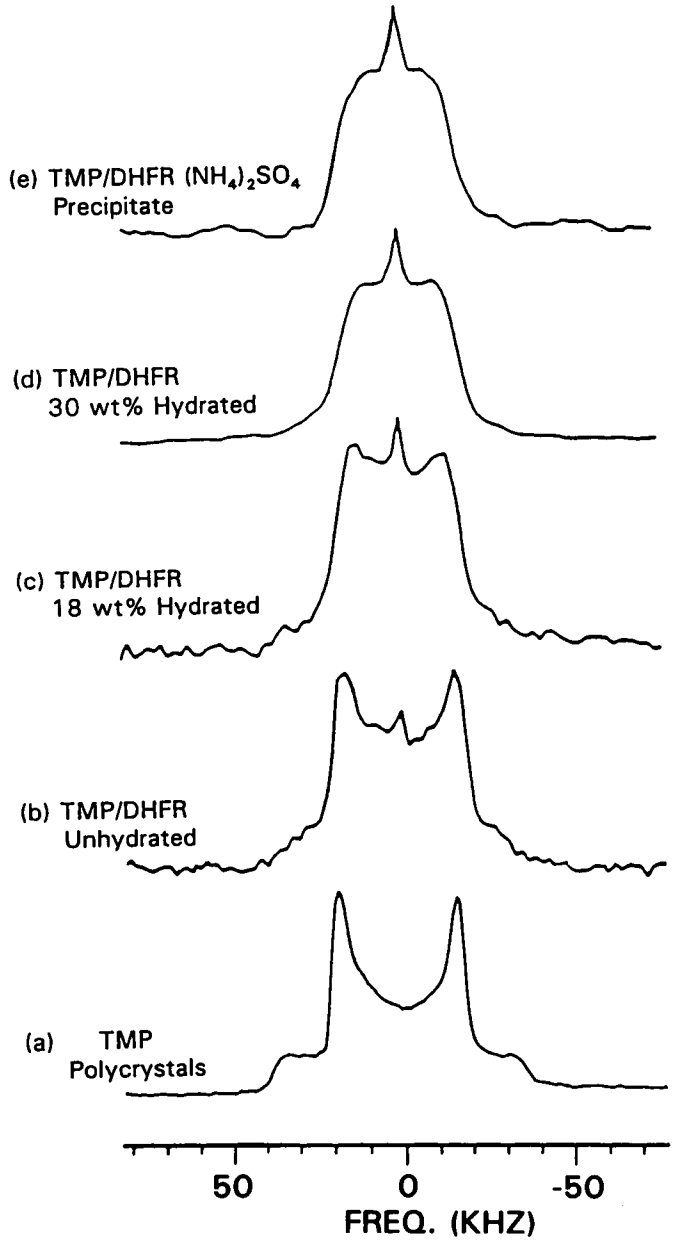

FIGURE 2 Deuterium NMR spectra of polycrystalline (3',4'-$^2H_6$)TMP (*a*), partially hydrated (3',4'-$^2H_6$)TMP/DHFR powder (*b–d*), and ammonium sulfate precipitated (3',4'-$^2H_6$)TMP/DHFR complex (*e*). All spectra were taken at 20°C as described in the text.

methoxyl groups can move in synchrony with the motion of the surrounding side chains.

(*b*) A minimum of 30 wt% hydration is needed to restore the dynamic behavior of TMP in the protein complex. The transformation of the deuterium spectrum from the rigid pattern to the narrower, motionally averaged spectrum appears to be gradual, suggesting a gradual relaxation of the protein molecule upon gradual increase in the hydration level. Rupley, Careri and coworkers have employed a variety of physical and spectroscopic techniques to characterize the effect of hydration on the time-averaged and dynamic properties of partially hydrated lysozyme powder (2). They detected the presence of three discontinuities occurring at 7, 20, and 28 wt% hydration, which they interpreted as due to the presence of a hierarchy of preferential hydration sites. At the lowest hydration level, below 7 wt%, water bound predominantly to the ionizable protein surface groups. The highest discontinuity point of 28 wt% corresponded to the hydration end-point, above which the non-ideality disappeared. This level of hydration corresponds to a coverage of 300 water molecules per protein, which is sufficient to cover 75% of the protein surface area with all the primary and secondary binding sites occupied. Between 20 and 28 wt% water condenses over the weakest interacting regions of the protein surface. The reaction of lysozyme with its hexasaccharide substrate develops sharply at 17–20 wt% hydration, and parallels the hydration dependence of the surface motion of the ESR probe TEMPONE. Marchetti et al. observed a broad ^{113}Cd resonance in a CP/MAS spectrum of lyophilized ^{113}Cd-substituted parvalbumin powder, which they interpreted as due to the presence of local disorder (20). Upon hydrating the protein powder to 38 wt%, the linewidth of the ^{113}Cd resonance reduced by a factor of 4, demonstrating the restoration of considerable local structure and dynamics. Our result is consistent with these observations and provides the most direct evidence

