Binding of Methotrexate to *Escherichia coli* Dihydrofolate Reductase as Measured by Visible and Ultraviolet Resonance Raman Spectroscopy

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Abstract: Raman solution spectra are shown which demonstrate protonation of the pteridine ring when MTX is bound to *Escherichia coli* DHFR. Bands are observed in the Raman spectra of the MTX–DHFR complex due to the other structural features of the complex. In addition, solution spectra of methotrexate, aminopterin, methopterin, and folic acid are presented. Tentative band assignments are made.

Methotrexate (MTX, Figure 1) is useful in the treatment of the most common form of childhood leukemia, and for several other cancers;[1] the molecular site of action of this cytotoxic agent is the enzyme dihydrofolate reductase (DHFR; EC 1.5.1.3).[2] In clinical usage, methotrexate is given at very high dose levels followed by leucovorin "rescue" for cures[3] or at moderate levels in combination with other chemotherapeutic agents for the maintenance of remissions.[4] It is unclear at present why the neoplastic cells are more sensitive to methotrexate than normal cells, but there is general agreement that MTX exerts its biological effects through inhibition of DHFR.

This paper is a study of the binding of the antineoplastic agent, MTX, to its target receptor, DHFR, by Raman spectroscopy. MTX has a resonance-enhanced Raman spectrum, allowing dilute solutions to be examined. Recent studies using UV-difference spectroscopy[5,6] have indicated that binding to *Escherichia coli* DHFR is greatly enhanced by MTX as compared to dihydrofolate because MTX forms a salt (ionic) linkage to the enzyme. Similar studies of DHFR from a mouse tumor[7] and porcine liver[8] also indicate salt linkages to MTX. This linkage has been shown to occur between a nitrogen at the 1 position of MTX and the carboxyl group of asparate-27 in the *E. coli* enzyme.[9] While these UV-difference spectra illustrate the importance of ionic binding in the MTX–DHFR complex, they also highlight structural features which are not mimicked by simple protonation of the methotrexate. Additionally, x-ray crystallographic studies[9] of the complex show the MTX to be in a folded configuration with the benzoyl glutamic acid moiety closer to the pteridine ring than its extended free solution form.[10]

We present Raman solution spectra at pH 7 which clearly show the protonation of the pteridine ring when methotrexate is bound to DHFR. Additionally, as is the case of the UV-difference spectral experiments,[5,6] we observe bands in Raman spectra of the MTX–DHFR complex due to other structural features of the complex. Thus, we extend the list of enzymes[11] and enzyme complexes[12] studied by Raman spectroscopy, and show that vibrational spectroscopy may provide additional insight in understanding the biochemistry of the methotextrate–DHFR interaction.

Lastly, in this paper we present Raman spectra of methotrexate (Figure 2) and related compounds which heretofore have not been published.

Experimental Section

Raman spectra were obtained on two spectrophotometers. Visible excitation spectra were recorded on a Cary 82 spectrophotometer

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using the 514.5-nm line of a Model 165 Spectra-Physics argon ion laser. Near-UV excited Raman spectra were obtained using a computerized[13] SPEX spectrophotometer and the 363.8-nm line of a Spectra-Physics Model 170 argon ion laser. For the UV spectra, multiple scans were taken and baseline correction and digital smoothing[14] techniques were used to facilitate interpretation of band positions and shifts. Because the solutions were often strongly fluorescent, a variety of sample holders, slit settings, and laser powers were used. A Varian spinning liquid cell was used to permit high-visible laser power (ca. 500–1500 mW) without significant sample heating. UV laser power was typically 150 mW. The Raman spectra of certain model compounds which did not require the spinning cell were recorded on 0.2-mL samples in a 3-mm i.d. tube.

Methotrexate (gift of Dr. H. B. Wood, Jr., NCI), aminopterin (Merck Sample Collection), and methopterin (gift of Dr. E. W. Cantrall, Lederle) were all purified by chromatography on DEAE-Sephadex[15] prior to use to remove fluorescent impurities. The MTX–DHFR complex was prepared using DHFR isolated from *E. coli,* strain MB-1428.[16,17] The enzyme–MTX complex exhibited the same ultraviolet absorption spectrum before and after Raman spectroscopy showing that the complex was not affected by the spectroscopy. All other chemicals were obtained commercially and used without further purification.

