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# Hydrogen-1 Nuclear Magnetic Resonance Study of the Complexes of Two Diastereoisomers of Folinic Acid with Dihydrofolate Reductase†

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ABSTRACT: The $^1H$ chemical shifts for the formyl and benzoyl protons of the individual diastereoisomers of folinic acid bound to dihydrofolate reductase have been measured. For the tightly bound biologically active 6*S*,α*S* isomer, the "bound" signals were assigned by using transfer of saturation methods. In this case, only one of the two rotameric states of the formyl group in folinic acid (form I) is bound to the enzyme. The H3′ and H5′ benzoyl protons have identical shifts in the bound state (as do the H2′ and H6′ protons). This equivalence is attributed to flipping of the benzoyl ring about the N10–C4′ and C1′–CO bonds in the bound state. In the case of the biologically inactive 6*R*,α*S* isomer, both rotameric forms (I and II) bind to the enzyme. The "bound" shifts for the formyl and aromatic protons are different in the complexes with the 6*S*,α*S* and 6*R*,α*S* isomers, indicating that the pteridine ring and benzoyl moiety are binding in different environments in their enzyme complexes. The glutamic acid moiety is probably binding at the same site in the two complexes.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduction of 7,8-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid by using NADPH as a coenzyme. The enzyme is of considerable pharmacological interest, being the target for several "antifolate" drugs such as methotrexate, trimethoprim, and pyrimethamine. We have been using high-resolution NMR to study the complexes formed by the enzyme with its coenzyme and substrate analogues with the aim of understanding the factors controlling the specificity of the ligand binding (Birdsall et al., 1977; Feeney et al., 1977, 1980a,b; Roberts et al., 1977, 1978; Cayley et al., 1979; Hyde et al., 1980a,b; Wyeth et al., 1980).

The complexes formed with the substrates folate and 7,8-dihydrofolate can be studied with ease, but complexes formed with the product 5,6,7,8-tetrahydrofolate have proved difficult to characterize because of the instability of the latter com-

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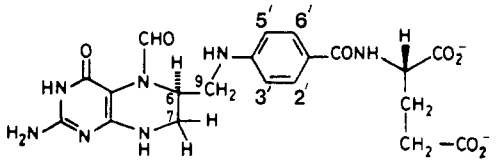
pound under aerobic conditions. To obtain some information about the binding of tetrahydrofolates, we have now studied the complex of the enzyme with folinic acid (5-formyl-5,6,7,8-tetrahydrofolic acid). Unlike tetrahydrofolic acid, folinic acid is very stable to oxygen under neutral and mildy alkaline conditions (Blakley, 1969). The natural diastereoisomer of folinic acid has structure **1**. It has two asymmetric



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carbons, one at C6 and the other at the $\alpha$ carbon of the glutamic acid moiety. In naturally occurring folinic acid, C6 has the *S* configuration and the glutamate residue has the *S* configuration of an L-amino acid. Commercial folinic acid is a diastereoisomeric mixture with C6 in both *S* and *R* configurations and the $\alpha$ carbon of the glutamate in the *S* configuration. Both isomers of folinic acid bind to dihydrofolate reductase, the natural 6*S*,$\alpha$*S* isomer binding much more tightly ($K_a = 1 \times 10^8$ $M^{-1}$) than the 6*R*,$\alpha$*S* isomer ($K_a = 10^4$ $M^{-1}$) (B. Birdsall and A. S. V. Burgen, unpublished results). Because the active form binds so tightly to the enzyme, this interaction could be of physiological importance within cells where significant concentrations of 5-formyl-5,6,7,8-tetrahydrofolate analogues are present. It should also be noted that folinic acid is used in chemotherapy as a "rescue" agent in high dose treatment with methotrexate (Bertino, 1977).

## Experimental Procedures

*Materials.* (6*RS*,$\alpha$*S*)-Folinic acid was obtained commercially from Sigma Chemical Co. The 6*S*,$\alpha$*S* and 6*R*,$\alpha$*S* diastereoisomers have been separated by chromatography and crystallization as described below.

