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A ^{1}H n.m.r. study of the role of the glutamate moiety in the binding of methotrexate to *Lactobacillus casei* dihydrofolate reductase

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1 The binding of a series of amide derivatives of methotrexate to *Lactobacillus casei* dihydrofolate reductase has been studied by inhibition constant measurements and by ^{1}H n.m.r. spectroscopy.

2 Amide modification of the α-carboxylate of methotrexate was found to prevent interaction of the γ-carboxylate with the imidazole of His 28.

3 Estimates of the contributions to the binding energy from the α-carboxylate-Arg 57 and γ-carboxylate-His 28 interactions have been made from a combination of inhibition and n.m.r. data.

Introduction

Methotrexate is well known as a powerful and clinically useful inhibitor of dihydrofolate reductase (Blakley, 1969; Bertino, 1971). During the thirty years since its original synthesis (Seeger *et al.*, 1949), a large number of analogues of methotrexate has been studied (see, for example, Roth & Cheng, 1982 and references therein). However, it is only in the last few years, with the advent of structural information on dihydrofolate reductase, that it has become possible to identify the molecular bases of the observed structure-activity relationships. The availability of this structural information (for references see Gronenborn, *et al.*, 1981; Bolin *et al.* 1982; Roberts, 1983), which comes from X-ray crystallography and, in solution, n.m.r. spectroscopy, allows one to investigate in detail the contribution of individual interactions between methotrexate and the enzyme to the overall binding energy.

The glutamate moiety of methotrexate appears to contribute to binding primarily through interactions of its carboxylate groups with the enzyme. For example, the dimethyl or diethyl esters of methotrexate bind 200–400 fold less tightly than the parent compound to the dihydrofolate reductases from *Lactobacillus casei* and L1210 lymphoma cells (Chaykovsky, *et al.*, 1974; Johns *et al.*, 1974). In the crystal structures of the complexes of methotrexate with *Escherichia coli* dihydrofolate reductase or of methotrexate and NADPH with the *L. casei* enzyme (Matthews *et al.* 1977; 1978; Bolin *et al.*, 1982) the α-carboxylate of the glutamate moiety forms an ion-pair with the guanidinium group of Arg-57, a residue which is highly conserved in prokaryotic and eukaryotic dihydrofolate reductases (Volz, *et al.*, 1982, and references therein). In the complex with the *L. casei* enzyme, the γ-carboxylate forms a second ion-pair, with the imidazole of His 28; earlier ^{1}H n.m.r. studies showed that this interaction is accompanied by an increase of about 1 unit in the pK of this imidazole (Roberts *et al.*, 1974; Birdsall *et al.*, 1977; Wyeth, *et al.*, 1980). The corresponding residue in the *E. coli* enzyme is Ala 29, and the γ-carboxylate appears to interact only with the solvent (Bolin *et al.*, 1982). It is likely that interaction of the γ-carboxylate with the enzyme is not confined to the *L. casei* enzyme, since basic amino acid residues are found at positions homologous to His 28 in the enzymes from *Streptococcus faecium* and from a number of vertebrate species (Volz *et al.*, 1982, and references therein). Modification of the γ-carboxyl alone effects binding to the enzyme from pigeon liver or L1210 cells as well as from *L. casei* (Piper & Montgomery, 1979; Rosowsky *et al.*, 1981).

In order to obtain a quantitative estimate of the contributions of these interactions to the binding energy, we have studied the binding of the amide derivatives of methotrexate, in which either the α- or the γ-carboxylate, or both, is replaced by a carboxamide. The difference in binding energy between methotrexate and these derivatives can only be inter-

preted in terms of the contribution of the α- and γ-carboxylate to binding if all the compounds bind in the same way, with the sole exception of the interaction which has been 'blocked' by modification. To assess this, we have also compared the mode of binding of methotrexate and its amides by ^{1}H n.m.r. spectroscopy, paying particular attention to the behaviour of His 28.

Methods

Methotrexate was obtained from Lederle (U.S.A.) and Nutritional Biochemicals Corp. and used without further purification. The methotrexate analogues (II–V) (Figure 1) were synthesized using the following procedures.

