# NEW BIOMEDICAL TECHNOLOGIES

# Thermodynamic Parameters of Binding of Mouse 2G3 Antitheophylline Monoclonal Antibodies to Theophylline

**Yu. N. Pokid'ko and R. G. Vasilov**

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The binding of 2G3 mouse antitheophylline monoclonal antibodies (affinity constant $K_{aff}=2\times10^{10}$ liter/mol) to theophylline is studied using isothermal titration microcalorimetry. Thermodynamic parameters of the binding are: enthalpy change $\Delta H=-0.23$ kcal/mol; Gibbs free energy change $\Delta G=-16.42$ kcal/mol; entropy change $\Delta S=0.054$ kcal/(mol×K). Regression analysis shows a two-site kinetic binding model. A great contribution of entropy component to the free energy change of 2G3 antibody binding to theophylline is indicative of an entropy-dependent process. The entropy-dependent nature of the binding presumably determines the binding kinetics.

**Key Words:** *monoclonal antibodies; theophylline; binding; microcalorimetry*

Isothermal titration microcalorimetry allows one to record directly the heat effects of the antigen-antibody binding [7] and to calculate thermodynamic parameters of this process [21]. Compared to other methods based on determination of the concentration of free and bound antibodies or antigens, the thermodynamic method provides more information on physical mechanisms underlying the binding. Each microcalorimetry experiment makes it possible not only to measure directly the enthalpy of the reaction, but also to calculate thermodynamic parameters of the binding, namely, free energy change ($\Delta G$) and entropy change ($\Delta S$) [21].

High specificity of antigen-antibody binding is due to complementary architecture of the antigen binding site (paratope) and antigenic determinant (epitope). This complementarity of physical and chemical properties of the interacting surfaces is responsible for the formation of hydrogen, electrostatic, and hydrophobic bonds. These bonds are characterized by the following binding energies: electrostatic interaction 5 kcal/mol, hydrogen bond 4 kcal/mol, hydrophobic interaction 4 kcal/mol, and van der Waals interaction 1 kcal/mol [17]. The maximum contribution to the antigen—antibody interaction is made by van der Waals interactions (about 90% of the total numbers of contacts); hydrogen bonds and ionic interactions between polar groups contribute 8-10 and 0-2%, respectively [15]. Only 20-23% of the total antigen-binding surface formed by hypervariable domains of an antibody and only 25-40% amino acid residues in these regions participate in antigen binding [15].

It was assumed that the antigen—antibody interactions are primarily hydrophobic [2,12-14,16,22]. Hydrophobic interaction can be accompanied by both positive [12,16,22] and negative [1,14,19,20,23] entropy change.

Other investigators showed that the antigen-antibody interaction is an enthalpy-dependent process with an opposite entropy contribution [6,10,18]. Microcalorimetric study of interactions between antigen and antibodies carrying point mutations in the

*Lekbiotekh* Research and Technical Center, Moscow

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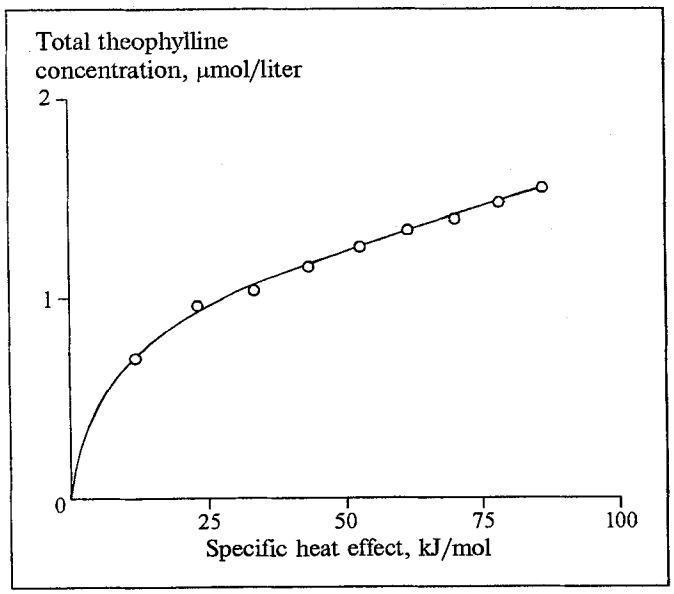


**Fig. 1.** Exothermic heat effect of the binding of 2G3 antitheophylline monoclonal antibodies to theophylline as a function of total theophylline concentration (circles) and the most realistic two-site binding model determined by nonlinear regression analysis (curve).

hypervariable Fv domains have demonstrated that enthalpy changes promoting the binding are compensated by unfavorable entropy changes [3,8,9]. Association of these antibodies with antigens is accompanied by van der Waals interactions and hydrogen bond formation [3].