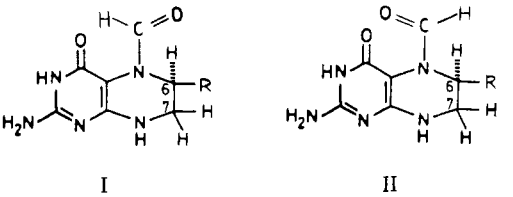
The natural isomer, (6*S*,$\alpha$*S*)-folinic acid (Cosulich et al., 1952), has been directly interrelated with the natural isomer (6*R*,$\alpha$*S*)-5,10-methenyl-5,6,7,8-tetrahydrofolate (Fontecilla-Camps et al., 1979a,b). $^2H_2O$ (99.85 atom % $^2H$) was obtained from Norsk Hydroelectrisk. The preparation and purification of the normal and selectively deuterated dihydrofolate reductase from *Lactobacillus casei* MTX/R were carried out as described previously (Dann et al., 1976; Feeney et al., 1977). The purified enzyme was lyophilized twice from $^2H_2O$ solution to remove most of the exchangeable protons and then redissolved to give approximately 1 mM enzyme solutions in $^2H_2O$ containing 3 mM dioxane reference, 50 mM potassium phosphate, and 500 mM potassium chloride at pH* 6.9 (pH* indicates uncorrected meter reading). The folinic acid samples were either weighed out as solids and added to the enzyme solutions to form the appropriate complexes or added to the enzyme solutions as microliter volumes of a 28 mM stock solution.

*Separation of the Diastereoisomers of (6RS,$\alpha$S)-Folinic Acid.* A slurry of TEAE-cellulose (from 80 g of DEAE-cellulose; Porath, 1957) in water was gravity settled in a 2.8-cm diameter column to a height of ~90 cm. The column was run at 4 °C and was washed first with 1% aqueous sodium hydroxide (1 L), then with 0.4 M aqueous ammonium hydrogen carbonate (1.5 L), and finally with $4 \times 10^{-3}$ M aqueous ammonium hydrogen carbonate (1.5 L). A solution of calcium (6*RS*,$\alpha$*S*)-5-formyl-5,6,7,8-tetrahydrofolate (325 mg) in $4 \times 10^{-3}$ M ammonium hydrogen carbonate (5 mL) was washed on to the column and eluted with an ammonium hydrogen carbonate gradient ($4 \times 10^{-3}$ M,/2 L, to 0.4 M,/2 L) of flow rate ~60 mL/h. Ten-milliliter fractions were collected, and fractions of similar rotations were pooled. Repeated crystallization of the groups of pooled fractions led to two diastereoisomeric fractions. The fraction with the negative rotation was expected to be the biologically active one (Cosulich et al., 1952). The active isomer had $[\alpha]_D$ −33.5° and the inactive had $[\alpha]_D$ >+50° in the buffer system used, estimating weight on the basis of $\epsilon$ (285 nm, pH 7) $37.2 \times 10^3$ (Uyeda & Rabinowitz, 1965). The samples were therefore considerably purer than those separated by Cosulich et al. (1952), and the value for the active isomer compares well with the more recent value of $[\alpha]_D$ −25.2° (Fontecilla-Camps et al., 1979a,b) which when "normalized" to $\epsilon$ (285 nm, pH 7) $37.2 \times 10^3$ was $[\alpha]_D$ −29.5°.

*NMR Experiments.* The $^1H$ NMR spectra were obtained at 270 MHz by using a Bruker WH270 spectrometer operating in the Fourier-transform mode. Spectral widths of 4200 Hz were used, and the free induction decays were accumulated in 8K data points (acquisition time of 0.975 s) and then weighted with an exponential function (line broadening 2 Hz) to improve the signal-to-noise ratio in the spectrum obtained after Fourier transformation. The transfer of saturation experiments (Forsén & Hoffman, 1963) were carried out by using the $^1H$ decoupler to provide the second irradiating frequency. The pulse sequence used in these experiments was $(t-\tau-\pi/2-AT-T)_n$ where the selective irradiation at a chosen frequency was applied during the time interval $t$ (0.8–1.0 s) and where $\tau$ is a short delay (2 ms) to allow for electronic recovery after the selective irradiation is removed. The acquisition time $AT$ was usually 0.975 s, and an additional delay $T$ of 3.0 s allowed for most of the magnetization to recover before the sequence was repeated (Cayley et al., 1979; Hyde et al., 1980a).