Methotrexate α-monoamide (II) (Piper *et al.*, 1982), N-[N'-(2,4-diamino-6-pteridinyl) methyl-N'-methyl-4-aminobenzoyl]-L-isoglutamine, and methotrexate γ-monoamide (III) (Piper *et al.*, 1982), N - [N' - (2,4-diamino - 6 - pteridinyl)methyl - N' - methyl - 4 - aminobenzoyl] - L - glutamine, were synthesized from N - benzyloxycarbonyl - L - glutamic acid and N - benzyloxy - carbonyl - L - glutamine respectively, as described by Antonjuk *et al.* (1983).

Methotrexate diamide (IV) was prepared from commercial methotrexate (Lederle, U.S.A.) as follows. To a stirred solution of methotrexate (100 mg) in dimethylformamide (2 ml) at −15° to −20°C was added triethylamine (0.09 ml), followed by isobutyl chlorocarbonate (0.09 ml). After 20 min at the same temperature, ammonia gas was bubbled in for 10 min. Water (1 ml) was added, and the reaction mixture was evaporated at 45°C under reduced pressure, yielding a residue which was crystallized from a mixture of dimethylformamide and water. This product (85 mg) was treated with isobutyl chlorocarbonate followed by ammonia as described above. On working up and crystallization from dimethylformamide-water, methotrexate diamide was obtained as yellow crystals, m.p. 193–196°C, mass spectrum (ammonia chemical ionisation) m/e 453 (7%, MH^+); 436 (47%, $MH\text{-}NH_3$), 326 (25%), 325† (100%), 324 (10%), 146 (36%), 129 (11%), 101 (72%), ^{13}C n.m.r. δ (solvent, CD_3SOCD_3 with δ39.6 p.p.m.) 27.5 (β-C), 31.9 (γ-C) 39.3 (NCH_3), 53.3 (α-C), 54.9 (CH_2N), 111.2 (C-3'), 121.5, 121.6 (C-1' and C-10' or C-4a), 129.0 (C-2'), 146.9 (C-6), 149.2 (C-7), 151.0 (C-4'), 154.1 (C-9 or C-8a), 162.1, 162.8 (C-2 and C-4), 166.2 (-C_6H_4CONH-), 174.2, 174.4 p.p.m. ($CONH_2$). Anal., Found: C, 49.85; H, 5.65; N, 28.6% $C_{20}H_{24}O_3N_{10}$, $2H_2O$ required C, 49.2; H, 5.8; N, 28.7%.

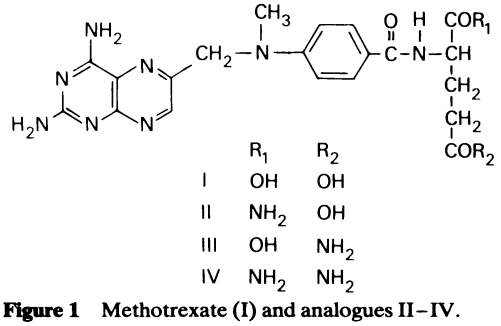

Figure 1 Methotrexate (I) and analogues II–IV.

Methotrexate dimethylester was prepared by esterifying methotrexate with methanol HCl by a modification of the method of Pfiffner *et al.* (1947) as described previously (Wyeth *et al.*, 1980).

Dihydrofolate reductase was isolated and purified from *L. casei* as described previously (Dann *et al.*, 1976). The enzyme was lyophilized twice from ^2H_2O solution to exchange all the exchangeable protons for deuterons, and was then examined as a 1 mM solution in a 50 mM potassium phosphate, 500 mM potassium chloride ^2H_2O buffer containing 1 mM dioxane as an internal reference. Complexes of the inhibitors with the enzyme were formed by adding accurately weighed amounts of solid inhibitor, corresponding to slightly more than one molar equivalent, to the enzyme solution.