Results

Methotrexate–Dihydrofolate Reductase Complex. The Raman spectra of the methotrexate-*E. coli* dihydrofolate reductase (MTX–DHFR, 5 × 10⁻³ M) binary complex are shown in Figure 2a (514.5-nm excitation) and Figure 3a (363.8-nm excitation). We have recorded the Raman spectrum of MTX in neutral (Figure 2b, 514.5-nm excitation) and acidic (Figure 3b, 363.8-nm excitation) media in order to deconvolute the features of the MTX–DHFR complex. We obtained the Raman spectrum of DHFR, but the limited solubility, ca. 10⁻³ M, and lack of resonance enhancement resulted in a poor signal-to-noise ratio. Table I summarizes the Raman band positions obtained from repeated scans of the DHFR, MTX, and MTX–DHFR solutions.

The Raman spectrum of neutral MTX solution shows an intense band at 1356 cm⁻¹ which is shifted to 1366 cm⁻¹ in the 0.01 N HCl solution. The pK_1 for N-1 protonation for methotrexate is 5.71 at 25 °C.[18] Both our work with model compounds (see next section) and the literature[19a] support an assignment of the bands in the 1300–1400-cm⁻¹ region to a pteridine ring vibration. The Raman spectra of naphthalene, 1-azanaphthalene, 2-azanaphthalene, and several diazanaphthalenes have been reported[19a] and show extremely intense bands in the 1350–1400-cm⁻¹ region which in all cases were assigned to a ring stretching mode. Since substitution of one or two nitrogen atoms for carbon atoms has little effect upon the position of the ring stretching fundamental in the 1350–1400-cm⁻¹ region, substitution of four nitrogen atoms should
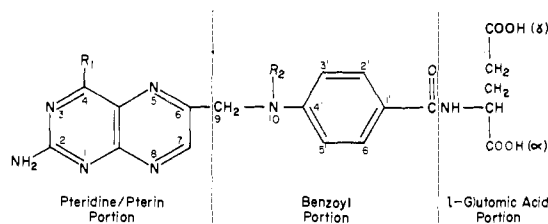
Figure 1. Numbering system for the folates: $R_1 = NH_2$, $R_2 = CH_3$: Methotrexate (MTX); $R_1 = NH_2$, $R_2 = H$: aminopterin (APT); $R_1 = OH$, $R_2 = CH_3$: methopterin (MTP); $R_1 = OH$, $R_2 = H$: folic acid (FA).

lead to a strong ring stretching vibration in the same region.

The Raman spectrum of the MTX-DHFR complex shows a pteridine ring stretch at 1366 cm^{-1}, which is very similar in position, shape, and intensity to the spectrum of protonated MTX. Furthermore, the appearance of a new band at 1407 cm^{-1} in both the complex and MTX at pH 2, which is absent in unprotonated MTX, strengthens the hypothesis of protonation accompanying binary complex formation.

Most notable among the other differences between the MTX at pH 7 and MTX-DHFR spectra is the presence of a strong 659-cm^{-1} band which is only present in the complex. A band at 680 cm^{-1} is observed, however, in the UV excited spectrum of the pH 2 MTX solution. Bands in this region may be assigned to various ring deformation modes for heterocyclic aromatics,[19d] and the differences observed between the two spectra are indicative of some yet undefined structural change which occurs when MTX binds to DHFR.

The Raman spectrum of the MTX-DHFR complex excited by visible light (514.5 nm) is noticeably different from the UV-excited spectrum. Bands appear in the visible excitation spectrum which may be protein related such as the 1006-cm^{-1} phenylalanine ring stretch.[11] In addition, observed scattering maxima at 1662 and 1250 cm^{-1} are probably the enzyme amide I and amide III bands, respectively.[11]

Spectral Comparison of Methotrexate (MTX), Aminopterin (APT), Methopterin (MPT), and Folic Acid (FA). The Raman spectra of the various folates are quite complex, and we will not attempt in this paper to make rigorous assignments. Comparison, however, of MTX, APT, MPT, and FA, Table II, shows several interesting features which permit some tentative band assignments.

