Multinuclear NMR Characterization of Two Coexisting Conformational States of the *Lactobacillus casei* Dihydrofolate Reductase–Trimethoprim–$NADP^+$ Complex†

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ABSTRACT: The complex of *Lactobacillus casei* dihydrofolate reductase with trimethoprim and $NADP^+$ exists in solution as a mixture of approximately equal amounts of two slowly interconverting conformational states [Gronenborn, A., Birdsall, B., Hyde, E. I., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1981) *Mol. Pharmacol. 20*, 145]. These have now been further characterized by multinuclear NMR experiments, and a partial structural model has been proposed. ^1H NMR spectra at 500 MHz show that the environments of six of the seven histidine residues differ between the two conformations. The characteristic ^1H and ^{31}P chemical shifts of nuclei of the coenzyme in the two conformations of the complex are identical in analogous complexes formed with a number of trimethoprim analogues, indicating that the nature of the two conformations is the same in each case. The pyrophosphate ^{31}P resonances have been assigned to the two conformations, and integration of the ^{31}P spectrum shows that the ratio of conformation I to conformation II varies from 0.4 to 2.3 in the complexes with the various trimethoprim analogues, the ratio for the trimethoprim complex itself being 1.2. Transferred NOE experiments, together with the ^1H and ^{13}C chemical shifts, indicate that in conformation II of the complex the nicotinamide ring of the coenzyme has swung away from the enzyme surface into solution; this is made possible by changes in the conformation of the pyrophosphate moiety. In conformation I, by contrast, the nicotinamide ring remains bound to the enzyme. ^{13}C and ^{15}N experiments show that trimethoprim is protonated on N1 in both conformations of the ternary complex. Analysis of the ^1H chemical shifts of trimethoprim in terms of ring current effects shows that in conformation I of the ternary complex trimethoprim retains the same conformation as in its binary complex, but ^{13}C, ^{15}N, and ^{19}F [using 2,4-diamino-5-(3,5-dimethoxy-4-fluorobenzyl)pyrimidine] experiments show that the environment of both the pyrimidine ring and benzyl ring is affected by the proximity of the coenzyme. Less information is available about the conformation of the inhibitor in conformation II of the complex, but its environment is similar to that in the binary enzyme–inhibitor complex. The implications of the existence of these two conformations of the enzyme for understanding cooperativity in binding between $NADP^+$ and trimethoprim are briefly discussed.

Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine], a valuable antibacterial drug, is an inhibitor of dihydrofolate reductase, and a substantial effort has been devoted to the study of structure–activity relationships among related 2,4-diaminopyrimidine inhibitors of this enzyme [for a review, see Roth & Cheng (1982)]. With the advent of structural information on trimethoprim binding to the enzyme, both from crystallography (Baker et al., 1981, 1983; Matthews et al., 1983; Stammers et al., 1983) and from NMR spectroscopy (Birdsall et al., 1977, 1983; Cayley et al., 1979; Gronenborn et al., 1981a,b,c; Roberts et al., 1981; Roberts, 1983a), it has become possible to begin to interpret these relationships in molecular detail [see, e.g., Roth & Cheng (1982), Li et al. (1982), Baker et al. (1983), Matthews et al. (1983), Birdsall et al. (1983), Roberts (1983a), and Stuart et al. (1983)].

The binding of the coenzyme ($NADP^+$ or NADPH)[1] to form a ternary complex has a substantial effect not only on the affinity of trimethoprim and other inhibitors but also on the structure–activity relationship (Birdsall et al., 1980a,b; Baccanari et al., 1982; Roberts, 1983a). In some cases, these effects have been shown to be accompanied by changes in protein conformation (Roberts, 1983a; Clore et al., 1984). We have shown that the ternary complex of trimethoprim and $NADP^+$ with the *Lactobacillus casei* dihydrofolate reductase exists in solution as a mixture of two conformational states (Gronenborn et al., 1981a,b). These two states, which are approximately equally populated, interconvert slowly (ca. 6 s^{-1} at 30 °C) so that separate resonances from the two are observed for several ^1H and ^{31}P nuclei in the complex. We were able to show that the mode of interaction of the nicotinamide ring of the coenzyme with the enzyme is appreciably different in the two states.

In addition to the significance of this conformational effect for our understanding of structure–binding constant relationships (Gronenborn et al., 1981b; Roberts, 1983a,b), this complex offers a rare opportunity for detailed characterization of a conformational equilibrium in a protein in solution. We now report further NMR experiments that allow us to propose a structural model for the conformational equilibrium and show that the position of the equilibrium is a sensitive function of ligand structure. A preliminary account of part of this work has been presented (Bevan et al., 1983).

Materials and Methods

Dihydrofolate reductase was isolated and purified from *Lactobacillus casei* MTX/R as described previously (Dann et al., 1976). Its concentration was determined by assay of

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[1] Abbreviations: thio$NADP^+$, thionicotinamide adenine dinucleotide phosphate (oxidized); NMN^+, nicotinamide mononucleotide (oxidized); PADPR-OMe, 2′-phosphoadenosine 5′-diphosphoribose methyl β-riboside; $APADP^+$, acetylpyridine adenine dinucleotide phosphate; NOE, nuclear Overhauser effect; EDTA, ethylenediaminetetraacetic acid.

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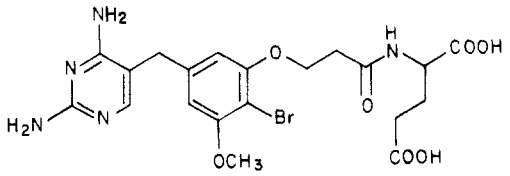
its catalytic activity, by measurements of absorbance at 280 nm, and by fluorometric titration with methotrexate (Dann et al., 1976).

[2-^{13}C]Trimethoprim was synthesized by Dr. S. Daluge as described previously (Roberts et al., 1981); the synthesis of [*1,3,N*^{2}-$^{15}N_3$]trimethoprim (by Dr. L. Kuyper) followed the same route but employed uniformly ^{15}N-enriched guanidine (U.S. Services, Inc.). [*carboxamide*-^{13}C]NADP$^+$ was synthesized as described by Way et al. (1975). [2′,6′-$^2H_2$]Trimethoprim was prepared by exchange as described by Cayley et al. (1979).

2,4-Diamino-5-(3,4,5-trimethoxybenzyl)-6-methylpyrimidine (6-methyltrimethoprim) and 2,4-diamino-5-(3,4,5-trimethoxybenzyl)-6-aminopyrimidine (6-aminotrimethoprim) (Roth et al., 1980) were gifts from Dr. B. Roth, Wellcome Research Laboratories, Research Triangle Park, NC. 2,4-Diamino-5-(3,5-dimethoxy-4-fluorobenzyl)pyrimidine (4′-fluorotrimethoprim) (Kompis & Wick, 1977) and Ro 16-3034 (I)



I

(Birdsall et al., 1984) were gifts from Dr. I. Kompis, Hoffman-La Roche and Co., Basel, Switzerland.

