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PROTONATION OF METHOTREXATE BOUND TO THE CATALYTIC SITE OF DIHYDROFOLATE REDUCTASE FROM LACTOBACILLUS CASEI.

Lennie Cocco*, Carroll Temple, Jr.**, John A. Montgomery**, Robert E. London† and Raymond L. Blakley*.

*The Department of Biochemistry, University of Iowa, Iowa City, IA 52242, **The Southern Research Institute, Birmingham, AL 35205, and †The Los Alamos Scientific Laboratory, University of California, Los Alamos, NM 87545.

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SUMMARY:

[2-^{13}C]Methotrexate bound to dihydrofolate reductase from Lactobacillus casei has been studied by nuclear magnetic resonance. The chemical shift for enzyme-bound methotrexate over the range pH 6.0 to 9.0 is constant and close to that for free, protonated methotrexate. Furthermore, the ultraviolet spectrum of bound methotrexate is the same from pH 6.4 to pH 10.0 and considerably different from that of free, unprotonated methotrexate. These data indicate that N-1 of bound methotrexate has $pK_a > 10$. From this and from the measured apparent association constants of methotrexate and folate it can be concluded that the charge interaction between the protonated N-1 of bound methotrexate and the active site carboxyl accounts for the particularly tight binding of the inhibitor.

INTRODUCTION:

The antineoplastic agent methotrexate arrests the proliferation of cells primarily due to the metabolic consequences of its very tight binding to dihydrofolate reductase (EC 1.5.1.3). Although the molecular basis of this tight binding has been a matter of debate, a great deal of evidence, has accumulated to indicate that, when bound to the reductase, methotrexate has N-1 of the pteridine ring protonated (1-8). This despite a pK_a of 5.7 for this protonation in the absence of enzyme (9,10). It has been generally assumed that the protonated pteridine ring of enzyme-bound methotrexate interacts electrostatically with an anionic group on the enzyme, and that this is at least partly responsible for the tightness of methotrexate binding. However, there have been few estimates of the magnitude of this contribution to the total binding energy.

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Hood and Roberts (11) addressed the question of the contribution of methotrexate protonation to overall binding energy by carrying out difference spectroscopy on the complex of methotrexate with dihydrofolate reductase from L. casei In their experiments excess methotrexate mixed with reductase was present in the sample cell, whereas the methotrexate and reductase solutions were kept separate in the reference cell. A plot of the extinction difference, at a selected wavelength, versus pH gave a bell-shaped curve (concave up). The data were fitted to an appropriate equation for deprotonation of free and bound methotrexate with different acid dissociation constants, and binding of protonated and unprotonated methotrexate to the enzyme with different association constants. This gave a pK_a value of 5.35 for free methotrexate and 8.55 for bound methotrexate. At pH 7.5 the association constant calculated for binding of unprotonated methotrexate was 1.75×10^7 M^{-1}, and since that observed for folate was 9.8×10^4 M^{-1}, two-thirds of the additional binding energy for methotrexate appears to arise from the difference in binding of neutral methotrexate and neutral folate. In view of the similarity of the structures this is an unexpected result.

We have reported (10) that the chemical shift in nuclear magnetic resonance spectra of [2-^{13}C]methotrexate bound to dihydrofolate reductase from Streptococcus faecium indicates that bound methotrexate remains protonated to at least pH 10, which results in quite different association constants from those calculated by Hood and Roberts. To determine whether this discrepancy is due to differences in the reductase from the two bacterial species, we now report NMR results on [2-^{13}C]methotrexate bound to L. casei dihydrofolate reductase. We also report UV spectra of methotrexate bound to the reductase.

MATERIALS AND METHODS

A culture of methotrexate-resistant L. casei was kindly provided by Dr. J.H. Freisheim. The reductase was purified by a modification of the procedure of Gundersen et al. (12). The active fractions from a G75 column were applied to a pteroyllysine Sepharose column (3.5 x 11 cm) and affinity chromatography was carried out according to Pastore et al. (13). The final preparation was homogeneous on polyacrylamide gel electrophoresis and had a specific activity of 29 IU per mg under conditions used for assay of the S. faecium reductase (14).