We observe that all four compounds exhibit a very strong band at 1607 ± 1 cm^{-1}. Since the model compounds pterin-6-carboxylic acid and 6-cyclopropyl-2,4-diaminopteridine do not exhibit a band in this region whereas N-(p-aminobenzoyl)-1-glutamic acid and p-(N,N-dimethylamino)benzoic acid show an intense 1607-cm^{-1} band, we assign this band to a benzoyl ring stretch. This assignment agrees with literature values[19b] for a $\nu_{(C=C)}$ of a para-disubstituted phenyl ring which typically are in the region 1550 to 1630 cm^{-1}.[22a] Hence, we specifically assign the 1607 ± 1-cm^{-1} band to the $\nu_{(C=C)}$ of the para-disubstituted phenyl ring.

The second region of interest, 1410–1330 cm^{-1}, does not exhibit a common feature for all four folates studied. Rather, the two amino folates, MTX and APT, show two scattering maxima: one very strong at 1356 cm^{-1} and the other not as strong at 1380 cm^{-1}. The two pterins, MPT and FA, show only one band in common, a very strong 1338-cm^{-1} band. Only two of the model compounds, pterin-6-carboxylic acid and 2,4-diamino-6-cyclopropylpteridine, show intense bands in this spectral region. Because of its limited solubility in neutral or acid solution, the spectrum of pterin-6-carboxylic acid was recorded from a 0.2 N NaOH solution. We observed a very intense band at 1332 cm^{-1} and a strong band at 1390 cm^{-1}. The latter band is the well-described[19c] symmetric phenyl-
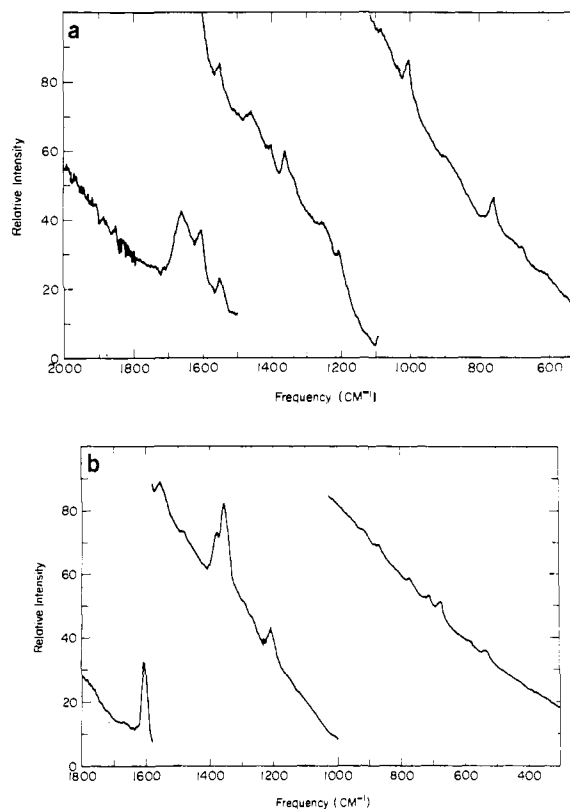

Figure 2. (a) Raman spectrum of the MTX-DHFR complex: concentration = 5×10^{-3} M, pH 7.2, excitation = 514.5 mn, power = 1500 mW, slit = 12 cm^{-1}, pen period = 1 s, scan speed = 1 cm^{-1}/s, buffer = 0.01 M Tris-HCl (pH 7.2) + 0.01 M NaCl. (b) Raman spectrum of 0.1 M MTX in 0.01 M KCl; excitation = 514.5 nm, power = 1000 mW, slit = 10 cm^{-1}, pen period = 1 s, scan speed = 2.5 cm^{-1}/s.

