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MICROCALORIMETRIC STUDY OF THE BINDING OF METHOTREXATE AND ITS METABOLITES TO THYMIDYLATE SYNTHASE

A new method for affinity constant determination

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Direct microcalorimetric measurements allow determination of both the ΔH and association constant of biological complexes if K_a value does not exceed $10^6\ M^{-1}$. For higher K_a values, ΔH can obviously be determined; this paper describes an original microcalorimetric method that permits determination of such high association constants. This method is based on the analysis of the competitive effect between two ligands having the same binding site in their receptor. As an example, the affinity constant for thymidylate synthase of a novel antifolate, CB 3717, was found to be $1.4 \cdot 10^7\ M^{-1}$ using methotrexate polyglutamate MTX-G2 ($K_a = 2.3 \cdot 10^5\ M^{-1}$) as competitor.

Introduction

Various microcalorimetric methods have been set up to determine the thermodynamic parameters of interactions between drugs and their target proteins. If the affinity of the ligand is not too high as compared to experimental protein concentration, a direct enthalpy titration method is available [1]. When this method is not suitable, and when the affinity of the ligand is high enough, one can use a technique based on the competitive effect between the studied compound and another compound whose binding parameters are known [2]. We present here a microcalorimetric method that allows association constant determination when the affinity of the ligand is not compatible with the two methods mentioned above.

We chose as an example the interaction between methotrexate and its metabolite methotrexate–G2, and thymidylate synthase. This drug is one of the most widely used anticancer agents, and acts by a tight-binding inhibition of

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dihydrofolate reductase [3]. Methotrexate can also inhibit thymidylate synthase [4], an enzyme essential for DNA biosynthesis.

The purpose of our study was to compare the affinity of methotrexate and methotrate–G2 for thymidylate synthase to that of a novel thymidylate synthase inhibitor, 10–propargyl–5,8–dideazafolate (CB 3717) [5], in the absence and in the presence of 5–fluoro–2'–deoxyuridine 5'–monophosphate (FdUMP), the specific inhibitor of the enzyme.

Materials and methods

Thymidylate synthase was extracted form a methotrexate-resistant strain of *Lactobacillus casei* [6] and purified according to the procedure described by Lyon *et al.* [7].

Microcalorimetric measurements were performed with a flow calorimeter apparatus (LKB Bioactivity Monitor 2277) at 37° in 0.1 *M* phosphate buffer, $pH = 7.40$. The phosphate buffer was chosen as an experimental medium in order to avoid possible interference by proton exchange which may occur during the binding; indeed, phosphate buffer has a very low heat of ionization.

Before the outset of each experiment, the baseline was established by pumping a ligand buffer solution into the first circuit of the calorimeter at a flow rate of 20 $ml \cdot h^{-1}$, and pumping the buffer alone into the second circuit at the same flow rate. The sensitivity used was 10 μW full-scale, with a background noise of 0.1 μW. The enzyme (2 to $3 \cdot 10^{-5}$ *M*) was dialyzed against the experimental buffer, and 1 or 2 ml were injected into the buffer flow, while the ligand solution was pumped into the second circuit. Whenever necessary, data were corrected from dilution and neutralization heat values.

The experimental heat quantities were corrected in the event of heat of dilution, and were expressed per mole of proteine (Q). These measurements are used to determine the enthalpy variation (ΔH), the association constant (K_a), and consequently the free energy variation (ΔG) and the entropy variation (ΔS) of the formed protein-drug complexes (1).

The simplest equilibrium complex, with stoichiometry equal to one between a protein (P) and a binding ligand (L) can be written:

$$P + L \rightleftarrows PL$$

If P_t and L_t represent the total protein and ligand concentration respectively, and (PL) the complex concentration at equilibrium, the expression of the formation equilibrium constant is:

$$K_a = \frac{(PL)}{(P_t - (PL)) \cdot (L_t - (PL))} \quad (1)$$

If L_i is a variable of the total ligand concentration L_t, and (PL_i) the corresponding complex concentration, the experimental heat measurement Qi $(J \cdot l^{-1})$ can be written:

$$Q_i = \Delta H \cdot (PL_i) \quad (2)$$

The mathematical expression of the theoretical heat saturation curve $Q_i = f(L_i)$, with P_t constant, is obtained from relationships (1) and (2):

$$Q_i = \frac{1/K_a + L_i + Pt - \sqrt{(1/K_a + L_i + P_t)^2 - 4 \cdot L_i \cdot P_t}}{2} \Delta H \quad (3)$$

The ΔH of K_a can be calculated from 4 or 5 experimental Q_i values obtained at constant P_t and variable L_i (with and L_i range of P_t to $10 \times Pt$). The K_a, ΔH, and if need be, stoichiometry values are determined by an iterative mathematical treatment of this experimental enthalpic titration curve [8].

