Photo-CIDNP Studies of the Influence of Ligand Binding on the Surface Accessibility of Aromatic Residues in Dihydrofolate Reductase†

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ABSTRACT: The surface accessibility of the histidine, tyrosine, and tryptophan residues of *Lactobacillus casei* dihydrofolate reductase has been determined from 360-MHz ^1H photochemically induced dynamic nuclear polarization (photo-CIDNP) NMR experiments. In the absence of ligands, four (or perhaps five) of the seven histidine residues and at least one of the four tryptophan residues are accessible to a flavin dye molecule. One of the five tyrosine residues is also slightly accessible. Of the accessible histidine residues, one becomes inaccessible on the binding of $NADP^+$ and one on the binding of *p*-aminobenzoyl glutamate. These have been assigned to residues which interact directly with these two ligands. One histidine residue (probably His-22) shows an increase in accessibility on addition of folate or methotrexate to the enzyme·$NADP^+$ complex. In addition, the binding of several ligands, notably trimethoprim, leads to an increase in the accessibility of a tryptophan residue. This is clear evidence for ligand-induced conformational changes in dihydrofolate reductase and allows us to identify some of the residues involved.

^1H NMR spectroscopy can provide valuable information about proteins and their interactions in solution, provided that resonances of individual residues can be resolved from the envelope of many hundred overlapping signals seen even for small proteins. This resolution problem is one of the major experimental difficulties in the study of, for example, protein–ligand interactions by ^1H NMR, and several approaches to spectral simplification have been used. Campbell & Dobson (1979) have introduced a number of methods for selecting subspectra based on differences in multiplicity or relaxation times, while Markley et al. (1968) and Feeney et al. (1977) have used selective deuteration.

Recently, Kaptein (1978) and Kaptein et al. (1978a,b) have developed a method based on laser photochemically induced dynamic nuclear polarization (photo-CIDNP) for obtaining NMR signals selectively from those histidine, tyrosine, and tryptophan residues which are on the surface of a protein. A solution of the protein is examined in the presence of a flavin dye which is excited to its triplet state by brief illumination with a laser. The photoexcited dye reacts reversibly, by electron transfer or hydrogen abstraction, with any accessible histidine, tyrosine, or tryptophan residues, leading to *transient* radical formation. Since the recombination is spin selective, this causes nuclear polarization in the side chains of these residues. The resulting large changes in intensity in the NMR signals of these surface residues can best be observed by subtracting a normal spectrum ("dark" spectrum) from one obtained immediately after illumination with the laser ("light" spectrum). In these difference spectra, a histidine residue gives absorption lines for the C2 and C4 protons and emission lines for the β-CH_2 protons, tyrosine gives an emission line for the C3′ and C5′ protons and absorption for the β-CH_2 protons, and tryptophan gives absorption lines for the C2, C4, and C6 protons and emission for the β-CH_2 protons (Kaptein, 1978). The method is valuable because it often gives simple spectra in which signals from individual amino acids on the surface of the protein can be identified. The relative intensities of the signals reflect the relative accessibility of these residues to the dye and thus provide useful structural information about proteins. Several studies of this type have been reported by Kaptein and co-workers (Kaptein, 1978; Kaptein et al., 1978a,b) on bovine pancreatic trypsin inhibitor, lysozyme, ribonucleases A and S (Lenstra et al., 1979) and the gene 5 protein (Hilbers et al., 1978). We have now used this method to study the influence of substrate and inhibitor binding on the accessibility of aromatic residues on the surface of dihydrofolate reductase (EC 1.5.1.3), which catalyzes the reduction of dihydrofolate to tetrahydrofolate by using NADPH as coenzyme. This enzyme is of considerable pharmacological interest as the target for antifolate drugs such as methotrexate and trimethoprim (Hitchings & Burchall, 1965; Blakley, 1969). Information about changes in accessibility of surface residues on the enzyme caused by the binding of substrate analogues could help to identify the residues involved in the binding.

Materials and Methods

^2H_2O (99.85 atom % ^2H) was obtained from Norsk Hydroelectrisk, methotrexate and aminopterin were from Nutritional Biochemicals Corp., $NADP^+$, trimethoprim, folate, and *p*-aminobenzoyl glutamate were from Sigma Chemicals, and 3-*N*-(carboxymethyl)lumiflavin was kindly supplied by Dr. F. Müller (Wageningen).

Dihydrofolate reductase was isolated and purified from a methotrexate-resistant strain of *Lactobacillus casei* as described by Dann et al. (1976). The enzyme was lyophilized twice from ^2H_2O solution to remove most of the exchangeable protons and then redissolved to give ~1 mM enzyme solutions in ^2H_2O containing 3 mM dioxane as a reference, 50 mM potassium phosphate, and 500 mM potassium chloride at pH* 6.7 (uncorrected meter reading). The ligands were either dissolved in the same buffer and added in microliter volumes or added as solids. For the tightly binding ligands such as methotrexate and trimethoprim, complexes were formed by adding 0.9–1.1 molar equiv of the ligands to the enzyme solutions. Microliter volumes of a concentrated solution of the lumiflavin dye in ^2H_2O were added to the enzyme solutions to give a final dye concentration of 2×10^{-4} M.