NMR Spectroscopy. ^{1}H saturation transfer experiments were carried out at 270 MHz by using a Bruker WH270 spectrometer as described previously (Cayley et al., 1979; Hyde et al., 1980a; Gronenborn et al., 1981b). ^{1}H NMR spectra of the protein were obtained at 500 MHz by using a Bruker AM500 spectrometer. Samples (0.35 mL) contained 0.5–1 mM enzyme, 1–6 molar equiv of inhibitor and NADP$^+$, 50 mM potassium phosphate, pH* 6.5, 500 mM KCl, 1 mM EDTA, and 1 mM dioxane, in ^2H_2O (the notation pH* indicates a meter reading uncorrected for the deuterium effect on the glass electrode). Spectra were acquired with quadrature detection, with spectral widths of 4.2 (270 MHz) or 8 kHz (500 MHz), and with pulse intervals of 0.5–1.0 s. Line broadening of 2 Hz was normally used; when required , resolution enhancement was by Gaussian multiplication (Ferrige & Lindon, 1978). Chemical shifts are given with respect to internal dioxane (3.71 ppm downfield from 4,4-dimethyl-4-silapentane-1-sulfonate).

^{13}C (50.3 MHz), ^{15}N (20.1 MHz), ^{19}F (188.3 MHz), and ^{31}P (81 MHz) spectra were obtained by using a Bruker WM200 spectrometer. For ^{13}C spectra, samples (3.0 mL) contained 0.5 mM enzyme and 0.45–0.4 mM inhibitor and coenzyme in the same buffer as used for the ^{1}H experiments. The same sample composition was used for ^{31}P experiments, except that 50 mM Bis-Tris [2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)-1,3-propanediol], pH* 6.5, replaced the phosphate buffer. For ^{19}F spectra, 0.4 mL of 1 mM enzyme in the phosphate buffer was used. For ^{15}N spectra, the enzyme concentration was increased to 1.3 mM, and the solvent was 90% H_2O–10% ^2H_2O. Spectral widths of 4–10 kHz, pulse angles of 30–90°, and pulse intervals of 1–4 s were employed; proton noise decoupling was used for ^{13}C, ^{15}N, and ^{31}P spectra. The signal-to-noise ratio of the ^{15}N spectra was improved by polarization transfer from protons with the refocused INEPT pulse sequence (Morris & Freeman, 1979; Morris, 1980). For simultaneous observation of the *N*1-H and 2-*N*H_2 signals, compromise conditions were used, corresponding to those appropriate for a triplet with $^1J_{NH}$ = 90 Hz.

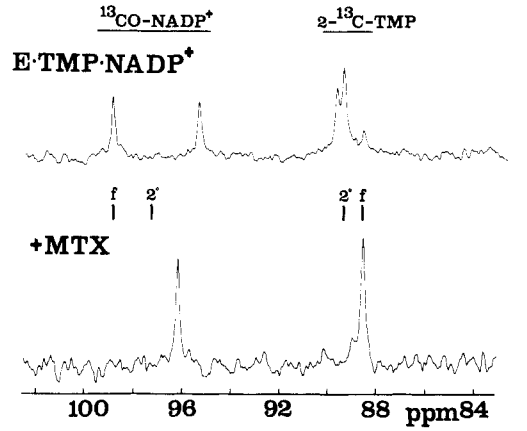


FIGURE 1: (Top) The 50.3-MHz ^{13}C NMR spectra of the complex between *L. casei* dihydrofolate reductase, [*carboxamide*-^{13}C]NADP$^+$, and [2-^{13}C]trimethoprim. The lower field pair of resonances arise from NADP$^+$ and the higher field pair from trimethoprim (there is also a signal at 88.5 ppm from a small amount of free trimethoprim). (Bottom) The same sample after addition of excess methotrexate. The "sticks" indicate the ^{13}C chemical shifts of NADP$^+$ and trimethoprim free (f) and in their respective binary complexes (2°).

The following chemical shift references were used: ^{13}C, external dioxane (50% in ^2H_2O); ^{15}N, external $^{15}NH_4Cl$ (0.1 M in 90% H_2O–10% ^2H_2O containing 0.1 M HCl); ^{19}F, free 4′-fluorotrimethoprim; ^{31}P, external inorganic phosphate, pH* 8.0 (2.94 ppm downfield from H_3PO_4). Sample temperatures (5–45 °C) were regulated to ±1 °C by a stream of heated or cooled dry air.

Results

A clear demonstration of the existence of two conformational states of the *L. casei* dihydrofolate reductase–trimethoprim–NADP$^+$ complex is given by the top spectrum in Figure 1. This is part of the ^{13}C spectrum of the complex formed with [2-^{13}C]trimethoprim and [*carboxamide*-^{13}C]-NADP$^+$. The sample contained enzyme, trimethoprim, and NADP$^+$ in proportions very close to 1:1:1; a slight excess of trimethoprim was present, and there is thus a small resonance at 88.51 ppm from free [2-^{13}C]trimethoprim. All the remaining four signals arise from the enriched carbon atoms of the *bound* ligands (this was confirmed directly by the addition of methotrexate; see below). It is clear that the individual labeled carbon atoms of both trimethoprim and NADP$^+$ each experience two distinct environments in the ternary complex. Examination of the ^{1}H spectrum of a sample having essentially identical enzyme to ligand ratios showed that the concentration of free enzyme was undetectably small. Taken together with the 1:1:1 ratio of enzyme, trimethoprim, and NADP$^+$ present in the sample, this indicates that only a 1:1:1 complex is present and rules out significant amounts of a 1:2:2 complex. We conclude that the two sets of signals in Figure 1 correspond to the two conformational states of the 1:1:1 enzyme–trimethoprim–NADP$^+$ complex described earlier (Gronenborn et al., 1981b).

The two resonances from bound [2-^{13}C]trimethoprim appear at 89.59 and 89.32 ppm (compared to 89.26 ppm in the binary complex; Roberts et al., 1981). To assign these to conformations I and II as defined earlier (Gronenborn et al., 1981b), we make use of our observation that the ternary complex containing the thionicotinamide coenzyme analogue, thio-NADP$^+$, in place of NADP$^+$ exists predominantly (>90%) in conformation II (Groneborn et al., 1981b). In the ^{13}C spectrum of the enzyme–[2-^{13}C]trimethoprim–thioNADP$^+$ complex, a single resonance from bound [2-^{13}C]trimethoprim was observed at 89.33 ppm, thus assigning the corresponding

Table I: Nicotinamide Proton Chemical Shifts of $NADP^+$ Bound to *L. casei* Dihydrofolate Reductase

	chemical shift (ppm)[a]							
	conformation I				conformation II			
complex	2	4	5	6	2	4	5	6
E–trimethoprim–$NADP^+$[b]	0.91	1.08	0.61	0.86	−0.10	0.12	0.01	−0.05
E–Ro 16-3034–$NADP^+$	0.83	1.03	*c*	0.83	−0.12	0.11	*c*	−0.04
E–6-aminotrimethoprim–$NADP^+$	0.88	1.08	0.77	0.71	−0.17	−0.07	−0.11	−0.21
E–6-methyltrimethoprim–$NADP^+$	0.88	1.11	0.76	0.84	*d*	−0.10	0.02	−0.21

[a] Chemical shifts for the 2-, 4-, 5-, and 6-protons of the bound coenzyme, determined by saturation transfer at 270 MHz, are expressed relative to those in NMN^+ to correct for the effects of intramolecular base stacking in the free coenzyme (Hyde et al., 1980a); error ±0.05 ppm. [b] From Gronenborn et al. (1981b). [c] Not determined. [d] Obscured by the free coenzyme signal.

signal in the enzyme–trimethoprim–$NADP^+$ complex (that closer to the resonance position in the binary complex) to conformation II.