## Results

The $^1H$ NMR spectra of the 6*S*,$\alpha$*S* and 6*R*,$\alpha$*S* diastereoisomers of folinic acid in the absence of dihydrofolate reductase have been found to be identical (Feeney et al., 1980c). However, the spectra of the folinic acid isomers did provide unequivocal evidence for the presence of two slowly interconverting conformations with unequal populations at room temperature. The two forms have been shown to correspond to two conformations of the formyl group arising from hindered rotation about the C–N bond as indicated in rotamers I and II (Feeney et al., 1980c). In each case, the steric interactions



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prevent the formyl group from taking up a fully planar conformation with respect to the ring system. The two conformers have quite distinct $^1H$ NMR spectra which have been fully analyzed (Feeney et al., 1980c). Figure 1 shows the aromatic region of the $^1H$ spectrum of folinic acid. The two formyl signals and two pairs of AA′BB′ multiplets from the *p*-aminobenzoyl moiety are clearly resolved. The more populated rotamer has been assigned to structure I where the formyl proton is oriented toward the 4-oxo group of the pteridine ring.

To facilitate the detection of signals from folinic acid bound to dihydrofolate reductase, selectively deuterated enzyme was used in some of the experiments. This enzyme had all of its aromatic protons except the 2,6 protons of tyrosine replaced

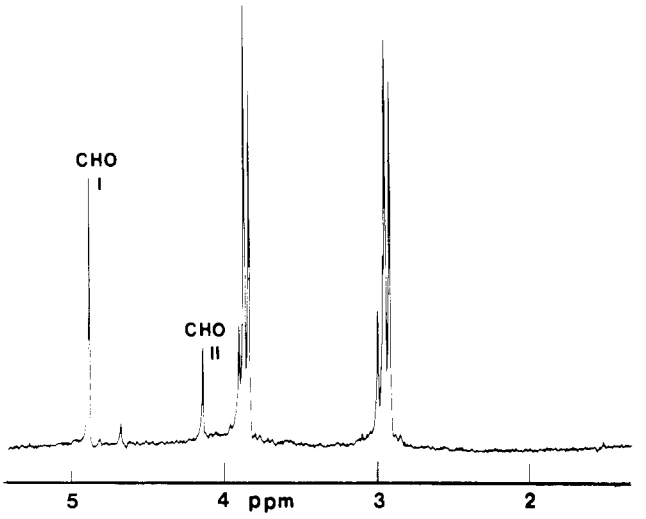


FIGURE 1: Low-field region of the 270-MHz $^1H$ NMR spectrum of 2 mM (6*RS*,α*S*)-folinic acid in $D_2O$ solution containing 50 mM potassium phosphate and 500 mM potassium chloride at 60 °C. The chemical shift scale is referenced to dioxane. (The dioxane signal is 3.71 ppm downfield from that of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).)

by deuterium (Feeney et al., 1977) so that the aromatic region of its $^1H$ NMR spectrum was much simplified, as illustrated in Figure 2a.

(6*R*,α*S*)-*Folinic Acid–Enzyme Complex.* The $^1H$ NMR spectra from a series of solutions containing deuterated enzyme (1.25 mM) in the presence of different amounts of the biologically inactive 6*R*,α*S* diastereoisomer of folinic acid are shown in Figure 2. Signals from the two conformations of the formyl proton and from one pair of the benzoyl aromatic protons could be detected. The chemical shifts of these signals changed progressively as the concentration of folinic acid was increased, indicating that there is fast exchange between free and bound species. The signals were all somewhat broad, suggesting that there was an exchange contribution to the line widths. Three of the five tyrosine resonances were influenced by the binding. In particular, the high-field resonance (E) shifted upfield by 0.17 ppm from its position in the free enzyme. There was no substantial difference in the enzyme spectrum when more than 1.5 equiv of folinic acid was present, indicating that the enzyme was essentially saturated with folinic acid in a 1.5:1 mixture. From the known binding constant of this isomer ($K_a = 10^4$ $M^{-1}$; B. Birdsall and A. S. V. Burgen, unpublished results), it can be calculated that in the 1:1 mixture more than 70% of the folinic acid is bound to the enzyme. The bound ligand shifts can be calculated from the observed ligand shifts in the 1:1 mixture. The formyl protons in the bound species are shifted by −0.10 ppm (I) and +0.13 ppm (II), and the H3′,H5′ benzoyl protons are shifted by +0.22 ppm relative to their positions in the free state. It is interesting that *both* rotameric forms of the inactive 6*R*,α*S* isomer are binding to the enzyme. Although the prevailing signal-to-noise ratio makes it difficult to measure accurate intensities, it appears that there is no large change (>10%) in the populations of forms I and II when they are bound to the enzyme.