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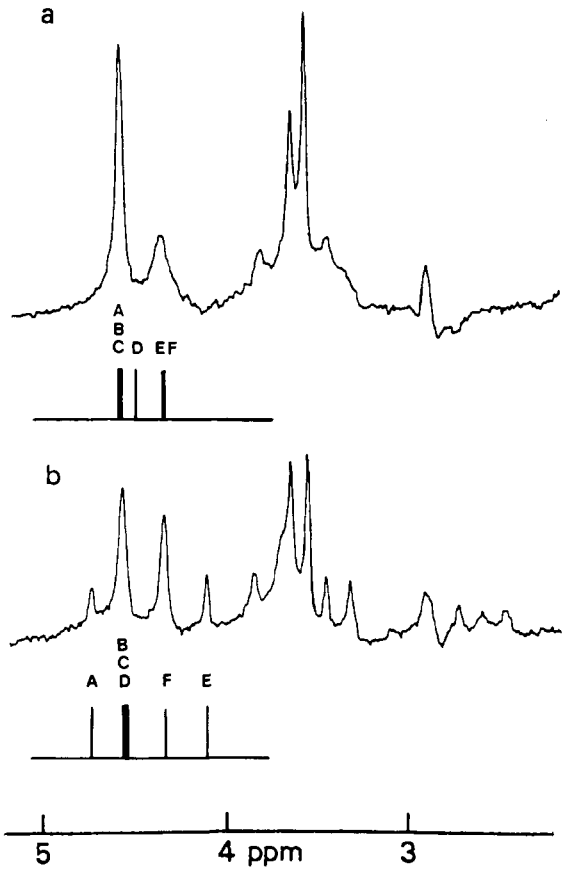

FIGURE 1: Aromatic region of the ^1H CIDNP spectra at 360 MHz for *L. casei* dihydrofolate reductase at pH* 6.75 in various complexes. (a) Enzyme alone; (b) enzyme·trimethoprim. The shift positions of the histidine C2 protons in the dark spectra are also given.

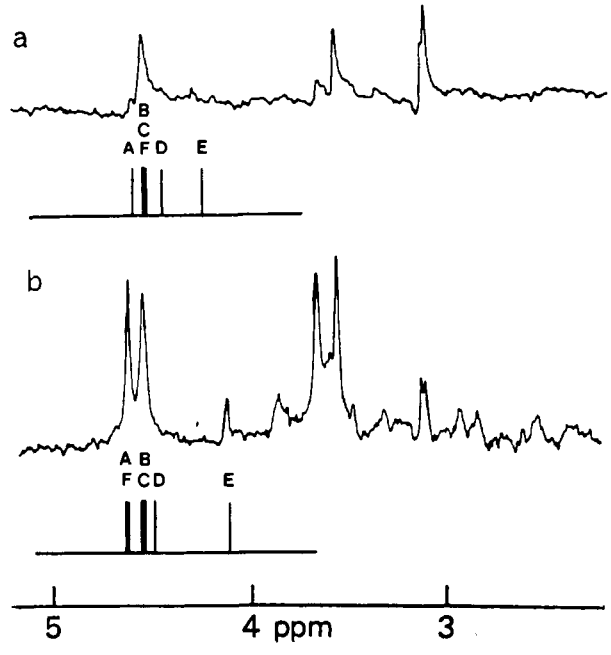

FIGURE 2: Aromatic region of the ^1H CIDNP spectra at 360 MHz for *L. casei* dihydrofolate reductase at pH* 6.75 in various complexes. (a) Enzyme·folate; (b) enzyme·aminopterin. The shift positions of the histidine C2 protons in the dark spectra are also given.

The ^1H NMR spectra were obtained at 360 MHz by using a Bruker HX-360 spectrometer operating in the Fourier transform mode. A Spectra Physics Model 171 argon ion laser was used as the light source, and the laser beam was directed into the sample via a computer-controlled shutter and mirror