Of the two signals from [*carboxamide*-^{13}C]$NADP^+$ in the ternary complex, at 98.86 and 95.27 ppm (compared to 97.26 ppm in the binary complex; Way et al., 1975), the lower field one corresponds exactly to the resonance position of free [*carboxamide*-^{13}C]$NADP^+$. The sample composition, together with the measured binding constant of $NADP^+$ in the ternary complex (Birdsall et al., 1980a), indicated that all the coenzyme should be bound to the enzyme. This was confirmed directly by addition of 2 molar equiv of methotrexate to the sample used for the top spectrum in Figure 1. Methotrexate displaces the trimethoprim from the complex, leading to the formation of the enzyme–methotrexate–$NADP^+$ complex. The spectrum obtained after methotrexate addition is shown as the bottom spectrum in Figure 1. A single resonance from the displaced [2-^{13}C]trimethoprim is observed at the position characteristic of free trimethoprim, and a single [*carboxamide*-^{13}C]$NADP^+$ resonance is seen at 96.24 ppm, the position characteristic of the enzyme–methotrexate–$NADP^+$ complex (Way et al., 1975). The absence of any ^{13}C resonance at 98.86 ppm after methotrexate addition shows that the signal at this position in the top spectrum cannot arise from free $NADP^+$ but must represent $NADP^+$ in one of the two conformations of the enzyme–trimethoprim–$NADP^+$ complex.[2]

The nicotinamide proton resonance of the bound coenzyme in conformation II of the ternary complex are also very close to those in the free coenzyme, whereas the corresponding resonances from conformation I are shifted substantially downfield (Gronenborn et al., 1981b; see below). It is thus likely that the [*carboxamide*-^{13}C]$NADP^+$ signal from the complex that coincides with that of free $NADP^+$ also originates from conformation II of the complex.

Coenzyme Resonances in Ternary Complexes with Trimethoprim Analogues. Since the nicotinamide ^{1}H and pyrophosphate ^{31}P resonances of $NADP^+$ have characteristically different chemical shifts in conformations I and II of the enzyme–trimethoprim–$NADP^+$ complex (Gronenborn et al., 1981b), they provide a convenient means of establishing the existence of the two conformations in ternary complexes formed with structural analogues of trimethoprim.

Nicotinamide ^{1}H chemical shifts of the bound coenzyme have been determined in a number of such ternary complexes by transfer of saturation experiments, and the results are shown in Table I. For each complex, two sets of nicotinamide ^{1}H resonances were located, one shifted downfield >0.6 ppm from the corresponding signals of $NMN^+$and the other within 0.2 ppm of the NMN^+ signals. As shown in Table I, the chemical

[2] In our earlier studies of [*carboxamide*-^{13}C]$NADP^+$ binding to the enzyme (Way et al., 1975), we used an $NADP^+$ to enzyme ratio of 2:1, so that this second resonance from bound $NADP^+$ was obscured by the signal from the free $NADP^+$.

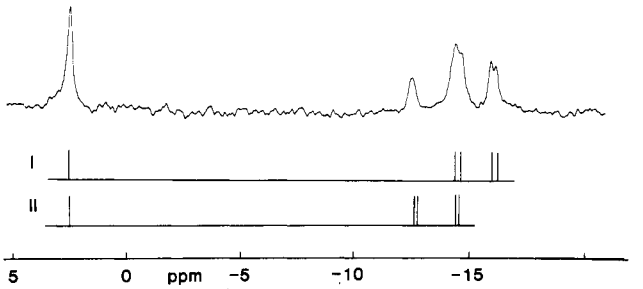


FIGURE 2: The 81-MHz ^{31}P NMR spectrum of the *L. casei* dihydrofolate reductase–$NADP^+$–6-methyltrimethoprim complex. The "stick diagrams" represent the pyrophosphate ^{31}P resonances of the two individual conformational states. The spectrum was obtained with noise-modulated proton decoupling.

Table II: Assignment of ^{31}P Resonances of $NADP^+$ in the Enzyme–Trimethoprim–$NADP^+$ Complex[a]

	chemical shift (ppm)	
assignment	conformation I	conformation II
adenosine 2′-phosphate	2.7	2.7
adenosine 5′-phosphate[b]	−16.4	−14.9
nicotinamide riboside 5′-phosphate[b]	−14.9	−12.9

[a] Identical chemical shifts are observed in complexes containing 6-aminotrimethoprim, 6-methyltrimethoprim, 4′-fluorotrimethoprim, or Ro 16-3034 in place of trimethoprim. [b] Pyrophosphate resonances.

shifts of these two sets of signals are similar in all the complexes, and by comparison with the chemical shifts in the enzyme–trimethoprim–$NADP^+$ complex (Gronenborn et al., 1981b), they can be assigned to conformations I and II, respectively, of the ternary complex. This demonstrates that the conformational equilibrium exists in ternary complexes formed with trimethoprim analogues and that the nature of the two conformational states is, at least as far as the $NADP^+$ nicotinamide ring is concerned, closely similar in all cases.

Further evidence for the similarity of the two conformations in all the complexes comes from the ^{31}P spectra. Figure 2 shows the 81-MHz ^{31}P spectrum of the enzyme–6-methyltrimethoprim–$NADP^+$ complex; in the pyrophosphate region of the spectrum (−10 to −20 ppm), two pairs of ^{31}P signals can be discerned, as shown in the "stick diagram" below the spectrum. These four pyrophosphate ^{31}P signals have the same chemical shift in each of the enzyme–trimethoprim analogue–$NADP^+$ complexes. As shown earlier (Gronenborn et al., 1981b), the resonance at −12.9 ppm and one of the two at −14.9 ppm arise from conformation II; in this conformation the ^{31}P–^{31}P coupling is small and unresolved in the spectrum shown in Figure 2 [see Gronenborn et al. (1981b)]. We have now been able to connect the individual ^{31}P resonances in the two conformational states by means of a ^{31}P transfer of saturation experiment, which demonstrated that the ^{31}P nucleus that has a resonance at −12.9 ppm in conformation II resonates at −14.9 ppm in conformation I. The pyrophosphate ^{31}P

Table III: Fractional Populations of the Two Conformations of *L. casei* Dihydrofolate Reductase–$NADP^+$–Trimethoprim Analogue Complexes

complex	fractional populations[a] conformation I	conformation II
E–$NADP^+$–trimethoprim	0.55	0.45
E–$NADP^+$–6-aminotrimethoprim	0.70	0.30
E–$NADP^+$–6-methyltrimethoprim	0.55	0.45
E–$NADP^+$–4′-fluorotrimethoprim	0.30	0.70
E–$NADP^+$–Ro 16-3034	0.40	0.60

[a] Determined by integration of ^{31}P spectra as described in the text; error ±0.05.

chemical shifts in conformation I are identical (to within 0.1 ppm) with those in the enzyme–methotrexate–$NADP^+$ complex, and we assume that the same assignment to the adenosine and nicotinamide riboside 5′-phosphates (Hyde et al., 1980b) holds in this case. The full assignments of the ^{31}P resonances are summarized in Table II, where it can be seen that *both* pyrophosphate ^{31}P nuclei differ in chemical shift, by 1.5–2.0 ppm, between the two conformations of the ternary complex.

The ^{31}P spectrum provides a convenient method for determining the relative proportions of conformations I and II in these ternary complexes, by integration of the appropriate pyrophosphate resonances. The spectrum in Figure 2 was obtained with a 45° pulse angle and a 4-s pulse interval; further decreases in pulse angle or increases in pulse interval did not change the relative areas of the two sets of pyrophosphate resonances, so that reliable estimates of the proportions of the two conformations can be obtained under these conditions. The proportions measured for several enzyme–$NADP^+$–trimethoprim analogue complexes are given in Table III. It is apparent that while the *nature* of the two conformations remains the same, their proportions depend quite markedly on the structure of the inhibitor.