Enthalpy of the antigen—antibody coupling can be directly measured by titration microcalorimetry [7], while $\Delta G$ and $\Delta S$ for this interaction can be calculated from the enthalpy values obtained in microcalorimetric experiments at two different temperatures. The free energy change can be estimated from the association constant ($K_{ass}$) by the formula:

$$\Delta G = -k \times T \times \ln(K_{ass}), \quad (1)$$

where $k$ is the Boltzmann constant, i.e., $1.38 \times 10^{-23}$ J/K, and $T$ is the absolute temperature (°K). $\Delta G$ can be expressed in $kT$ units per a pair of interacting molecules at 20°C (293 K): 1 $kT = 4.04 \times 10^{-21}$ J or in $RT$ units ($R = N \times k$, where $N$ is the Avogadro constant, $6.02 \times 10^{23}$ mol$^{-1}$ and $R$ is the molar gas constant 8.314 J/K=1.9865 cal/K) [21] and calculated per mol.

The left part of equation (1) is a dimensionless value. This requires an appropriate transformation of the right part of this equation. To this end the usual dimension of $K_{ass}$ liter/mol is converted into (mol×fraction)$^{-1}$ units [21], taking into account a parameter of liquid medium, 55.56 mol $H_2O$/liter. Thus, for interaction in an aqueous medium:

$$1 \text{ mol/liter} = 1/55.56 \text{ mol} \times \text{fraction}^{-1} = 0.018 \text{ mol} \times \text{fraction},$$

i.e., 1 liter/mol = 55.56 (mol×fraction)$^{-1}$ [21].

Entropy change upon binding can be calculated from enthalpy ($\Delta H$) and free energy ($\Delta G$) changes using the following formula:

$$\Delta G = \Delta H - T \times \Delta S, \quad (2)$$

where $T$ is absolute temperature, K.

**TABLE 1.** Parameters of the Maximum Realistic Model of the Binding of 2G3 Antitheophylline Monoclonal Antibodies to Theophylline

| Parameter | Value | Statistical error | 95% confidence interval |
|---|---|---|---|
| $B_{max1}$, J/mol | $9.8 \times 10^2$ | $8.1 \times 10$ | $7.8 \times 10^2$ $1.2 \times 10^3$ |
| $K_{diss1}$, mol/liter | $6.8 \times 10^{-6}$ | $1.7 \times 10^{-6}$ | $2.7 \times 10^{-6}$ $1.1 \times 10^{-5}$ |
| $B_{max2}$, J/mol | $3.9 \times 10^9$ | not measured | |
| $K_{diss2}$, mol/liter | $5.3 \times 10^{-1}$ | not measured | |

**Note.** Fitness criteria for the chosen two-site binding model: number of degrees of freedom=6, coefficient of correlation $R^2$=0.9986, absolute sum of deviation squares=2640, Sy.x=20.98.

## MATERIALS AND METHODS

*Preparation of antibodies.* Mouse antitheophylline monoclonal 2G3 antibodies, $IgG2_\alpha$ isotype, were obtained and purified as described previously [5]. The purity of the antibodies was tested by electrophoresis in polyacrylamide gel. Affinity constant ($K_{aff}$) was determined by enzyme-linked immunoenzyme assay [4,5].

*Thermodynamic study of the binding of monoclonal antitheophylline antibodies with theophylline using step-by-step titration isothermal microcalorimetry.* A Thermal Activity Monitor (TAM) LKB-2277 microcalorimeter (LKB) was used. Theophylline solution was pumped with a Hamilton MicroLab micropump (LKB).

Purified monoclonal antibodies (1 ml, 20 mg/ml) were dialyzed against phosphate buffered saline containing 0.1% sodium azide first at 4°C for 6 h and then at 25°C for 6 h. Theophylline (Sigma) was dissolved in the last portion of dilapidate (80 μg/ml). The solution was vigorously agitated in a vortex shaker and incubated at 25°C for 6 h (temperature of the working zone of the microcalorimeter) with slow stirring. The initial concentrations of antibodies and antigens were measured spectrophotometrically at 280 nm. One milliliter of dialyzed antibody solution was placed into the working cell of the microcalorimeter; the cell being weighted before and after filling. The pumps were calibrated before each experimental series, the cell being weighted before and after each experimental series.

*Chemical calibration of the microcalorimeter.* A standard reaction for chemical calibration of this type of microcalorimeters was as follows:

solution 1: 1.1 mM Tris and 0.6 mM Tris-HCl in 0.1 M KCl;
solution 2: 0.11 mM HCl in 0.1 M KCl.

Mixing of these solutions at an optimal rate of 1 μl/sec (determined for the given device from the signal/noise ratio) yields a thermal effect of 50 mcal [11].

