Biochemical Pharmacology, Vol. 32, No. 6, pp. 1059–1062, 1983.
Printed in Great Britain.

0006-2952/83/061059-04 $03.00/0

INTERACTION OF METHOTREXATE METABOLITES WITH BEEF LIVER DIHYDROFOLATE REDUCTASE—I

BINARY COMPLEX STUDY

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(*Received* 16 *July* 1982; *accepted* 25 *October* 1982)

Abstract—In order to explain the difference of inhibition of dihydrofolate reductase (DHFR) by methotrexate (MTX) and its metabolites 7-hydroxymethotrexate [7OH (MTX)] and polyglutamate derivatives [MTX (G_1) and MTX (G_2)], direct determinations of binding parameters to beef liver DHFR were performed. Association constants are calculated by fluorescence titrations and thermodynamic parameters by microcalorimetric measurements. The parameters of interaction are nearly identical for MTX and polyglutamate derivatives but are different for MTX and 7OH (MTX). For this last derivative electrostatic forces are less predominant and a larger modification of its conformation appears in the enzyme during the complex formation.

The most frequently used folic acid analog methotrexate (2,4-diamino-N_{10}-methylpteroylglutamate) (MTX) is an effective agent in a wide variety of malignancies. The basic action of MTX, the inhibition of dihydrofolate reductase (DHFR) [5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase (EC 1.5.1.3)], has been appreciated for two decades. Otherwise MTX has long been considered to be insignificantly metabolized. However its use in high doses prompted the isolation of important amounts of a hepatic metabolite 7-hydroxymethotrexate [7OH (MTX)] in plasma [1, 2]. Neither the absence of 7OH (MTX) in the cells nor its presence have been proved. By means of enzymatic measurements [2] 7OH (MTX) has been found to be approximately 200-fold weaker as an inhibitor of DHFR than MTX.

On the other hand, in recent years the existence of intracellular polyglutamate metabolites that have potential antifolate activity was demonstrated *in vitro* and *in vivo* by several laboratories. Numerous reports indicated that MTX polyglutamates are at least as effective as MTX in inhibiting DHFR [3, 4].

In order to explain these differences of inhibition between MTX and its metabolites direct determination by physical methods of binding parameters to one mammalian DHFR, beef liver DHFR, have been performed in this work. Previous studies [2] on MTX metabolite binding were carried out by enzymatic assays using bacterial enzymes. Indeed, DHFRs from humans and animals may differ in their relative sensitivities to inhibitors. However it is very difficult to prepare human DHFR in large amounts [5]. On the other hand, it is conventional to distinguish between bacterial and mammalian enzymes. Moreover, with regard to DHFR from malignant human tissues, a noteworthy analogy must be noted between sequences for beef liver and L1210 DHFR [6, 7].

MATERIALS AND METHODS

Reagents. DHFR was prepared from beef liver as described by Kaufman and Kemerer [8]. 7OH (MTX) was isolated from the urine of a rabbit receiving high doses of MTX and was purified on DEAE-cellulose according to the method of Fabre *et al.*† Its purity as checked by u.v. spectrophotometry and HPLC was greater than 99%.

MTX was purchased from Lederle Laboratory and purified by the John and Ti Li Loo method [9] (purity greater than 99%). MTX (G_1) and MTX (G_2) were gifts from Dr Montgomery.‡ DHF was purchased from Sigma.

Fluorescence measurements. Fluorescence measurements were carried out with a spectrofluorimeter [Perkin–Elmer MPF 3L (automatic correction and thermostated cell holder)] at 10°. The excitation wavelength was 286 nm corresponding to the maximum of the corrected excitation spectrum. Slits were 5 mm/6 mm for the excitation and emission wavelengths respectively. About 40 μl of DHFR stock solutions were diluted to 6 ml by phosphate buffer (0.1 M, pH = 6.8). An aliquot was taken as a reference and used to test the absence of fluorescence and activity evolution during the experimental time scale. Increasing quantities (1–30 μl) of the solutions in the same buffer as the products studied were sequentially added as 1-μl fractions and the fluorescence intensity was monitored after each addition. The dilution effect may be considered as negligible. No modification in the spectrum shape was noted when ligand was added, so we always measured the fluorescence intensity at 325 nm. On the other hand, we verified that the fluorescence intensity of a DHFR