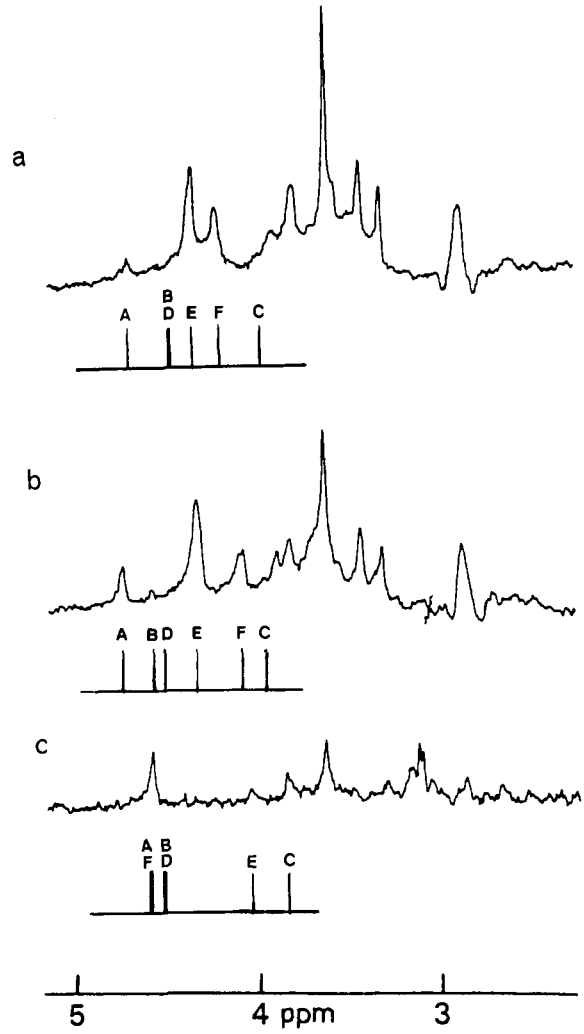

FIGURE 3: Aromatic region of the ^1H CIDNP spectra at 360 MHz for *L. casei* dihydrofolate reductase at pH* 6.75 in various complexes. (a) Enzyme·$NADP^+$; (b) enzyme·trimethoprim·$NADP^+$; (c) enzyme·folate·$NADP^+$. The shift positions of the histidine C2 protons in the dark spectra are also given.

through the bottom of the probe. Free induction decays were obtained alternatingly for light and dark conditions and subtracted to give the CIDNP spectrum. A 0.6-s light pulse was used, with a 7-s delay between successive 90° pulses to give a total repetition time of 14 s for the complete cycle. Twenty scans were accumulated for each spectrum. More extensive signal averaging to improve the quality of the spectra was not attempted because the spectrometer had to be used in an unlocked mode and also because the dye is bleached in prolonged experiments. These short experiments have the advantage of minimizing any structural perturbation of the enzyme by laser illumination. A full description of the experimental method has been given elsewhere (Kaptein, 1978).

Results and Discussion

In the aromatic regions of the photo-CIDNP difference spectra of *L. casei* dihydrofolate reductase and its complexes (see Figures 1, 2, and 3), the most prominent signals can be identified as the resonances of the C2 and C4 protons of histidine residues with some weaker bands coming from tryptophan residues. The β protons of these histidine and tryptophan residues give rise to the large emission signals in the aliphatic region of the spectrum (see Figure 4). Only one very small emission signal (at 2.77 ppm) was observed in the aromatic region, showing that one of the five tyrosine residues is slightly accessible to the flavin.

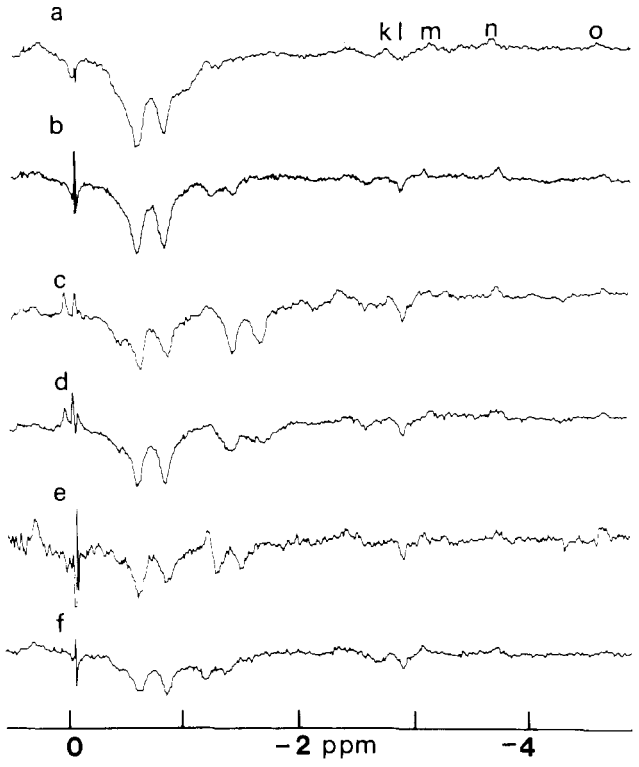

FIGURE 4: Aliphatic region of the ^1H CIDNP spectra at 360 MHz for *L. casei* dihydrofolate reductase at pH* 6.75 in various complexes. (a) Enzyme alone; (b) enzyme·$NADP^+$; (c) enzyme·trimethoprim; (d) enzyme·trimethoprim·$NADP^+$; (e) enzyme·aminopterin; (f) enzyme·folate.

We have previously reported extensive studies of the histidine C2 proton resonances of dihydrofolate reductase and its complexes (Birdsall et al., 1977), and we shall use here the same nomenclature for the histidine residues, pending the completion of the assignment of these resonances to individual residues in the sequence [see Wyeth et al. (1980), Matthews (1979), and the discussion below]. Birdsall et al. (1977) were only able to detect C2 proton resonances for six (H_A–H_F) of the seven histidine residues in the sequence (Freisheim et al., 1978); the signal of the seventh residue, H_G, has now been identified in spectra of the complexes with inhibitors or coenzyme (Wyeth et al., 1980).

We have compared dark spectra obtained before and after illumination or with and without dye. These experiments gave no evidence for any structural perturbation of the protein or for extensive binding of the dye. Thus, all the C2 proton resonances from histidine residues had the same chemical shifts as those reported earlier (Birdsall et al., 1977; Wyeth et al., 1980).