Transferred NOE Experiments. We have examined the conformation about the nicotinamide ribose glycosidic bond of the bound coenzyme by means of transferred NOE (TNOE) experiments (Albrand et al., 1979; Hyde et al., 1980a; Clore & Gronenborn, 1982; Feeney et al., 1983). In these experiments, nuclear Overhauser effects (NOEs) between nuclei in the bound coenzyme are transferred to the more easily detected nuclei in excess free coenzyme by virtue of the exchange of coenzyme molecules between the bound and the free states; the negative NOEs arising from nuclei in close proximity in the enzyme–ligand complex are readily distinguished from the positive effects encountered in small molecules. NOE measurements can give information on the glycosidic bond conformation since the latter determines the distances between the nicotinamide ribose 1′ proton (N1′) and the N2 and N6 protons on the nicotinamide ring. Thus, in the enzyme–methotrexate–$NADP^+$ complex (in which the coenzyme appears to bind in the same way as in conformation I of the enzyme–trimethoprim–$NADP^+$ complex; Gronenborn et al., 1981b) a TNOE is observed between N1′ and N2 but not between N1′ and N6, indicating an anti conformation about the glycosidic bond (Albrand et al., 1979; Hyde et al., 1980a).

In order to observe the TNOE from conformation II of the complex selectively, we made use of the observation (Gronenborn et al., 1981b) that the dissociation rate constant of $NADP^+$ is significantly greater in conformation II than in conformation I, so that at low temperature (5 °C) transfer of saturation (and hence TNOE) can only be observed for protons of the coenzyme in conformation II of the complex. The results of a TNOE experiment on the enzyme–Ro 16-3034–$NADP^+$ complex at 5 °C are shown in Figure 3. Irradiation at 2.41 ppm, the resonance position of N1′ of $NADP^+$ in conformation II of the complex, leads to a decrease in intensity of *both* the N2 and the N6 resonances. This indicates that *both* these protons can approach closely to N1′ in the bond coenzyme; the most probable interpretation is that there is a mixture of syn and anti conformations, although we cannot, from this experiment, rule out the possibility of a single conformation of the "low-anti" type. Quantitatively similar TNOE effects have been observed for the N2 and N6 protons of the coenzyme in the dihydrofolate reductase–thio$NADP^+$ complex, for which quantitative analysis showed that the data were consistent with an equilibrium mixture of two or more conformations about the glycosidic bond in the bound coenzyme (Feeney et al., 1983). We conclude that this is also the case in conformation II of the ternary complex.

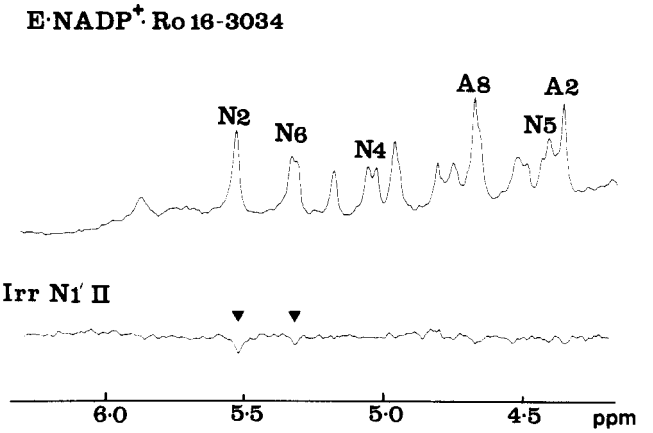


FIGURE 3: Part of the 270-MHz ^1H NMR spectra of a solution containing the *L. casei* dihydrofolate reductase–$NADP^+$–Ro 16-3034 complex together with a 2-fold molar excess of free $NADP^+$: (top) control spectrum, with the resonances of free $NADP^+$ indicated; (bottom) difference between the control spectrum and one obtained after irradiation at the resonance position of N1′ in conformation II of the complex. The arrows indicate the decreased intensity of the N2 and N6 resonances due to the transferred NOE effect.

Resonances of Trimethoprim and Its Analogues. The experiments described above, which extend those of Gronenborn et al. (1981b), show clearly that the mode of binding of the coenzyme differs between the two conformations of the ternary complex. To establish how trimethoprim binding differs between the two conformations, we have studied a number of resonances of trimethoprim and its analogues. The 2-^{13}C chemical shift of trimethoprim is very similar in the two conformations of the ternary complex (Figure 1) and much closer to the chemical shift of protonated trimethoprim (87.97 ppm) than to that of neutral trimethoprim (95.06 ppm; Roberts et al., 1981). This suggests that trimethoprim is protonated in both conformations of the complex.

To obtain unambiguous evidence for this, we have examined the ^{15}N spectrum of the enzyme–$NADP^+$–[1,3,N^2-$^{15}N_3$]trimethoprim complex. In Figure 4 this spectrum is compared with that of the binary complex and that of the free protonated trimethoprim. The spectra of the enzyme complexes were obtained by the INEPT technique (Morris & Freeman, 1979; Morris, 1980), in which the ^{15}N signal is enhanced in intensity by transfer of polarization from a proton through scalar coupling. The pulse timings used were those appropriate for a one-bond ^{15}N–^1H scalar coupling (~90 Hz), and the observations of an INEPT enhancement of the N1 resonance in the binary complex and in both conformations of the ternary complex show unequivocally that a proton is directly bonded to this nitrogen—i.e., that the pyrimidine ring is protonated.

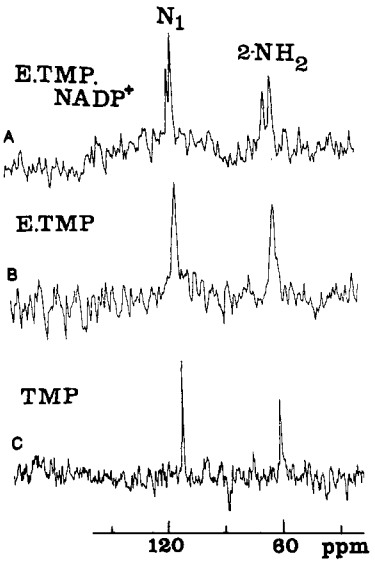


FIGURE 4: The 20.2-MHz ^{15}N NMR spectra of [1,3,N^2-$^{15}N_3$]trimethoprim (A) in its ternary complex with dihydrofolate reductase and $NADP^+$, (B) in its binary complex with dihydrofolate reductase, and (C) in 0.1 M HCl. Spectra A and B were obtained by the INEPT method; this could not be used for spectrum C, since the nitrogen-bound protons of free trimethoprim exchange rapidly with the aqueous solvent. Noise-modulated proton decoupling was used in each case. Only the $^{15}N1$ and 2-$^{15}NH_2$ resonances are shown.

Table IV: ^{13}C, ^{15}N, and ^{19}F Chemical Shifts of Trimethoprim and 4′-Fluorotrimethoprim Bound to *L. casei* Dihydrofolate Reductase

		chemical shift (ppm)[a]		
			enzyme–inhibitor–$NADP^+$ complex	
compound	nucleus	enzyme–inhibitor complex	I[b]	II[b]
trimethoprim	2-^{13}C	89.26	89.59	89.32
	1-^{15}N	111.3	113.2	111.0
	2-$^{15}NH_2$	59.7	62.0	58.3
4′-fluorotrimethoprim	4′-F	0.72	4.50	1.02

[a] For chemical shift reference compounds, see Materials and Methods. [b] Conformations I and II; direct evidence for the assignment of the resonances to individual conformations is available for ^{13}C and ^{19}F but not for ^{15}N; see text.