For chemical calibration of the given microcalorimeter 1 ml of solution 1 was placed into the working cell, while solution 2 was placed into the pumping system for the titration solution. After thermal equilibrium in the working area had been reached, the pump was turned on, and solution 2 (1 ml) was infused into the cell filled with solution 1 at a rate of 1 μl/sec. A 300-fold amplified output signal was recorded at a rate of 1 record per sec. The same interrogation rate was used in all subsequent experiments.

During chemical calibration the microcalorimeter recorded the liberation of 6 mJ, which corresponded to 1.43 mcal. Thus, the transmission coefficient was 34.865.

After thermal equilibrium has been attained and the power of the output signal had become stable, the recorded signal was set to zero and the device was switched to computer-operated mode according to the previously designed algorithm.

During step-by-step titration the baseline signal was recorded for 120 min, after that 30 μl theophylline was injected into the cell containing the antibody solution at intervals of 90 min and a rate of 1 μl/sec. This volume was chosen on the basis of constructive features of the microcalorimeter, errors due to pump functioning, the number of experimental points, and the experiment duration. When selecting the rate of pumping, the signal/noise ratio for the calorimeter and working characteristics of the pump were taken into account. The interval between the consecutive theophylline injections depended on technical characteristics of the microcalorimeter and was determined by the relaxation time, thermal processes of binding, and the rate of attaining quasistationary state of the studied process.

Molar concentrations of antibodies and theophylline were recalculated for dilution.

*Processing of thermograms.* For each step the heat was determined by standard methods by linear regression of the initial and final baselines.

The following binding models were considered: one-site model, two-site binding models (for identical independent and for nonidentical/linked binding sites), dose—effect dependence with and without a slope (Hill curves), competition of two binding sites, mono- and two-phase exponential decay, mono- and two-phase exponential association, exponential growth, and sigmoidal (Boltzmann) dependence. The parameters of analyzed models were evaluated statistically (at a probability of 95%) using nonlinear regression analysis with the asymptotic interval estimation. This approach has been extensively employed for rigorous and statistically substantiated choice of the model and calculation of its parameters. The models were compared without preliminary assumption and speculations regarding the test model and in accordance with the usual hierarchy of statistical criteria for choosing the best fitting model from models with different numbers of variables.

*Calculation of thermodynamic characteristics of antigen-antibody binding.* Using the most realistic model of binding kinetics, the enthalpy change ($\Delta H$) was determined at 25°C (298 K, temperature of the working cell). $K_{ass}$ was equal to $K_{aff}$ measured previously [5], and the corresponding value of free energy change ($\Delta G$) was calculated using equation (1) [17]. Entropy change was calculated from formula (2).

## RESULTS

The total exothermic effect of theophylline binding by antitheophylline monoclonal antibodies 2G3 as a function of theophylline concentration and the curve for the maximum realistic model are shown on Fig. 1 and the fitness criteria are presented in Table 1.

The model termed as a two-site binding model is described by the following equation derived from the mass action law:

$$Y=B_{max1}\times X/(K_{diss1}+X)+ B_{max2}\times X/(K_{diss2}+X), \quad (3)$$

where $X$ is the total theophylline concentration (mol/liter), $B_{max1}$ and $B_{max2}$ are the maximum bindings at the first and second antigen-binding sites (J/mol), respectively, and $K_{diss1}$ and $K_{diss2}$ are the total concentrations of theophylline required for the half-maximum binding (mol/liter). This model is characteristic of a two-site binding. Moreover, when the system described by the above model attains a quasistationary equilibrium, a kinetic equilibrium is established between free antigen molecules and antibodies and the $(\text{antibody—antigen})_2$ complexes, the concentration of the $(\text{antibody—antigen})_1$ complexes tends to zero.

From the data presented in Table 1 and the equation $K_{ass}=K_{aff}=2\times10^{10}$ liter/mol$=1.1\times10^{12}$ (mol$\times$ fraction)$^{-1}$ [5] free energy change $\Delta G$ was estimated [21]:

$$\Delta G\approx-16.42 \text{ kcal/mol}=-27.74\ kT \text{ units.}$$

The experimental heat effect of theophylline binding by antitheophylline monoclonal antibodies 2G3 was:

$$\Delta H=-0.98 \text{ kJ/mol}=-0.23 \text{ kcal/mol.}$$

The positive heat effect of binding results in a decrease of the heat energy of the system and therefore the enthalpy change in this system is negative.

Using the obtained values of free energy and enthalpy changes, we calculated the entropy change for binding at 25°C (298 K) from equation (2):

$$\Delta S=(\Delta H-\Delta G)/T=0.054 \text{ kcal/(mol}\times\text{K)}=0.227 \text{ kJ/(mol}\times\text{K)}.$$

Thus, association of mouse antitheophylline monoclonal antibodies 2G3 with theophylline is accompanied by negative enthalpy change and positive entropy change. A considerable contribution into Gibbs free energy change is made by entropy change, which probably determines the binding kinetics.

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