Photo-CIDNP Spectra. (1) Enzyme Alone. In the aromatic region of the CIDNP spectrum of the enzyme alone (Figure 1a) the intense signals at 4.29 and 4.58 ppm (C2 protons) and 3.54 and 3.65 ppm (C4 protons) arise from accessible histidine residues. The weaker signals at 2.86, 3.38, and 3.83 ppm are from at least one tryptophan residue. At this pH* value, the C2 proton resonances of histidines A, B, and C are at 4.58 ppm, that of H_D is at 4.48 ppm, and those of H_E and H_F are at 4.29 ppm (Birdsall et al., 1977), as indicated in Figure 1a. The absence of a signal at 4.48 ppm in the CIDNP spectra clearly indicates that histidine D is inaccessible to the dye. Because there are signals in the CIDNP spectra at both 4.29 and 4.58 ppm, it is not possible to deduce if any of the other histidines are inaccessible. We cannot use the relative intensities of the signals as an aid to interpretation because residues with different accessibilities will have signals of different intensities. However, these signals, particularly that at 4.29 ppm, are somewhat asymmetrical, suggesting that there is more than one histidine residue contributing to each signal.

In the aliphatic region of the spectrum (Figure 4a) two intense broad emission signals are observed at −0.54 and −0.80 ppm (arising from the β-CH_2 protons of the accessible histidine and tryptophan residues). The protons in each β-CH_2 group will be magnetically nonequivalent, and the two observed signals probably arise from the nonequivalent protons within each residue. The nonequivalence of the ^1H shielding between β-CH_2 protons for histidine and tryptophan residues in simple peptides is often of this order of magnitude (~0.25 ppm). The line broadening on the resonances at −0.54 and −0.80 ppm could then be due to small shift differences between the protons in the different β-CH_2 groups and/or to unresolved spin–spin coupling.

The small resonances at −2.78, −2.90, −3.18, −3.74, and −4.69 ppm, labeled k–o in Figure 4, most probably arise from aliphatic protons of the protein which are involved in cross-relaxation with polarized nuclei in the accessible histidine and tryptophan residues. The emission signal (l) must come from protons cross-relaxed by the histidine or tryptophan β protons. The highest field signal, o, corresponds to a single methyl resonance which is clearly resolved in the dark spectrum. These methyl protons are shielded ~2 ppm by the ring current of a neighboring aromatic ring, possibly with the accessible histidine or tryptophan residue giving the cross-relaxation effect.

(2) Enzyme·Trimethoprim Complex. In the dark spectrum of this complex at pH* 6.75 we can resolve five signals for the C2 protons of the seven histidines, with signals from histidines B, C, and D still being partially overlapped (Birdsall et al., 1977; Wyeth et al., 1980). In the CIDNP spectrum (Figure 1b) a large signal at 4.39 ppm indicates that histidine F is accessible while the signal at 4.63 ppm could arise from one or more of histidines B, C, and D. Small signals occurring at the resonance positions of histidines A and E show that they are only partially accessible to the dye. No signal is observed for histidine G (at 5.80 ppm; not shown in Figure 1b), showing that this residue is inaccessible to the dye. The C4 protons of the accessible histidines resonate at 3.56 and 3.66 ppm.

The four signals between 2.47 and 2.91 ppm in the CIDNP spectrum (Figure 1b) correspond to at least two accessible tryptophan residues, since a single tryptophan can give only three signals in the aromatic region of the CIDNP spectrum (Kaptein, 1978). In the CIDNP spectrum of enzyme alone there is evidence for only one accessible tryptophan residue, suggesting that a tryptophan has *increased* its accessibility on trimethoprim binding.

The aliphatic region of the CIDNP spectrum (Figure 4c) contains four emission signals from β-CH_2 protons: two of these signals have the same chemical shifts as observed in the spectrum for the enzyme alone, but the other signals are at higher field. The latter signals could be associated with the tryptophan(s) whose accessibility has increased on trimethoprim binding. The observed chemical shifts are substantially (0.5–0.8 ppm) to higher field of those of β-CH_2 protons of tryptophan or histidine in simple peptides.

(3) Enzyme·Folate Complex. The complex formed by adding excess folate (2 molar equiv) to the enzyme gives a CIDNP spectrum (Figure 2a) which indicates that most of the aromatic residues are inaccessible to the dye. We observe a single histidine C2 proton resonance at 4.58 ppm (from histidines B, C, or F), together with a single C4 proton reso-

nance at 3.55 ppm. The small signals in the range 2.9–3.65 ppm could arise from a partially accessible tryptophan residue.

The resonance at 3.02 ppm arises from the 3′ and 5′ protons of free folate; no resonance of bound folate was observed. The presence of excess (free) ligand leads to an overall decrease in the intensity of the CIDNP spectrum of the protein, presumably due to competition between folate and enzyme for the low concentration of excited state flavin molecules.

In the aliphatic region of the spectrum (Figure 4f), four β-CH_2 resonances are seen, as with trimethoprim. From the aliphatic region, it is clear that two aromatic residues are accessible to the dye in this complex, and from the aromatic region it seems that these are a single histidine and a tryptophan.