Both N1 and the 2-amino group show separate signals for the two conformations of the ternary complex separated by 2.2 and 3.7 ppm, respectively. These shift differences are however small compared to that between the protonated and neutral species [90.3 ppm; see Städeli et al. (1980)], and the chemical shifts in both conformations (113.2 and 111.0 ppm for N1 and 62.0 and 58.3 ppm for 2-NH_2) clearly correspond to a protonated molecule [cf. chemical shifts of 109.2 (1-^{15}N) and 57.7 ppm (2-$^{15}NH_2$) for free trimethoprim at pH 3.5].

The ^{13}C and ^{15}N chemical shifts, summarized in Table IV, demonstrate that there is a difference in the environment of the pyrimidine ring of trimethoprim between the two conformations of the ternary complex. To obtain analogous information for the benzyl ring of the bound inhibitor, we have used the analogue 4′-fluorotrimethoprim, in which the *p*-methoxy group has been replaced by a fluorine. Two ^{19}F resonances were observed for the enzyme–$NADP^+$–4′-fluorotrimethoprim complex, at 1.02 and 4.50 ppm relative to free 4′-fluorotrimethoprim, with an intensity ratio of about 4:1. These resonances can be assigned to conformations I and II of the complex by comparison with the ^{31}P spectrum of the same complex. This shows that conformation II predominates (see Table III), so that the larger ^{19}F resonance (at 1.02 ppm) can be assigned to conformation II of the complex.[3]

Table V: ^1H Chemical Shifts of Trimethoprim and Its Analogues Bound to *L. casei* Dihydrofolate Reductase

	chemical shift (ppm)[a]			
	enzyme–inhibitor complex[b]		enzyme–inhibitor–$NADP^+$ complex[c]	
compound	H6	H2′,6′	H6	H2′,6′
6-methyltrimethoprim[d]	−0.49	−0.29	−0.60 (II) −0.89 (I)	−0.44 (II) −1.53 (I)
6-aminotrimethoprim		−0.30		−0.32 (II) −1.37 (I)
Ro 16-3034	−0.93	−1.63	−1.04	−1.69
trimethoprim	−0.86	−0.79	−1.02[e]	−1.70[e]

[a] Determined by saturation transfer at 270 MHz; expressed relative to the corresponding resonance of the free inhibitor, with upfield shift negative. [b] Trimethoprim data from Cayley et al. (1979); remaining data from A. W. Bevan, C. Pascual, B. Birdsall, G. C. K. Roberts, and J. Feeney, unpublished work. [c] Assignment to conformations I and II is indicated where direct evidence is available. [d] For this compound, H6 refers to the 6-methyl proton resonances. [e] From Gronenborn et al. (1981b).

^1H transfer of saturation experiments on the enzyme–trimethoprim–$NADP^+$ complex at 45 °C (Gronenborn et al., 1981b) revealed only a single H6 and a single H2′,6′ resonance[4] from the bound coenzyme. Since saturation transfer experiments give no information on resonance intensity, this observation could be interpreted in three alternative ways: (a) the 6-proton and 2′,6′-protons each have the same environment and, therefore, chemical shift in both conformations of the ternary complex; (b) the chemical shift difference between the two conformations is sufficiently small to be averaged out by the interconversion of the two conformational states (ca. 40 s^{-1} at 45 °C; Gronenborn et al., 1981a,b); (c) the dissociation rate constant of trimethoprim from one of the two conformations is too slow for transfer of saturation to be observed at 45 °C [cf. the analogous observation with $NADP^+$ at 5 °C; see above and Gronenborn et al. (1981b)]. No resonances could be detected in the difference between the ^1H spectra of complexes containing trimethoprim and [2′,6′-$^2H_2$]trimethoprim, presumably as a result of line broadening due to relatively slow "flipping" of the benzyl ring about its symmetry axis [see Cayley et al. (1979)].

In order to decide which interpretation of the transfer of saturation results with trimethoprim is more likely to be correct, we have studied the binding of 6-methyl- and 6-aminotrimethoprim. These two analogues bind less tightly than the parent compound, thus allowing us to carry out ^1H transfer of saturation experiments at lower temperatures. The results of these experiments are summarized in Table V; for both analogues, two distinct resonance positions were located for the 2′,6′-protons of the bound inhibitor, and two positions were also found for the 6-methyl protons of bound 6-methyltrimethoprim. For the 2′,6′-protons, the two observed resonance positions could correspond, for example, to a rapidly flipping benzyl ring (averaged H2′,6′ chemical shifts) in different environments in conformations I and II or to a slowly flipping benzyl ring (separate H2′ and H6′ resonances) that has the same environment in both conformations. Analogous saturation transfer experiments with the complex of enzyme,

[3] The larger intensity ratio observed in the ^{19}F spectra than in the ^{31}P spectra most probably reflects a difference in the ^{19}F spin–lattice relaxation rate between the two conformational states, since relatively rapid pulsing was used in acquiring the ^{19}F spectra.

[4] In free trimethoprim, the two ortho protons on the benzyl ring, H2′ and H6′, are equivalent and give rise to a single resonance.

6-methyltrimethoprim, and thioNADP$^+$ (which favors conformation II of the ternary complex) revealed a single resonance position for H2′,6′, at 0.44 ppm. This indicates that the two H2′,6′ resonance positions observed for the NADP$^+$ complex do in fact correspond to the two conformations of the complex. It also allows us to assign the signals at −0.60 (6-methyl) and −0.44 ppm (H2′,6′) to conformation II and those at −0.89 (6-methyl) and −1.53 ppm (H2′,6′) to conformation I (see Table V). The similarity of both the H2′,6′ chemical shifts in the 6-aminotrimethoprim complex indicates that the analogous assignment applies in that case. The substantial upfield shift of the single detectable H2′,6′ resonance of trimethoprim in the ternary complex suggests, by comparison with the 6-methyltrimethoprim shifts, that this and the single H6 resonance arise from conformation I of the complex.

We have also carried out saturation transfer experiments on the ternary complex containing Ro 16-3034 (I). This compound has an asymmetrically substituted benzyl ring, with a carboxylate-containing substituent designed to interact with Arg-57 and His-28 (Birdsall et al., 1984). The flipping of its benzyl ring when bound to the enzyme will thus be impeded. Some difficulty was encountered in these experiments, since Ro 16-3034 binds to the enzyme significantly more tightly than trimethoprim does. However, in experiments carried out at 50 °C, saturation transfer effects were observed at −1.04 (for H6) and at −1.69 ppm (for the *o*-benzyl protons).[5] These chemical shifts are closely similar on the one hand to those seen for trimethoprim in the ternary complex, and assigned to conformation I, and on the other hand to those of Ro 16-3034 itself in its binary complex with the enzyme (see Table V).

The ^{1}H chemical shifts of trimethoprim in conformation I of the ternary complex have been analyzed in terms of ring current shift contributions, to obtain information on the conformation of the bound trimethoprim molecule, as described earlier for the binary complex (Cayley et al., 1979; Birdsall et al., 1983). This analysis involves two basic assumptions: (i) We assume that the observed chemical shift effects on the protons of trimethoprim arise solely from the magnetic anisotropy effects of nearby aromatic rings, including those of trimethoprim itself. Only one aromatic amino acid residue, Phe-30, is close enough to have a significant effect, but in conformation I of the ternary complex we must also consider the nicotinamide ring of NADP$^+$. (ii) We assume that the aromatic ring of Phe-30 and the nicotinamide ring of NADP$^+$ have the same spatial relationship to the pyrimidine ring of trimethoprim as they do to the corresponding part of methotrexate in the refined crystal structure of the enzyme–methotrexate–coenzyme complex (Filman et al., 1982). This complex involves—at least nominally [see Filman et al. (1982)]—the reduced form of the coenzyme; no crystal structures of complexes in which the coenzyme is definitely in the oxidized state are yet available.

