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^{1}H and ^{31}P NMR Characterization of Two Conformations of the Trimethoprim-$NADP^{+}$-Dihydrofolate Reductase Complex

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SUMMARY

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The ternary complex between *Lactobacillus casei* dihydrofolate reductase, the coenzyme $NADP^{+}$, and the antibacterial drug trimethoprim was studied by ^{1}H and ^{31}P NMR spectroscopy. The C_2-H resonances of two of the histidine residues of the protein were each split into two signals of approximately half-proton intensity in the ^{1}H spectrum of this complex. Studies of the temperature-dependence of the lineshape of these histidine signals showed that the splitting is due to the coexistence of approximately equal amounts of two slowly interconverting (6 sec^{-1} at 31°) conformational forms of the complex. Two sets of proton resonances from the bound coenzyme were identified by the use of selectively deuterated coenzyme and by transfer of saturation experiments. Conformation I was characterized by nicotinamide proton resonances shifted substantially (0.6-1.1 ppm) to low field from their positions in the free coenzyme, while in Conformation II the changes in chemical shift on binding were much smaller (≤0.12 ppm). Only a single set of ^{1}H resonances from the bound trimethoprim was observed in transfer of saturation experiments at 45°, perhaps because of relatively rapid interconversion between the two conformational states at this temperature. However, the addition of $NADP^{+}$ produced a large (1.1 ppm) upfield shift of the 2′,6′-proton resonance of trimethoprim relative to its position in the binary complex. In the ^{31}P spectrum of the bound coenzyme, two sets of signals were seen for the pyrophosphate phosphorus nuclei. As judged from both the ^{1}H and ^{31}P spectra, the complexes of enzyme, trimethoprim, and $NADP^{+}$ or $NHDP^{+}$ (the hypoxanthine analogue) are mixtures of Conformations I and II, whereas the complexes formed with the thionicotinamide or acetylpyridine analogues of the coenzymes ($TNADP^{+}$ and $APADP^{+}$) are exclusively in Conformation II. The enzyme-methotrexate-$NADP^{+}$ complex is exclusively in Conformation I. Comparing the ^{31}P spectra of the enzyme-trimethoprim-$TNADP^{+}$ and enzyme-methotrexate-$NADP^{+}$ complexes, the two conformational states were found to differ in the conformation of the pyrophosphate backbone of the bound coenzyme, as indicated by the ^{31}P-^{1}H and ^{31}P-^{31}P spin-spin coupling constants. This system appears to be an example of a two-state conformational equilibrium which can be "switched" by the binding of ligands of different structure. The nature of the two conformational states and their implications for structure-activity analysis are discussed.

INTRODUCTION

The widely used antibacterial drug trimethoprim (1), in common with other "antifolate" drugs such as methotrexate and pyrimethamine, produces its biological effects by inhibition of the enzyme dihydrofolate reductase (2, 3). This enzyme is required to maintain the cellular levels of tetrahydrofolate derivatives, which are involved in a number of reactions of one-carbon metabolism, notably thymidylate biosynthesis. Trimethoprim owes its usefulness as an antibacterial agent to the fact that it binds much more tightly to the bacterial than to the mammalian enzyme (4). The relatively low molecular weight of dihydrofolate reductase (17-25,000 in most species; refs. 2, 5) makes it possible to study this drug-receptor system by a variety of physical and chemical techniques. The three-dimensional structures of the *Escherichia coli* enzyme-methotrexate complex (6) and the *Lactobacillus casei* enzyme-methotrexate-NADPH

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complex (7, 8) have been determined by x-ray crystallography, and the interactions of the enzyme with its substrates, inhibitors, and coenzyme have also been studied in some detail by NMR spectroscopy (e.g., refs. 9–17).

We have been using NMR to provide the conformational information required for a detailed understanding of the structure-activity relationships among dihydrofolate reductase inhibitors. It has become clear that structurally closely related compounds can bind to this enzyme in quite different ways (see, e.g., refs. 15 and 16). The most striking example of this is the finding (18–20) that, although the substrate folate and the inhibitor methotrexate are structurally very similar, in their complexes with the enzyme the pteridine ring differs in orientation with respect to the coenzyme by approximately 180°. In addition, the presence of the coenzyme not only increases the binding of most substrates and inhibitors, but also changes their relative affinity, i.e., the specificity of the enzyme (21, 22). There is a good deal of evidence that many of these effects are mediated by changes in conformation of the enzyme (9–17). In the course of our studies of ternary enzyme-coenzyme-inhibitor complexes, we found that the enzyme-trimethoprim-$NADP^+$ complex exists in solution as a mixture of approximately equal amounts of two slowly interconverting conformations.

MATERIALS AND METHODS

Materials. Dihydrofolate reductase was isolated from *L. casei* MTX/R and purified as described by Dann *et al.* (23); its concentration was determined from its absorbance at 280 nm, by assaying its catalytic activity, and by fluorimetric titration with methotrexate (23). The coenzymes $NADP^+$, $NHDP^+$,[2] $TNADP^+$, and $APADP^+$ were obtained from Sigma Chemical Co., Ltd. (London, England).

