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^{13}C NMR Evidence of the Slow Exchange of Tryptophans in Dihydrofolate Reductase Between Stable Conformations

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SUMMARY:

^{13}C NMR spectra are reported for dihydrofolate reductase of Streptococcus faecium labeled with [γ-^{13}C]tryptophan. Two of the four tryptophans generate unusual resonances indicating slow exchange of the residues between alternative stable conformations. Since 3', 5'-dichloromethotrexate sharpens two of the resonances, it apparently locks the corresponding residues into one conformation.

INTRODUCTION:

One of the unique contributions which nuclear magnetic resonance techniques can make in the study of enzymes and other biologically important proteins is the elucidation of dynamic processes. The time scales for these processes cover a wide range: many seconds for the isomerism of imino acid peptide bonds (1); 10^{-1}-10^{-7} s for the 180° flipping of Phe and Tyr residues about their C^{β}-C^{γ} bonds (2-4); 10^{-8} s for protein tumbling (5,6); to as short as 10^{-12} s for internal motions of side chains (5-7). Of particular interest are the rates of conformational transitions associated with substrate binding or catalytic activity. In this report we present evidence that two of the four tryptophan residues in dihydrofolate reductase of Streptococcus faecium are in slow exchange between nonequivalent microenvironments, and that the equilibria between the corresponding enzyme conformers are markedly affected by the binding of ligands. We further propose a molecular basis for this conformational heterogeneity.

MATERIALS & METHODS:

Dihydrofolate reductase was obtained from Streptococcus faecium var Durans strain A grown in medium containing [γ-^{13}C]tryptophan (90 atom%, Kor Isotopes, Cambridge, Mass.) The enzyme was purified by published procedures (8).

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Proton noise decoupled ^{13}C-NMR spectra were obtained on a Varian XL-100-15 spectrometer operating in the Fourier Transform mode. Spectra were obtained with a 500 Hz spectral width and 512 data points using an 80° pulse width and no pulse delay.

A spectrum was also obtained at the Purdue University Biochemical Magnetic Resonance Laboratory using a Nicolet Technology Corporation 360 MHz spectrometer. For this measurement the enzyme (0.54 mM) was at 8° in 0.05 M potassium phosphate buffer (pH 7.3) containing 10% D_2O, 0.3 M KCl and 1 mM EDTA.

RESULTS AND DISCUSSION:

Dihydrofolate reductase from S. faecium contains four tryptophan residues (9). Figure 1 shows the proton decoupled ^{13}C NMR spectra of the uncomplexed [γ-^{13}C]-tryptophan labeled reductase at several temperatures and of the binary complex of enzyme with 3',5'-dichloromethotrexate, an inhibitor.

In figure 1a (uncomplexed enzyme at 5°C) the two sharp downfield peaks (1 and 2) at 111.1 and 110.4 ppm (relative to TMS) exhibit shifts similar to those observed for Trp C^{γ} in hen egg white lysozyme, cytochrome c, hemoglobin and myoglobin (10-12), as well as in several peptides (13). Peak integration suggests that peaks 1 and 2 each correspond to a single Trp, the double peaks 3a and 3b (109.8 and 110.0 ppm) to a third, and peak 4, the broad upfield resonance (105.5 ppm), to the fourth. Although differential nuclear Overhauser enhancement (NOE) values may invalidate the use of peak intensities in such estimations, there is little evidence for such effects for the aromatic, non-protonated ^{13}C resonances studied in lysozyme (6). Further, measurements of the spin lattice relaxation times for the ternary DHFR-methotrexate-NADPH complex are identical for all of the resonances (14) so that the NOE values will be similar.

The double structure (3a, 3b) of one of the Trp resonances is informative. Since all the ^{1}H nuclei are decoupled, scalar ^{1}H-^{13}C spin coupling cannot be responsible. Furthermore, the low natural abundance of ^{13}C (1.108%) at other positions in the indole ring, along with the low probability of through-space ^{13}C-^{13}C coupling, eliminate the possibility of a doublet arising from this source of scalar coupling. The remaining possibility is that peaks 3a, 3b correspond to two conformational states of this residue which have different

