appreciably between deoxy- and oxy-Mb for this resonance. The small value (0.2–0.3 ppm) of the change in chemical shift (Δ) exhibited by resonance H-8 mainly derives from an unusual upfield shift at low pH. This indicates a strong shielding of the protonated form of this residue which could derive from the close proximity to an aromatic ring, such as Phe-46 if H-8 is His-64 or the porphyrin ring if H-8 is His-97, or to a shielding H-bonding group (water or oxygen) when the ring is protonated. These results indicate a similar degree of conservation of microenvironment in the myoglobin molecule for several species, which might be expected for such important residues which are conserved through evolution.

In the absence of any other information, the similarity of the pK_a value of resonance H-9 to that determined for His-64 (Hayashi et al., 1976; Saito et al., 1977) must be considered the firmest piece of corroborative evidence on which to base an assignment, and hence it is tentatively concluded that His-64 is the preferable of the two possible assignments. In view of the potential importance of these resonances, if they do indeed correspond to the near-heme histidine residues, we are currently investigating approaches to their definitive assignment.

Added in Proof

For further details of assignments for resonances H-1 to H-7, see Botelho & Gurd (1978) and Botelho et al. (1978).

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Interpretation of Nuclear Magnetic Resonance Spectra for *Lactobacillus casei* Dihydrofolate Reductase Based on the X-ray Structure of the Enzyme–Methotrexate–NADPH Complex†

David A. Matthews

ABSTRACT: The three-dimensional molecular structure of *Lactobacillus casei* dihydrofolate reductase complexed with NADPH and methotrexate has been used to interpret published magnetic resonance spectra for this enzyme. Proton resonances from histidine residues and ^{19}F resonances from fluorine-labeled fluorotyrosine and fluorotryptophan dihydrofolate reductase have been assigned in several cases to specific amino acids in the primary sequence. Furthermore, the ^{31}P signals from the pyrophosphate moiety of bound NADPH have been assigned and the large upfield shift for ^{13}C-labeled (at the carboxamide carbon) $NADP^+$ upon binding to the reductase has been explained in terms of desolvation effects.

Dihydrofolate reductase (DHFR)[1] is the NADPH-dependent enzyme that reduces dihydrofolate to tetrahydrofolate. Fully reduced folates have an essential role in purine and pyrimidine biosynthesis. DHFR is thought to be the intracellular receptor for drugs such as methotrexate (MTX) and trimethoprim that have proven clinically useful in antineoplastic and antibacterial chemotherapy, respectively. Because of its low molecular weight (~20 000) and the relative ease with which the enzyme can be isolated from a variety of sources, DHFR is being studied by a number of physical and chemical methods. Five DHFRs have been completely sequenced, and spectroscopic studies utilizing fluorescence,

† From the Department of Chemistry, University of California, San Diego, La Jolla, California 92093 *Received October 10, 1978; revised manuscript received January 11, 1979* This work supported by Research Grant CA 17374 from the National Cancer Institute.

[1] Abbreviations used: DHFR, dihydrofolate reductase; MTX, methotrexate.

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circular dichroism, UV, and nuclear magnetic resonance techniques have been reported. Recently the three-dimensional structures for *Escherichia coli* DHFR complexed with MTX (Matthews et al., 1977) and for a *Lactobacillus casei* DHFR ternary complex with MTX and NADPH (Matthews et al., 1978, 1979) were solved at 2.5 Å resolution using X-ray diffraction methods. Structural information obtained from these diffraction experiments provides a framework within which the interpretation of spectroscopic results can be made at the atomic level.

Because it is possible under favorable circumstances to resolve and study nuclear magnetic resonance signals from individual atoms in proteins of this size, NMR provides a potentially rich source of information about molecular structure and dynamics in solution. In the absence of X-ray structural information about a particular protein the full realization of this potential is substantially reduced and in most instances a purely phenomenological description of the NMR data must suffice. The purpose of this communication is to use the known three-dimensional structure for *L. casei* DHFR in order to extend the discussion of published NMR results for this enzyme and where possible to assign particular resonances to specific residues in the amino acid sequence.

In recent years *L. casei* DHFR has been extensively studied using NMR spectroscopy at the University of California, San Diego (Pastore et al., 1974a, 1976) and at the National Institute for Medical Research in London (Roberts et al., 1974; Way et al., 1975; Feeney et al., 1975, 1977a; Birdsall et al., 1977; Kimber et al., 1977, 1978). In many instances proton resonance shifts have been noted upon binding substrates, coenzymes, and a variety of inhibitors. Following similar successes with a number of other low-molecular-weight proteins (Markley, 1975), it has been possible to resolve the C(2)-H peaks of the histidine imidazole rings in DHFR by ^{1}H NMR (Birdsall et al., 1977). The effects of pH and of the binding of substrates and inhibitors on the chemical shifts of the C(2) protons were reported. In the complicated region containing overlapping signals from aromatic protons it has been possible to simplify the spectra and to separate individual resonances by deuterating potentially interfering nuclei (Feeney et al., 1977a). ^{31}P resonances from NADPH (Feeney et al., 1975) and ^{13}C signals from isotopically labeled coenzyme (Way et al., 1975) and inhibitors (Pastore et al., 1976) have been used as NMR probes for directly monitoring ligand binding to *L. casei* DHFR. Finally, in recently reported work from the London group, fluorine-labeled tyrosine and tryptophan amino acids have been incorporated into the enzyme, and the ^{19}F resonances were studied both in the apoenzyme and in various enzyme-inhibited complexes (Kimber et al., 1977, 1978).

Certain resolved NMR signals can now be assigned to individual amino acids based on structural arguments. In addition, some chemical shift data can be rationalized in terms of proximity effects from specific neighboring groups. This kind of detailed analysis was not possible prior to the completion of the X-ray structure determination. Nevertheless, in several instances, Roberts, Feeney, and co-workers were able to infer possible molecular interpretations for some of the chemical shift data they reported. It will be seen below that most of their specific interpretations are in good agreement with the X-ray data. Before proceeding further some comments should be made concerning *L. casei* DHFRs that are being studied by various workers.