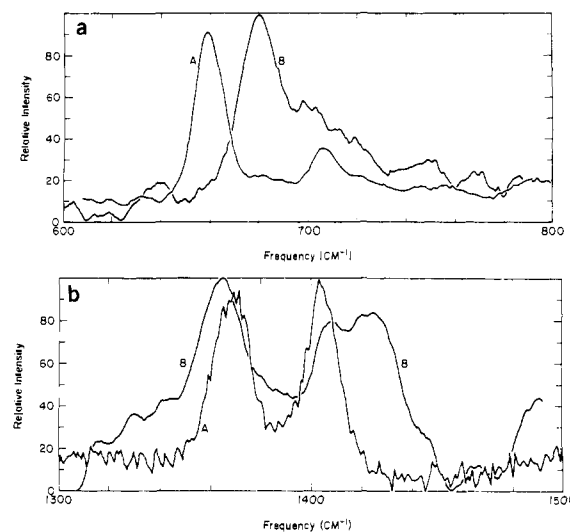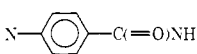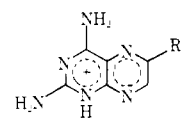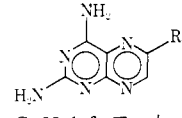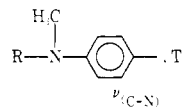Figure 3. Raman spectra of (A) 5×10^{-3} M MTX-DHFR complex; excitation = 363.8 nm, power = 150 mW, slit = 8 cm^{-1}; (B) MTX saturated in 0.01 N HCl, excitation = 363.8 nm, power = 150 mW, slit = 10 cm^{-1}: (a) 600–800 cm^{-1}; (b) 1300–1500 cm^{-1}.

carboxylate stretch. The former band is similar in position and intensity to the 1338-cm^{-1} band observed in MPT and FA. Since these compounds exist in the keto form in most aqueous solutions[18] and lactams show medium to strong Raman C-N stretching modes near 1350 cm^{-1},[19e,24] we assign these bands to a C-N vibration of the pterin ring. The secondary amide group (Figure 1) is in the trans configuration[23,24] and, hence, would not contribute to the 1338-cm^{-1} band. The Raman

Table I. Observed Frequencies of the Methotrexate-Dihydrofolate Reductase Complex (MTX-DHFR), 5×10^{-3} M; DHFR, 1×10^{-3} M; and MTX, 10^{-1} M

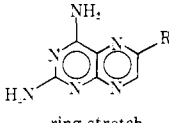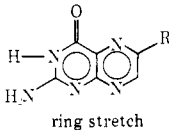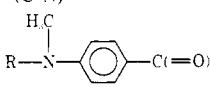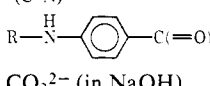| MTX–DHFR UV: λ 363.8 nm Visible: λ 514.5 nm	DHFR[a] λ 514.5 nm	MTX UV: λ 363.8 nm, pH 2 Visible: λ 514.5 nm, pH 0.3[b]	MTX λ 514.5 nm N = pH 6.5 B = pH 12	Tentative assignment
1660 vs,[c] visible	1662 s			Amide I[d]
			1607 vs, B	$\nu_{(C=C)}$ in N—⟨○⟩—C(=O)NH
1609 s, visible		1608 visible	1605 vs, N	
			1557 m, N	
1553 m, visible		1560 visible	1555 m, B	MTX, Amide II, Trp[d,e]
			1540 sh, B/N	
			1480 w, B/N	
1462 m, visible	1460 m			C–H def., CH₃ deg. str., CH₂ sym[d]
			1450 ± 10 vw, N	
		1425 visible		
		1423 s, UV		
1409 w, visible				
1405 s, UV		1408 s, UV		
			1380 s, N	
			1379 s, B	
1369 s, UV		1368 visible		ring stretch[e]
1363 s, visible		1365 s, UV		
			1356 vs, B/N	ring stretch[e]
1335 sh, visible				C–H def., Trp[d]
			1290 w, N	
~1268 w, UV		1264 w, UV	1260 w, N	Amide III, MTX
1250 w, visible				Amide III
~1229 w, UV				
1210 w, visible			1210 m, B/N	R—N—⟨○⟩— , Tyr, Phe[d,e] $\nu_{(C-N)}$
	1157 w			
1083 w, visible				
1006 w, visible	1003 m			Phe, enzyme[d]
			915 vw, N	
890 w, visible				Gly
	878 vs			Borate buffer
			870 vw, N	
			770 w, B/N	
761 s, visible				Trp[d]
	745 m			Enzyme buffer
			710 w, B	
706 m, UV		703 w, UV	708 w, N	
			676 w, B	
675 w, visible		680 s, UV	674 w, N	
659 s, UV				
			535 w, B	
			532 w, N	
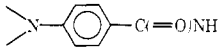
[a] In a buffer consisting of 10^{-1} M borate, 10^{-1} M KCl, 10^{-3} M dithiothreitol, and 10^{-4} M EDTA. [b] ±10 cm⁻¹ on visible bands; visible spectrum recorded from 1150 to 1700 cm⁻¹. [c] m = medium, w = weak, vw = very weak, vs = very strong, s = strong, sh = shoulder. [d] Reference 11. [e] Reference 19.

