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Effects of temperature on the interaction of dihydrofolate reductase with some of its ligands [1]

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Abstract

We have studied the interaction of bovine liver dihydrofolate reductase with methotrexate and trimetrexate at different temperatures by a flow-microcalorimetric method. Results indicate that dihydrofolate reductase undergoes a partial unfolding below 37°C, and that the binding of antifolate compounds induces a protein refolding. On the other hand, the cofactor NADPH has a stabilizing effect on the thermal transition of the protein, and this protective effect is more pronounced in the range 25–37°C.

INTRODUCTION

Dihydrofolate reductase (DHFR) is an NADPH dependent enzyme which catalyzes the reduction of dihydrofolate to tetrahydrofolate. The protein is the molecular target of numerous antifolate compounds such as methotrexate (MTX) (Fig. 1), a widely used anticancer drug which acts as a competitor to dihydrofolate. We have previously determined, by isothermal microcalorimetry, the thermodynamic parameters of the interaction of MTX with DHFR without NADPH (binary system) and in the presence of NADPH (ternary system) [1].

Trimetrexate (TMQ) (Fig. 1) is a second-generation non classical DHFR inhibitor which overcomes MTX-transport resistance. We recently compared the thermodynamic behavior of TMQ and MTX towards DHFR [2]. The aim of the present work was to study these interactions at different temperatures in the range 10–37°C, with and without NADPH. Indeed,

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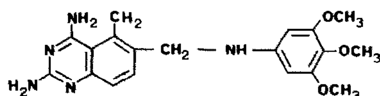

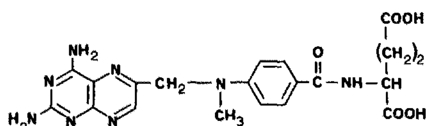

Fig. 1. Structural formulas of trimetrexate and methotrexate.

DHFR presents poor thermal stability [3], and structural modifications appear below the physiological temperature of 37°C; this phenomenon should influence the pharmacological effect of DHFR inhibitors.

MATERIALS AND METHODS

DHFR was extracted from bovine liver and purified by affinity chromatography [4]; its purity was checked after each extraction. Its molecular weight is 22500 daltons.

TMQ was a gift of Warner Lambert Company (Ann Arbor, MI), MTX was purchased from Lederle and Specia Laboratories, and NADPH was a Sigma product.

Microcalorimetric measurements were carried out with an LKB flow microcalorimeter, the Bioactivity Monitor 2277. The reactants were mixed after thermal stabilization in two separate circuits. Reactants were pumped at a flow rate of 20 $ml\,h^{-1}$ in each circuit. The sensitivity used was 10 μW full-scale, and in these conditions the background noise was less than 0.1 μW.

Binding experiments were performed at 10, 25 and 37°C in 0.1 M phosphate buffer, pH 6.80. Before the outset of each experiment, the baseline was recorded by pumping ligand buffer solutions into the first circuit and pumping the buffer alone into the second one. When the baseline was stable, 1 ml of 10^{-5} M protein solution was introduced into the buffer flow, and heats of binding were determined by integration or by deviation measurements. The calorimeter was periodically calibrated both electrically and chemically.

The affinity of the antifolate compounds for DHFR was so high that saturation of the protein was reached when the ligand/DHFR concentration ratio was higher than 5, with a protein concentration of about 10^{-5} M. Experimental heat quantities obtained at saturation were expressed per

mol of DHFR, and were equal to the enthalpy variation (ΔH). They were, if necessary, corrected for protein dilution and buffer ionization heat values.

To determine association constants (K), we measured heat quantities for different ligand/protein concentration ratios. Results were calculated according to an iterative method [5] which allows determination of $1/K$ with great accuracy when

$$100 \times \left(\begin{array}{c} \text{protein} \\ \text{concentration} \end{array} \right) > 1/K > \left(\begin{array}{c} \text{protein} \\ \text{concentration} \end{array} \right) / 100$$

The higher the affinity constant, the easier it is to determine ΔH values.