DHFR has been independently isolated from MTX-resistant strains of *L. casei* at three different laboratories (Gundersen et al., 1972; Pastore et al., 1974b; Dann et al., 1976). The crystal structure work (Matthews et al., 1978) was carried out using DHFR as isolated and characterized by Pastore et al. (1974b). The electron density map for this enzyme was subsequently interpreted using the amino acid sequence (Bitar et al., 1977) for DHFR from a second independently isolated MTX-resistant strain of *L. casei*, the one first reported by Gundersen et al. (1972). It is possible that the DHFRs isolated from these two mutant strains may be different although the fit of the primary sequence to the electron-density map was very good in most places (Matthews et al., 1978). Thus if any differences do exist they must be very minor. A third independently derived MTX-resistant strain of *L. casei* serves as the enzyme source for NMR experiments reported by the English workers (Dann et al., 1976). The amino acid composition for this mutant appears to differ slightly from that calculated using the known sequence of the mutant described by Gundersen et al. (1972). However, this apparent difference is by no means certain since amino terminal sequence comparisons for these two strains show that, for at least the first 50 residues, the enzymes are in fact identical with the possible exception of a change from Asn to Asp at residue 8 (Batley & Morris, 1977). Furthermore the tryptophan compositions which were thought to differ by one are now known to be the same (Kimber et al., 1978).

Crystal Structure of *L. casei* Dihydrofolate Reductase

An initial model for the DHFR–MTX–NADPH ternary complex was constructed from a 2.5-Å electron density map phase by standard isomorphous replacement techniques (Matthews et al., 1978). Subsequently, the initial model for the polypeptide chain was rebuilt into a 2.0-Å resolution map using calculated phases and partially refined by difference Fourier methods (details to be reported elsewhere). The following discussion and assignment of observed resonances to specific amino acids are based on this partially refined high-resolution structure.

The pH of the crystallization medium was 7.3 as measured with a standard glass electrode. Figure 1 shows the α-carbon backbone of the enzyme and how MTX and NADPH bind at the active site.

Proton Resonances from Histidine

Birdsall et al. (1977) have studied the effects of binding substrates and inhibitors to *L. casei* DHFR on the histidine C(2) proton resonances of the enzyme. They identify signals from six protons (H_A–H_F) in agreement with the amino acid analysis of Dann et al. (1976). The *L. casei* enzyme that we have studied crystallographically has seven histidines. As discussed above, a question as to the actual existence of this apparent difference in histidine content will be resolved once the primary sequence of the mutant being studied in London is completed. In the following, we assume that each of the signals arises from one of the seven crystallographically identifiable histidine residues that exist in the known X-ray structure for *L. casei* DHFR.

Usually electron density for a histidine side chain in a 2.0-Å resolution map can be fit equally well by either of two side chain conformations differing in rotation about C_β–C_γ by 180°. At this resolution, nitrogens cannot be distinguished from carbons, so although the density envelope for the imidazole ring may be well defined there are two alternative choices for the orientation of the ring within that density. If one of the two choices is preferable in that one or both ring nitrogen atoms are in a position to hydrogen bond with nearby backbone or side chain atoms, we have assumed that the conformation

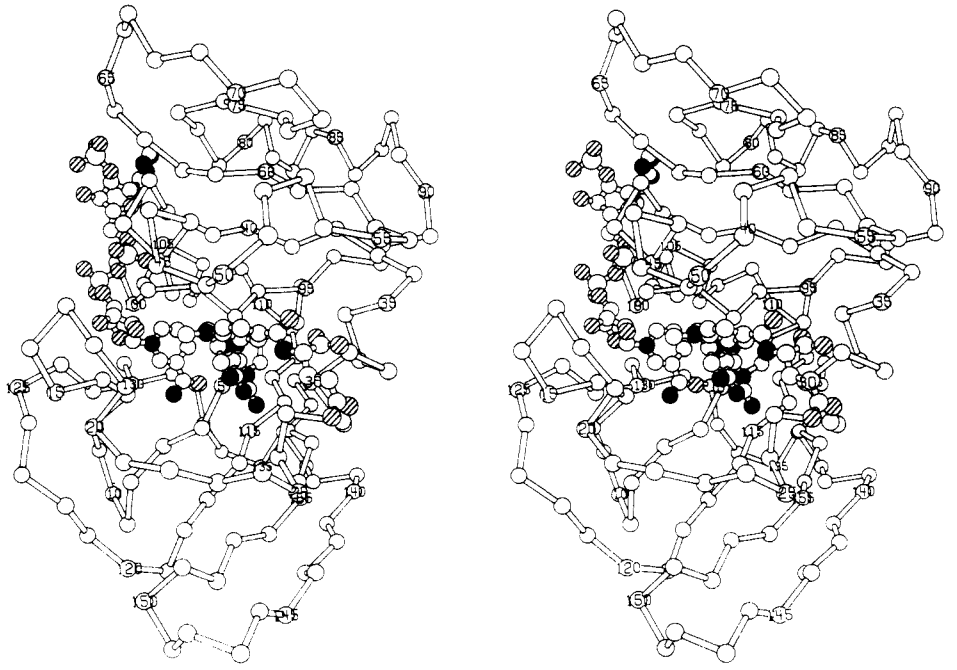

FIGURE 1: Stereo drawing of the 162 α-carbon atoms in *L. casei* dihydrofolate reductase. Also shown are bound molecules of NADPH and methotrexate. Oxygen and nitrogen atoms are indicated by shading and blackening, respectively.

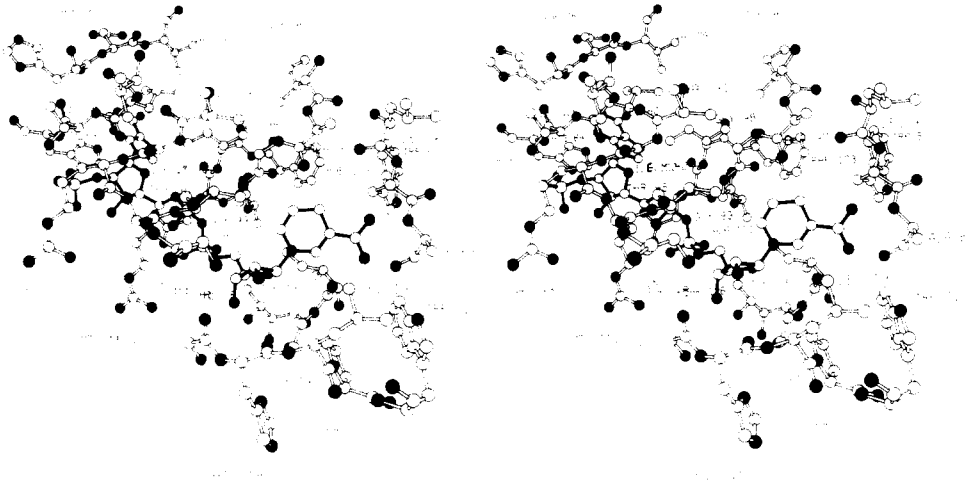

FIGURE 2: View of NADPH bound to the surrounding protein. Oxygen and nitrogen atoms are indicated by blackening and shading, respectively.

permitting hydrogen bond formation will be favored.