A Bruker WH 270 spectrometer was used to obtain 270 MHz ^{1}H n.m.r. spectra at 11°C, and the pH*-dependence of the histidine C2-proton resonances was determined and analysed as described previously (Wyeth *et al.*, 1980; Gronenborn *et al.*, 1981). The notation pH* denotes a pH meter reading in ^2H_2O solution without correction for the deuterium isotope effect on the glass electrode.

The concentration of the inhibitors required to inhibit the enzyme activity by 50% (I_{50}), was measured at pH 7.5 using a modification of the assay described by Mathews *et al.* (1963) with 150 mM KCl, 180 μM NADPH and 90 μM dihydrofolate. Apparent K_i values were calculated using the relationship $I_{50} = [S]K_i/K_m$, (where $[S]$ is the dihydrofolate concentration), using the K_m value for dihydrofolate, 0.36 μM, reported by Dann *et al.* (1976). Equilibrium constants for the formation of binary complexes of the inhibitors with the enzyme were measured fluorimetrically as described by Birdsall *et al.* (1980).

†The *m/e* 325 ion (and/or the 326 ion) is found also in the ammonia chemical ionization mass spectra of various monoamide monoester derivatives of methotrexate (Antonjuk *et al.*, 1983), and is given the structure:

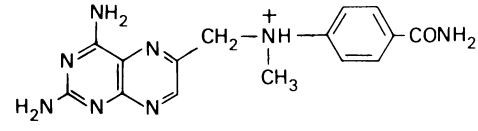

Table 1 Inhibition of *L. casei* dihydrofolate reductase by methotrexate and its amide derivatives (compounds I–IV)

Compound	I_{50}(M)[a]	K_i(pM)[b]	ΔG_{app} (kJ.mol^{-1})[c]
Methotrexate (I)	2.7×10^{-9}	10.8	62.6
Methotrexate α-amide (II)	2.6×10^{-7}	1040	51.3
Methotrexate γ-amide (III)	2.4×10^{-8}	96	57.2
Methotrexate α,γ-diamide (IV)	2.4×10^{-7}	960	51.5

[a] Concentration required for 50% inhibition under the standard conditions described in Methods. Standard errors $< 10\%$ in each case.
[b] Inhibition constants, calculated from I_{50} values as described in Methods.
[c] Binding energy; $\Delta G_{app} = -RT \ln(1/K_i)$

Results

Inhibition constants

The I_{50} values of compounds I–IV are given in Table 1, together with the inhibition constants (K_i values) and binding energies (ΔG_{app}) calculated from them. These values were necessarily determined in the presence of the coenzyme NADPH, and thus reflect the binding of the inhibitor to the enzyme-NADPH complex.

We attempted to determine the equilibrium constants for formation of the binary enzyme-inhibitor complexes by fluorescence quenching (Birdsall *et al.*, 1980). However, the binding of methotrexate itself and of its γ-amide was too tight ($K_a > 10^8$ M^{-1}) to be determined in this way, although a value of 3.5 (± 0.4) $\times 10^7$ M^{-1} could be obtained for the α-amide (II).

The values in Table 1 show that the γ-amide of methotrexate (III) binds nine times less well than the parent compound, while the corresponding α-amide (II) binds 100 times less well. However, when both carboxylate groups are converted to amides (compound IV), the inhibition constant is very similar to that of the α-amide; the effects of forming amides at the α- and γ-positions are clearly not additive.

Effects of inhibitor binding on histidine residues

The seven histidine residues of *L. casei* dihydrofolate reductase have been studied in some detail by n.m.r. (Birdsall *et al.*, 1977; Wyeth *et al.*, 1980; Gronenborn *et al.*, 1981), and assignments for all their C2-proton resonances are now available (Wyeth *et al.*, 1980; B. Birdsall, J. Feeney and G.C.K. Roberts, unpublished work), although some of these remain tentative. The pK values of the individual histidine residues can be estimated by determining the pH-dependence of these resonances (see Jardetzky & Roberts, 1981).