(*4*) *Enzyme·p-Aminobenzoyl Glutamate (PABG) Complex.* PABG binds relatively weakly to the enzyme ($K = 0.83 \times 10^3$ M^{-1}), and the rate of dissociation of the complex is sufficiently fast that there is usually rapid exchange (on the NMR time scale) between free and bound species in the equilibrium. Thus, when increasing amounts of PABG are added to the enzyme (spectra not shown), the C2 proton signal of histidine F (initially at 4.41 ppm) progressively changes its chemical shift and decreases in intensity as this residue becomes inaccessible. At the early stages of the PABG titration, the signal at 4.41 ppm becomes partially resolved into two broad signals, which suggests that the C2 proton of histidine E as well as that from histidine F has a resonance at this position in the free enzyme. Increasing the PABG concentration eventually removes both signals, indicating that both histidines E and F are inaccessible in the presence of this ligand. There is good evidence that histidine F is His-28 and that this residue interacts with the γ-carboxyl group not only of *p*-aminobenzoyl glutamate but also of folate, methotrexate, and aminopterin as well (Birdsall et al., 1977; Matthews et al., 1978; Matthews, 1979; Wyeth et al., 1980). It is therefore probable that histidine F is also inaccessible in the folate, methotrexate, and aminopterin complexes, although in these cases its signal cannot be separately resolved.

(*5*) *Enzyme·Aminopterin and Enzyme·Methotrexate Complexes.* These complexes give very similar CIDNP spectra and can be considered together. Figure 2b shows the spectrum of the enzyme·aminopterin complex. There is a large signal at 4.68 ppm at the frequency position of the C2 protons in histidines A and F; as in the folate complex, histidine F is most probably inaccessible (see above), so that this signal must arise from histidine A. The signal at 4.58 ppm shows that histidines B and/or C are also accessible while the small signal at 4.14 ppm shows that histidine E is almost completely inaccessible. Histidines D and G are inaccessible. The histidine C4 protons resonate at 3.66 and 3.55 ppm while the signals at 3.86, 2.88, and 2.80 correspond to one or two accessible tryptophan residues.

The aliphatic region of the CIDNP spectrum of the aminopterin complex (Figure 4e) is very similar to that of the trimethoprim complex, with an additional pair of β-CH_2 emission signals at −1.22 and −1.43 ppm. These probably arise from a tryptophan residue which becomes more accessible in many of the complexes than in the enzyme alone.

No signals from bound aminopterin or methotrexate were detected in the CIDNP spectra.

(*6*) *Enzyme·NADP$^+$ Complex.* The CIDNP spectrum of this complex (Figure 3a) contains signals at 4.28 and 4.42 ppm from the C2 protons of histidines F and E (A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments) which must be accessible to the flavin. Very small signals at 3.95 and 4.78 ppm indicate that histidines C and A are only very slightly accessible while the absence of C2 proton signals for histidines B, D, and G shows these residues to be inaccessible to the dye. The C4 proton resonances of the accessible residues are at 3.65 ppm and possibly 3.84 ppm. The other signals in the spectrum correspond to one or two accessible tryptophan residues.

In the CIDNP spectra of free NADP$^+$, strong absorption signals are observed for the A2 and A8 protons. Because the resonance positions of the A2 and A8 protons in bound NADP$^+$ coincide with histidine signals at 4.42 and 3.65 ppm (Hyde et al., 1980), we cannot rule out the possibility that signals from bound NADP$^+$ appear in the CIDNP spectrum. However, from consideration of the intensity ratio of the aromatic signals and the β-CH_2 signals, it seems probable that the intense signals at 4.42 and 3.65 ppm arise mainly from histidine signals. It is worth noting that no signals for bound NADP$^+$ were observed in any of the ternary complexes involving NADP$^+$, substrate analogues, and the enzyme.

In the aliphatic region of the spectrum (Figure 4b) strong emission lines for the β-CH_2 protons are again detected at −0.55 and −0.80 ppm while weak emission lines are seen at −1.22 and −1.39 ppm corresponding to a β-CH_2 in a residue with low accessibility.

Attempts to obtain CIDNP spectra of complexes containing NADPH were unsuccessful since the dye was bleached within a few seconds of illumination.

(*7*) *Enzyme·Trimethoprim·NADP$^+$ Complex.* The CIDNP spectrum of the enzyme·trimethoprim·NADP$^+$ complex (Figure 3b) shows that histidines B, D, and G are inaccessible in this complex. The C2 proton signal for histidine C, which is known to move upfield to 3.95 ppm on addition of NADP$^+$ (A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments), appears as a very weak signal, showing that histidine C has a low degree of accessibility. Histidines E (4.39 ppm) and F (4.14 ppm) retain the accessibility they showed in the enzyme·NADP$^+$ and enzyme·trimethoprim binary complexes.

The signal of histidine A (4.82 ppm) is clearly apparent in Figure 3b, having an appreciably greater intensity than that in the enzyme·NADP$^+$ binary complex (Figure 3a). Addition of trimethoprim to the enzyme·NADP$^+$ complex thus *increases* the accessibility of histidine A.

Several signals from tryptophan protons are observed, but the signals at 2.47–2.73 ppm are less intense than those in the enzyme·trimethoprim complex, suggesting that one of the tryptophans has become less accessible on forming the ternary complex (although still more accessible than in the enzyme alone). As with the binary complex of NADP$^+$, it is not possible to exclude the possibility that the A2 and A8 protons of bound NADP$^+$ are contributing to the signals at 4.39 and 3.65 ppm.