The residue identified as H_F by the English workers has a pK of 6.5 in the free enzyme. Addition of MTX or any inhibitor or substrate having a *p*-aminobenzoyl L-glutamate group increases the pK of H_F to 7.2. It was proposed earlier by Roberts et al. (1974) that this increase in pK could arise from an interaction between a carboxyl group of the glutamate and this histidine residue. In the X-ray structure of the DHFR–MTX–NADPH complex the side chain imidazole of His-28 hydrogen bonds with the γ-carboxyl of MTX (Matthews et al., 1978), thus H_F is assigned as His-28.

The X-ray structural results also explain why resonances for the aromatic protons of benzoyl L-glutamate are shifted upfield when this moiety binds to *L. casei* DHFR (Roberts et al., 1974). Lining the substrate binding pocket on the side opposite to that occupied by His-28 is Phe-49. The aromatic ring of Phe-49 is nearly parallel to the phenyl group of MTX in the enzyme–MTX–NADPH ternary complex (Matthews et al., 1978) and is positioned so that its center lies above protons ortho and meta to the glutamate group of the inhibitor. Magnetic shielding from ring currents in the side chain of Phe-49 would account for the observed shifts of 0.41 and 0.48 ppm for protons ortho and meta to the glutamate moiety, respectively.

A second binding site for *p*-aminobenzoyl L-glutamate itself was shown not to be competitive with MTX and binding affects a single histidine residue—H_C (Birdsall et al., 1977). We suggest that this second site coincides with the hydrophobic crevice used by the enzyme to bind the adenine portion of NADPH. This is a nonspecific hydrophobic pocket (Figure 2) with the side chain of His-64 lining one side (Matthews et al., 1979). The imidazole ring is parallel to the plane of adenine in the ternary complex so that if the phenyl group of *p*-aminobenzoyl L-glutamate were bound in a similar manner as adenine, a ring current induced upfield shift of the C(2) proton resonance of histidine-64 would be expected. In the presence of 50 mM *p*-aminobenzoyl L-glutamate the resonance from H_C is 0.35 ppm to high field of its position in the apoenzyme or the enzyme–MTX complex. Histidine-64 would then be H_C. H_C is unaffected by binding of MTX (Birdsall et al., 1977) as might be expected since His-64 is no closer than 20 Å to any portion of the substrate specificity crevice.

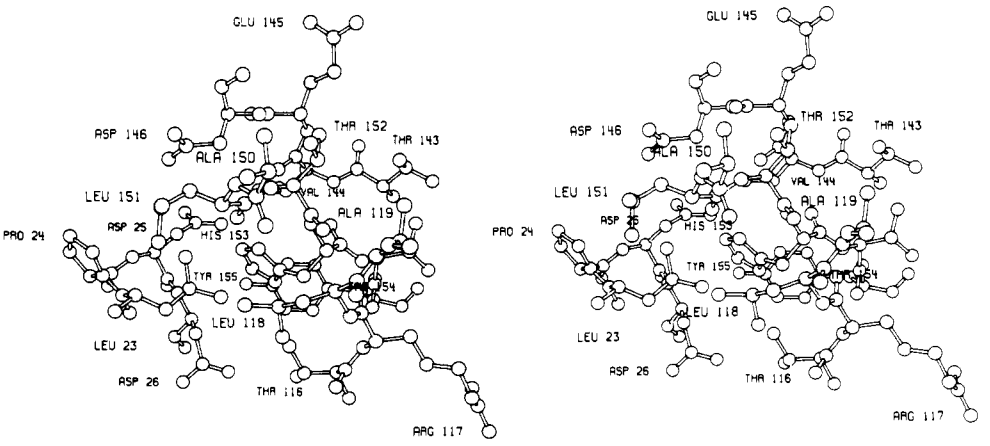

FIGURE 3: Protein environment in the neighborhood of His-153.

Because of its close proximity to and potential hydrogen bonding interaction with the 2′-phosphate of NADPH (Matthews et al., 1979), the pK of His-64 should be increased when the coenzyme or suitable analogues bind to *L. casei* DHFR.

Histidine residues H_B and H_D are unaffected by the binding of folate, dihydrofolate, folinic acid, trimethoprim, MTX, and aminopterin, suggesting that they are far removed spatially from the active site and reside in a region of the enzyme not conformationally sensitive to the binding of substrates and inhibitors. H_B and H_D are assigned to His-89 and His-77. The side chain imidazole of His-77 is hydrogen bonded with the backbone carbonyl of Gln-65 and resides close to the side chain carboxyls of Glu-66 and Asp-78. Thus the environment of His-77 is such that the protonated form of the imidazole side chain can be stabilized by a hydrogen bond and a charge interaction with two carboxyl groups. The pK of H_D is 7.7, highest of all the histidine resonances identified by Birdsall et al. (1977) and 0.5 pK unit higher than that for H_B. His-77 is thus assigned as H_D. A portion of the adenine binding pocket is formed by the peptide bond between His-77 and Asp-78. Thus it is quite possible that binding of NADPH will perturb the C(2) proton resonance of His-77. However, no detailed study of histidine resonances in the presence of bound coenzyme has yet been published.

The above discussion now suggests the assignment of H_B as His-89. This residue is located near the end of helix αE in a partially solvent accessible environment. Both His-77 and His-89 are more than 20 Å from any part of the substrate binding site.

We turn now to the proton resonances from histidines H_A and H_E. All substrates and inhibitors examined caused downfield shifts in the resonance of H_A by 0.10–0.16 ppm, and no correlation with special structural features of the various ligands is apparent (Birdsall et al., 1977). Methotrexate, aminopterin, and trimethoprim but not folate, dihydrofolate, and folinic acid produced analogous upfield shifts for histidine H_E. In addition, the pK of histidine residue H_E is decreased 0.27–0.62 unit on binding compounds containing the 2,4-diaminopyrimidine ring. On the other hand, 2-amino-4-hydroxypteridines increase the pK of this histidine by 0.20–0.25 unit (Birdsall et al., 1977). Histidines H_A and H_E are assigned to residues 18 and 22. Both of these residues are located in a flexible loop which connects the amino terminal β strand βA with helix αB. This loop plays an important role in binding the nicotinamide mononucleotide portion of NADPH and undergoes some conformational rearrangement concomitant with coenzyme binding (Matthews et al., 1978, 1979).