spectrum of 2,4-diamino-6-cyclopropylpteridine in acidic medium exhibits one very strong band centered at 1358 cm⁻¹. On the basis of the model compound, we assign this vibration in MTX and APT to the 2,4-diaminopteridine ring vibration. The band at 1380 cm⁻¹ observed in the neutral aminofolates is not observed in the model compound reflecting the effect of acidity on the pteridine framework.

A third region of interest, 1215–1180 cm⁻¹, in the folate spectra is defined. We observe a 20-cm⁻¹ difference between the N-10 tertiary amines (Figure 1), MTX and MPT, and the N-10 secondary amines, APT and FA. Model compounds for the aminobenzoyl moiety show the same trends. A tertiary amine, *p*-(*N,N*-dimethylamino)benzoate, shows a moderate 1201-cm⁻¹ band of medium intensity in addition to the expected 1607 cm⁻¹, $\nu_{(C=C)}$, and 1383 cm⁻¹, $\nu_{sym(COO-)}$, bands, whereas a secondary amine, *p*-(*N*-methylamino)benzoate, shows a medium intensity 1185-cm⁻¹ band in addition to the strong 1609- and 1383-cm⁻¹ bands. The absolute position and magnitude of the spectral shift are strikingly similar among the various tertiary and secondary amines including folates and

Table II. Observed Frequencies of Methotrexate (MTX), Aminopterin (AP), Methopterin (MP), and Folic Acid (FA) in Aqueous Solutions, λ 514.5 nm

MTX B = 0.01 M NaOH N = 0.01 M KCl	APT[a] B = 0.01 M NaOH N = 0.01 M KCl	MPT B = 1 M NaOH N = 0.01 M KCl	FA B = 1 M NaOH	Assignments[b]
1607 vs, B	1609 vs, B	1609 vs, B		
1605 vs, N	1605 vs, N	1607 m, N	1608 vs, B	$\nu_{(C=C)}$ in [structure: (CH3)2N—C6H4—C(=O)NH]
1555 m, N			1570 w, B	
1557 m, B	1562, w, N	1560 m, B		
	1545 w, B			
1540 sh, B/N	1542, w, B	1545 m, N		
1480 w, B/N	1484 w, N	1475 m, N		
1450 vw, N	1455 w, N			
		1420 m, B		
1380 s, N	1380 sh, N			
1379 s, B	1380 s, B			
1356 vs, B/N	1356 vs, B			[structure: pteridine NH2 ring] ring stretch
	1355 vs, N			
		1348 m, N		[structure: pterin O ring] ring stretch
		1338 vs, B	1338 vs, B	
		1304 s, N	1312 sh, B	
1290 w, N				
	1275 w, N	1270 vw, B		
1260 w, N				$\nu_{(C-N)}$ in [structure: R—N(CH3)—C6H4—C(=O)]
1210 m, B/N		1211 w, B		
		1210 m, N		
	1189 m, B		1191 m, B	$\nu_{(C-N)}$ in [structure: R—N(H)—C6H4—C(=O)]
	1188 m, N			
		1068 vw, B	1067 m, B	CO_3^{2-} (in NaOH)
		929 w, B		
915 vw, N				
870 vw, N	870 w, N		868 m, B	
770 w, B/N		770 w, B		
		740 vw, B		
710 w, B				
708 w, N				
676 w, B		680 w, B		
674 w, N	680 w, N	678 m, N	682 m, B	
535 w, B				
532 w, N	533 w, N	535 vw, B	535 w, B	