We have measured the ^{1}H spectra of the complexes of enzyme with compounds II–IV as a function of pH* over the range (5.0–8.0) in which the enzyme is stable. Figure 2 shows a typical set of titration curves

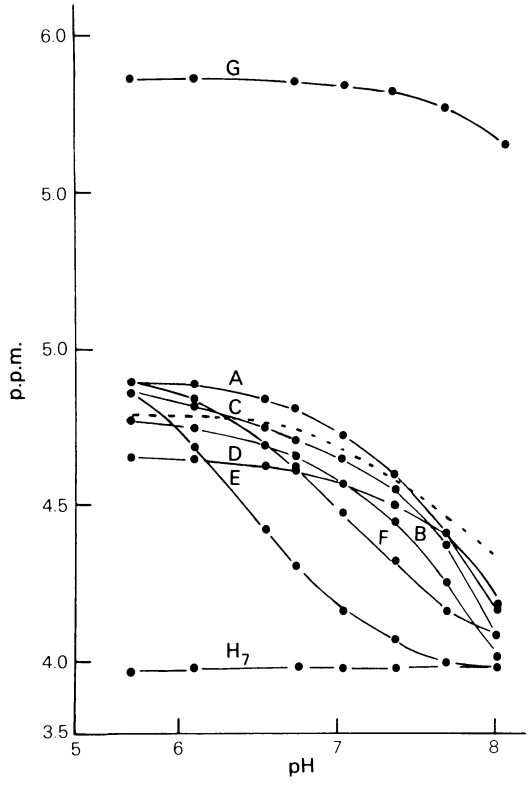

Figure 2 The ^{1}H chemical shifts of the seven histidine C2 protons (A to G) plotted as a function of pH in the 1:1 complex of dihydrofolate reductase with the methotrexate α-amide (II). The dotted line is the pH titration curve for His F (His 28) in the complex of dihydrofolate reductase with methotrexate.

obtained by plotting the C2-proton chemical shifts as a function of pH*, in this case for the complex with the α-amide (II). The pK values estimated from these data (and the corresponding data for III and IV) by non-linear regression methods are given in Table 2, together with the values for the enzyme alone and its complex with methotrexate (Birdsall *et al.*, 1977; Gronenborn *et al.*, 1980).

Examination of Table 2 reveals that, although resonances A and G show some change in chemical shift on inhibitor binding, the only two histidine residues whose pKs change are His 18 and His 28, giving rise to resonances E and F, respectively. The pK values of these two histidines in the various complexes are summarized in Table 3.

The marked increase in pK of His 28 produced by methotrexate binding, due to the interaction with the γ-carboxylate revealed in the crystal structure, is essentially abolished when the γ-carboxylate is converted to a carboxamide in compounds III or IV. In complexes with the latter two compounds, the pK of His 28 is not significantly different from its value in the enzyme alone. However the binding of the α-amide (II), which retains a free γ-carboxylate group, does not produce the large increase in pK of this residue seen in the complex with methotrexate itself. This cannot be explained on the simple hypothesis that conversion of the α-carboxylate to an amide interferes only with the α-carboxyl-Arg 57 interaction.

Further indications of the mode of binding of the methotrexate derivatives can be obtained from the pK of His 18 and from the chemical shift of the pteridine 7-proton resonance. The decrease in pK of His 18 accompanying methotrexate binding (Table 3) appears to reflect an inhibitor-induced conformational change (Birdsall *et al.*, 1977; Wyeth *et al.*, 1980; Gronenborn *et al.*, 1981); this decrease in pK appears to be essentially the same for the α- and γ-amides as for methotrexate itself. However, significantly larger decreases are observed for both the α,γ-diamide and the α,γ-dimethyl ester, indicating a significantly different mode of binding.