The upper limit for the determination of K value is about $10^6 M^{-1}$ in our experimental conditions. If, however, the association constant is higher than this upper limit value, enthalpy variation and stoichiometry value can still be measured very accurately by this method.

Ternary system experiments were performed with a FdUMP concentration 10–fold higher than that of thymidylate synthase.

If the affinity constant K_1 of a ligand L is too high to be directly determined with the previously described method, one can calculate it by the use of a competitive effect between the ligand and a competitor C whose association constant (K_2) and enthalpy variation ΔH_2 can be measured separately. This original method can be summarized as follows:

$$\begin{array}{ccc} P + L & \rightleftarrows & PL \\ + & K_1 & \\ C & & \\ \downarrow\uparrow K_2 & & \\ PC & & \end{array}$$

We can thereby determine K_1 if K_2 is known. Let us call:

P_t the total protein concentration, L_t the total concentration of the ligand L, C_t the total concentration of the competitor C, PL the concentration of the complex protein-ligand at equilibrium, PC the concentra-

tion of the complex protein-competitor at equilibrium, ΔH_1, ΔH_2, K_1 and K_2 the enthalpy variations and association constants of the complexes PL and PC respectively

$$K_1 = \frac{PL}{(P_t - PL - PC)\cdot(L_t - PL)} \tag{4}$$

$$K_2 = \frac{PC}{(P_t - PL - PC)\cdot(C_t - PC)} \tag{5}$$

The heat. Q in $J\cdot l^{-1}$, exchanged when L and C bind simultaneously to the protein can be written:

$$Q = PL \cdot \Delta H_1 + PC \cdot \Delta H_2 \tag{6}$$

From this relationship, one can obtain PL:

$$PL = \frac{Q - PC\cdot\Delta H_2}{\Delta H_1} \tag{7}$$

K_2 can be written:

$$K_2 = \frac{PC}{\left(P_t - \frac{Q - PC\cdot\Delta H_2}{\Delta H_1} - PC\right)\cdot\left(C_t - PC\right)} \tag{8}$$

and thus:

$$(PC)^2\left(1 - \frac{\Delta H_2}{\Delta H_1}\right) + PC\left(\frac{\Delta H_2\cdot C_t}{\Delta H_1} - P_t + \frac{Q}{\Delta H_1} - C_t - \frac{1}{K_2}\right) + C_t\left(P_t - \frac{Q}{\Delta H_1}\right) = 0 \tag{9}$$

Only one PC value is compatible, and this value allows PL, and then K_1 to be determined.

Results and discussion

We studied the interaction between thymidylate synthase and methotrexate, methotrexate–G2 and 10–propargyl–5,8–dideazafolate (CB 3717) in binary system (enzyme/inhibitor) and in the presence of FdUMP (ternary system). Results are presented in Table 1.

Table 1 Enthalpy variation (ΔH, in $kJ \cdot mol^{-1}$) and association constant (K_a, in M^{-1}) of the thymidylate synthase/inhibitor complexes

Inhibitor	Binary system		With FdUMP	
	ΔH	K_a	ΔH	K_a
Methotrexate	undetectable	undetectable	–45±2	$9.1 \cdot 10^3$
Methotrexate–G2	–23±3	6 600	–61±2	$2.3 \cdot 10^5$
CB 3717	undetectable	undetectable	–93±3	$1.4 \cdot 10^7$

Our results show that only methotrexate–G2 interacts directly with thymidylate synthase, while both methotrexate and 10–propargyl–5,8–dideazafolate require the presence of FdUMP to bind to the target enzyme. Moreover, in ternary system all the studied compounds exhibit a large exothermic effect when they bind to thymidylate synthase.