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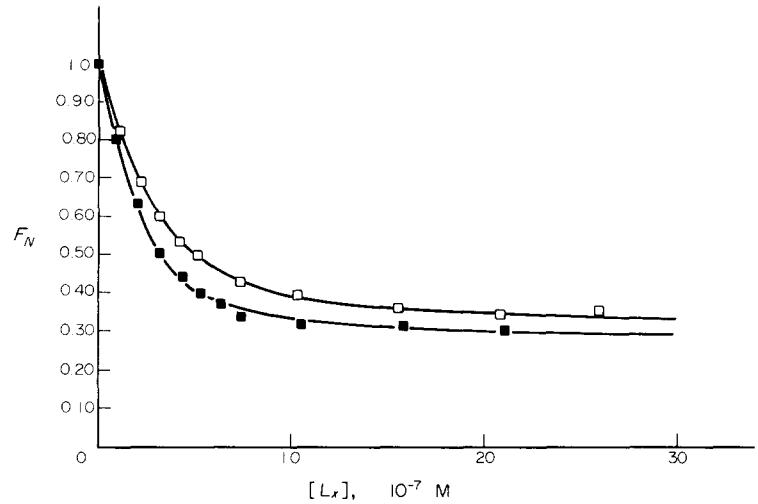

Fig. 1. Fluorescence inhibition of DHFR (F_N) vs molar concn ($[L_x]$) of added ligand: (■) DHF, (□) 7OH (MTX).

solution was a linear function of its concn in the range studied.

The Raman effect was also quite negligible under experimental conditions. Concerning the correction of the inner filter effect, the fluorescence intensity of a TRP solution with the same absorbance (less than 0.2) was sequentially recorded after each addition of the same quantity of the products studied.

Determination of association constants by fluorescence measurements. The fluorescence intensity (F_N) at 325 nm, normalized with regard to that of the DHFR solution without any ligand added, was plotted vs the molar concn ($[L_x]$) of added ligand (Figs. 1 and 2). Corrections for inner filter effects, carried out in the case of 7OH (MTX) and DHF, were not necessary for other products used at much lower concns. At least the residual fluorescence intensity (F_b) of the enzyme was determined when it was saturated by ligand.

Results were analyzed as previously described [10] and used to calculate K_a (the apparent association constant).

Microcalorimetry. The flow calorimeter used was an LKB apparatus type 10700-1. It was set in a thermostatically controlled enclosure; pumps were operated at a flow rate of $v_1 = 2.77 \times 10^{-6}$ l/sec for the ligand and $v_2 = 11.11 \times 10^{-6}$ l/sec for the enzyme.

The heat of measurement (Q) was determined according to a previous paper [11].

The heats of deprotonation of phosphate buffer under our experimental conditions of temp and ionic strength [12] resulting from a possible pK shift of the ligand during the complex formation were of the same order of magnitude as our uncertainties concerning ΔH values. Consequently they were not taken into account.

Contrary to fluorescence, fast microcalorimetric experiments can be performed at 37° without thermal

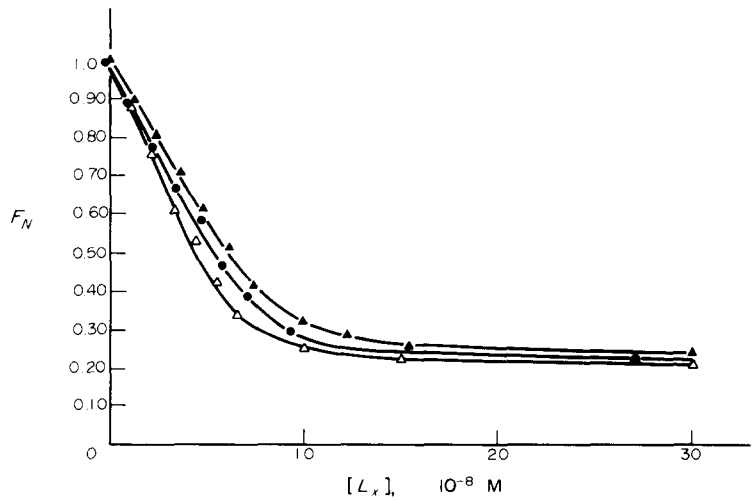

Fig. 2. Fluorescence inhibition of DHFR (F_N) vs molar concn ($[L_x]$) of added ligand: (▲) MTX, (●) MTX (G_1), (△) MTX (G_2).