Table I: Magnetic Resonance Assignments for *L. casei* Dihydrofolate Reductase

type	resonance	assignment
histidine C(2) proton (Birdsall et al., 1977)	H_A	His-18 or His-22
	H_B	His-89
	H_C	His-64
	H_D	His-77
	H_E	His-18 or His-22
	H_F	His-28
	unobsd	His-153
3-fluorotyrosine (Kimber et al., 1977)	Y_N^F	Tyr-29
6-fluorotryptophan (Kimber et al., 1977)	W_K^F	Trp-5
	W_L^F	Trp-133
	W_M^F	Trp-21
	W_N^F	Trp-158

Moreover this loop is close to the substrate binding crevice and it is likely that conformational changes occur in the loop when substrates and inhibitors such as MTX bind. A specific assignment of H_A (or H_B) as either His-18 or His-22 cannot be justified at this time.

The seventh and remaining histidine in the mutant *L. casei* DHFR that we have studied crystallographically is His-153. The fact that the English workers report only six histidine resonances for their mutant *L. casei* reductase could signify a real difference in histidine content for these two mutants or alternatively for some reason the signal from His-153 was unobserved in the magnetic resonance spectrum.

It can be seen from Figure 3 that His-153 exists in an unusual environment. Although the side chain carboxyl of Asp-25 is nearby, the imidazole side chain is otherwise completely buried in the hydrophobic core of the protein. This should result in a broadened resonance that will not titrate in the pH range investigated (5.5–7.5). Furthermore, the imidazole ring resides just above the aromatic side chain of Tyr-155 so that the C(2) proton resonance of His-153 may be shifted upfield into the envelope of aromatic protons. For these reasons it may have escaped identification although the alternative hypothesis that this histidine is not present in the mutant *L. casei* DHFR being studied in London cannot be dismissed. Assignments for the histidine resonances are summarized in Table I.

Phosphorus-31 Resonances from Bound Coenzyme

Both NADPH and $NADP^+$ form tightly bound binary complexes with DHFR. Proton resonance experiments with these complexes have proven difficult to interpret because of problems separating signals arising from the bound ligand from those of the parent protein. Alternative approaches have

included monitoring ^{31}P signals from the coenzyme (Feeney et al., 1975) and enriching the carboxamido carbon of nicotinamide with ^{13}C (Way et al., 1975), thus providing a previously nonexistent nuclear probe.

In the presence of enzyme, the 2′-phosphate signal of NADPH is shifted downfield 2.19 ppm, while the two resonances of the pyrophosphate group are both shifted upfield but to different extents (0.16 and 2.69 ppm). Moreover, the chemical shift of the resonance assigned to the 2′-phosphate of NADPH is independent of pH within the range of 4.5–7.5. Feeney et al. (1975) concluded that the 2′-phosphate group binds to DHFR in the dianionic form and that its pK must be reduced by at least 3 units from its value in the free coenzyme. Narrow lines in the single-resonance ^{31}P spectrum of the NADPH–enzyme complex enabled the authors to observe the effects of ^{31}P–O–C–^{1}H spin–spin coupling. The magnitude of this three-bond coupling constant is a sensitive function of the dihedral angle θ which defines the conformation about the $C_{5'}$–$O_{5'}$ bond. The phosphorus nuclei in the pyrophosphate group couple to their respective $C_{5'}$ protons to different extents. It was concluded that one nucleotide changes conformation about the $C_{5'}$–$O_{5'}$ bond by at least 50° upon binding to DHFR (Feeney et al., 1975).

Enzyme Interactions with the 2′-Phosphate Group. NADPH binds to *L. casei* DHFR in an open conformation, occupying an elongated shallow groove at the carboxyl end of an extended parallel β pleated sheet structure (Figure 1). The binding site for nicotinamide overlaps with a second deeper crevice that serves to bind substrate. From Figure 2 it is evident that a variety of hydrogen bonds, van der Waals, and charge interactions are responsible for correct positioning of NADPH on the enzyme surface.

Of particular interest in connection with this discussion is the environment of the 2′-phosphate group. One of the 2′-phosphate oxygens hydrogen bonds with a side chain NH from His-64. Four of the six histidine residues in *L. casei* DHFR have pKs around 7.5, while the remaining two exhibit slightly more acidic pK values close to 6.5 (Birdsall et al., 1977). It was shown above that His-64 belongs to the former group. Moreover, because of its close proximity to the doubly negatively charged 2′-phosphate, the pK of His-64 is almost certainly increased in the presence of bound NADPH. Since the crystals used in this analysis were buffered at pH 7.3, His-64 will be somewhat below its pK which means it will be more than half protonated, and this net positive charge on the imidazole side chain will further enhance binding of NADPH.

The side chain hydroxyl of Thr-63 hydrogen bonds with a second 2′-phosphate oxygen. In addition the guanidinium group of Arg-43 closely approaches two of the phosphate oxygens. Note also that the 2′-phosphate group resides about 5 Å from the amino terminus of a two-turn α helix, αC. The polarity of the electric field associated with a helix dipole is such that negatively charged groups like phosphate will be stabilized if they bind near the amino end of the helix (Hol et al., 1978). The mutual influence of positive electric fields and nearby residues capable of providing hydrogen bonds to the 2′-phosphate oxygen atoms could readily account for the 100-fold decrease in association constant for NADPH compared to NADH (Birdsall et al., 1977). Furthermore, tight anchoring of the adenosine ribose through its 2′-phosphate substituent may provide a mechanism for selecting a strained conformation around the $C_{5'}$–$O_{5'}$ bond when the enzyme–coenzyme complex is formed as discussed below.