Having made these assumptions, we can use the Johnson–Bovey (1958) equations[6] to calculate the effect of the aromatic rings on the trimethoprim H6 and H2′,6′ chemical shifts as a function of the conformation of the trimethoprim molecule. For comparison with these calculations, the measured H6 and H2′,6′ chemical shifts of bound trimethoprim must be expressed relative to those of the model compounds 2,4-diamino-5-methylpyrimidine and 1-alkyl-3,4,5-trimethoxybenzene, respectively, so as to allow for any ring current effects in free trimethoprim (Cayley et al., 1979; Birdsall et al., 1983).

In conformation I of the ternary complex, the trimethoprim ^{1}H chemical shifts (referred to these model compounds) are (H6) −1.26 ppm and (H2′,6′) −1.51 ppm. There will be three ring current contributions to the H6 chemical shift—from the benzyl ring of trimethoprim, from Phe-30, and from the nicotinamide ring of NADP$^+$. Only the first of these will depend upon the conformation of trimethoprim. With assumption ii above, the contributions of Phe-30 and the nicotinamide ring are calculated to be −0.19 and −0.07 ppm, respectively. The "internal" contribution (from the benzyl ring) is thus −1.00 ppm; this is very similar to the "internal" contribution calculated in the same way for the binary complex (−0.91 ppm; Birdsall et al., 1983). Calculations of the "internal" contribution to the H6 chemical shift as a function of trimethoprim conformation (Birdsall et al., 1983) showed that upfield shifts of this magnitude were only obtained in a very restricted region of conformational space. It follows that the trimethoprim conformation in state I of the ternary complex must be similar to that in the binary complex.

To define the trimethoprim conformation more precisely, we must include the H2′,6′ chemical shift in the calculation. Only a single H2′,6′ resonance position from conformation I of the complex was identified by saturation transfer. However, because of the indirect nature of this experiment, this can be interpreted either as the resonance from a single ortho proton (the other being within 0.1 ppm of the resonance of the free ligand and, hence, undetectable by saturation transfer) or as the resonance of both ortho protons, averaged by flipping of the benzyl ring about its axis. Within the constraints of assumptions i and ii there is no conformation of trimethoprim that gives calculated H2′,6′ chemical shifts within ±0.1 ppm of −1.5 and −0.2 ppm, as required by the first of these interpretations, and simultaneously gives the correct chemical shift for H6. We conclude, therefore, that the resonance located by saturation transfer is at the average of the chemical shifts of H2′ and H6′. The trimethoprim analogue Ro 16-3034, in which "flipping" of the benzyl ring is impeded, shows a single ortho proton resonance position, with the same chemical shift as seen for the average H2′,6′ resonance in trimethoprim. This indicates that at least one of the ortho proton resonances is at this position in Ro 16-3034 and thus probably in trimethoprim too. It follows that the 2′- and 6′-protons of trimethoprim must have closely similar chemical shifts.

As shown in Figure 5, there is only a very small region of conformational space in which the calculated chemical shifts of H6, H2′, and H6′ are simultaneously in agreement (to ±0.05 ppm in each case) with the experimental values, where H2′ and H6′ are taken to have identical chemical shifts (−1.51 ppm). This is centered on (τ_1,τ_2) values of (186°, 72°) and is essentially identical with the "consensus solution" (191°, 73°) obtained from ^{1}H NMR studies of the binary complexes with the *L. casei* and *Escherichia coli* enzymes (Birdsall et al., 1983). In this conformation, one of the ortho protons (arbitrarily denoted H6′) experiences a large upfield shift from the ring current of Phe-30, while the other experiences a very similar upfield shift from the nicotinamide ring of the coenzyme. If, instead of assuming that H2′ and H6′ have identical chemical shifts, we assume that their *average* shift is −1.51 ppm and that they can be separated by up to 0.1 ppm

[5] The two ortho proton resonances of free Ro 16-3034 are accidentally equivalent, so that we cannot identify which ortho proton resonates at 1.69 ppm in the bound state.

[6] Correction of the Johnson–Bovey equations for benzene for use with a pyrimidine ring leads to only a ≤7% change in the calculated ring-current shifts (Giessner-Prettre et al., 1976). This is less than the intrinsic uncertainties in the calculations and comparable to the experimental error in the observed chemical shift changes and has been neglected.

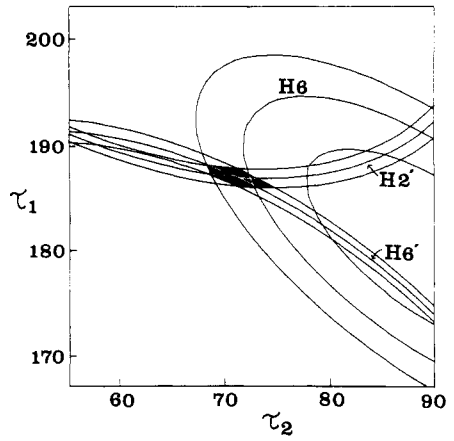


FIGURE 5: Calculation of the conformation of trimethoprim in conformation I of the *L. casei* dihydrofolate reductase–$NADP^+$–trimethoprim complex. The three sets of contours show the calculated chemical shifts of H2′, H6′, and H6 as a function of the trimethoprim torsion angles τ_1 (C4–C6–C7–C1′) and τ_2 (C5–C7–C1′–C2′). In each case, the central contour represents a chemical shift equal to the experimental estimates, the outer contours differing from this by ±0.05 ppm. The shaded area indicates the region of conformational space in which all three calculated chemical shifts agree with the experimental values to ±0.05 ppm. Note that the diagram represents only a small part of conformational space; no other solutions were found in a search of the full space.

(the limit set by the observation of a single saturation transfer peak with Ro 16-3034), we find a slightly larger region of conformation space (ca. ±7° in each dihedral angle) is consistent with the observed shifts, but this is centered about the same conformation as shown in Figure 5.

The accuracy of this conformational solution depends, of course, on the correctness of the assumptions upon which it is based. As noted earlier (Birdsall et al., 1983), the observation of a large (>0.8 ppm) "internal" contribution to the upfield shift of H6 is, by itself, sufficient to define a small region of conformational space around the conformation observed in the binary complex. It follows that small differences in the orientation of the pyrimidine ring in the binding site will not greatly affect the calculated conformation, provided that they do not lead to large increases in the shift contribution from the nicotinamide ring or from Phe-30. For any trimethoprim conformations approximately consistent with the H6 shift, the position of the nicotinamide ring must be similar to that seen in the crystal if it is to produce a large upfield shift of H2′. Changes in the dihedral angle about the nicotinamide glycosidic bond of ±10° did not alter the calculated trimethoprim conformation, showing that our conclusions do not depend upon the nicotinamide ring occupying *precisely* the same position as that in the crystal structure of the enzyme–methotrexate–coenzyme complex.