^1H NMR spectroscopy. Samples contained approximately 1 mM enzyme in 0.35 ml of ^2H_2O containing 1 mM dioxan, 1 mM EDTA, 500 mM KCl, 50 mM potassium phosphate, pH* 6.5, 1–3 mM methotrexate or trimethoprim, and 1–6 mM coenzyme. The 270 MHz ^1H NMR spectra were obtained by the Fourier transform method using a Bruker WH270 spectrometer. Up to 5000 transients were averaged, using an acquisition time of 0.5–1.0 sec (4096 or 8192 data points, 4.2 kHz spectral width). The free induction decay was multiplied by an exponential function to improve the signal-to-noise ratio, leading to a line-broadening of 2 Hz, and if 4096 data points had been collected, the data table was filled to 8192 points with zeros before Fourier transformation. The procedure for transfer of saturation experiments was as described earlier (15). Sample temperatures (2–55°) were maintained to ±1°. 470 MHz spectra were obtained under essentially identical conditions, using the spectrometer of the Oxford Enzyme Group. Chemical shifts were measured relative to internal dioxan (3.71 ppm downfield from 2,2-dimethyl-silapentane-5-sulphonate), with downfield shifts positive. The chemical shifts of the bound coenzymes are expressed relative to the corresponding mononucleotides (15). Lineshape simulations employed a program written by Dr. J. G. Batchelor (see ref. 24).

^{31}P NMR spectroscopy. Samples contained approximately 1 mM enzyme in 1.4 ml of ^2H_2O containing 1 mM EDTA, 500 mM KCl, 50 mM bis-tris [2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)-1,3-propane-diol], pH* 6.5, 1–3 mM methotrexate or trimethoprim, and 0.5–1.0 mM coenzyme. The 40.5 MHz ^{31}P spectra were obtained by Fourier transform method using a Varian XL-100-15 spectrometer. Spectra were acquired in the block averaging mode; typically 300 blocks each of 200 spectra were averaged, using an acquisition time of 0.5 sec for a spectral width of 2 kHz. Noise-modulated proton decoupling was employed unless otherwise noted. The free induction decay was multiplied by an exponential to improve the signal-to-noise ratio, leading to a linebroadening of 1.6 Hz. Chemical shifts are expressed relative to inorganic phosphate, pH* 8.0 (2.94 ppm downfield from H_3PO_4) with downfield shifts positive.

RESULTS AND DISCUSSION

Evidence for two interconverting conformations. Fig. 1 shows the histidine C2-proton resonances of *L. casei* dihydrofolate reductase in its complex with $NADP^+$ and trimethoprim and, for comparison, those of the corresponding complex containing methotrexate rather than trimethoprim. It can be seen that resonances F and E, which have been assigned to His 28 and (tentatively) His 18, respectively, (12–14, 25) appear as "doublets" in the spectrum of the trimethoprim complex but not in that of the methotrexate complex. (The different position of resonance F in the two spectra can be accounted for by the increase in pK of His 28 due to its interaction with

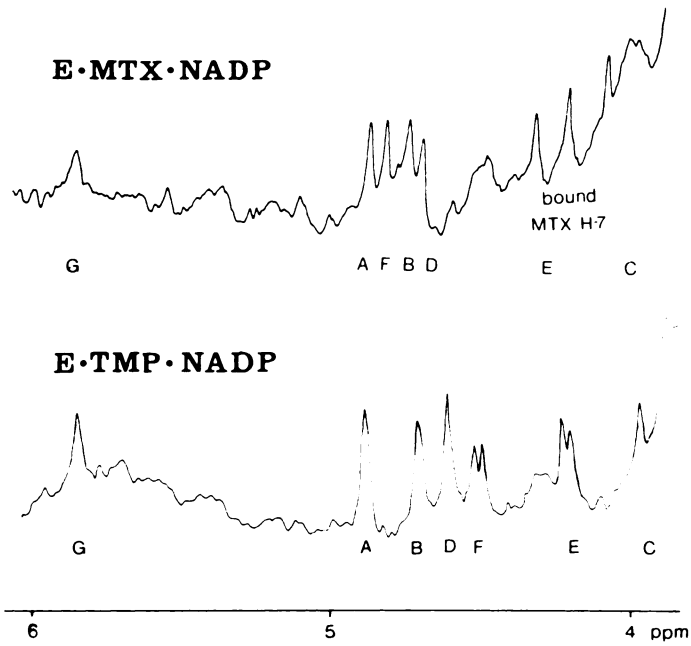

FIG. 1. *Histidine C2-proton resonance region of the ^1H NMR spectrum of L. casei dihydrofolate reductase in its complexes with methotrexate (MTX) and $NADP^+$ (top) or trimethoprim (TMP) and $NADP^+$ (bottom)*

[2] The abbreviations used are: $NHDP^+$, $TNADP^+$, and $APADP^+$, the hypoxanthine, thionicotinamide, and acetylpyridine analogues of $NADP^+$, respectively; pH*, a pH reading uncorrected for the isotope effect on the glass electrode.

the glutamate moiety of methotrexate (7, 9), which has no counterpart in trimethoprim.)