Assignment of the Pyrophosphate ^{31}P Resonances. Crystal structure studies of mono- and dinucleotides (Arnott & Hukins, 1969) and spectroscopic observations of nucleotides in solution (Sarma & Mynott, 1973) have established that the preferred value for θ, the dihedral angle about the $C_{5'}$–$O_{5'}$ bond, is 180 ± 20°. In this conformation phosphorus is gauche to both $C_{5'}$ hydrogens. Three NAD^+-dependent dehydrogenases have been studied crystallographically in the presence of bound coenzyme. In all instances the dinucleotides have values for θ close to 180°. We recently reported that NADPH binds to *L. casei* DHFR with a conformational angle, θ_a, equal to 120° thus placing P_a cis to one of the $C_{5'}$ hydrogens (Matthews et al., 1978). This structural result permits specific assignment of the two pyrophosphate ^{31}P resonances in the enzyme–NADPH complex since Feeney et al. (1975) have argued that the large spin–spin coupling constant for the ^{31}P signal at −13.94 ppm arises from a strained conformation about a $C_{5'}$–$O_{5'}$ bond in the bound dinucleotide. Thus the resonance at −13.94 ppm is assigned to P_a and the high field signal at −16.47 ppm must arise from P_n.

[carboxamido-^{13}C]NADP$^+$ ([^{13}CO]NADP$^+$) Resonance

When 1 equiv of [^{13}CO]$NADP^+$ is added to a solution of *L. casei* DHFR, a single resonance is observed to occur in the ^{13}C spectrum, 1.6 ppm to high field of the signal from free [^{13}CO]$NADP^+$ (Way et al., 1975). Subsequent additions of MTX or folate form ternary complexes in which the chemical shift for bound [^{13}CO]$NADP^+$ moves considerably further upfield to positions 2.62 and 4.33 ppm from free $NADP^+$, respectively. Way et al. (1975) dismissed as unlikely the possibility that ring currents from a nearby aromatic side chain could cause such large upfield shifts. They suggested that the more likely origin for the bound shifts lies in the effects of electric fields from nearby charged groups on the ^{13}C shielding.

Feeney, Roberts, and co-workers have provided ^{31}P NMR data indicating that any differences in binding between NADPH and $NADP^+$ must be strongly localized at the binding site for the nicotinamide ring (Feeney et al., 1977b). Because the dinucleotide backbone must be held the same way in enzyme-bound NADPH and $NADP^+$, it is unlikely that the reduced and oxidized nicotinamide rings could be positioned very differently from one another. With the exception of Asp-26 there are no charged amino acid side chains within 15 Å of the carboxamide carbon as it exists in the enzyme–MTX–NADPH complex so that this side chain remains the most likely source of possible electric field perturbations at the carboxamide carbon in the enzyme–MTX–$NADP^+$ complex as well.

The large upfield shifts observed for [^{13}CO]$NADP^+$ upon binding to binary complexes of enzyme–MTX and enzyme–folate cannot readily be explained by ring current effects. The indole ring of Trp-21 is the only aromatic side chain which interacts directly with nicotinamide in the enzyme–MTX–NADPH complex. Figure 2 shows that one would not expect the ^{13}C chemical shift for $NADP^+$ to be greatly affected by a ring current mechanism involving Trp-21 if the oxidized and reduced cofactors are bound in a roughly analogous manner. We now consider the possible effects of Asp-26 on the ([^{13}CO]$NADP^+$) resonance in the bound coenzyme.

Ultraviolet (Erickson & Mathews, 1972; Poe et al., 1974; Hood & Roberts, 1978) and Raman (Saperstein et al., 1978) spectroscopic studies and calorimetric measurements (Subramanian & Kaufman, 1978) have shown that MTX is protonated when bound to DHFR. Recent X-ray structural studies have indicated that the charge interaction occurs between the protonated pteridine of MTX (at N_1) and the side chain carboxyl of Asp-26 (Matthews et al., 1977, 1978). However, no protonation of the pteridine ring occurs when

folate binds to DHFR (Hood & Roberts, 1978; Subramanian & Kaufman, 1978). In the enzyme–folate complex the protonation state of the Asp-26 side chain is uncertain. However, because of reduced solvent accessibility to the aspartate side chain in binary complexes with substrate or inhibitors (Matthews et al., 1977) and complete solvent exclusion in the corresponding ternary complexes with NADPH (Matthews et al., 1978), the pK of Asp-26 in these enzyme complexes may be considerably elevated compared to the pK value for a completely solvent accessible aspartate side chain. Thus the formal charge on the Asp-26 side chain in the DHFR–folate–NADPH complex is probably small. However, as noted above, the upfield shift of the carboxamide carbon resonance is much larger in the DHFR–folate–$NADP^+$ complex than in the corresponding ternary complex with MTX. Although these results do not totally eliminate a possible role for Asp-26 in producing the observed upfield shifts at the carboxamide carbon of bound $NADP^+$, they suggest that other considerations may be important.

What additional factors then could explain the observed resonance shift for the carboxamide carbon when $NADP^+$ binds to DHFR? It can be anticipated that any differences in hydrogen bonding at the carboxamide group in bound and completely solvated forms of $NADP^+$ will be expressed as differences in ^{13}C shieldings. Protic solvents will increase polarization of the carbonyl bond producing decreased shielding with increasing acid strength (Stothers, 1972). The nicotinamide portion of NADPH binds to DHFR in a hydrophobic cleft. Solvent access to nicotinamide in the binary E–NADPH complex is even further restricted in the ternary complex with MTX (Matthews et al., 1978). The chemical shift of the carboxamide carbon in free $NADP^+$ should thus move upfield upon formation of the DHFR–$NADP^+$ complex and move even further upfield on addition of MTX to give the ternary complex. This agrees with the experimental spectra. The greater upfield shift in the ternary complex with folate compared to MTX must reflect differences in how the two pteridine rings are oriented with respect to the nicotinamide ring of $NADP^+$. A more quantitative evaluation of these upfield shifts induced by desolvation must await an exact description of possible hydrogen bonding between the carboxamide group of $NADP^+$ and the enzyme as well as the identification and location of any bound water molecules in the vicinity of the catalytic site. Nevertheless the magnitude of the shifts are well within the range for ^{13}C-labeled carbonyl groups upon transfer from water to more aprotic solvents (Stothers, 1972). Finally, we note that, when 6-fluorotryptophan is incorporated into DHFR, changes in chemical shift for the ^{19}F resonance of Trp-21 that occur upon addition of MTX and NADPH are explained in terms of successively reduced solvent accessibility (see below) and the same picture emerges concerning a buried hydrophobic environment for the Trp-21 side chain. Since the indole side chain of Trp-21 and the carboxamide group of bound NADPH are together in the active site of DHFR, it is satisfying that rationalization of two different sets of chemical shift data from nearby nuclei can be based on a common characterization of this portion of the active site.