The aliphatic region of the CIDNP spectrum (Figure 4d) is very similar to that for the trimethoprim binary complex except that the high-field β-CH_2 proton signals (−1.36 and −1.68 ppm) are less intense in the ternary complex, thus correlating with the different intensities seen for signals from tryptophan aromatic protons in the region 2.47–2.73 ppm.

(*8*) *Enzyme·Folate·NADP$^+$, Enzyme·Methotrexate·NADP$^+$, and Enzyme·Aminopterin·NADP$^+$ Complexes.* These three ternary complexes give essentially the same CIDNP spectrum (Figure 3c). Large signals at 4.67 and 3.66 ppm correspond to C2 and C4 protons in histidine A (excluding histidine F on the same grounds as in the binary complexes). This is particularly interesting in the case of the folate complex since

histidine A is inaccessible in the enzyme·folate binary complex and virtually inaccessible in the binary complex with $NADP^+$. The small signal at ~4.14 ppm corresponds to a slightly accessible histidine E. Signals are observed in the region 2.6–3.9 ppm corresponding to at least one accessible tryptophan residue and probably the C2 proton of histidine C at 3.95 ppm (A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments).

In the aliphatic region of the spectra two sets of β-CH_2 emission lines are again observed, indicating that at least two aromatic residues are accessible [probably one histidine (histidine A) residue and one or two tryptophan residues].

In no case did we observe signals from protons in the bound ligands, indicating that the adenine ring of the coenzyme and the benzoyl ring of the substrates and inhibitors are inaccessible to the dye.

Interpretation of the Spectra in Terms of Residue Accessibility. The photo-CIDNP experiment has clearly been successful in producing a very substantial simplification of the ^1H NMR spectrum of the protein. Of the 25 aromatic residues, only 7 (or perhaps 8) have been seen in the photo-CIDNP difference spectra. A further advantage of this method of spectral simplification is that it selects *surface* residues, which are of course the most likely to be involved in interactions with ligands.

Interpretation of the CIDNP spectra will obviously depend on the factors determining the "accessibility" of the protein residues to the dye. As noted in the introduction, there appear to be two potential mechanisms for the transient radical formation which leads to the CIDNP effect: electron transfer and hydrogen atom abstraction. Electron transfer presumbably requires the aromatic ring of the dye to make contact with that of the protein residue, so that the histidine, tryptophan, or tyrosine ring would have to be free from obstruction by other residues, at least on one side. For hydrogen atom abstraction from an NH or OH group, on the other hand, only this group need be accessible, although hydrogen bonding of this group might be sufficient to render the residue "inaccessible" as far as the photo-CIDNP experiment is concerned. Thus, in comparing the results of the solution experiments with the crystal structure of the enzyme·methotrexate·NADPH complex (Matthews et al., 1978, 1979), we have estimated the accessibility of the aromatic residues in the crystal structure by two different criteria. We have counted the number of atoms from other residues (a) on either side of the plane of the ring in a cylinder of 5-Å radius and 5-Å height and (b) within 5 Å of a NH or OH group which might be involved in a hydrogen atom abstraction reaction with the dye.

These qualitative estimates of accessibility, which are all that can be made in the absence of a more detailed understanding of the mechanisms involved in the photo-CIDNP effect, in general correlate very well with the solution studies. For example, the observation of only one very weak emission line in the aromatic region of the CIDNP spectra shows that only one of the five tyrosine residues is at all accessible to the dye, in any of the complexes, and this is entirely consistent with their inaccessibility in the crystal structure. It must be borne in mind, however, that the crystal structure is that of the enzyme·methotrexate·NADPH complex, a complex which we have not been able to study by the photo-CIDNP method (due to a photochemical reaction between the flavin dye and NADPH).

Tryptophan Residues. In the CIDNP spectrum of enzyme alone there is evidence for at least one accessible Trp residue. In all the complexes studied a further tryptophan becomes accessible. This implies that the enzyme undergoes a conformational change on ligand binding which increases the accessibility of a tryptophan residue. This residue is most accessible in the trimethoprim·enzyme complex. The β-CH_2 protons of this tryptophan are shifted appreciably (0.2–0.8 ppm) to higher field in the different complexes, with both protons being similarly affected. It is possible that the β-CH_2 protons of this tryptophan are over the ring of an aromatic residue in the protein. The chemical shifts of these β-CH_2 protons vary in the different complexes, suggesting small conformational differences which would perturb the ring current shifts. If the two accessible tryptophan residues are the same in all complexes, then the accessibilities indicated by the crystal structure of the methotrexate·NADPH·enzyme complex suggest that tryptophan-21 and -133 are the two most likely candidates. In both cases, there are one or more methyl groups near the ring which could give rise to the high-field signal at −4.69 ppm.