The "splittings" on resonances E and F (in Hertz) are directly proportional to magnetic field strength, and thus represents a chemical shift difference. Therefore, the two histidine residues from which these signals originate each exist in two different environments. The approximately equal intensity of the two components of resonances E and F indicates that approximately equal amounts of the two species are present in solution. One possibility is that the two species are hitherto undetected isoenzymes of dihydrofolate reductase, of the kind found in *E. coli* RT500 (26). This possibility can be ruled out by showing that the two species interconvert. This was done by examining the temperature dependence of these two histidine resonances over the range 0–50° at both 270 MHz and 470 MHz. The results at 470 MHz are shown in Fig. 2, where it can be seen that each doublet broadens somewhat and then collapses into a single fairly sharp signal as the temperature is increased. (The progressive downfield shift of the resonances with increasing temperature arises from the temperature-dependence of their pK values.) These changes in lineshape *cannot* be accounted for by a model in which there is no interconversion of the two species responsible for the splitting of resonances E and F. In particular, the increase in linewidth with increasing temperature at the lower temperatures clearly indicates that exchange between the two species is occurring. As indicated in Fig. 2, the temperature dependence of the lineshape of these two resonances *can* be satisfactorily accounted for by a two-site exchange model. The correctness of this model is confirmed by the finding that, at any given temperature, the four estimates of the rate constant (from simulation of the two resonances E and F, each at two frequencies, 270 and 470 MHz) agree to within ±15%. At 31°, the rate constant is 6 (±0.9) sec^{-1}. The collapse of the doublet of resonance F at a lower temperature than that of resonance E simply reflects the smaller chemical shift difference between the two environments for F than for E (0.044 ppm versus 0.063 ppm at 5°).

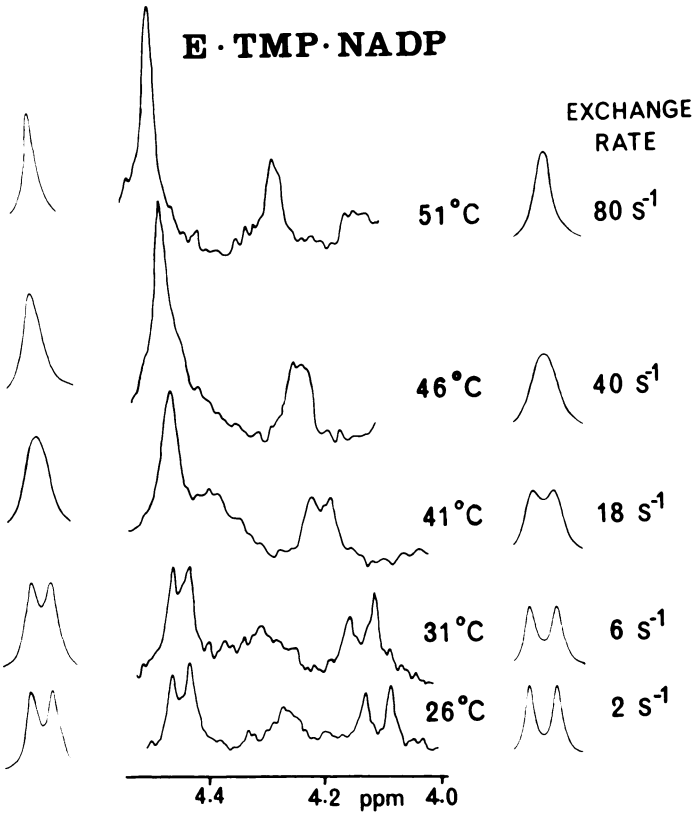

FIG. 2. *The histidine C2-proton resonances E (right) and F of the dihydrofolate reductase-trimethoprim (TMP)-NADP$^+$ complex as a function of temperature*

The simulated line-shapes, together with the value of the rate constant used in the simulation, are shown at each side.

These experiments thus demonstrate that the trimethoprim-NADP$^+$-dihydrofolate reductase complex exists in solution as a mixture of approximately equal amounts of two conformational forms, which differ in the environment of two histidine residues (His 28 and probably His 18), and which interconvert at a rate of about 6 sec^{-1} at 31°. The activation parameters for the interconversion, calculated from the temperature dependence of the estimated rate constants, are $\Delta H^{\ddagger} = 25$ (±3) kcal/mol, $\Delta S^{\ddagger} = 28$ (±8) cal °K^{-1} mol^{-1}. It is interesting that there is no detectable temperature dependence of the relative populations of the two forms, implying that the enthalpy difference, as well as the free energy difference, between them is very small. In order to characterize further the structural difference between these two conformations, we examined the ^{1}H and ^{31}P resonances of the bound ligands.

^{1}H resonances of the coenzyme. NADP$^+$ binds sufficiently tightly to the enzyme to be in slow exchange on the NMR timescale, so that separate signals are seen for protons of the free and bound coenzyme (15). At the low-field end of the ^{1}H spectrum of the enzyme-trimethoprim-NADP$^+$ complex, three resonances can be seen, and these can be identified as those of the 2-, 4-, and 6-protons of the nicotinamide ring of the bound coenzyme by comparison with the spectra of otherwise identical samples containing selectively deuterated coenzyme (Fig. 3). These resonances are close to the positions of the corresponding ^{1}H resonances in the enzyme-NADP$^+$ binary complex (15). However, comparison of their intensity with that of the histidine C2-proton resonance G (Fig. 3) shows clearly that these coenzyme signals arise from only half the total bound coenzyme in the ternary complex; that is, they arise from only one of the two conformational states of the complex. The conformation giving rise to these low field nicotinamide proton resonances is denoted Conformation I.