Magnetic Resonance Studies of Tyrosine Residues

Dihydrofolate reductase from *L. casei* has five tyrosine residues. Two approaches have been used by Feeney, Roberts, and co-workers in order to resolve tyrosine resonances from the large number of overlapping signals that occur in the aromatic region of the ^1H spectrum in a protein of this size. Feeney et al. have prepared a selectively deuterated DHFR in which all the aromatic protons except the 2,6 protons of the tyrosine residues have been replaced by deuterium (Feeney et al., 1977a). Owing to rapid rotation about the C_β–C_γ bond, the 2 and 6 protons have the same chemical shift so that in favorable cases five absorption bands are observed. The chemical shifts of these resonances were studied in a variety of binary and ternary enzyme complexes with substrates, inhibitors, and coenzymes.

In a second series of experiments the *L. casei* organism was grown in a medium containing 3-fluorotyrosine. Purified DHFR from this source provided an enzyme having ^{19}F selectively incorporated into each tyrosine side chain at the 3 position (Kimber et al., 1977). Thus the resolution problem is circumvented since the only resonances that occur in the ^{19}F NMR spectrum arise from the five labeled tyrosines. Enzyme spectra were reported for 3-fluorotyrosine DHFR complexes containing a number of different bound ligands.

The five tyrosine residues occur at positions 29, 46, 68, 85, and 155 in the primary sequence for *L. casei* DHFR (Bitar et al., 1977). In one instance it is possible to correlate a ^{19}F resonance with a specific tyrosine (see below); however, a complete assignment of all the spectra will not be attempted for several reasons. (1) The range of chemical shifts for the 2,6-proton resonances is only 0.32 ppm in the apoenzyme, while ligand induced shifts are never more than 0.15 ppm. (2) With only a single exception (Tyr-29), none of the tyrosine side chains interact directly with either methotrexate or NADPH, thus implying that conformation changes almost exclusively are responsible for observed shifts in the positions of the ^1H and ^{19}F absorption bands when ligands bind. Since X-ray structures for these complexes are not yet available, this discussion must be related to the reductase–MTX–NADPH structure and detailed arguments concerning possible conformational changes in the other complexes examined with NMR must be made with great caution. (3) Finally, all five tyrosine side chains exist in rather complicated protein environments. Each tyrosine side chain is in close proximity to hydrophobic groups, possible hydrogen bond donor and/or acceptor groups and charged amino acid side chains. Furthermore tyrosines 29 and 155 as well as tyrosines 46 and 68 are in van der Waals contact with each other. Thus a rationalization of chemical shift data on the basis of structure alone is difficult.

One of the five fluorotyrosine resonances, Y_N^F, is especially affected on forming binary complexes with folate or substrate analogues. The corresponding resonance shifts downfield 1.06–2.68 ppm when the substrate site is occupied as compared with shifts of $\leq$0.37 ppm for the other three resonances influenced by ligand binding (Kimber et al., 1977). The largest shift (2.68 ppm) is observed in the presence of MTX and the 2,4-diaminopyrimidine fragment is responsible for the major part of this substantial change (Kimber et al., 1977).

The origin of fluorine shielding has been attributed to electric field effects dominated by the second-order electric field effect which always leads to downfield shifts (Feeney et al., 1966; Kimber et al., 1977). Thus in general, buried fluoroamino acids in proteins will have chemical shifts to low field of the same residue in a more solvent accessible environment.

On the basis of the above discussion, Y_N^F is assigned to Tyr-29 (Figure 4). The 3 position of Tyr-29 is only 3.5 Å from the 2-amino group of bound MTX. Assuming no major reorientation of the tyrosine side chain upon substitution of fluorine for hydrogen at the 3 position, the fluorine atom will thus reside quite close to the 2-amino group and probably

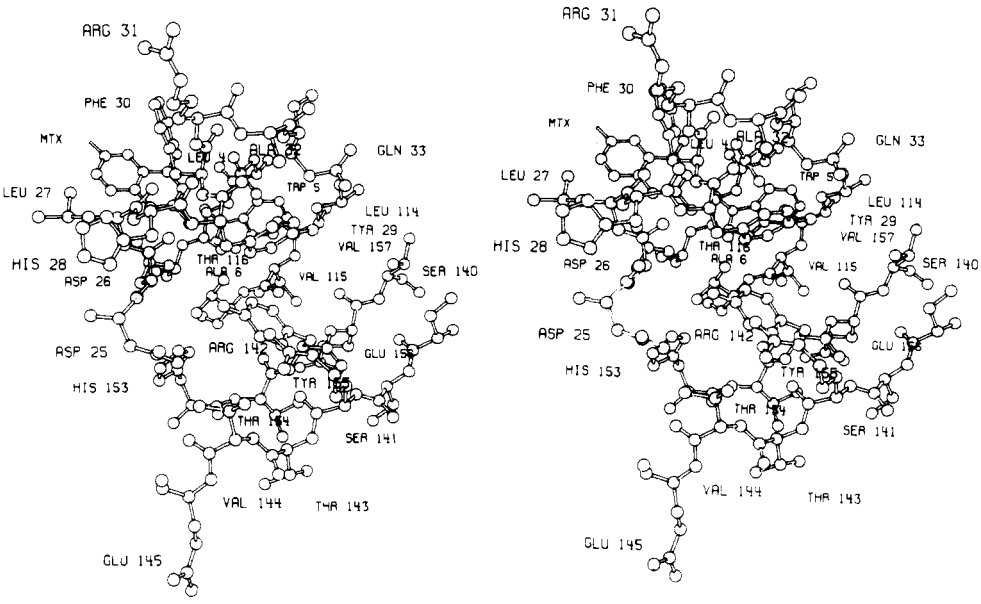

FIGURE 4: Protein environment in the neighborhood of Tyr-29. Also shown is the pteridine portion of bound methotrexate.