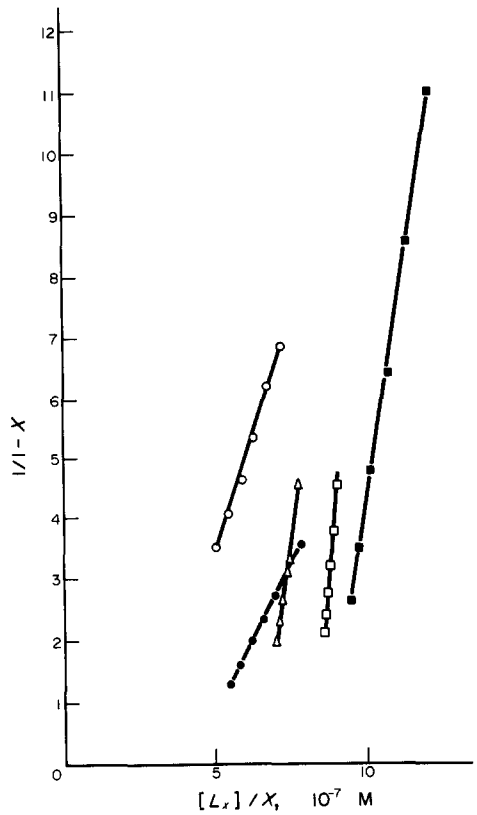

Fig. 3. Curve of the function $1/1 - X = f([L_x]/X)$. X = fractional concn of occupied sites. (△) MTX, (▲) MTX (G_1), (□) MTX (G_2), (●) 7OH (MTX), (○) DHF.

Table 1. Study of ligand fixation by fluorescence quenching measurements

Ligand	C (M)	F_b	K_a (M^{-1})	$n[P_T]$ (M)
MTX	3.30×10^{-5}	0.20	3.8×10^{8}	6.5×10^{-8}
MTX (G_1)	3.70×10^{-5}	0.19	3.1×10^{8}	8.6×10^{-8}
MTX (G_2)	3.54×10^{-5}	0.23	5.1×10^{8}	8.1×10^{-8}
DHF	3.18×10^{-4}	0.28	1.6×10^{7}	3.0×10^{-7}
7OH (MTX)	3.12×10^{-4}	0.31	1.0×10^{7}	2.8×10^{-7}

C = initial molar concn of ligand stock solution.
K_a = apparent association constant.
$n[P_T]$ = molar concn of binding sites in the medium.

denaturation of the enzyme. Mean ΔH values can be calculated by several measurements at enzyme saturation ($\Delta H = Q[P_T]$, where $[P_T]$ = molar protein concn, which was 10-fold lower than that of ligand).

Moreover, with fractional saturations complex stoichiometry can be determined; in our case, assuming that this stoichiometry is equal to one, this method allows us to check the protein purity. The thermodynamic parameters ΔG and ΔS were obtained from classical thermodynamic relationships.

RESULTS AND DISCUSSION

Fluorescence

Whatever the product studied the curves of the function of $1/1 - X$ vs $[L_x]/X$ were linear (Fig. 3). All the results are listed in Table 1. We can see that the apparent association constants of the polyglutamate derivatives MTG (G_1) and MTX (G_2) are of the same order as that of MTX. On the contrary the apparent association constant of 7OH (MTX) is about 40-fold lower than that of MTX and even slightly lower than that of the natural substrate DHF.

Microcalorimetry

In Table 2 we list the thermodynamic parameters at 10 and 37° for the binding of MTX, metabolites of MTX and the natural substrate DHF. At 10°, the large negative enthalpic values observed for MTX as well as for polyglutamates suggest that the interaction under experimental conditions is dominated by electrostatic rather than hydrophobic forces. This observation is in good agreement with the fact that MTX binds to the enzyme [13] in the protonated form. Consequently polyglutamate derivatives are likely to be in the same charge state.

The negative ΔS values which indicate an increase in the orderliness of the system during complexation are thermodynamically unfavourable and the formation of complexes is only enthalpically driven.

The enthalpic contribution to the complex formation reactions is less important in the case of DHF and above all in the case of 7OH (MTX), for which a favourable entropic variation is seen. We can assume therefore that as for DHF [13] 7OH (MTX) binds to the enzyme in its neutral form.