[a] Spectrum of APT in base recorded from 1150 to 1700 cm^{-1} only. [b] See also ref 19.

model compounds. We can only make the most tentative assignment of bands in this region to a C-N stretch involving N-10 with C-9 and the methyl carbon in MTX, MTP, and dimethylaminobenzoate. Presumably, the N-phenyl stretch does not make a strong contribution to these bands since it is typically near 1280 cm^{-1} for para-substituted anilines.[22b]

In summary, we advance the following: the 1615–1600-cm^{-1} region reflects a $\nu_{(C=C)}$ of the phenyl ring, the 1410–1330-cm^{-1} region reflects the skeletal backbone of the pteridine/pterin ring, and the 1215–1180-cm^{-1} region is sensitive to substitution at N-10.

Demonstration of Resonance Raman Effect for the Folates. The intensity of the 1338-cm^{-1} Raman band of folic acid dissolved in a carbonate buffer shows a three-fold increase in relation to the nonresonant CO_3^{2-} band at 1067 cm^{-1} when the excitation wavelength is changed from 514.5 to 488.0 nm. Since folic acid as well as the other pterins and aminofolates has a strong electronic absorption at 368 nm (log ϵ = 3.96 in 0.1 N NaOH),[20] it is not surprising that resonance enhancement occurs.[21]

In acid solutions, however, the solutions are much less colored because of the blue shift in absorption to 307 nm.[18,20] Reduced resonance enhancement coupled with low solubility might account for the extremely low S/N in the acid spectra of MTX when using 514.5-nm excitation. In comparison, when 363.8-nm excitation is used, Figure 3b, the S/N is higher as a result of the greater overlap between the laser excitation frequency and the electronic absorption frequency.[21]

Discussion

In the past few years, direct physical evidence for ionic binding of the 4-aminofolates to DHFR has been obtained.[5,6] This evidence has relied on careful measurements of changes in the electronic absorption strengths upon complexation of methotrexate[5] and aminopterin[6] to DHFR. Such changes when plotted vs. wavelength were shown to closely mimic the differences in electronic absorption strengths between the aminofolate, e.g., methotrexate, buffered to pH 7 and 1.5. While the evidence is quite striking, this "proof" of the ionic binding hypothesis relies on a simplistic interpretation of all the complex changes observed in these electronic absorption spectra.

The Raman spectra that we have presented similarly do not constitute proof of the ionic binding hypothesis; however, they do support it using a totally different spectroscopic technique. The Raman spectrum of MTX in 0.01 N HCl is similar in band positions and intensities to the spectrum of MTX–DHFR complex at pH 7.0. Unlike the UV absorption experiment,[3,4] we have direct evidence that this protonation is directed toward the pteridine ring because of the Raman spectral changes in the 1410–1330-cm^{-1} region upon MTX–DHFR binding. The lack of measurable change in the 1615–1600 and 1215–1180 cm^{-1} regions strongly implies that protonation does not affect the aminobenzoyl moiety.

We have concentrated on only three regions of the MTX–DHFR spectrum. The lack of suitable models has prevented interpretation of several other interesting features. For example, the band at 1425 cm^{-1} present in Figure 3b, MTX in 0.01 M HCl, is absent in the MTX–DHFR spectrum. Clearly, more information is yet to be gleaned from these spectra.

Conclusion

Striking similarities have been observed in the Raman spectrum of MTX in 0.01 N HCl and that of the MTX–DHFR complex at pH 7. Furthermore, these features are not observed in the spectrum of MTX at pH 7 or 13. Our data support the view that MTX and other aminofolates are bound to DHFR in solution by an ionic linkage between a protonated nitrogen of the pteridine ring and an adjacent carboxylate of the folates as working hypotheses to aid in our understanding of these molecules.

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