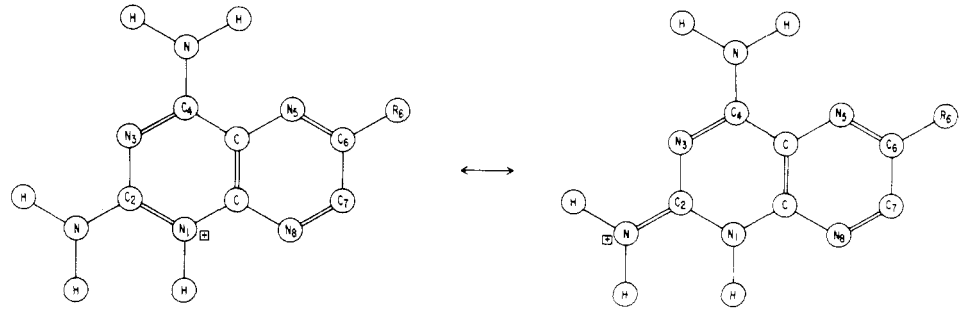

FIGURE 5: Resonance structures showing delocalization of the positive charge onto the 2-amino substituent of methotrexate following protonation at N_1.

interact via a hydrogen bond. Furthermore, the positive charge produced at N_1 of MTX upon binding to DHFR is partially delocalized onto the 2-amino group by resonance structures such as that shown in Figure 5. At least three direct effects will contribute to the observed downfield shift of $Y_N{}^F$ upon binding substrate or substrate analogues.

(1) Tyr-29, in the absence of bound ligands, would appear partially accessible to solvent in accord with its ^{19}F chemical shift of only 0.38 (±0.10 ppm) downfield from the ^{19}F signal of 3-fluorotyrosine in denatured enzyme (see Figure 4). Ligand binding in close proximity to the 3 position of the tyrosine side chain will further restrict solvent access to the fluorine nucleus thus shifting the signal downfield.

(2) The probable hydrogen bond between the 2-amino group of MTX and fluorine at position 3 will further deshield the F nucleus.

(3) A first-order electric field effect arising from the partial positive charge on the 2-amino substituent wil produce a further downfield shift of $Y_N{}^F$. Note that the downfield shift caused by hydrogen bonding is really an electric field effect so that effects 2 and 3 are closely related.

The pteridine ring of bound folate is unprotonated and the downfield shift is only 1.06 ppm compared to 2.68 ppm for the corresponding binary complex with MTX. The resonance from $Y_N{}^F$ is shifted downfield by 1.02 ppm in the enzyme–NADPH complex. This coenzyme induced shift could arise because the nicotinamide portion of NADPH resides within 9 Å of the 3 position of Tyr-29 and will restrict solvent access to the tyrosine side chain. The change in chemical shift on forming the ternary complex DHFR–MTX–NADPH (3.32 ppm) is approximately the sum of the effects of MTX (2.68 ppm) and NADPH (1.02 ppm). This result led to speculation that ligand effects on this resonance are direct ones resulting from the proximity of MTX and NADPH to $Y_N{}^F$ (Kimber et al., 1977). The above arguments support that hypothesis.

Resonances from 6-Fluorotryptophan-Labeled DHFR

Analogues of *L. casei* DHFR containing 6-fluorotryptophan have been prepared and studied by ^{19}F nuclear magnetic resonance (Kimber et al., 1977). The spectra show four resolved resonances. Initially the high field signal was assigned to two overlapped ^{19}F resonances, suggesting a total tryptophan content of five. More recently, sequence studies suggest that the enzyme used by the English workers contains only four tryptophans in agreement with the number in the *L. casei* enzyme that we have studied crystallographically (Kimber et al., 1978). The resonances are labeled $W_K{}^F$–$W_N{}^F$ going from low to high field.

The major change in chemical shift on forming the binary complex with MTX is a large downfield shift (1.13 ppm) of one resonance, $W_M{}^F$, while the other resonances are only slightly perturbed (≤0.28 ppm). The same resonance is shifted downfield 2.27 ppm in the presence of bound NADPH. Three of the four tryptophan residues in *L. casei* DHFR are clustered together at one corner of the molecule (Figure 6). The indole side chains of Trp-133 and Trp-158 are in van der Waals contact, far removed from the active site. Backbone atoms of Trp-5 are near the pyrimidine ring of MTX but the side chain points away from the active site. However, Trp-21 (Figure 2) resides on the inner surface of the large cavity in

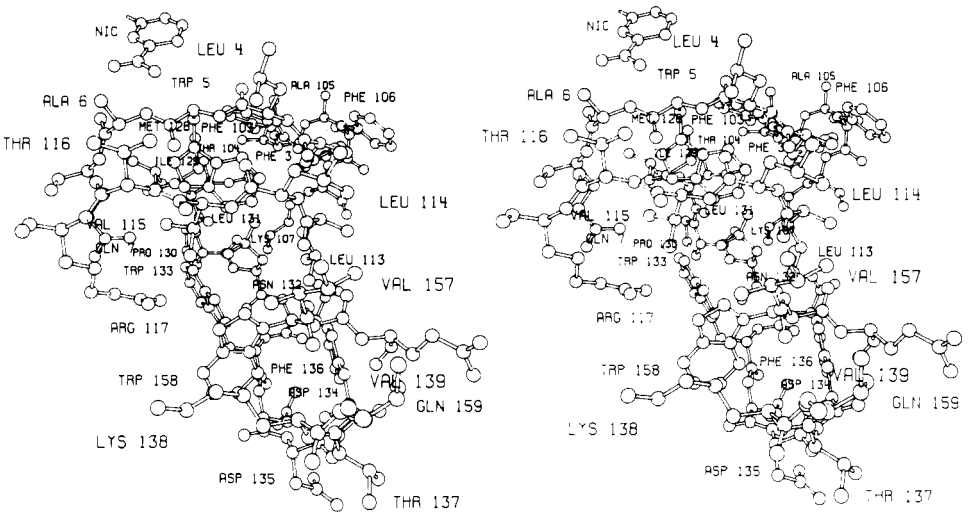

FIGURE 6: Protein environment in the neighborhood of Trp-5, Trp-133, and Trp-158.

which MTX and the nicotinamide portion of NADPH are bound. The side chain is in van der Waals contact with the carboxamide portion of the coenzyme in the ternary complex. It is suggested that Trp-21 is W_M^F. This assignment also explains the direction of the observed chemical shift upon binding MTX or NADPH. Second-order electric field effects are the most likely cause of chemical shifts of this magnitude and resonances of buried fluoroamino acids should appear to low field of those in more solvent accessible environments. A totally vacant active site would permit solvent access to the specificity crevice and the indole side chain of Trp-21 at N(1), C(6), and C(7). Approximately half of the solid angle around this residue would be open to solvent consistent with the observed chemical shift (0.5 ppm upfield of 6-fluorotryptophan) for W_M^F in the absence of bound ligands. Bound MTX reduces solvent accessibility to only one-quarter of the solid angle about C(6), while in the ternary complex water is totally excluded. Thus in passing from the apoenzyme to the ternary complex with MTX and NADPH, Trp-21 becomes progressively more buried resulting in successively larger downfield shifts of the fluorine resonance. The above argument supports the suggestion made by Kimber et al. (1977) that W_M^F corresponds to an active-site tryptophan that is in close proximity to the nicotinamide portion of bound NADPH.