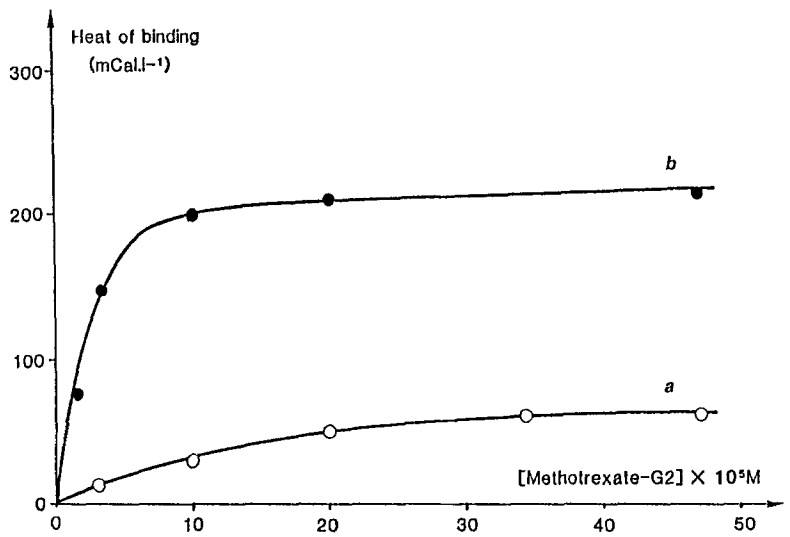

Fig. 1 Fractional saturation heat curves of methotrexate–G2 at 37°C, in 0.1 *M* phosphate buffer *pH* 7.40:, a) in binary system, b) in the presence of FdUMP

If one takes into account the above mentioned restrictions, only methotrexate and methotrexate–G2 affinity constants are directly measurable by the enthalpic titration method. Figure 1 shows the fractional saturation heat curves obtained for methotrexate–G2 in binary and ternary systems.

The binding heats of interaction between various inhibitor concentrations and a fixed thymidylate synthase quantity (1 ml $3.0 \cdot 10^{-5}$ *M*) were analyzed according to the enthalpic titration method.

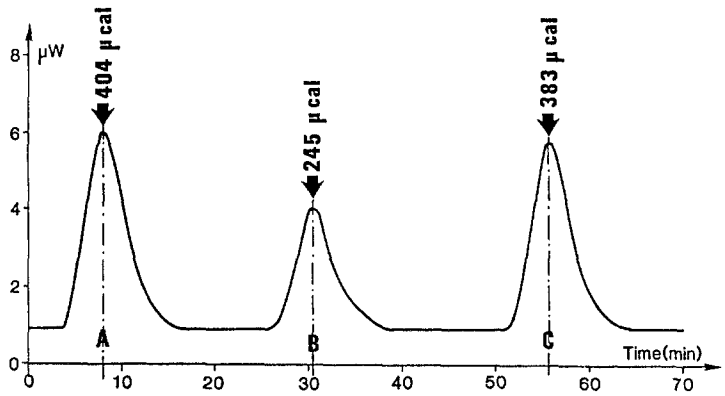

Fig. 2 Microcalorimetric experimental recording of competition between methotrexate–G2 and CB 3717 for thymidylate synthase binding site. Interaction of 1 ml of thymidylate synthase $0.895 \cdot 10^{-5}$ *M* with:, A: CB 3717 $0.965 \cdot 10^{-5}$, B: methotrexate–G2 $1.087 \cdot 10^{-4}$ *M* C: CB 3717 $0.965 \cdot 10^{-5}$ *M* and methotrexate –G2 $1.087 \cdot 10^{-4}$ *M*. Concentrations are the final ones in the reaction medium ($V = 2$ ml)

To determine CB 3717 association constant, we used the competitive method. A competition between this compound and methotrexate–G2 for the thymidylate synthase binding site was analyzed (Fig. 2). This experiment proves that CB 3717 is a competitor of methotrexate–G2 for the thymidylate binding site; a K_a ratio of 60 in favour of CB 3717 was obtained. Thus, with the precedently measured K_a for methotrexate–G2, we were able to determine the association constant for CB 3717: $K_a = 1.4 \cdot 10^7\ M^{-1}$.

In conclusion, the microcalorimetric method presented in this paper allowed the determination of the association constant for the complexes thymidylate synthase-inhibitors. This original competitive method complements two other microcalorimetric methods described precedently (1, 2) in the field of protein-ligand interaction studies.

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Zusammenfassung – Direkte mikrokalorimetrische Messungen erlauben, sowohl die Bildungsenthalpie ΔH wie auch die Gleichgewichtskonstante K_a biologischer Komplexe zu bestimmen, sofern der K_a-Wert $10^6 M^{-1}$ nicht übersteigt. Für höhere K_a-Werte kann ΔH durchaus direkt ermittelt werden. In dieser Arbeit wird eine neuartige mikrokalorimetrische Methode beschrieben, die auch eine Bestimmung sehr hoher K_a-Werte zulässt. Diese Methode fusst auf der Analyse eines kompetitiven Effekts zweier Liganden, welche die gleiche Bindungsstelle am Rezeptor besetzen können. Als Beispiel wird K_a für die Bindung eines neuen Folsäureantagonisten, CB 3717, an Thymidylat Synthase, unter Verwendung von Methotrexat Polyglutamat MTX-G2 als kompetitiver Ligand, bestimmt.