The synthesis of [2-^{13}C]methotrexate and [2-^{13}C]folate has been described (10), and the procedures used for determining the chemical shift of ^{13}C in

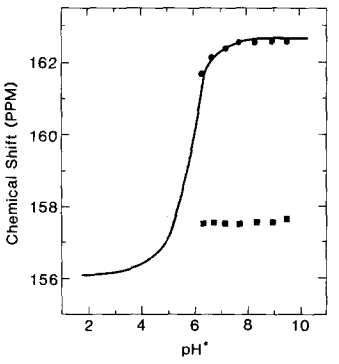

Figure 1. Dependence on pH* of the ^{13}C chemical shift of [2-^{13}C]methotrexate. The continuous line shows the chemical shift of methotrexate C-2 in absence of enzyme. ● ■ Chemical shifts at 50.30 MHz of [2-^{13}C]methotrexate (1.0 mM) in the presence of dihydrofolate reductase from L. casei (0.5 mM). Other details as previously described (10).

enzyme-bound methotrexate at various pH values were the same as used for methotrexate bound to the S. faecium enzyme (10). Under these conditions the binary complex of methotrexate with the reductase was stable up to pH 9.5, while the folate complex was stable to pH 8.5.

The spectrum of methotrexate bound to the reductase was determined at 24°C with a Cary 14 spectrophotometer set to give 0.1 absorbance unit at full scale. Standard 1 cm cells in both reference and sample compartments contained the buffered reductase solution. In the sample cell the solution also contained methotrexate at a much lower concentration than enzyme.

Fluorimetric determination of association constants was carried out on reductase in 50 mM potassium phosphate buffer containing 0.3 M KCl as previously (15), but with computer-assisted calculations based on the equation in the form used by Torikata et al. (16).

RESULTS AND DISCUSSION:

The dependence of the chemical shift of [2-^{13}C]methotrexate in the presence of L. casei dihydrofolate reductase is shown in Figure 1. Two resonances are seen in the NMR spectrum, one of which has a chemical shift at each pH similar to that for free methotrexate. The other has a chemical shift (157.51 ppm) which is unchanged over the pH range 6.0 to 9.0, although at pH 9.5 there is a slight downfield shift of this resonance. This result is essentially the same as that previously observed for [2-^{13}C]methotrexate in the presence of S. faecium dihydrofolate reductase (10) and, as in that case, is interpreted to mean that there is no change in the state of protonation of bound methotrexate over the pH range explored. The chemical shift for the bound methotrexate is much closer to

that for free, protonated methotrexate (156.19 ppm) than that for free, unprotonated methotrexate (162.54 ppm, Fig. 1). As we have previously argued in the case of the S. faecium reductase complex, this indicates that bound methotrexate remains protonated at pH values up to 9.5. As in the case of S. faecium (10) [2-^{13}C]folate showed no change in chemical shift due to binding.

Since this result is in conflict with the conclusions of Hood and Roberts (11), we performed a difference spectrum titration with methotrexate and S. faecium reductase essentially according to the method used by Hood and Roberts. Enzyme and methotrexate were present in separate compartments in the reference beam, but in the sample beam buffer was in one compartment and a mixture of enzyme and methotrexate (equimolar) in the other. When the absorbance difference at an appropriate wavelength (400 nm) was plotted as a function of pH, we obtained essentially the same result as Hood and Roberts. However, when the actual spectrum of bound methotrexate was obtained (as described in Methods and Figure 2) this spectrum was unchanged between pH 6 and pH 10. Further, methotrexate bound to L. casei dihydrofolate reductase had exactly the same spectrum as obtained with the S. faecium enzyme. This spectrum is shown in Figure 2, and it may be seen that there was no significant change in it between pH 6.4 and 10.0. These results clearly support the conclusion, derived from the NMR study, that reductase-bound methotrexate is protonated at pH values up to 10.

It may be noted that although the UV-spectrum of enzyme-bound methotrexate differs markedly from that of free methotrexate at the same pH, it also differs significantly from that of free protonated methotrexate. This, together with the difference in chemical shift of bound [2-^{13}C]methotrexate from that of free [2-^{13}C]methotrexate at low pH, clearly indicates that the interaction of methotrexate with residues at the active site is more complex than simple protonation of N-1. It should be noted that the chemical shift observed for methotrexate bound to S. faecium reductase is 157.09 ppm, whereas that for methotrexate bound to the L. casei enzyme is 157.51 ppm. This undoubtedly reflects some difference in the interaction of methotrexate with the active site of L. casei reductase