The chemical shift of resonance W_K^F is 4.52 ppm to low field of the resonance position for 6-fluorotryptophan in the denatured enzyme. A large downfield shift of this magnitude could occur for a completely buried fluoroamino acid. The side chain of Trp-5 is deeply buried in a hydrophobic pocket formed by the side chains of Phe-3, Gln-7, Phe-103, Leu-143, Val-115, Met-128, Leu-131, and Trp-133. Since the 6 position of the indole side chain of Trp-133 is partially accessible and that of Trp-158 totally accessible to solvent, and the resonances W_M^F and W_N^F are shifted only 1.34 ppm downfield and 1.08 ppm upfield, respectively, from the position in denatured enzyme, the best assignment for W_K^F is to Trp-5. This assignment is further supported by the following discussion.

Addition of MTX or NADPH to 6-fluorotryptophan-labeled *L. casei* DHFR leads to a narrowing of the signals, and it is observed that resonances W_K^F and W_L^F are doublets with a splitting of about 17 Hz (Kimber et al., 1977). Since the spectra were taken under conditions of proton noise decoupling, the splittings cannot arise from ^1H–^{19}F spin–spin interaction. Recent experiments have demonstrated that the splitting is not a chemical shift difference owing to different conformational states, but rather results from a ^{19}F–^{19}F through-space coupling between fluorine nuclei on adjacent tryptophan side chains, and that the 6 positions of the respective indole rings must be less than 4 Å apart in the folded protein (Kimber et al., 1978).

In the *L. casei* DHFR ternary complex the 6 position of Trp-5 is 5.4 and 10.0 Å from the corresponding position of Trp-133 and Trp-158, respectively. Furthermore, the 6 positions of Trp-133 and Trp-158 are separated by 8.7 Å in the crystal structure. Reference to Figure 6 shows that not only are the C(6)–Trp-5 to C(6)–Trp-158 and C(6)–Trp-133 to C(6)–Trp-158 distances too great to account for the observed splitting but also the C(6)–F bonds would, in the absence of large conformation changes induced by fluorine substitution, be pointing in directions about 90° relative to one another thus making the fluorine–fluorine distances even larger than the distances separating the respective C(6) carbon atoms. Moreover, the environments around the 6 position of Trp-133 and Trp-158 are sufficiently free of steric constraints that substitution of hydrogen by fluorine is not likely to cause even minor side chain rearrangement from the conformations observed in the crystal structure in spite of the increased atomic radius of fluorine compared to hydrogen. These observations suggest that the ^{19}F spin–spin coupling is occurring between fluorine atoms on Trp-5 and Trp-133. Thus W_L^F and W_N^F are assigned as Trp-133 and Trp-158, respectively.

The distance of 5.4 Å separating the 6 positions of Trp-5 and Trp-133 and the relative position of their respective indole rings suggest that in order to explain the observed spin–spin splitting a slight conformational change, probably at Trp-5, must occur when fluorine is incorporated into the tryptophan side chains of *L. casei* DHFR. It could be argued that the enzyme conformation in the crystal is slightly altered relative to its form in solution; however, this is extremely unlikely especially for a residue like Trp-5 that is deeply buried in the hydrophobic core of the protein. Furthermore, the crystal structure suggests an obvious reason and a possible test for the postulated conformational change at Trp-5 upon replacement of hydrogen by fluorine.

Leu-113 closely approaches the indole ring of Trp-5 and the contact distance between C_β of Leu-113 and the 6 position of Trp-5 is only 3.2 Å. When fluorine is substituted for hydrogen, the increased bulk of the former could cause the tryptophan side chain to rotate about C_α–C_β. Simultaneous rotations about C_β–C_γ must occur in order to prevent unfavorable van der Waals contacts with C_γ of Met-128. If, according to this argument, rotations of 30 and 60° occurred about C_α–C_β and

C_β–C_γ of Trp-5, respectively, fluorine atoms bonded to the C(6) carbons of Trp-5 and Trp-133 would now reside less than 4 Å apart.

It is quite possible that the side chain of Trp-133 may change conformation slightly because of changes in orientation of Trp-5 occurring upon fluorine substitution. This is because at their closest approach in the crystal structure of the ternary complex [C(7)–Trp-5 and C(7)–Trp-133] the respective side chains are only 3.9 Å apart. Methyl groups from Val-115 and Leu-131 are situated above and close to the indole rings of Trp-5 and Trp-133, respectively (see Figure 6). Proton resonances from these methyl groups in the native enzyme are most probably shifted upfield by the ring current mechanism. An examination of the high field ^1H resonances in 6-fluorotyptophan DHFR might well reveal changes in this region indicative of some movement of Trp-5 and possibly Trp-133 relative to the side chains of Val-115 and Leu 131, respectively.

The above discussion serves to emphasize the caution which must accompany structural arguments based on enzymes that have been chemically modified. Although the through-space spin–spin coupling between two fluorotryptophans in DHFR is an interesting and significant observation, the phenomenon in this case results from changes in the tertiary structure of the enzyme caused by the chemical modification itself. Thus arguments based on how these fluorine resonances and their splittings may change in the presence of bound ligands will have at best only limited relevance to the natural enzyme.

Conclusion

L. casei DHFR is now one of only several proteins that have been extensively studied by high-resolution NMR techniques. In order for these types of experiments to contribute to a detailed understanding of ligand binding and enzyme mechanism it is necessary that the spectra of various enzyme–ligand complexes be understood in terms of effects on individual residues. Twenty resonances from individual amino acids have been identified and described by researchers in London. Some success has been achieved in assigning observed magnetic resonance signals (primarily from histidine and fluorotryptophan residues) to specific amino acids based on a correlation of the experimental spectra with the results of X-ray diffraction experiments.

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