A second set of nicotinamide proton resonances, from Conformation II, has been observed further upfield, and much closer to the resonance positions of free NADP$^+$. These are also indicated in Fig. 3; their intensities cannot be reliably estimated, since they overlap the resonances of some slowly exchanging NH protons. The chemical shifts and assignments of both sets of nicotinamide proton resonances have been independently confirmed by transfer of saturation experiments (15, 27). In these experiments, irradiation of a resonance of the bound ligand leads to a decrease in intensity of the corresponding resonance of the free ligand as the free and bound ligand molecules exchange. The position of the "bound" reso-

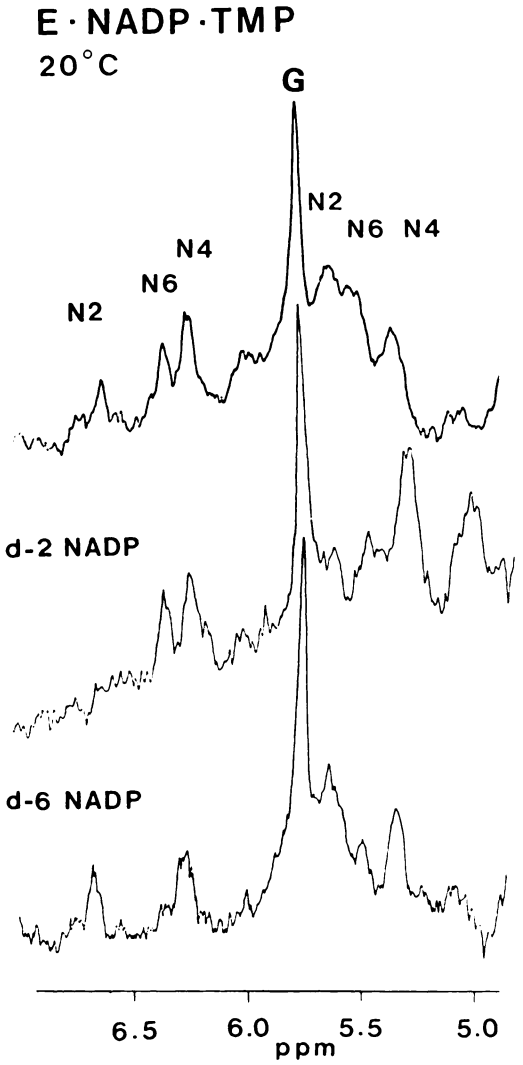

FIG. 3. *The low-field region of the ^1H NMR spectrum of the dihydrofolate reductase-trimethoprim (TMP)-NADP$^+$ complex*

From the *top*, the samples contained normal NADP$^+$, [2-^{2}H-nicotinamide]-NADP$^+$, and [6-^{2}H-nicotinamide]-NADP$^+$. The resonances labeled *N2*, *N4*, and *N6* are those of the 2, 4, and 6-protons, respectively, of the bound coenzyme in Conformations I (at low field), and II (at higher field) of the complex. The resonance labeled *G* is a histidine C2-proton signal.

nances can be identified by systematic irradiation through the relevant region of the spectrum. A transfer of saturation from the bound to the free state was readily observed for the nicotinamide protons in Conformation II at both 11 and 40°, but transfer from the same protons in Conformation I could only be detected at 40°; as a consequence, only the resonance positions corresponding to Conformation II were detected in our earlier transfer of saturation experiments (15). This observation implies that the dissociation rate constant of the coenzyme is greater from Conformation II than from Conformation I. The resonance positions of the adenine protons of the bound coenzyme have also been determined by transfer of saturation; these positions are the same as those in the binary enzyme-NADP$^+$ complex (15), and there is no evidence for the existence of two sets of adenine proton resonances.

The chemical shifts of the nicotinamide proton signals in the two conformations of the enzyme-trimethoprim-NADP$^+$ complex are summarized in Table 1, together with the corresponding values for a number of other complexes, determined by transfer of saturation experiments. In Conformation I, the nicotinamide ring of NADP$^+$ or its hypoxanthine analogue, NHDP$^+$, is clearly bound to the enzyme in an environment which produces large downfield shifts (0.61–1.08 ppm) of the proton resonances. The precise origins of these shifts are not yet understood, but they can serve as a "fingerprint" of a particular environment for the nicotinamide ring. The chemical shift changes in Conformation I of the enzyme-trimethoprim-NADP$^+$ complex are, with the exception of that of the nicotinamide 2-proton, very similar to those in the enzyme-methotrexate-NADP$^+$ complex, so that the nicotinamide environment must be similar in the two complexes. In contrast, the chemical shift changes on coenzyme binding to form Conformation II of the complex are very much smaller. Similar extremely small shifts are seen when the acetylpyridine (APADP$^+$) or thionicotinamide (TNADP$^+$) analogues of the coenzyme bind to the enzyme-trimethoprim complex.

These results are consistent with the idea that the two conformations detected for the enzyme-trimethoprim-NADP$^+$ complex exist for a number of related complexes, but that their relative proportions vary substantially with

TABLE 1

Nicotinamide proton chemical shifts of coenzyme bound to L. casei dihydrofolate reductase

Complex[a]	Chemical shift[b] (ppm)							
	Conformation I				Conformation II			
	2	4	5	6	2	4	5	6
E·trimethoprim·NADP$^+$	0.91	1.08	0.61	0.86	−0.10	0.12	0.01	−0.05
E·trimethoprim·NHDP$^+$	0.90	1.06	ND[c]	0.86	0.08	0.13	ND[c]	ND[c]
E·methotrexate·NADP$^+$[d]	0.53	0.92	0.79	0.73	—	—	—	—
E·trimethoprim·TNADP$^+$	—	—	—	—	−0.01	−0.10	0.01	−0.07
E·trimethoprim·APADP$^+$	—	—	—	—	−0.05	−0.12	−0.04	−0.14

[a] For abbreviations see footnote 1.