Table I: Summary of the Accessibility of the Histidine Residues

complexes	histidine residues[a] A	B	C	D	E	F	G
enzyme alone		+		0		+	0
enzyme·trimethoprim	+	+		0(?)	+	+	0
enzyme·folate	0	+		0	0	0(?)	0
enzyme·PABG	+	+		0	0	0	0
enzyme·methotrexate	+	+		0	±	0(?)	0
enzyme·aminopterin	+	+		0	±	0(?)	0
enzyme·$NADP^+$	±	0	±	0	+	+	0
enzyme·$NADP^+$·trimethoprim	+	0	±	0	+	+	0
enzyme·$NADP^+$·folate	+	0	±	0	±	0(?)	0
enzyme·$NADP^+$·methotrexate	+	0	±	0	±	0(?)	0
enzyme·$NADP^+$·aminopterin	+	0	±	0	±	0(?)	0

[a] Braces link residues whose resonances overlap. The following notation is used: (+) accessible; (0) inaccessible; (±) only slightly accessible; [0(?)] resonance overlapped but believed (from the spectra of related complexes) to be inaccessible.

Histidine Residues. The full value of the histidine accessibility measurements (summarized in Table I) will only be realized when the unequivocal assignments of the various resonances have been made. Only two of the C2 proton assignments are known with reasonable certainty, namely, histidine C (His-64) and histidine F (His-28). These assignments are based on studies of ligand binding, $Cr(CN_6)^{3-}$-induced line broadening, and deuterium exchange and on comparisons of the NMR data with crystal structure data on the enzyme·methotrexate·NADPH complex (Wyeth et al., 1980; Matthews, 1979).

Histidine C is accessible in all complexes which do not contain $NADP^+$ but is almost totally inaccessible in the presence of coenzyme. In the crystal structure of the ternary complex the imidazole ring of His-64 overlaps the adenine ring of NADPH and also forms a hydrogen bond to the 2′-phosphate group (Matthews et al., 1978, 1979). NMR studies of the effects of coenzyme binding on the C2 proton of histidine-64 and on the adenine protons and the 2′-phosphate group of the coenzyme (A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments; Hyde et al., 1980) clearly show that both of these structural features are maintained in all the coenzyme complexes studied here. Between them they account for the inaccessibility of histidine-64 (histidine C) to the dye when coenzyme is bound.

Histidine-28 interacts directly with the γ-carboxylate group of the glutamic acid moiety of methotrexate in the ternary complex (Matthews et al., 1978). NMR studies again show that this interaction exists in all complexes containing a ligand having a *p*-aminobenzoyl L-glutamate moiety (Birdsall et al., 1977; A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments). This is in agreement with the present photo-CIDNP results, which show that histidine F is accessible in the trimethoprim complexes but not in that with *p*-aminobenzoyl glutamate.

Histidine G is inaccessible in all the complexes studied; because its C2 proton signal is very broad in the dark spectrum of the enzyme alone, we cannot determine its accessibility in the absence of ligands. Histidine D is inaccessible in the enzyme alone and in all other complexes where it has a well resolved signal in the dark spectrum. It is also probably inaccessible in the complex with trimethoprim although the possibility that its signal is present under that of histidine C (4.63 ppm) cannot be completely excluded. However, in the ternary complex of trimethoprim and $NADP^+$, histidine D is definitely inaccessible. Histidine B is found to be inaccessible in all complexes where it has a well resolved signal in the dark spectrum. In the spectra of enzyme alone and in those of the binary complexes with the inhibitors or substrate, there is always a signal at ~4.58 ppm (from histidine C) which would overlap the signal from histidine B if this were present. However, in studies of the deuterium exchange of the histidine C2 protons in the enzyme·methotrexate complex, histidines B and G exchanged considerably more slowly than the other histidine residues (Wyeth et al., 1980). Although accessibility as defined by deuterium exchange is no doubt rather different from accessibility as defined by photo-CIDNP, this strongly suggests that histidine B is in fact inaccessible in all the complexes (and that the signal in the CIDNP spectra at 4.58 ppm in the binary complexes arises solely from histidine C).

Thus, in all complexes where histidines B, D, and G can be clearly identified, we find that they are inaccessible. Histidine D, while inaccessible to the flavin, is significantly accessible to the solvent, as judged by deuterium exchange (Wyeth et al., 1980). The two most inaccessible histidines in the crystal structure of the ternary complex are 89 and 153, both of which are obstructed on both sides of the imidazole ring by other protein residues. It seems likely that these correspond to residues B and G, although the individual assignments cannot yet be made. This assignment agrees with that proposed by Matthews (1979). The remaining two histidines (A and E) show different accessibilities in the various complexes. Histidine E is partially accessible in the enzyme alone and in the binary and ternary complexes of the enzyme with $NADP^+$ and trimethoprim but is almost completely inaccessible in complexes containing PABG, folate, methotrexate, or aminopterin.

The behavior of histidine A is particularly interesting in that, like the tryptophan residue discussed above, it provides direct evidence that ligand-induced conformational changes take place, since there is an *increase* in the accessibility of the residue on ligand binding. Evidence for the involvement of this residue in a conformational change was hitherto indirect (Birdsall et al., 1977). Histidine A is almost completely inaccessible in the binary complex of the enzyme with $NADP^+$ but becomes accessible in the ternary complexes with folate, methotrexate, or aminopterin. Likewise, histidine A is inaccessible in the binary complex with folate but becomes accessible when $NADP^+$ is added to the complex. An interesting difference between folate and methotrexate binding is that methotrexate, unlike folate, does not cause histidine A to become inaccessible in the binary complex. In addition to other evidence for conformational differences between the enzyme·folate and enzyme·methotrexate complexes (Birdsall et al., 1977; Feeney et al., 1977; Kimber et al., 1977; Roberts et al., 1977), there is now clear evidence for a substantial difference in orientation between substrate and inhibitor when bound to the enzyme (Charlton et al., 1979; Hitchings, 1979).