Histidine Residues. The first evidence for the existence of two conformations of the dihydrofolate reductase–trimethoprim–$NADP^+$ complex came from the observation (Gronenborn et al., 1981a,b) that the C2-proton resonances of two of the histidine residues appear as two signals at low temperature and show characteristic broadening and coalesce into single resonance as the temperature is increased. With the improved chemical shift dispersion and sensitivity of a 500-MHz spectrometer, we have now found that, in fact, six of the seven histidine residues have different environments in the two conformations of the complex. Figure 6 shows the histidine C2-proton resonances in the resolution-enhanced 500-MHz spectrum of the complex at 11 °C. Resonance C (His-64; Wyeth et al., 1980) is clearly a single peak, but the other six resonances show "splittings", summarized in Table VI, ranging

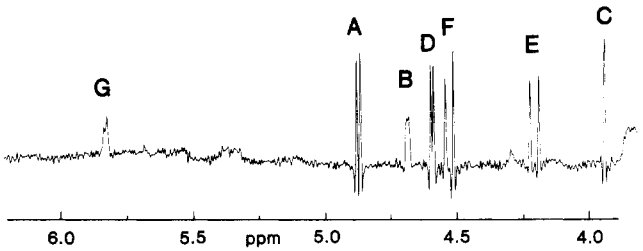


FIGURE 6: Part of the deconvoluted 500-MHz ^1H NMR spectrum of the *L. casei* dihydrofolate reductase–$NADP^+$–trimethoprim complex, showing the C2-proton resonances of the seven histidine residues (labeled A–G).

Table VI: Chemical Shift Differences for the Histidine C2–H Resonances between Conformations I and II of the Dihydrofolate Reductase–Trimethoprim–$NADP^+$ Complex

resonance	assignment[a]	chemical shift difference (ppm)[c]
A	His-22	0.013
B	His-89[b]	0.008
C	His-64	0
D	His-77[b]	0.010
E	His-18	0.033[d]
F	His-28	0.029[d]
G	His-153	0.010

[a] Wyeth et al. (1980); B. Birdsall, J. Feeney, and G. C. K. Roberts (unpublished work). [b] Tentative assignment. [c] ±0.004 ppm. [d] These values are somewhat smaller than those quoted by Gronenborn et al. (1981b), which were obtained at a slightly different pH and temperature.

from 0.008 to 0.033 ppm (4–16.5 Hz).

Discussion

The conformational equilibrium that we have identified in the *L. casei* dihydrofolate reductase–trimethoprim–$NADP^+$ complex clearly involves the active site of the enzyme. There are substantial differences in the environment of the bound ligands between the two conformations. However, the conformational effects are not restricted to the active site region, as indicated by the observation that the environments of six of the seven histidine residues are also affected. As judged by the crystal structure of the enzyme–methotrexate–coenzyme complex (Bolin et al., 1982), only two histidine residues are likely to be in contact with the bound $NADP^+$ or trimethoprim: His-64, whose environment does not differ between the two conformations, forms part of the binding site for the adenine ring of the coenzyme and the carbonyl oxygen of His-18 forms hydrogen bonds to the nicotinamide ribose. In addition, the carbonyl of His-77 forms a hydrogen bond to a fixed water molecule, which in turn hydrogen bonds to N1 of the adenine ring (Bolin et al., 1982). The remaining four histidines are between 6.5 and 16 Å from either $NADP^+$ or trimethoprim.

The differences in the protein structure between the two conformations appear to determine the nature of the difference in ligand environment between the two conformations, since complexes formed with a range of structural analogues of trimethoprim (this work) or $NADP^+$ (Gronenborn et al., 1981b) clearly exist in the same two conformations, although the equilibrium constant between the two varies between <0.1 and 2.3 in the different complexes. In addition, comparison of the coenzyme dissociation rate constant with the rate of interconversion of conformations I and II indicates that, at least at low temperature, the rate-limiting step in the conformational interconversion is associated with a rearrangement of the protein structure (Gronenborn et al., 1981b). At present, although we cannot describe these changes in protein conformation in any detail, we can give a reasonably detailed

description of the conformation and environment of the coenzyme and inhibitor in the two conformations of the ternary complex.

Conformation and Environment of the Bound Coenzyme. In conformation I, the binding of the coenzyme appears to be very similar to that in the binary enzyme–$NADP^+$ and ternary enzyme–$NADP^+$–methotrexate complexes (Hyde et al., 1980a,b), as judged from the ^1H and ^{31}P chemical shifts. The adenine ^1H and 2′-phosphate ^{31}P chemical shifts are identical in conformation I of the enzyme–$NADP^+$–trimethoprim complex and the binary enzyme–$NADP^+$ complex, while there are only modest differences in the large changes in pyrophosphate ^{31}P and nicotinamide ^1H chemical shifts that accompany coenzyme binding. Thus the changes in nicotinamide proton chemical shift on binding in conformation I of the ternary complex range from 0.61 to 1.08 ppm, while the difference between these values and those for the enzyme–$NADP^+$ complex are only 0.13–0.30 ppm. For all but one of these signals, the shifts in conformation I of the trimethoprim complex are closer to those in the enzyme–$NADP^+$–methotrexate complex than to those in the binary complex. This is also the case for the carboxamide-^{13}C resonance, which shows the largest differences in chemical shift; the signal in conformation I is 1.99 ppm upfield of that in the binary complex and 0.97 ppm upfield of that in the enzyme–$NADP^+$–methotrexate complex. All the chemical shift and coupling constant (Gronenborn et al., 1981b) data are consistent with the suggestion that, in conformation I of the enzyme–$NADP^+$–trimethoprim complex, the environment and conformation of the coenzyme are similar to those in the enzyme–$NADP^+$–methotrexate complex.

Turning to a comparison of conformations I and II of the enzyme–$NADP^+$–trimethoprim ternary complex, we note that the adenine ^1H and 2′-phosphate ^{31}P chemical shifts are identical in the two conformational states. This shows that the part of the protein forming the adenosine 2′-phosphate binding site is unaffected by the conformational equilibrium, a conclusion that is supported by the lack of effect on the resonance of His-64.

The nicotinamide ring, by contrast, clearly has a very different environment in the two conformations. As noted previously (Gronenborn et al., 1981b), the much smaller changes in nicotinamide proton chemical shifts associated with the binding of $NADP^+$ to conformation II, compared with those resulting from binding to conformation I, suggest that the nicotinamide is interacting more loosely with the protein in conformation II. The transferred NOE experiments reported in this paper suggest that the potential function for rotation about the nicotinamide glycosidic bond of $NADP^+$ bound to the enzyme in conformation II of the ternary complex is similar to that in the free coenzyme, leading to a mixture of anti and syn conformations [see Feeney et al. (1983)]. This suggests that the nicotinamide riboside portion of the coenzyme is not interacting with the protein at all in conformation II of the complex but has, rather, swung away from the protein so as to interact only with solvent. Strong support for this proposal comes from the observation that the ^{13}C resonance of bound [*carboxamide*-^{13}C]$NADP^+$ assigned to conformation II of the complex has exactly the same chemical shift as in the free coenzyme.