[b] Chemical shifts of the 2-, 4-, 5-, and 6-protons of the bound coenzyme are expressed relative to those in the corresponding mononucleotides. This corrects for the variable effects of intramolecular base stacking in the free coenzyme (15). Mononucleotide chemical shifts are given in ref. 15.

[c] ND, not determined.

[d] From ref. 15.

ligand structure. Both conformations are seen (in roughly equal amounts) with the enzyme-trimethoprim-$NADP^+$ and -$NHDP^+$ complexes, but the enzyme-methotrexate-$NADP^+$ complex appears to exist only in Conformation I, and the ternary complexes containing trimethoprim and the coenzyme analogues with modified nicotinamide rings, $TNADP^+$ and $APADP^+$, only in Conformation II. It is clear that the two conformations differ substantially in the environment of the coenzyme nicotinamide ring. The ^{31}P NMR experiments described below show that this difference extends to the pyrophosphate group of the coenzyme.

^1H resonances of trimethoprim. Transfer of saturation experiments have also been carried out to locate the resonance positions of the pyrimidine 6- and benzyl 2′,6′-protons of trimethoprim bound to the enzyme in the ternary complex with $NADP^+$. As in the binary trimethoprim complex (28), the dissociation rate constant of trimethoprim from the complex was such that the experiments could only be successfully carried out at 45°. For each proton, a plot of the intensity of the "free" resonance as a function of the irradiation frequency showed only a single minimum, centred at 2.6 ppm for the 6-proton and 1.2 ppm for the 2′,6′-protons. These minima were, however, considerably broader (~80 Hz) than those observed for $NADP^+$ (~40 Hz) in the same complex at the same temperature [the frequency ranges giving transfer of saturation are considerably larger than the true linewidth (29)]. A single broad minimum such as this would be observed if the chemical shift difference for both the trimethoprim 6- and 2′,6′-proton signals between Conformations I and II were small enough for the two signals to be virtually coalesced at 45°. At this temperature, the rate of interconversion is about 40 sec^{-1} (cf. Fig. 2), so coalescence would be observed for signals separated by approximately 40 Hz (0.15 ppm), a small shift difference compared to that between free and bound trimethoprim (see below). However, we cannot rule out the alternative explanation that the dissociation rate constant of trimethoprim from one of the conformations of the complex is too slow for transfer of saturation to be observed at this temperature (as for $NADP^+$ from Conformation I at 11°), so that we can only detect the resonances of one conformation.

These experiments thus provide no additional information about the difference between Conformations I and II. However, they do show that $NADP^+$ produces a substantial change in the environment of the bound trimethoprim in at least one of the conformations. When trimethoprim binds to the enzyme, the 6-proton and 2′,6′-proton resonances shift upfield by 1.1 and 0.6 ppm, respectively (28). On binding to the enzyme-$NADP^+$ complex, these shifts are 1.0 and 1.7 ppm. Our interpretation (28) of the shifts in the binary complex attributed that of the 6-proton largely to the ring current of the benzyl ring of trimethoprim, and that of the 2′,6′-protons to the ring current of Phe 30. On this basis, the similar shift of the 6-proton in the ternary complex suggests that there is no large change in the conformation of bound trimethoprim on adding $NADP^+$. The much larger shift of the 2′,6′-protons would arise if they were closer to Phe 30 in the ternary complex, but it is at least as likely that an additional, separate, shift contribution has become significant; one possibility is the ring current of the nicotinamide ring of the coenzyme itself.

^{31}P resonances of the coenzyme. The three ^{31}P resonances of the coenzyme (from the 2′-phosphate and pyrophosphate) have proved useful in characterizing coenzyme binding to dihydrofolate reductase (16, 30). When $NADP^+$ binds to the enzyme or to the enzyme-methotrexate complex, the 2′-phosphate resonance shifts substantially downfield on binding, whereas both the pyrophosphate signals shift upfield, but one much more than the other, so that their nonequivalence is increased and the doublet splitting ($^2J_{PP}$ = 21 Hz) is clearly evident. This is shown for the enzyme-methotrexate-$NADP^+$ complex in Fig. 4.

The pyrophosphate region of the ^{31}P spectrum of the enzyme-trimethoprim-$NADP^+$ complex (Fig. 4) is more complicated. There appear to be four doublets, one at −12.9 ppm (doublet splitting ill-resolved), two at −14.9 ppm, and one at −16.4 ppm, and these must represent the two pyrophosphate signals from the coenzyme in each of two environments, presumably Conformations I and II. Strong support for this interpretation comes from the comparison with the enzyme-trimethoprim-$TNADP^+$ and enzyme-methotrexate-$NADP^+$ complexes also shown in Fig. 4. The methotrexate complex appears, from the ^1H NMR experiments described above, to exist only in Conformation I, and its ^{31}P spectrum shows only two pyrophosphate signals, at −14.9 and −16.5 ppm. Similarly, the enzyme-trimethoprim-$TNADP^+$ complex,