If $1/K$ is lower than the ratio (protein concentration)/100, it is still possible to determine the K value by microcalorimetry if the studied inhibitor has another ligand competitor for the same protein binding site, and if the K and ΔH of the second compound are known. There are many antifolate compounds for which the affinity constant is about 10^5 M^{-1}. The method consists in evaluating the K ratio by using the experimental heat measured when the ligand and its competitor bind simultaneously to the same protein binding site [6]. Moreover, the method allows the competitive effect to be demonstrated. Inhibition constant measurements were carried out by a pH-stat titration method [7]. The binding parameters in the ternary system were determined by using a NADPH concentration corresponding to DHFR saturation in the protein circuit.

From the K and ΔH values, ΔG and ΔS were calculated according to the classical thermodynamic relationships. All the results are given with their confidence interval of the mean ($p = 0.05$), and they were obtained from three to eight separate experiments.

RESULTS AND DISCUSSION

Study of complex formation at 37°C

We first measured the thermodynamic parameters of the interactions at physiological temperature. Results in the binary system (without NADPH) and in the ternary system (with NADPH) are presented in Table 1. Only the MTX K value in the binary system ($K = 1.6 \times 10^7$ M^{-1}) was directly measurable by the enthalpic titration method previously mentioned [5]. Other constants were obtained by the microcalorimetric competitive method [6] or by inhibition measurements [7].

Results show that, in the absence as well as in the presence of NADPH, TMQ and MTX show very close thermodynamic parameters. Their affinity constants at 37°C in the presence of NADPH, which is a direct reflection of their inhibitory potency, were equal. In all cases the complex formation is

TABLE 1

Thermodynamic parameters of the interaction of MTX And TMQ with DHFR at 37°C

	K (M^{-1})	ΔH (kJ mol^{-1})	ΔG (J mol^{-1})	ΔS (J K^{-1} mol^{-1})
Binary complexes				
MTX	2×10^{7}	-105 ± 4	-43 ± 5	-200 ± 10
TMQ	2×10^{8}	-97 ± 3	-50 ± 6	-152 ± 9
Ternary complexes				
MTX	3×10^{10}	-72 ± 3	-62 ± 5	-35 ± 2
TMQ	3×10^{10}	-67 ± 5	-62 ± 5	-16 ± 2

driven by favorable enthalpy variation, but the entropy variations are unfavorable.

Analyses using differential scanning calorimetry have shown that modifications by temperature of the ternary structure of proteins are generally associated with large positive variations of ΔH, ΔS and heat capacity (ΔC_p). In the binary system, the binding of TMQ and MTX to DHFR induces a conformational change of the protein, i.e., a restructuring to a more stable form.

The measured ΔH (ΔH^2_{mes}) would in fact be the sum of binding ΔH (ΔH^2_b) and the restructuration ΔH induced by the ligand (ΔH^2_r). In the same way, the measured values of ΔS (ΔS^2_{mes}) would be the sum of binding ΔS (ΔS^2_b) and restructuration ΔS induced by the ligand (ΔS^2_r). Thus, we can write

$$\Delta H^2_{mes} = \Delta H^2_b + \Delta H^2_r$$

$$\Delta S^2_{mes} = \Delta S^2_b + \Delta S^2_r$$

This hypothesis is confirmed by the fact that ΔH and ΔS negative values are lower in the ternary system; indeed, NADPH would induce a favorable DHFR conformational change which partially protects the protein from structural modifications occurring below 37°C.

In the ternary system, enthalpy and entropy variation values (ΔH^3_{mes} and ΔS^3_{mes}) may reflect a third parameter of the phenomenon: the possible interaction of NADPH with the ligands (ΔH^3_{int} and ΔS^3_{int}) because the cofactor and the antifolate compounds bind to very close protein sites. Thus we can write

$$\Delta H^3_{mes} = \Delta H^3_b + \Delta H^3_r + \Delta H^3_{int}$$

$$\Delta S^3_{mes} = \Delta S^3_b + \Delta S^3_r + \Delta S^3_{int}$$

TABLE 2

Thermodynamic parameters of the interaction of MTX and TMQ with DHFR at 10°C

	K (M^{-1})	ΔH (kJ mol^{-1})	ΔG (J mol^{-1})	ΔS (J K^{-1} mol^{-1})
Binary complexes				
MTX	3×10^8	-44 ± 3	-46 ± 5	$+7\pm2$
TMQ	6×10^9	-23 ± 3	-53 ± 7	$+106\pm10$
Ternary complexes				
MTX	3×10^{10}	-58 ± 3	-58 ± 5	0 ± 2
TMQ	3×10^{10}	-45 ± 5	-57 ± 5	$+42\pm4$