(6*S*,α*S*)-*Folinic Acid–Enzyme Complexes.* The natural 6*S*,α*S* isomer binds much more tightly ($K_a = 10^8$ $M^{-1}$) to the enzyme than does the 6*R*,α*S* isomer ($K_a = 10^4$ $M^{-1}$) (B. Birdsall and A. S. V. Burgen, unpublished results). Because of this large difference in binding constants, it is possible to use a mixture of the diastereoisomers to study the binding of the 6*S*,α*S* isomer. Thus, for an enzyme solution (1 mM) in

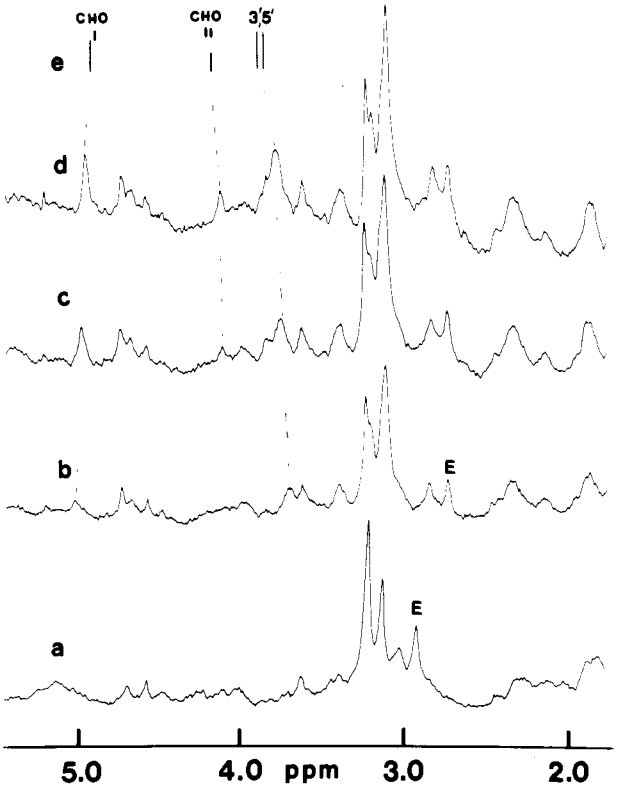


FIGURE 2: Low-field region of the 270-MHz $^1H$ NMR spectrum at 1.25 mM solution of [2,6-$H_2$]tyrosine selectively deuterated dihydrofolate reductase at pH 6.9 and 25 °C. (a) Alone; (b) with 1 molar equiv of (6*R*,α*S*)-folinic acid; (c) with 1.5 molar equiv of (6*R*,α*S*)-folinic acid; (d) with 2.0 molar equiv of (6*R*,α*S*)-folinic acid; (e) the frequency positions of the formyl and H3′ and H5′ benzoyl protons in free (6*R*,α*S*)-folinic acid.

the presence of 2 molar equiv of the diastereoisomeric mixture, essentially only the 6*S*,α*S* isomer is bound, with the 6*R*,α*S* isomer remaining free in solution. This is illustrated in the $^1H$ NMR spectrum shown in Figure 3c. In the presence of less than 2 molar equiv of the diastereoisomeric mixture, both forms bind to the enzyme. In the presence of 1 molar equiv of the mixture, no NMR signals could be detected at the frequency positions of free folinic acid protons (see Figure 3b). The chemical shifts of the tyrosine signals of the enzyme indicate that the enzyme is fully saturated with folinic acid, both isomers being bound under these conditions. At concentrations of the folinic acid mixture which are slightly less than 2 molar equiv, very broad signals are observed near the frequency positions of signals in free folinic acid (not shown in Figure 3). At concentrations of the folinic acid mixture which slightly exceed 2 molar equiv, the free folinic acid signals become very sharp (see Figure 3c). Thus, when more than 1 molar equiv of the natural 6*S*,α*S* isomer is present, the 6*R*,α*S* isomer is no longer binding at the site responsible for the line broadening of its signals. This indicates that the two isomers are binding competitively at this site.