If the adenosine ring remains in its binding site while the nicotinamide swings out into solution, there must clearly be changes in conformation about the bonds linking the two, and the ^{31}P spectra allow us to begin to define these. First, a change in ^1H–^{31}P spin coupling to the nicotinamide 5′-phosphate shows that the dihedral angle about the C5′–O bond of the nicotinamide ribose changes by about 50° on going from conformation I to II, to a gauche–gauche conformation identical with that observed in the free coenzyme (Gronenborn et al., 1981b). Second, *both* the ^{31}P resonances of the pyrophosphate moiety shift downfield by 1.5–2.0 ppm on going from state I to II (Table II), and the two-bond ^{31}P–^{31}P coupling decreases (Gronenborn et al., 1981b). Phosphate ^{31}P chemical shifts appear to be rather insensitive to the proximity of charged groups (Gorenstein et al., 1976) but very sensitive to small changes in O–P–O bond angle (Gorenstein, 1977, 1981; Ribas Prado et al., 1979); the change in the two-bond coupling suggests that a change in P–O–P bond angle may also be involved. In phosphate diesters, the O–P–O bond angle is clearly correlated with torsion angles about the P–O bonds (Perahia & Pullman, 1976; Gorenstein et al., 1977), and this is likely also to be true in pyrophosphates. Thus, in addition to the rotation about the C5′–O bond at the nicotinamide end of the molecule, the transition from conformation I to conformation II seems also to involve rotation about the P–O bonds of both the pyrophosphate phosphorus atoms. These changes in coenzyme conformation do not, however, extend to the adenosine C5′–O bond, since the ^1H–^{31}P coupling to the adenosine 5′-phosphate remains unaltered (Gronenborn et al., 1981b).

The fact that the coenzyme is "partially bound" in conformation II of the complex raises the possibility that this conformation represents an intermediate between the enzyme–trimethoprim complex and conformation I of the ternary complex. However, at 11 °C the rate of dissociation of $NADP^+$ from conformation I is substantially faster than the rate of interconversion of the two conformations (Hyde et al., 1980a; Gronenborn et al., 1981b), indicating that this is most probably not the case.

Conformation and Environment of Bound Trimethoprim. The ^{13}C and ^{15}N experiments establish unambiguously that trimethoprim is protonated on N1 of the pyrimidine ring in both conformations of the ternary complex as it is in the binary complex (Roberts et al., 1981; A. W. Bevan, G. C. K. Roberts, J. Feeney, and L. Kuyper, unpublished work).

For all the nuclei (^1H, ^{13}C, ^{15}N, ^{19}F) of trimethoprim and its analogues for which we have been able to observe resonances from both conformations of the ternary complex, we find that one of the two resonances has a chemical shift close to that in the binary complex (Tables IV and V). In the case of the ^1H, ^{13}C, and ^{19}F resonances, there is direct evidence that this resonance arises from conformation II of the complex. In this conformation the nicotinamide ring of the coenzyme has swung away from the enzyme and, hence, away from the bound trimethoprim. It is thus reasonable that the environment of the trimethoprim in conformation II will be more similar to that in the absence of coenzyme than it will in conformation I. We therefore assume that the ^{15}N chemical shifts will follow the same pattern and assign the further downfield resonances of $^{15}N1$ and 2-$^{15}NH_2$ to conformation I.

The difference in 2-^{13}C chemical shift between conformations I and II is small (0.27 ppm), but the ^{15}N chemical shift differences are larger (2.2 and 3.7 ppm). ^{15}N chemical shifts are well-known to be very sensitive to intermolecular effects, such as hydrogen bonding and ion pair formation (Levy & Lichter, 1979; Martin et al., 1981; Witanowski et al., 1981). For example, the ^{15}N chemical shift of pyridinium chloride changes by 14 ppm on going from dimethyl sulfoxide to water solution (Duthaler & Roberts, 1978), and effects of similar

magnitude are seen for N3 of *N*-methylimidazolium ion (Schuster & Roberts, 1979). In the absence of a detailed understanding of these effects, we cannot interpret the much smaller ^{15}N shift differences between the two conformations in structural terms.

We have shown, however, that the ^1H chemical shifts of trimethoprim can be interpreted so as to determine the conformation of the trimethoprim molecule in its binary complex with the enzyme (Cayley et al., 1979; Birdsall et al., 1983). As outlined under Results, we have now used this approach to determine the conformation of the bound trimethoprim molecule in conformation I of the ternary complex. In this conformation, the nicotinamide ring of the coenzyme is probably in van der Waals contact with the inhibitor molecule (see below). However, the ^1H chemical shift evidence indicates that trimethoprim nonetheless retains the same conformation in state I of the ternary complex as it has in the binary complex.

Less direct information is available about its conformation in state II of the complex, since the proton resonances of trimethoprim in this state of the complex cannot be located by saturation transfer (presumably because its dissociation rate constant is too low). The 6-substituted analogues of trimethoprim, for which we can locate the resonances in conformation II, have a different conformation from trimethoprim in the binary complex [A. W. Bevan, G. C. K. Roberts, and J. Feeney, unpublished work; see also Baker et al. (1983)]. However, their H2′,6′ chemical shifts are similar in conformation II of the ternary complex and in the binary complex, again consistent with a similarity of conformation.

Coenzyme–Inhibitor Cooperativity. Trimethoprim binds a factor of 2 more tightly to the *L. casei* enzyme in the presence of $NADP^+$ than in its absence (Birdsall et al., 1980a). This cooperativity in binding is obviously likely to be different in the two conformations of the ternary complex. The enzyme–trimethoprim–thio$NADP^+$ and –A$PADP^+$ complexes exist to >90% in conformation II (Gronenborn et al., 1981b), and these coenzyme analogues show 4.7–6.1-fold cooperativity in binding with trimethoprim (Birdsall et al., 1980a). If in conformation II the nicotinamide ring is not in contact with the enzyme, the cooperativity in this conformation cannot originate from direct interactions between trimethoprim and the coenzyme but must arise from conformational changes induced by the binding of the rest of the coenzyme. Indeed, PADPR-OMe, in which the nicotinamide ring is replaced by a methoxy group, shows 4.8-fold cooperativity with trimethoprim. The nuclei of trimethoprim we have studied show relatively small chemical shift differences between the binary complex and conformation II of the ternary complex, so that we cannot yet pinpoint which part of the trimethoprim binding site is affected by these conformational changes.

Since the adenosine end of the coenzyme binds in the same way in both conformations of the ternary complex, it is likely that the same conformation changes are produced in both—and yet the coenzyme–trimethoprim cooperativity must be substantially less in conformation I than in conformation II. This suggests that the changes in trimethoprim environment produced by the nicotinamide ring binding are energetically unfavorable. The large downfield shift of the ^{19}F resonance of 4′-fluorotrimethoprim in conformation I points to a change in the surroundings of the para position of the benzyl ring. To assess this, we have built trimethoprim into the *L. casei* enzyme–methotrexate–coenzyme complex structure, placing the diaminopyrimidine ring in the same position as the corresponding part of methotrexate, with trimethoprim in the conformation determined from the ^1H chemical shifts. We have taken the position of the nicotinamide ring to be that in the crystal structure. Examination of this model and comparison with the *E. coli* enzyme–trimethoprim complex [Baker et al., 1981; see also Stuart et al. (1983)] indicate that the closest residue to the 4′-methoxy group is Ser-48, which also interacts, via bound water molecules, with the nicotinamide ring and its ribose (Filman et al., 1982). In addition, the nicotinamide ring is in close contact with one of the 7-CH_2 protons of trimethoprim and the nicotinamide ribose 2′-OH with the 3′-methoxy group of trimethoprim. Finally, Leu-19 makes contact with both the nicotinamide ring and the 3′-methoxy group. Comparison of the *E. coli* enzyme–methotrexate and *L. casei* enzyme–methotrexate–coenzyme complex (Bolin et al., 1982; Filman et al., 1982) suggests that this latter side chain changes its orientation on coenzyme binding. There are thus a number of interactions between the nicotinamide riboside portion of the coenzyme and trimethoprim, both direct and mediated by Leu-19 and Ser-48, which, if they were energetically unfavorable, could account for the lower cooperativity in conformation I of the ternary complex.

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