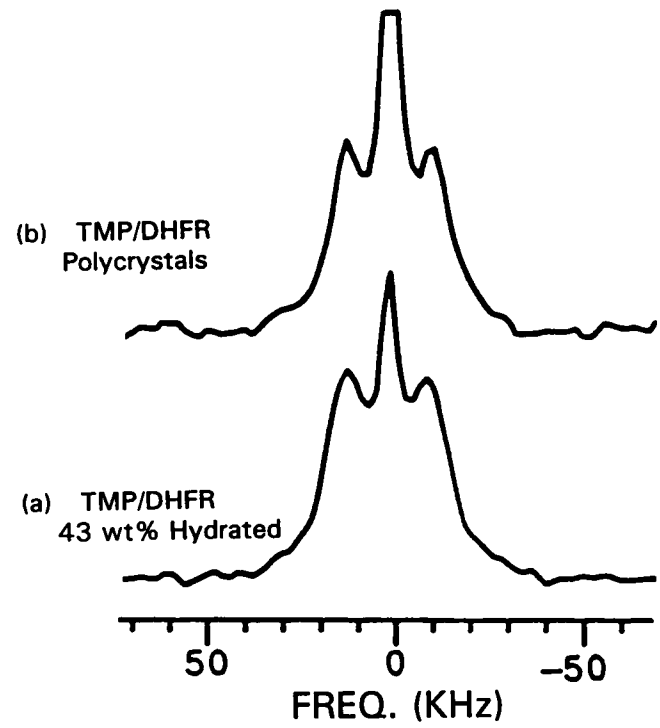

FIGURE 3 Deuterium NMR spectra of (4'-$^2H_3$)TMP/DHFR binary complex of: (*a*) 43 wt% hydrated powder; (*b*) polycrystalline samples. Spectra were taken at 20°C.