At concentrations of the folinic acid mixture which exceed 2 molar equiv, we have detected signals corresponding to the bound 6*S*,α*S* isomer which are shifted by −0.29 (formyl proton), +0.42 (H3′, H5′), and +1.03 ppm (H2′, H6′) from the corresponding signals in the spectrum of unbound folinic acid. The shifts of these signals do not change when excess folinic acid is added, indicating that there is slow exchange between bound and free species. The detection of only one signal for the bound formyl proton indicates that the 6*S*,α*S* isomer is bound in only one of its two rotameric forms. The assignments of the bound signals have been confirmed une-

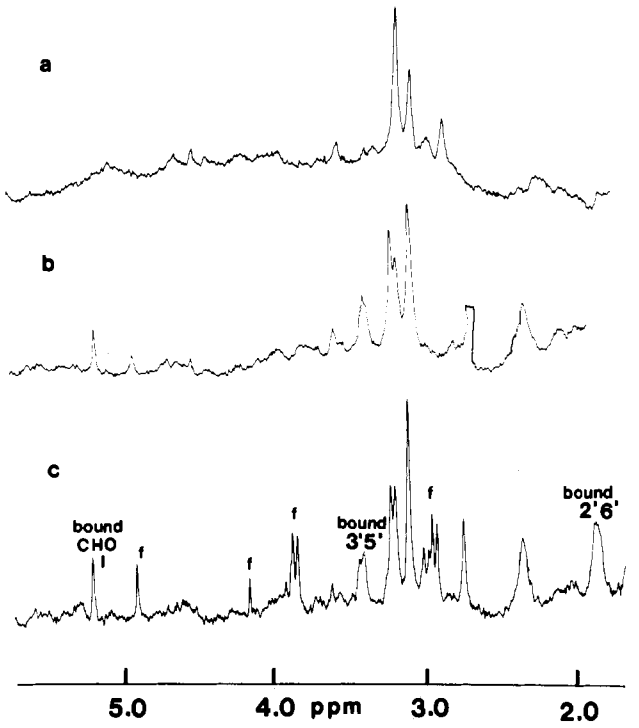


FIGURE 3: Low-field region of the 270-MHz $^1H$ NMR spectrum of a 1 mM solution of [2,6-$H_2$]tyrosine selectively deuterated dihydrofolate reductase at pH 6.9 and 25 °C. (a) Alone; (b) with 1 molar equiv of (6*RS*,α*S*)-folinic acid; (c) with 2 molar equiv of (6*RS*,α*S*)-folinic acid. The "bound" signals correspond to the formyl (I) and benzoyl protons of the 6*S*,α*S* isomer. The "free" signals (marked f) correspond to those protons in the free 6*R*,α*S* isomer.

quivocally by using transfer of saturation methods on samples containing an excess of the free (6*S*,α*S*)-folinic acid. Irradiating at the frequency positions corresponding to bound protons caused the signals of the corresponding protons in the free folinic acid to decrease in intensity.

Transfer of saturation for the benzoyl protons was not observed at any other frequency positions despite an exhaustive search. Thus the H3′ and H5′ protons have the same chemical shift in bound folinic acid as do the H2′ and H6′ protons. The two bound benzoyl proton signals have been connected by spin-decoupling experiments. Selective irradiation at the frequency of the bound H2′, H6′ protons caused collapse of the H3′, H5′ doublet. The sharpness of the decoupled signals confirmed that the H3′ and H5′ protons have identical chemical shifts.

The transfer of saturation experiments on the formyl proton signals not only confirmed the assignment of the bound formyl signal at 5.3 ppm but also established that the rotameric state of the bound formyl group is as shown in form I. When a solution containing 5 mM (6*RS*,α*S*)-folinic acid in the presence of 1 mM enzyme solution is used, the formyl signals from the free folinic acid are clearly visible, as seen in Figure 4a. Irradiation at the bound signal at 5.3 ppm causes a large decrease in intensity of the free formyl signal from form I (4.88 ppm) with no detectable effect on the free formyl signal from form II (4.16 ppm). Although there is a slow exchange process between the two rotameric states in free folinic acid, this is too slow to interfere with the transfer of saturation between the bound formyl proton and the free formyl proton of form I.