ΔH values at 37° are more exothermic than at 10°. The calculated variations of specific heats (ΔC_p) are the most negative for DHF and 7OH (MTX); this result lets us assume a more important change in the structure of protein complexed with these compounds, which could correspond to a decrease in the exposure of hydrophobic groups and to an increase in the intramolecular hydrophobic forces.

Table 2. Thermodynamic binding parameters of ligands to DHFR in phosphate buffer (pH = 7.4, $\mu = 0.15$)

	$\theta = 10°$			$\theta = 37°$	
Ligand	ΔG (kJ.mole^{-1})	ΔH (kJ.mole^{-1})	ΔS (kJ.mole^{-1}.K^{-1})	ΔH (kJ.mole^{-1})	ΔC_p (kJ.mole^{-1}.K^{-1})
MTX	−46.4	−83 ± 4	−129	−100 ± 4	−0.63
MTX (G_1)	−45.9	−88 ± 4	−148	−100 ± 4	−0.44
MTX (G_2)	−47.1	−83 ± 4	−127	−97 ± 4	−0.52
DHF	−39.0	−45 ± 3	−20	−74 ± 3	−1.07
7OH (MTX)	−37.9	−28 ± 2	+35	−62 ± 3	−1.26

K_a (the binding constant for ΔG and ΔS determinations) were obtained from fluorescence titrations at 10°.
Protein concn $[P_T] = 1.44 \times 10^{-5}$ M.

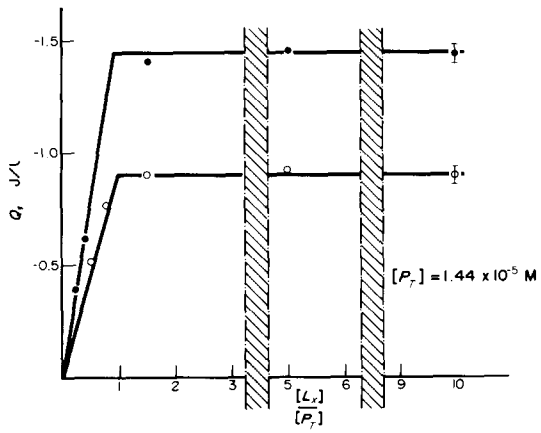

Fig. 4. Experimental heats of measurement (Q) vs molar concn of added ligand ($[L_x]$)/molar protein concn ($[P_T]$): (●) MTX, (○) 7OH (MTX).

In Fig. 4 are plotted the fractional saturation heat curves of MTX and 7OH (MTX). In both cases the number of sites is nearly 0.9; thus we can check that the percentage of protein purity is nearly 90% assuming that the true stoichiometry is equal to one. The particular results observed with 7OH (MTX) lead us to verify the identity of its binding site with MTX and DHF binding sites. We have tried therefore to demonstrate a possible competitive effect [14].

At 37° the ΔH values measured for the binding of MTX and DHF to DHFR in the presence of 7OH (MTX) are -39 and $-12\,kJ.mole^{-1}$ respectively. These heats exactly correspond both to the binding of MTX ($-100\,kJ.mole^{-1}$) or DHF ($-74\,kJ.mole^{-1}$) and the releasing of 7OH (MTX) ($+62\,kJ.mole^{-1}$); they constitute an element in favour of a similarity of binding sites.

In conclusion the thermodynamic binding parameters show that the polyglutamate metabolite binding behaviour is nearly identical to that of MTX. On the contrary 7OH (MTX) presents a less important association constant and probably binds in its neutral form.

Moreover the ratio between the association constants of MTX and 7OH (MTX) is nearly 40 while the ratio of the enzymatic inhibition effects is nearly 200 [2]. This difference might be due to the influence of the ligand binding on NADPH in the ternary complex. The more important variation of conformation showed by large negative ΔC_p values for 7OH (MTX) would be in favour of this hypothesis. Consequently we are now studying these ternary complexes.

Acknowledgements—We are very grateful to Dr Sari for his important assistance in the microcalorimetry and H. Bouteille for his technical assistance. We also thank Dr Montgomery for his generous supply of polyglutamate derivatives. This work was supported by an INSERM contract No. CRL 802002 and by a grant from La Fédération des Centres de Lutte contre le Cancer.

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