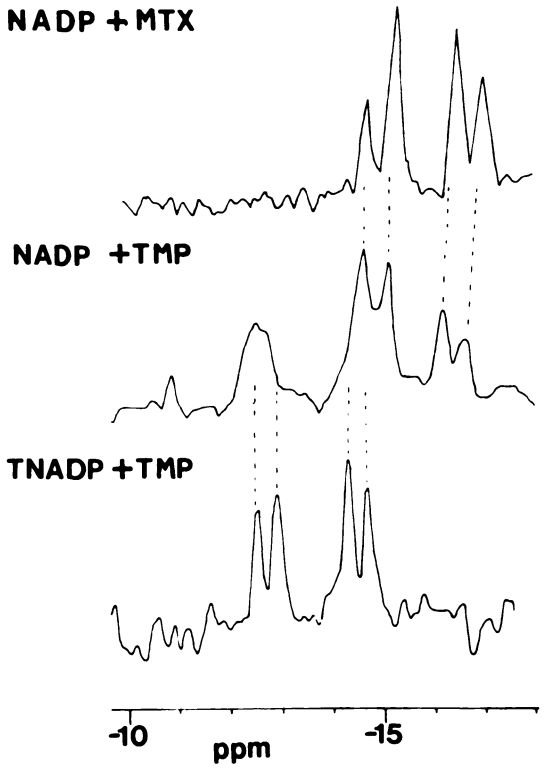

FIG. 4. *The pyrophosphate region of the ^1H-decoupled ^{31}P NMR spectra of the complexes of dihydrofolate reductase with methotrexate (MTX) and $NADP^+$ (top), trimethoprim (TMP) and $NADP^+$ (center), and trimethoprim and $TNADP^+$ (bottom)*

representative of Conformation II, also has only two pyrophosphate signals, but at −12.8 and −14.8 ppm. We conclude that Conformation I is characterized by ^{31}P signals at −14.9 and −16.4 ppm, and Conformation II by signals at −12.9 and −14.9 ppm (see Table 2). It follows that at least one of the pyrophosphate phosphorus atoms has a different environment in the two conformational states. The 2′-phosphate, on the other hand, shows a single resonance at the same chemical shift in all three complexes, so that there is no indication that its environment differs in the two conformations.

The ^{31}P resonances of the coenzyme bound to dihydrofolate reductase are sufficiently narrow for the effects of ^{1}H-^{31}P spin-spin coupling to be clearly detectable in single resonance spectra (30). The pyrophosphate regions of the single resonance ^{31}P spectra of the enzyme-methotrexate-$NADP^+$ and enzyme-trimethoprim-$TNADP^+$ complexes, representing Conformations I and II, respectively, are shown in Fig. 5. The ^{1}H-^{31}P couplings cannot be studied in the enzyme-trimethoprim-$NADP^+$ complex itself, because of the overlap of the signals from the two conformations at −14.9 ppm. Considering first the methotrexate complex, comparison with the ^{1}H-decoupled spectrum in Fig. 4 shows that the effects of ^{1}H-^{31}P coupling can clearly be seen on the lower-field ^{31}P signal, but not on the higher-field signal. The coupling involved is a three-bond coupling between the ^{31}P and the two ribose C5′-protons; approximate estimates of the sum of the two ^{31}P-^{1}H coupling constants are 13 Hz for the low-field signal and <5 Hz for the high-field signal (Table 2). These are similar to the values obtained for the enzyme-methotrexate-NADPH complex (30). These three-bond coupling constants depend on the dihedral angle about the two C5′-O bonds of the coenzyme, and comparison of the dihedral angles calculated from the coupling constants (30, 31) with those observed for the NADPH complex in the crystal permits the assignment of the lower-field signal to the pyrophosphate phosphorus at the nicotinamide end of the molecule (16).[3] In view of the close similarity of the chemical shifts, it seems likely that the same assignment applies to the $NADP^+$ complex. It follows that in Conformation I the coenzyme has a pyrophosphate conformation similar to that seen in the crystal: the adenine pyrophosphate phosphorus is *gauche* to the two C5′-protons, whereas at the nicotinamide end, the dihedral angle about the C5′-O bond differs from this by about 50°.

Turning to the enzyme-trimethoprim-$TNADP^+$ complex, the single resonance ^{31}P spectrum in Fig. 5 is seen to be virtually identical to the ^{1}H-decoupled spectrum in Fig. 4. In this case, therefore, both pyrophosphate phosphorus nuclei have proton couplings of less than 5 Hz. It follows that in Conformation II the conformation about both C5′-O bonds is such as to place the phosphorus *gauche* to both C5′-protons. The conformation about the nicotinamide C5′-O bond thus differs by approximately 50° between Conformations I and II.

In addition to this difference in ^{31}P-^{1}H coupling between the two conformations, there is also a difference in the two-bond ^{31}P-^{31}P coupling. In the free coenzyme, this has a value of 20.8 Hz, and essentially the same value is observed in the complexes of the enzyme, methotrexate, and reduced (30) or oxidized coenzyme (Table 2). However, in Conformation II of the enzyme-trimethoprim-$NADP^+$ complex and in the enzyme-trimethoprim-$TNADP^+$ complex (see Figs. 4 and 5 and Table 2), this coupling constant is only 11 Hz. Only two other instances of a change in $^{2}J_{POP}$ accompanying the binding of a nucleotide to a protein have been reported: $^{2}J_{\alpha\beta}$ decreases from 19.7 to 17.1 Hz when ATP binds to adenylate kinase (32), and $^{2}J_{POP}$ of FAD, which is 21 Hz in the free state, is only 14 Hz when the coenzyme is bound to ferredoxin: $NADP^+$ oxidoreductase (33).