The chemical shifts for the formyl and benzoyl protons in the bound diastereoisomers of folinic acid are summarized in Table I. No information about the bound shifts of the aliphatic protons in folinic acid was obtained since these give rise

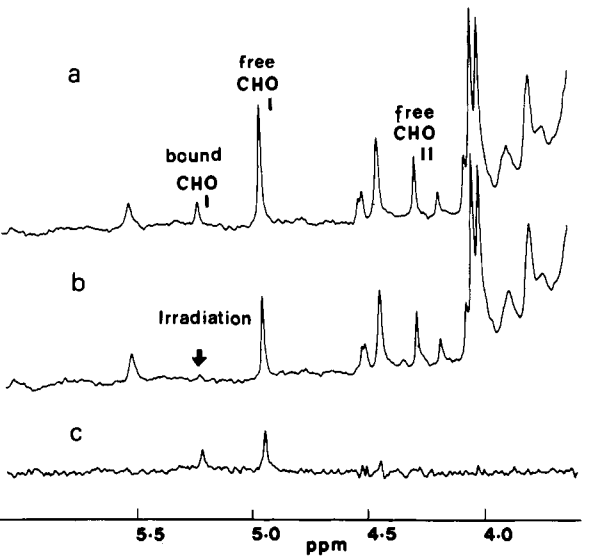


FIGURE 4: Low-field region of the 270-MHz $^1H$ NMR spectrum of 1 mM dihydrofolate reductase in the presence of 5 molar equiv of (6*RS*,α*S*)-folinic acid at pH 6 and 45 °C. (a) With irradiation outside the spectral region; (b) with selective gated irradiation at the frequency of the "bound" formyl signal; (c) the difference between (a) and (b) showing the decrease in intensity of the "free" formyl (I) signal.

Table I: $^1H$ Chemical Shifts (ppm) at 25 °C for Protons in the Diastereoisomers of Folinic Acid When Bound to Dihydrofolate Reductase[a]

| | formyl | | benzoyl | |
|---|---|---|---|---|
| compound | form I | form II | H3′,H5′ | H2′,H6′ |
| (6*S*,α*S*)-folinic acid | −0.29 | not bound | +0.42 | +1.03 |
| (6*R*,α*S*)-folinic acid | −0.10 | +0.13 | +0.22 | *b* |

[a] Shifts measured from the positions of the signals in free folinic acid. [b] These H2′,H6′ signals could not be measured accurately because of overlap with protein signals.

to very complicated multiplets which are obscured by protein resonances.

The effects of binding the diastereoisomers of folinic acid on the histidine residues of the enzyme have also been studied. At pH 6.5, the C2 proton signal of histidine-28 (labeled His F in previous publications; Birdsall et al., 1977) is shifted upfield by at least 0.2 ppm in both complexes. This is consistent with the p*K* of histidine-28 increasing by ~0.7 pK unit on complex formation (assuming that the chemical shift change is caused solely by a change in p*K*). This increase in p*K* has been shown to be a characteristic feature of binding of any molecule containing the (*p*-aminobenzoyl)glutamate moiety to the enzyme and is attributed to a direct interaction between His-28 and the γ-$CO_2^-$ of the glutamate (Wyeth et al., 1980).

## Discussion

The very different shifts observed for corresponding protons in the two diastereoisomers of folinic acid when bound to the enzyme clearly indicate that the pteridine ring and the benzoyl aromatic ring are in different environments in the two cases. The fact that both rotameric states of the 6*R*,α*S* isomer are bound without changing the population ratio of the two forms suggests that the pteridine ring in this isomer is binding in a less constrained site than that of the 6*S*,α*S* isomer. Because the two diastereoisomers of folinic acid bind competitively to the enzyme, it seems likely that their binding sites overlap. Both isomers have a similar effect on the p*K* of histidine-28,

which strongly indicates that the glutamic acid moiety is interacting with the enzyme in a broadly similar manner in the two complexes (Birdsall et al., 1977). Although some parts of the two isomers bind in different environments, it is interesting that they have identical effects on the tyrosine resonances of the enzyme. At least two of these tyrosines are not in direct contact with the expected binding site for substrate analogues (Matthews et al., 1978; Feeney et al., 1977), and the observed shifts are probably due to induced conformational changes which are the same for both isomers.