The chemical shift of the pteridine 7-proton resonance of the bound inhibitor is influenced by the magnetic anisotropy of the three nearby aromatic rings; Phe 30, Phe 49 and the benzoyl ring of the inhibitor. The chemical shift contributions from these aromatic rings to the pteridine 7 proton in various conformations of the methotrexate analogues were calculated using the Johnson and Bovey ring current shift equation (Johnson & Bovey, 1958). In these calculations it was assumed that the pteridine ring binds in the same binding site as found for the methotrexate pteridine ring in the crystal structure of the enzyme-methotrexate-NADPH complex (Matthews *et al.*, 1977; 1978) and all values of tor-

Table 2 pK values and imidazole C2-proton chemical shifts[a] of the histidine residues of *L. casei* dihydrofolate reductase, alone and in its complexes[b] with methotrexate and its amide derivatives (I–IV)

Resonance	*Histidine Residue*[c]	*Enzyme alone*[d]		E–I[d]		E–II		E–III		E–IV	
		pK	δ_{HA}	pK	δ_{HA}	pK	δ_{HA}	pK	δ_{HA}	pK	δ_{HA}
A[e]	22	7.5[g]	4.85	7.45	4.89	7.45	4.91	7.5	4.91	7.5	4.92
B[e]	89	7.4	4.80	7.35	4.78	7.3	4.78	7.4	4.79	7.3	4.78
C[e]	64	7.5[g]	4.88	7.5[g]	4.81	7.45	4.84	7.4	4.84	7.4	4.90
D[e]	77	7.9	4.65	7.7	4.66	7.85	4.65	7.8	4.66	7.7	4.66
E	18	6.57	4.92	6.21	4.95	6.28	5.03	6.14	5.15	6.03	4.89
F	28	6.67	4.99	7.8[e]	4.78	6.83	4.93	6.74	4.98	6.60	4.92
G[f]	153	–	5.51	–	5.87	–	5.88	–	5.87	–	5.89

[a] δ_{HA}, chemical shift (p.p.m. from dioxan, ±0.02) in the protonated form.
[b] Denoted, E–I, E–II, etc.
[c] From Wyeth *et al.*, 1980, and B. Birdsall, J. Feeney and G.C.K. Roberts, unpublished work; assignments of resonances B, D and G tentative.
[d] From Gronenborn *et al.*, 1981.
[e] Only incomplete titration curves could be obtained for these residues; pK values are estimated by assuming a total chemical shift change on titration of 1.0 p.p.m. (See Gronenborn *et al.*, 1981) ±0.07 units.
[f] Not enough of titration curve obtained to estimate pK (pK > 8).
[g] Non-Henderson-Hasselbach titration curves (see Gronenborn *et al.*, 1981).

sion angles (examined at 10° intervals) in the C6-C9-N10-C1′ fragment were considered. These calculations show that the chemical shift of the pteridine 7 proton resonance is very sensitive to the precise orientation of the benzoyl ring in the complex in many of the conformations. In the complexes of methotrexate or its γ-amide with the enzyme, the 7-proton signal appears at 4.15 p.p.m. However in the complex of the α-amide the resonance of this proton (assigned unambiguously by a transfer of saturation experiment) appears at 3.92 p.p.m. In the diamide complex the 7-proton signal is upfield of 3.9 p.p.m. (obscured by aromatic proton resonances of the protein), while in the diester complex it is at 4.25 p.p.m. The behaviour of this resonance clearly indicates that methotrexate derivatives in which the α-carboxylate is modified exhibit a significantly altered mode of binding, possibly involving changes in the orientation of the benzoyl ring in the different complexes.

Discussion

Binding of methotrexate γ-amide

Conversion of the γ-carboxylate of methotrexate to a carboxamide has two effects on the interaction of the inhibitor with dihydrofolate reductase. The binding is weaker by a factor of about nine, and the increase in pK of His 28 seen when methotrexate binds is abolished. In other respects (such as the pK of His 18, and the chemical shift of the pteridine 7-proton), the γ-amide behaves in the same way as methotrexate itself. It is reasonable to conclude that the modification of the γ-carboxylate has affected only the γ-carboxylate-His 28 interaction. We can thus use the difference in ΔG_{app} between methotrexate and its γ-amide (5.4 kJ.mol^{-1}) as an estimate of the contribution of this interaction to the overall binding energy.