The factors determining the magnitude of $^{2}J_{POP}$ are as yet ill-understood, but it seems likely that one important factor is the pyrophosphate conformation. An indication of this is the observation of $^{2}J_{POP}$ values as high as 33 Hz in a series of thymidine cyclic 3′,5′-pyrophosphates (34). A two-bond coupling would be expected to be sensitive to small changes in bond angle, here the P-O-P angle. In phosphate diesters there is a clear correlation between the O-P-O bond angle and the dihedral angle about the P-O bond (35, 36). If the same kind of correlation exists in pyrophosphates, this would provide a simple mechanism by which the conformation about the P-O bonds of the pyrophosphate group could influence $^{2}J_{POP}$. A likely interpretation of the decrease in this coupling in Conformation II is thus that one or more of the P-O bonds differ in dihedral angle between Conformations I and II of the complex. Since phosphate ^{31}P chemical shifts appear to be rather insensitive to the proximity of charged groups (37) but very sensitive to bond angle and, hence, conformational effects (38, 39), this conformational difference might also account for the marked difference in ^{31}P chemical shifts between the two conformations of the complex.

General discussion. The NMR experiments described here have not only demonstrated the existence of two slowly interconverting conformations of the enzyme-trimethoprim-$NADP^+$ complex, but have also allowed us to begin to characterize the differences between them in structural terms.

The most obvious of these is the mode of binding of $NADP^+$. Whereas the adenine end of the coenzyme appears to bind in the same way in both forms of the complex, the nicotinamide end clearly does not. The magnitudes of the chemical shift changes suggest that the nicotinamide ring may be interacting more loosely with the protein in Conformation II than in Conformation I. This difference in nicotinamide binding is associated with an appreciable change in the pyrophosphate "backbone" of the coenzyme, in particular with a rotation of about 50° about the C5′-O bond at the nicotinamide end of the molecule.

At low temperature, the rate-limiting step in the interconversion of the two conformations is clearly associated with a rearrangement of the protein conformation. The rate of dissociation of the coenzyme from the complex at 11° (measured by transfer of saturation; refs. 15, 27) is approximately 4 sec^{-1}, whereas the rate of interconversion at this temperature, estimated by extrapolation of

[3] D. A. Matthews and D. J. Filman, personal communication correcting the assignment in ref. 25.

TABLE 2

^{31}P chemical shifts[a] and coupling constants[b] of coenzyme bound to dihydrofolate reductase

Complex	Conformation I				Conformation II			
	δ2′P	δPP	$\sum^3J_{PH}$	$^2J_{PP}$	δ2′P	δPP	$\sum^3J_{PH}$	$^2J_{PP}$
E·trimethoprim·NADP⁺	2.7	−14.9 −16.4	ND[c]	21.0	2.7	−12.9 −14.9	ND[c]	11
E·trimethoprim·NHDP⁺	2.8	−15.0 −16.4	ND[c]	21.5	2.8	−12.7 −15.0	ND[c]	10
E·methotrexate·NADP⁺	2.7	−14.9 −16.5	~13 <5	21.0	—	—	—	—
E·trimethoprim·TNADP⁺	—	—	—	—	2.7	−12.8 −14.8	<5 <5	11
E·trimethoprim·APADP⁺	—	—	—	—	2.7	−12.7 −14.6	ND[c]	12

[a] In ppm from inorganic phosphate (pH* = 8.0), upfield shifts negative. δ2′P, chemical shift of 2′-phosphate resonance. δPP, chemical shifts of pyrophosphate resonances; assignment discussed in the text.

[b] In Hz. $\sum^3J_{PH}$, sum of the ^{31}P-O-C5′-^1H_A and ^{31}P-O-C5′-^1H_B coupling constants; estimated from linewidths with and without ^{1}H decoupling and from spectral simulation. Relaxation effects were taken into account as described earlier (30); the good agreement for the enzyme-methotrexate-NADPH complex between the dihedral angles calculated from these coupling constants (30) and those observed in the crystal (8, 25, footnote 3) confirms the correctness of the analysis. $^2J_{PP}$, two-bond ^{31}P-O-^{31}P coupling constant; measured directly from the ^{1}H decoupled spectra.

[c] ND, Not determined.

the data in Figure 2, is 0.5 sec^{-1}. The changes in protein conformation involved in this interconversion cannot yet be described in detail, since we have only been able to study a relatively limited number of individual residues by NMR (9–14). Of the sixteen aromatic residues studied in detail so far, four have different environments in Conformations I and II: His 18 and 28, discussed above, and two tryptophan residues,[4] one of which is Trp 21. The remaining five histidines and two tryptophans, along with the five tyrosines (10) are not affected. In the enzyme-methotrexate-NADPH complex (7, 8), the indole ring of Trp 21 is in contact with the nicotinamide ring of the coenzyme, and the carbonyl of His 18 forms a hydrogen bond to 03′ of the nicotinamide ribose. If we assume that NADP⁺ binds in a similar way to NADPH, the differences in environment of these two residues could therefore be fairly direct consequences of the different mode of binding of the coenzyme. If we further assume that trimethoprim binds as we have proposed earlier (28), His 28 would not, however, be in contact with either trimethoprim or NADP⁺ (in the methotrexate complex it interacts with the methotrexate glutamate γ-carboxyl; ref. 7), and for this residue an effect transmitted through the protein structure seems more likely.