The tight binding of only one rotameric state of the 6*S*,α*S* isomer provides a good example of a conformational selection process induced by binding to an enzyme. The fact that the rotameric equilibrium for the pteridine formyl group is perturbed so strongly indicates that the pteridine ring moiety is probably participating strongly in the binding to the enzyme. This of course is also implied by the large increase in binding of (6*S*,α*S*)-folinic acid ($K_a = 10^8$ $M^{-1}$) compared with that of (*N*-methyl-*p*-aminobenzoyl)glutamate ($K_a = 1.03 \times 10^3$ $M^{-1}$; Birdsall et al., 1978).

The observation of identical bound shifts for the H3′ and H5′ benzoyl protons (and for the H2′ and H6′ protons) is somewhat surprising. It seems most unlikely that the large identical bound shifts could arise from the two protons being rigidly held in fixed positions in the protein complex in environments which accidentally gave the same shielding. The observed equivalence of shielding could result if the benzoyl aromatic ring is "flipping" about the N10–C4′ and C1′–CO bonds in the bound state. Similar effects have been observed for the trimethoxybenzyl ring of trimethoprim bound to dihydrofolate reductase (Cayley et al., 1979). It is not possible to estimate the rate of ring flipping in bound folinic acid because the chemical shift difference between the two interconverting forms is not known. However, even if the shift difference is only 0.2 ppm (54 Hz), the observed equivalence would require a rate of rotation of >300 $s^{-1}$. Rotation about the N10–C4′ bond (which of course has partial double-bond character) in free folinic acid is unlikely to be faster than $10^4$ $s^{-1}$ at 25 °C. NMR studies on 4-(*N*,*N*-dimethylamino)-nitrosobenzenes (Matsubayashi et al., 1970) indicate that rotation about the N–C4 bond is <25 $s^{-1}$ at −60 °C, which taking $\Delta E^{\ddagger} = 10$ kcal $mol^{-1}$ corresponds to $< 2 \times 10^4$ $s^{-1}$ at 25 °C (Lunazzi et al., 1980). In the bound state, the interactions with the protein could influence the rate in a number of ways. If N10 deviates from $sp^2$ hybridization in bound folinic acid, the loss of planarity could result in a lower rotational energy barrier and an increased rate of flipping. Similar considerations apply to the C1′–CO bond. Protonation at N10 would also increase the rate of flipping (although there is no evidence to suggest that such protonation takes place). The steric interactions between the benzoyl ring and protein residues will, on the other hand, impede ring flipping. If the benzoyl ring is binding in a site similar to that in methotrexate in the enzyme·methotrexate·NADPH complex (Matthews et al., 1978), then the side chains of Leu-27, Phe-49, and Leu-54 will be in contact with the ring. At first sight, it is difficult to envisage how the benzoyl ring of bound folinic acid could flip about the N10–C4′ and C1′–CO bonds at the required rate, particularly as the molecule is tightly bound to the enzyme with multiple contacts involving both the glutamate and pteridine ends of the molecule. However, it is known that conformational fluctuations can take place within protein structures which can momentarily remove the steric interactions impeding ring flipping. This mechanism has been proposed to account for the flipping of tyrosine rings about their $C_\beta$–$C_\gamma$ bond in bovine pancreatic trypsin inhibitor (BPTI) (Gelin & Karplus, 1975; Wagner et al., 1975, 1976; Snyder et al., 1975) and a number of other proteins. The crystal structure of BPTI indicates that some of the tyrosine residues would not be able to flip if only the crystal conformation is available to the molecule.