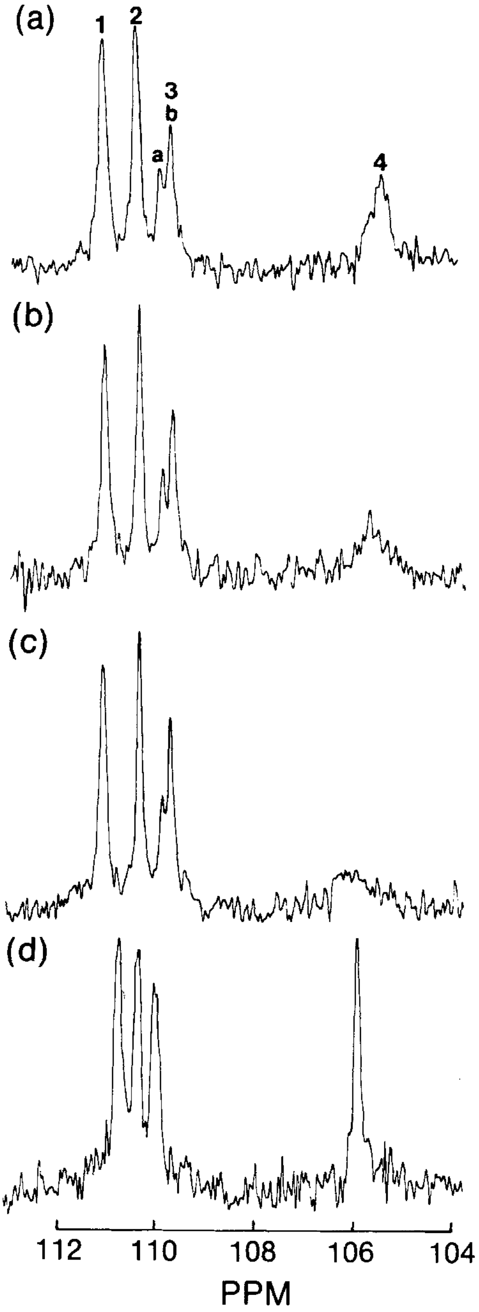

Figure 1. Proton-decoupled ^{13}C-NMR spectra at 25.2 MHz of the tryptophan C$^{\gamma}$ region of [γ-^{13}C (90 atom %)]tryptophan-labeled dihydrofolate reductase in 0.05 M phosphate buffer, pH 7.3, 0.5 M KCl, 0.02 % sodium azide, 1 mM sodium EDTA, and 10 % D_2O for the NMR lock. A, 0.85 mM dihydrofolate reductase alone at 5°, 144,324 transients; B, 0.97 mM dihydrofolate reductase alone at 15°, 63,309 transients; C, 0.85 mM dihydrofolate reductase alone at 25°, 116,470 transients; D, 0.97 mM dihydrofolate reductase and 1.0 mM 3',5'-dichloromethotrexate at 15° 59,365 transients.

chemical shifts and which are in slow exchange. The spectrum obtained at 90 MHz on the Nicolet spectrometer showed a chemical shift difference between peaks 3a and 3b of 0.21 ppm, the same as in the spectra obtained at 25.2 MHz. This means that the separation (in Hz) is proportional to field strength, a result required if chemical exchange is responsible for the double structure, but incompatible with spin coupling. Simulation of peaks 3a and 3b assuming such chemical exchange is consistent with a relative population for the two species of 2:3 and lifetimes of $\geq$ 0.5 s and 0.75 s, respectively.

The upfield resonance (peak 4) is unique, being ∿ 3 ppm further upfield than the most upfield Trp C^{γ} resonance observed in a recent series of studies by Allerhand and coworkers (11). Ring current shifts expected to arise from hydrophobic interactions with adjacent aromatic residues can explain only a part of the total shift observed, but other possible contributions include steric (15) and electric field induced shifts (16,17). As an example of the latter, the Trp 108 C^{δ}-H proton of lysozyme is observed to shift as the nearby Glu-35 is titrated (18). Further, theoretical calculations suggest that unsaturated carbons are more likely to exhibit significant chemical shifts in the proximity of nearby charges than saturated carbons (19).

The temperature dependence of the linewidth and shift of peak 4 (Fig. 1) are indicative of a slow exchange process. As the temperature is increased from 5° C to 25°C the system approaches an intermediate exchange rate so that the upfield peak is greatly broadened and shifted downfield more than 0.8 ppm. At temperatures greater than 25°, the decreased stability of the uncomplexed enzyme causes some precipitation. However a spectrum obtained at 30° follows the pattern established in Fig. 1. In the case of peak 4 explanation of the results by the exchange process requires two states with very different populations, the major component having a shift of ∿ 5 ppm upfield of peak 2, and the minor component a shift close to that of the downfield resonances.
If this is the case, the minor conformational species must be unobservable because it is present at too low a concentration and probably also because its

resonance is too severely broadened to be detectable. It may correspond to a conformer in which the tryptophan residue is more exposed to the solvent and hence exhibits a shift closer to that observed in small peptides (13). Using a slow exchange approximation to interpret the linewidth, the lifetime of the major species at 15°C is ∿ 0.03 s.