In dihydrofolate reductase (15, 16), as well as in liver alcohol dehydrogenase (40), different coenzyme analogues bind differently in their binary complexes. In the case of dihydrofolate reductase, the structure of the binary complex seems to be somewhat different for each of the structurally related ligands, an example of the familiar concept of "induced fit." The present observations on ternary complexes imply the existence, in addition, of a different kind of conformational change linked to ligand binding. The similarities in ^{1}H and ^{31}P chemical shifts between Conformations I and II formed with different ligands strongly suggest that the nature of these conformations is determined by the protein, with only their relative proportions being affected by the structure of the bound ligand.

This suggests that an equilibrium between Conformations I and II might exist in the absence of ligands. We have as yet found no evidence for this, but it would not be apparent in the histidine resonances if the equilibrium constant were far from one or if the rate of interconversion were faster than in the ternary complex. There is good evidence for the existence of two or more confor-

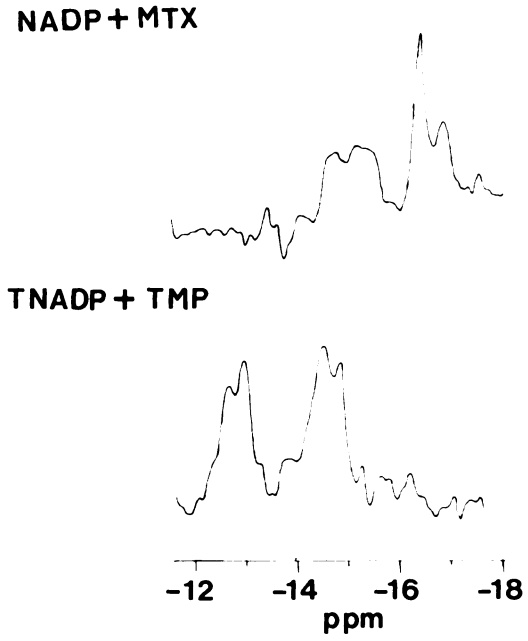

FIG. 5. *The pyrophosphate region of the ^{31}P NMR spectra of the complexes of dihydrofolate reductase with methotrexate (MTX) and NADP⁺ (top) and trimethoprim (TMP) and TNADP⁺ (bottom).*

Experimental conditions were identical with those for Fig. 4, except that no ^{1}H irradiation was employed.

[4] J. Feeney and B. Birdsall, unpublished experiments.

mational states of the dihydrofolate reductases from *L. casei* (41), *E. coli* (42), and *Streptococcus faecium* (17), but the structural relationship of these to the conformations identified here is unknown.

The existence of a conformational equilibrium in the ternary complexes has clear implications for attempts to understand the relationship between ligand structure and binding energy. The observation that ligand binding can change the position of the conformational equilibrium obviously implies that Conformations I and II differ in their affinity for ligands. The ratio of Conformation I to Conformation II varies, in the complexes we have examined so far, from ≥10:1 in the enzyme-methotrexate-$NADP^+$ complex, through approximately 1:1 in the enzyme-trimethoprim-$NADP^+$ complex, to ≤1:10 in the enzyme-trimethoprim-$TNADP^+$ complex. The substantial variation in this ratio indicates that the two conformations must also differ in their relative affinity for structurally related ligands, that is, in their specificity. Thus, whereas $NADP^+$ binds 1.8 times more tightly than $TNADP^+$ to the enzyme-trimethoprim complex in terms of the overall binding constant (21), the ratio of binding constants, K_{NADP^+}/K_{TNADP^+}, is ≥10 for Conformation I but 0.9–1.0 for Conformation II. This indication that the nicotinamide ring binding subsite is less specific in Conformation II is clearly consistent with the chemical shift evidence for "looser" binding in this conformation. A similar comparison shows that, for the enzyme-$NADP^+$ complex, $K_{methotrexate}/K_{trimethoprim}$ is 1100–1300 for Conformation I but ≤110 for Conformation II.

Of course, it is widely assumed that drugs can bind selectively to particular conformations of their receptor molecule, and that this selectivity is the basis of the distinction between different clases of drug acting on the same receptor. For example, the behavior of drugs interacting with neurotransmitter receptors can be rationalized by postulating that agonists select a different conformation of the receptor from that selected by antagonists. The experiments described in this paper show clearly that such conformational selection occurs and, moreover, that a given ligand can bind to the receptor in more than one way, as indicated by the quite different conformations of $NADP^+$ in Conformations I and II of the ternary complex.

The relations between structure and binding, for both the coenzyme analogues and the antifolates, are therefore more complex than they appear at first sight, since in each case they are due to the superposition of two distinct structure-activity relationships, one for each of the conformational states of the complex. It is not at all improbable that such complexities occur in many drug-receptor systems and account for the anomalies often found in apparently simple series of structural analogues. We are investigating this problem further in the case of dihydrofolate reductase with a series of trimethoprim analogues.

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