In the crystal structure of the enzyme·methotrexate·NADPH complex (Matthews et al., 1978) the most solvent-accessible histidine is histidine-22. If the enzyme·methotrexate·$NADP^+$ complex is similar to that containing NADPH (at least in this region of the protein), then we conclude that H_A = His-22. [By elimination, H_D and H_E correspond to His-18 and His-77; again the individual assignments are not known. The assignment of the histidine C2 proton signals is further discussed by Wyeth et al. (1980).] From a comparison of the crystal structure of the *Escherichia coli* dihydrofolate reductase·methotrexate complex (Matthews et al., 1978) and the *L. casei* enzyme·methotrexate·NADPH complex, Matthews et al. (1979) proposed that NADPH binding changes the conformation of residues 12–22 in such a way as to bring Leu-19 into contact with methotrexate in the ternary but not the binary complex. Although the details of this conformational change will doubtless differ from one complex to another, the present results, together with the assignment of H_A to His-22, strongly support the idea that this region of the protein undergoes ligand-induced conformational changes, not only on coenzyme binding but also on substrate or inhibitor binding.

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Inhibition of Microtubule Assembly by Phosphorylation of Microtubule-Associated Proteins†

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ABSTRACT: ^{32}P labeling of microtubular protein by endogenous protein kinase activity is shown to result from a net increase in protein-bound phosphate and is not the result of a phosphate exchange reaction between ATP and phosphoprotein. Protein phosphorylation is maximal in the presence of 0.5 mM Mg^{2+} and 0.25 mM ATP, resulting in approximately 2.8 nmol of phosphate/mg of protein. However, phosphorylation can be increased two-to threefold by cAMP. The protein substrates for phosphorylation in either the absence or presence of cAMP are the microtubule-associated proteins which copurify with tubulin and promote microtubule assembly. Phosphorylation of microtubule-associated proteins inhibits both the rate and extent of microtubule assembly when the protein is exposed to conditions which result in dissociation of rings. These results are taken to indicate that phosphorylation modifies MAPs so that they have a reduced ability to form an assembly-competent complex with tubulin.

Microtubules are involved in a number of dynamic cellular processes which require rapid modulation of microtubule assembly and function. The observation that purified microtubular protein is phosphorylated in a reaction which is stimulated by cAMP (Goodman et al., 1970; Sloboda et al., 1975; Rappaport et al., 1976; Sandoval & Cuatrecasus, 1976a; Sheterline, 1977) has generated considerable interest in phosphorylation as a possible mechanism for the regulation of microtubule-mediated functions. Microtubular proteins are also phosphorylated in vivo (Eipper, 1974; Sloboda et al., 1975), and many studies have shown that cAMP, a well-known modulator of protein phosphorylation, affects the number (Porter et al., 1974), organization (Kram & Tomkins, 1973; Willingham & Pastan, 1975; Nath et al., 1978), and function (Williams & Wolff, 1970; Hsie & Puck, 1971; Prasad & Hsie, 1971; Kirkland & Burton, 1972; Puck, 1977) of microtubules in intact cells.

Protein kinase activity consistently copurifies with microtubular protein prepared from a variety of sources including brain (Goodman et al., 1970; Eipper, 1974; Sloboda et al., 1975), chick embryonic muscle (Piras & Piras, 1974), and platelets (Ikeda & Steiner, 1979). While the protein kinase activity is clearly distinct from tubulin (Eipper, 1974; Rappaport et al., 1976; Sheterline, 1977), the major component of microtubules, a specific association between protein kinase and tubulin has been suggested based on the observation that protein kinase activity elutes with tubulin purified by colchicine–agarose affinity chromatography (Sandoval & Cuatrecasus, 1976a).

In microtubule preparations purified by cycles of assembly and disassembly, the major substrate for the cAMP-stimulated phosphorylation reaction is a group of high molecular weight microtubule-associated proteins (MAPs)[1] (Sloboda et al., 1975; Rappaport et al., 1976; Sheterline, 1977). These proteins stimulate microtubule assembly under polymerizing conditions and promote the formation of rings under depolymerizing conditions (Murphy & Borisy, 1975; Sloboda et al., 1976; Murphy et al., 1977). Nevertheless, the state of phosphorylation of microtubular proteins has yet to be correlated with any change in microtubule assembly, disassembly, or function in vitro.

In this paper, in vitro ^{32}P labeling of microtubule-associated proteins is shown to result from the addition of new phosphate moieties and does not result from a phosphate exchange re-

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; RB, reassembly buffer (0.1 M Mes, 0.5 mM $MgCl_2$, and 1.0 mM EGTA, pH 6.8); MAPs, microtubule-associated proteins; PEI, polyethylenimine.

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