An alternative mechanism of "flipping" must also be considered for a bound ligand such as folinic acid which is involved in multicontact binding to the protein. In this case, a transient complex might be formed in which some of the protein–ligand interactions are momentarily broken, thus allowing the flipping motion of the benzoyl ring in the "released" (*p*-aminobenzoyl)glutamate (PABG) moiety. However, a consideration of the rates of the processes involved indicates that this mechanism can be discounted in the case of folinic acid. From binding studies of PABG analogues, the dissociation rate constant for the PABG fragment of folinic acid is expected to be in the range $10^3$–$10^5$ $s^{-1}$: the reassociation rate process will of course be much faster since the equilibrium is very much in favor of the fully bound form. For example, if 1% of the partially bound form is present in the equilibrium, the reassociation rates would be $10^5$–$10^7$ $s^{-1}$, and the corresponding lifetimes of the partially bound forms ($10^{-5}$ to $10^{-7}$ s) would be inadequate to allow for the relatively slow rotation about the N10–C4′ bond. Thus it seems likely that the flipping motion leading to the equivalence of the aromatic protons in the *p*-aminobenzoyl moiety takes place when the folinic acid is fully bound to the enzyme and requires a transient "breathing" of the structure in the vicinity of the bound ligand.

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# Effect of Oxygen and Allosteric Effectors on Structural Stability of Oligomeric Hemocyanins of the Arthropod, *Limulus polyphemus*, and the Mollusc, *Helix pomatia*†

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ABSTRACT: The hemocyanins of arthropods and molluscs are allosteric proteins whose reactivity toward oxygen is influenced by the ionic composition and pH of the buffering medium. In general, the oxygen dissociation curves exhibited by these proteins can be described by models which invoke conformational transitions between high- and low-reactivity states. Since the degree of ligation and the concentration of allosteric effectors are both influential in determining the conformational state which the molecule will assume, it would seem likely that the intersubunit contacts which determine the structural stability of the oligomers might also be affected by these conditions. We have used a stopped-flow light-scattering technique and parallel sedimentation velocity experiments in order to probe the stability of the quaternary structure of *Helix pomatia* α-hemocyanin and *Limulus polyphemus* hemocyanin. We find that there are substantial oxygen- and NaCl-dependent differences in the time courses of dissociation of both hemocyanins when divalent cations are removed at high pH. Notably, *Limulus* oxy- and deoxyhemocyanin can exist in a 48-subunit aggregation state, but the oxygenated form is much more quickly dissociated into monomeric subunits when calcium ions are removed at high pH. A similar difference between the stability of oxy and deoxy forms of *Helix* α-hemocyanin was found. These results and an analysis of oxygen binding curves of the aggregated and dissociated forms lead us to the conclusion that although the allosteric units of these high molecular weight proteins appear to be restricted structural units, the overall stability and quaternary state of the high molecular weight aggregate are substantially affected by the conformational changes which accompany oxygenation. The rates of dissociation of *Limulus* oxyhemocyanin and *Helix* α-oxyhemocyanin were found to be approximately equal to the rates of dissociation when the deoxygenated molecules were rapidly mixed with air-equilibrated dissociation buffer. We conclude that the changes in conformational state which accompany oxygenation occur within the dead time of the stopped-flow apparatus and thus have first-order rate constants with a minimum value of 2000 $s^{-1}$.

The blue, copper-containing hemocyanins of the horseshoe crab *Limulus polyphemus* and the vineyard snail *Helix pomatia* are among the largest of respiratory proteins. *Limulus* hemocyanin, which has a sedimentation coefficient of 60 S and a molecular weight of $\sim 3.2 \times 10^6$, contains 48 oxygen binding sites (Johnson & Yphantis, 1978). *Helix* hemocyanin, which has a sedimentation value of 100 S and a molecular weight of $\sim 9 \times 10^6$, contains 180 oxygen binding sites (Van Holde & van Bruggen, 1971). Both hemocyanins show cooperative interactions between their subunits in the process of oxygen binding. In both cases, their functional properties can basically be described by the two-state model for allosteric transitions (Colosimo et al., 1974; Brouwer et al., 1977; van Driel et al., 1978; Zolla et al., 1978). This paper concerns the stability of these high molecular weight oligomers and the changes in quaternary constraints that accompany the interaction of these molecules with oxygen and with allosteric effectors.

The molecular architectures of the hemocyanins of the mollusc *H. pomatia* and the arthropod *L. polyphemus* are very different (Hendrickson, 1977; Bonaventura et al., 1977a). The hemocyanins of molluscs are constructed like hollow cylindrical drums partially closed at the ends by "collars" (Mellema & Klug, 1972; Siezen & van Bruggen, 1974). The cylinders are composed of 20 very long polypeptide chains, each of which

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