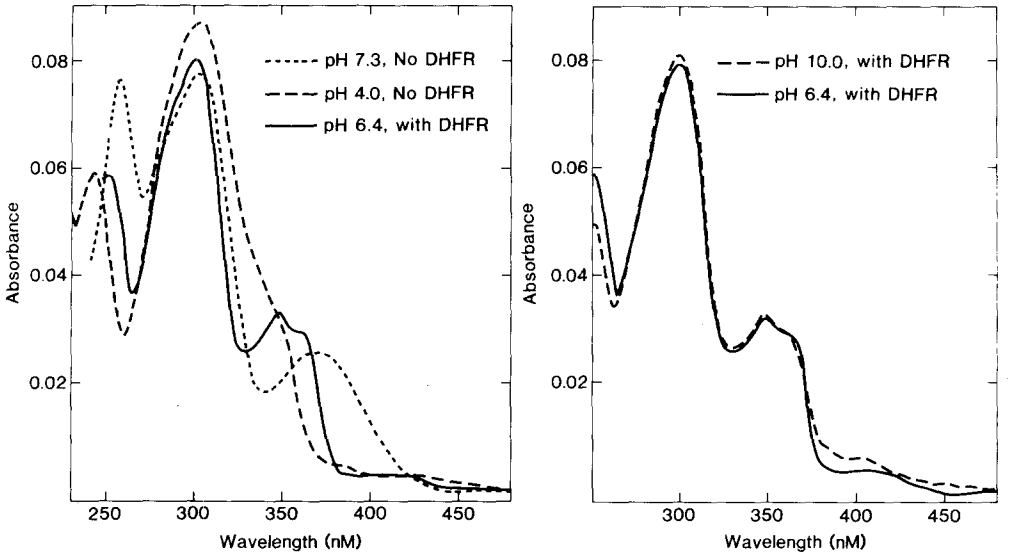

Figure 2. Ultraviolet-visible spectrum of free and enzyme-bound methotrexate at various pH values. In all cases the reference and sample cells contained identical solutions except for the presence of methotrexate (3.7 μM) in the sample cell. L. casei dihydrofolate reductase (DHFR) when present was at a concentration of 71 μM. Reagents present were as follows. Left panel: — — —, 50 mM sodium acetate buffer, pH 4.0; - - - - -, 10 mM potassium phosphate buffer, pH 7.3; ———, 10 mM potassium phosphate buffer, pH 6.4, and DHFR. Right panel: ———, 10 mM potassium phosphate buffer, pH 6.4, and DHFR; — — —, 10 mM potassium phosphate and 50 mM Tris chloride buffer, pH 10.0, and DHFR. All mixtures except the first also contained 0.5 M KCl, 1 mM EDTA and 0.02% sodium azide.

from its interaction with the active site of the S. faecium enzyme, a difference which is apparently too subtle to be reflected in the UV spectrum.

The source of the apparent pK_a of 8.55 in the method of Hood and Roberts is unclear, but one possibility is the assumption in their treatment that the concentration of bound methotrexate is given accurately by the concentration of enzyme present (i.e. the concentration of uncomplexed enzyme is zero). This assumption becomes increasingly inaccurate as the pH is raised. Since Hood and Roberts do not give the concentration of ligands used, the magnitude of this error cannot be assessed from their published data.

Fluorimetric titration gave the association constants for folate and methotrexate shown in Table 1. When combined with a pK_a of 5.7 for free methotrexate (9,10) and a pK_a of 10.5 for bound methotrexate the observed constants led, by the method previously used (11), to the association constants for unprotonated

Table 1. Association constants for the binding of folate and methotrexate to L. casei dihydrofolate reductase.

		Association Constants (M^{-1})		
pH	Ligand	Observed	Protonated species	Unprotonated species
7.5	Methotrexate	$2.0(\pm0.5)\times10^{8}$	1.3×10^{10}	2.1×10^{5}
7.5	Folate	$1.22(\pm0.04)\times10^{5}$		
8.5	Methotrexate	$4.3(\pm1.1)\times10^{7}$	2.7×10^{10}	4.3×10^{5}
8.5	Folate	$4.7(\pm0.9)\times10^{4}$		

methotrexate and protonated methotrexate shown in the Table. Since the calculated association constant for unprotonated methotrexate is about the same as the observed constant for folate at pH 7.5, and only nine-fold greater at pH 8.5, the large difference between the observed association constants for folate and methotrexate is almost entirely due to the capability for protonation at N-1 in the latter case.

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