Study of complex formation at 10°C

To confirm our hypothesis concerning protein restructuration, the same interactions were studied at 10°C. One can consider that at 10°C DHFR remains in its native form: the protein has not undergone thermal denaturation. Results obtained at 10°C are presented in Table 2.

The positive values of entropy at 10°C would correspond to hydrophobic interactions; these hydrophobic interactions were more pronounced for TMQ. Indeed, this compound is hydrophobic whereas MTX is hydrophilic.

Hence the highly negative ΔS values obtained at 37°C in the binary system would correspond to DHFR refolding induced by the ligands. The lower difference observed in the ternary system may reflect that NADPH protects DHFR from thermal unfolding.

Similarly, if we assume that between 10 and 37°C the value of ΔH of binding in the binary system ΔH_b^2 cannot vary sharply, then the difference between ΔH_{mes}^2 at 37°C and ΔH_{mes}^2 at 10°C may reflect the refolding of DHFR induced by the drug (ΔH_r^2). This difference was lower in the ternary system (ΔH_r^3), proving once again the protective effect of NADPH. Thus, the difference ($\Delta H_{mes}^2 - \Delta H_{mes}^3$) at 10°C can be attributed only to the ligand/NADPH interaction (ΔH_{int}^3).

Similarly, one could calculate all these parameters in the case of entropy variations.

Measurements of heat capacity variations

To investigate further the temperature corresponding to DHFR thermal transition, we measured MTX and TMQ ΔH values at 25°C with and without NADPH. Figure 2 presents the variations of ΔH as a function of temperature.

From these results, we determined ΔC_p variations between 10 and 25°C, and between 25 and 37°C (Table 3). They indicate that the stabilizing effect

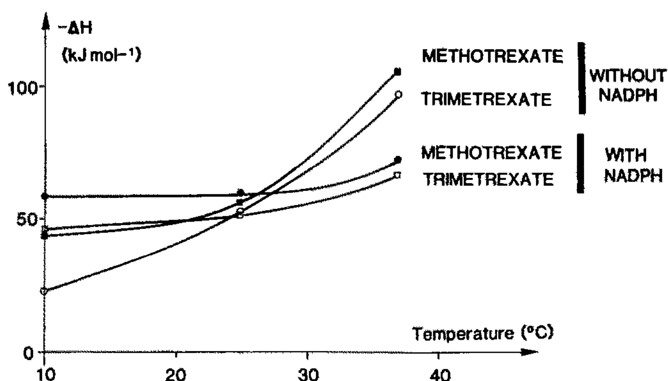

Fig. 2. Enthalpy variation of methotrexate and trimetrexate as a function of temperature.

TABLE 3

Heat capacity (kJ mol^{-1} K^{-1}) of the DHFR-ligand complexes

	10-25°C		25-37°C	
	Binary system	Ternary system	Binary system	Ternary system
MTX	-1	0	-4	-1.2
TMQ	-2	-0.5	-4	-1.2

of NADPH on the thermal transition of DHFR is clearly more pronounced in the 25-37°C temperature range. The large negative ΔC_p values show a change induced by the antifolate drugs in the protein structure to reach a more stable conformation.

CONCLUSIONS

Important modifications of the ternary structure of DHFR have been demonstrated. A partial unfolding appears at a temperature inferior to 37°C, and it is less pronounced when the protein is in the presence of its cofactor NADPH. These changes may occur near 37°C, and thus temperature variations in the physiological temperature range may influence the pharmacological behavior of DHFR.

This work will be continued in the 35-37°C temperature range by isothermal and differential scanning calorimetry with different DHFR because the thermal behavior of this protein is certainly dependent on the enzyme source.

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