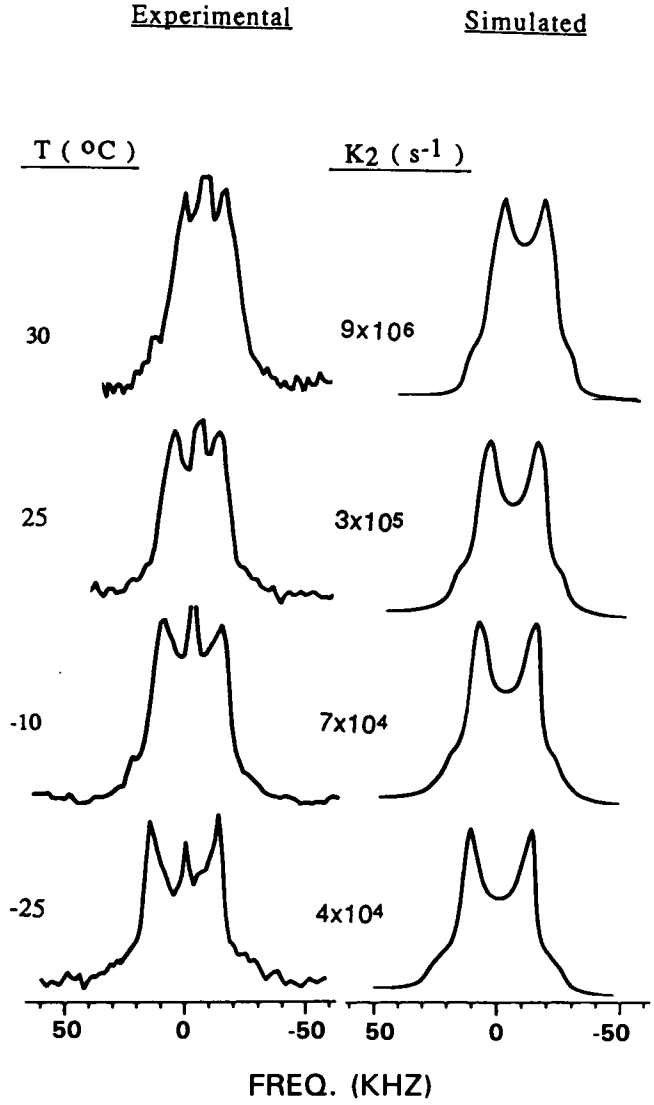

FIGURE 4 (*a*) ^2H NMR spectra of 43 wt% hydrated (4′-$^2H_3$)TMP/DHFR complex at various temperatures. (*b*) Computer simulated spectra of (*a*), assuming that the 4′-methoxyl group is vibrating at ±27° about the C4′-O bond at a rate given in the figure.

of the onset of dynamic processes on the protein moiety upon hydration. Apparently, the full restoration of protein dynamics occurs at a level corresponding to a fully hydrated protein at 30 wt% hydration for DHFR.

(*c*) The deuterium NMR spectrum of the fully hydrated (>30 wt%) sample is indistinguishable from that of the polycrystalline and ammonium sulfate precipitated samples. Since protein structure in the crystalline state is generally believed to be in the native conformation, our observations suggest that fully hydrated proteins and ammonium sulfate-precipitated proteins are also likely to be in native form. This is not too surprising, since 30 wt% hydration spans the lower part of the range of water content reported for a large variety of protein crystals (21). Thus, fully hydrated protein powder can be used for solid NMR studies of macromolecular structure and dynamics. Although it is much easier to prepare solid protein samples from ammonium sulfate precipitates, the loss of sensitivity due to dielectric loss and the large volume of ammonium sulfate co-precipitated with the protein make such practice undesirable.

(*d*) The *meta*- and *para*-methoxyl groups have different dynamic properties. X-ray crystal structure shows that the active site of DHFR is a cavity of 15 Å deep cutting across one face of the enzyme, and is lined with hydrophobic side chains (22, 23). TMP binds at the active site with its pyrimidine ring occupying the interior extremity of the cleft, where it is held by a combination of van der Waals forces, hydrogen bonds, and ionic interactions with the protein. The carboxyl group of Asp-27 closely approaches both N_1 and the 2-amino group of TMP, forming a salt bridge and hydrogen bonds with them. The trimethoxybenzyl side chain extends out toward the mouth of the binding cavity, making van der Waals contact with residues Leu-28, Ile-50, Phe-31, and Ile-94 from helices B and C near the entrance to the active site. The upper *meta*-methoxyl group is in van der Waals contact with Leu-54, while Phe-31 is positioned 3.9 Å from the carbon atom of the methoxyl group. This configuration could impose certain constraints on the mobility of this group. Although all three methoxyl groups are bound near the entrance to the active site cleft, their solvent accessibilities are different. The upper *meta*-methoxyl group is effectively sequestered from solvent, while the lower *meta*-methoxyl group is the most solvent-exposed of the three and is surrounded by a constellation of fixed water molecules. Two water molecules are found near the *para*-methoxyl group. Our data clearly detect the presence of different dynamic properties for the *para*- and *meta*-methoxyl groups. Since the steric hindrance between the two *meta*-methoxyl groups is substantially different, one expects that the upper methoxyl group should be much more rigid than that of the lower one. However, this cannot be substantiated from our deuterium spectrum. One possible explanation is that the Leu-54 and Phe-31 side chains are very flexible, so as to impose no constraints on the dynamic behavior of the upper *meta*-methoxyl group. Therefore, protein moieties pose a strong influence on the dynamics of the ligand in the protein complex.

Recently Searle et al. have reported a ^1H and ^{13}C solution NMR study of the dynamics of TMP bound to *Lactobacillus casei* DHFR (24). They employed the method of Lipari and Szabo (25) to analyze their ^{13}C relaxation data of the 4′-OCH_3 and 7-CH_2 of [7,4′-$^{13}C_2$]TMP in its complex with the enzyme, and obtained information on the rapid motions about the intervening bonds: C7-C1′, C4′-O, and O-CH_3. Their results agree well with ours qualitatively in terms of detecting the presence of large amplitude and fast motion for the methoxyl groups, but relatively slow motion for the benzyl ring. However, some discrepancies exist. A full analysis and comparison of our data will be presented elsewhere.

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