An alternative approach is to consider the magnitude of the ligand-induced changes in the pK of His 28. The increase in pK reflects a preferential interaction of the carboxylate with the protonated, rather than the neutral, form of the imidazole of His 28. If we make the reasonable assumption that any interactions (such as hydrogen-bonding) with the *neutral* imidazole are the same, in energetic terms, for both the carboxylate and the carboxamide, then we can use the difference in pK values to estimate the additional free energy associated with the binding of the carboxylate, rather than the carboxamide, to the protonated imidazole. The value obtained, 6.0 kJ.mol^{-1}, is close to that calculated above from the ΔG_{app} values, supporting the idea that the γ-carboxylate-His 28 interaction is predominantly Coulombic in nature and makes a contribution of some 5.7 kJ.mol^{-1} to the overall binding energy of methotrexate.

Table 3 pK values of histidines 18 and 28 of *L. casei* dihydrofolate reductase, alone and in its complexes with methotrexate and its derivatives

	pK values[a]	
	His 18	*His 28*
Enzyme alone	6.57	6.67
+ methotrexate (I)	6.21	7.8[b]
+ methotrexate α-amide (II)	6.28	6.83
+ methotrexate γ-amide (III)	6.14	6.74
+ methotrexate α,γ-diamide (IV)	6.03	6.60
+ methotrexate α,γ-dimethyl ester[c]	5.96	6.20

[a] From Table 2: ±0.04 units
[b] ±0.07 units
[c] From Wyeth *et al.* (1980).

Binding of methotrexate α-amide

The inhibition constants we have measured show, in agreement with earlier work (Piper & Montgomery, 1979; Rosowsky *et al.*, 1981; Domin, Cheng & Hakala, 1982), that modification of the α-carboxylate of methotrexate has a larger effect on binding to dihydrofolate reductase than does modification of the γ-carboxylate. This has generally been interpreted as indicating that the interaction of the α-carboxylate with the enzyme (specifically with Arg 57) makes a larger contribution than that of the γ-carboxylate to the overall binding energy.

However, the ^{1}H n.m.r. experiments show clearly that modification of the α-carboxylate is accompanied by disruption of the γ-carboxylate-His interaction. In addition, the altered chemical shift of the pteridine 7-proton suggests that the orientation of the benzoyl ring may also have changed. Either the simple absence of the α-carboxylate-Arg 57 interaction or additionally some unfavourable contact of the carboxamide with the enzyme appears to have led to a substantial change in the mode of binding of the benzoyl-glutamate moiety. This also provides an explanation for the observation that the α,γ-diamide binds as strongly as the α-amide since in the latter, the interaction of the γ-carboxylate with the enzyme has already been broken.

It follows that the decrease in binding energy produced by modification of the α-carboxylate does not correspond simply to the contribution of the α-carboxylate-Arg 57 interaction to the overall binding. The observed decrease in binding energy of 11.3 kJ.mol^{-1} must contain a component due to the disruption of the γ-carboxylate-His 28 interaction; using the estimate made above of the energy of this interaction, 5.7 kJ.mol^{-1}, we conclude that the contribution of the α-carboxylate interaction is about 5.6

kJ.mol^{-1}. The latter is an approximate value, since we cannot quantify the energetic consequences of any change in the mode of binding of the benzoyl ring. These estimates indicate that the α-carboxylate-Arg 57 interaction and the γ-carboxylate-His 28 interaction make roughly equal contributions to the overall binding energy. Some support for the estimate of the α-carboxylate-Arg 57 interaction energy comes from the work of Kuyper *et al.* (1982), who have prepared a series of trimethoprim analogues bearing a carboxylate group attached by a short alkyl chain. When the alkyl chain was of the appropriate length, the carboxylate was shown crystallographically to interact with Arg 57 of the *E. coli* enzyme, and the additional interaction energy (over that of trimethoprim itself, in the binary complex) amounted to 6.8 kJ.mol^{-1}.

Thus we have shown that the interaction of the α-carboxylate group of methotrexate with Arg 57 contributes approximately 10% of the overall binding energy while a roughly equal contribution is made by the γ-carboxylate interaction with His 28. These experiments illustrate the fact that the contribution of individual groups to the drug-receptor interaction cannot be estimated from a comparison of affinities alone; structural information is required before the affinities can be properly interpreted.

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