This slow exchange between enzyme conformations monitored by the tryptophan C^{γ} resonances is dramatically affected by the presence of ligands, a number of which have been found to sharpen the unusual resonances (14). The most pronounced sharpening of peaks 3 and 4 results from the addition of methotrexate or analogs such as 3',5'-dichloromethotrexate (Fig. 1d). The most probable interpretation of these data is that dichloromethotrexate binding prevents the slow exchange between conformers by locking the enzyme in a conformation characterized by an upfield-shifted tryptophan C^{γ} resonance.

^{19}F NMR of 6-fluorotryptophan-labeled dihydrofolate reductase from Lactobacillus casei gave broad resonances which sharpened in the presence of substrates or inhibitors (20). This was interpreted to indicate that in the absence of ligands the enzyme exists in a number of interconverting configurations, while the ligand complexes are essentially in a single conformation (21). While this conclusion is consistent with the present data, the effect which we have observed is significantly more specific, since only two of the Trp residues are involved. The greater conformational heterogeneity observed in the fluorotryptophan-labeled enzyme is perhaps related to the reported destabilization of the enzyme produced by the fluorine label (21). Although two of the fluorotryptophan resonances were split due to through-space ^{19}F-^{19}F coupling (22) no splitting was observed due to slow conformational processes analogous to those discussed here. A more analogous phenomenon has been observed for the Trp C^{δ}-H proton resonance corresponding to Trp 63 in lysozyme (23), where linewidth is again sharply reduced in the complex with inhibitor.

One goal of the present study is the elucidation of the molecular basis for the slow exchange processes observed by ^{13}C NMR. The best explanation for this behavior is the occurrence of a slow oscillation of the Trp residues between two stable conformations. This must be achieved by some form of restricted rotation of the indole ring about the C^{β}-C^{γ} bond, perhaps with some rotation about the C^{α}-C^{β} bond also. One model for such a system involves motion in which first one and then other face of the indole ring associates with a hydrophobic residue. This process would require relaxation of the enzyme tertiary structure, which is also required for the Phe and Tyr C^{β}-C^{γ} 180° ring flipping which occurs in various proteins (2-4, 24). In an alternative model the indole ring might undergo 180° flipping, analogous to that observed for the Phe and Tyr residues, but significantly different because of the bulk and asymmetry of the indole ring.

The functional and structural significance of the conformational heterogeneity monitored by the peak 3 and 4 Trp C^{γ} resonances is at present uncertain. In particular, it is unclear whether these effects are more or less localized to the Trp residues observed, or whether they involve a larger portion of the protein structure. In our previous NMR studies on dihydrofolate reductase labeled with [methyl-^{13}C]methionine (8) or [guanidino-^{13}C]arginine (25,26) no evidence was found that slow conformational transitions affect Met or Arg residues, and this might be construed as evidence that the transitions involving Trp peaks 3 and 4 are highly localized. On the other hand peak 1 in the spectrum of the [^{13}C]arginine-labeled enzyme does appear to monitor a conformational transition occuring on a shorter time scale and this might be interrelated in a complex fashion with the transitions involving the Trp peaks. Further, the greater complexity of the spectra obtained with enzyme labeled with [^{13}C]Arg and especially with [^{13}C]Met make it difficult to observe exchange-broadened resonances. However, it may be noted that simulation of the spectrum of the uncomplexed enzyme labeled with [^{13}C]Met indicated the presence of several resonances with linewidths of $\sim$ 40 Hz and at higher pH such broadening was even more evident. To determine whether these resonances are broadened by exchange requires further study.

It should be noted in this connection, however, that circular dichroic, magnetic circular dichroic and fluorescence techniques have suggested a direct involvement of one or more of the tryptophans of the *L. casei* reductase in interactions with substrates and methotrexate (27-31). In addition, chemical modification of Trp 21 inactivates the enzyme (32) and this residue is conserved in all reductase sequences investigated, so that Trp 21 is probably at the active site. One of the resonances changed by ligand binding (peaks 1, 3 or 4) therefore probably corresponds to Trp 21. More definite assignment of the resonances must await the outcome of further studies that are in progress.

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