Thermochimica Acta, 147 (1989) 119–126
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

A MICROCALORIMETRIC METHOD USING A MINIMAL QUANTITY OF REACTANT TO DETERMINE PROTEIN–LIGAND BINDING PARAMETERS

J.C. SARI, R. GILLI, V. PEYROT and C. BRIAND

Laboratoire de Physique Pharmaceutique, Faculte de Pharmacie, Bd Jean Moulin, 13005 Marseille (France)

(Received 11 November 1988)

ABSTRACT

The experimental conditions and equipment as well as the mathematical treatments of the results obtained with a novel isothermal flow microcalorimetric method which, with only a very small quantity of reactant, allows determination of the binding parameters of biological complexes are described. The apparatus is an LKB Bioactivity Monitor equipped with an original closed circuit system.

For example, to determine the binding parameters (association constant, enthalpy variation and stoichiometry values) of a protein–ligand complex, it took only 40 min and 2 ml of 10^{-5} M protein solution, when the enthalpy variation was 40 kJ mol^{-1}. It would have taken 10 ml of 10^{-5} M protein and 100 min of experimentation to obtain these results, with the same accuracy, by a classical method.

INTRODUCTION

Twenty years ago, isothermal microcalorimetry was already being used to determine the thermodynamic parameters (stoichiometry, association constant and enthalpy variation) of complexes of fundamental biological interest, e.g. adenosine triphosphate–magnesium [1].

However, the lack of sensitivity of Calvet's isothermal differential fluxmeter [2] made study of protein–ligand interaction impossible because 10^{-3} M reactant solutions were usually required to record an accurate thermic signal. Such protein concentrations were not compatible with their solubility and/or the difficulty in obtaining several milligrams of these expensive macromolecules.

During the last decade a new generation of fluxmeters has appeared. The isothermal aluminium block of Calvet's apparatus, into which the thermopiles were inserted, has been replaced by an air or water bath with a temperature control of 10^{-4} °C. The electrical background noise was there-

0040-6031/89/$03.50

fore significantly diminished and the thermic signal could be amplified about 100-fold.

Today, there are three fundamental types of fluxmeters: batch, perfusion and flow microcalorimeters, which differ in their reactant mixing systems [3]. The sensitivity of each type is equivalent. The recent LKB Bioactivity Monitor (BAM), a modified Wadso apparatus [4], allows recording of a heat flow lower than 1 ± 0.05 μW. However, if the reaction is instantaneous, the flow apparatus is better because batch or perfusion systems are time consuming (4–8 h) in obtaining a thermic equilibrium of reactants before their mixing. This is incompatible with the use of thermolabile macromolecules. With a flow system, at a flow rate of 20 ml h^{-1} for each pump, about 15–20 min is sufficient to run an experiment.

To measure protein–ligand affinity the sensitivity of microcalorimetry is comparable to that of other techniques such as UV spectroscopy, NMR, fluorescence, ORD and potentiometric titration. About 500 articles have been published on this subject. However, to promote microcalorimetry in the field of biochemistry, immunology or pharmacology, it is indispensable to further reduce the quantity of reactant used in each experiment.

CLASSICAL MICROCALORIMETRIC METHOD

The BAM flow apparatus is widely used to simultaneously determine stoichiometry, the formation association constant (K), the enthalpy variation (ΔH) and, consequently, the free enthalpy variation (ΔG) and entropy variation (ΔS) of biological complexes as different as protein–substrate [5], protein–drug [6] or protein–protein [7].

The most simple equilibrium complex, with stoichiometry equal to one between a protein (P) and a binding ligand (L) can be written $P + L \rightleftharpoons PL$.

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

$$K = \frac{(PL)}{[P_t - (PL)][L_t - (PL)]} \tag{1}$$

If L_i is a variable of the total ligand concentration L_t, and (PL_i) is the corresponding complex concentration, the experimental heat measurement

$$Q_i(\mathrm{J\,l^{-1}}) = \Delta H(PL_i) \tag{2}$$

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)

$$Q_i = \frac{1/K + L_i + P_t - \sqrt{(1/K + L_i + P_t)^2 - 4L_iP_t}}{2}\Delta H \tag{3}$$

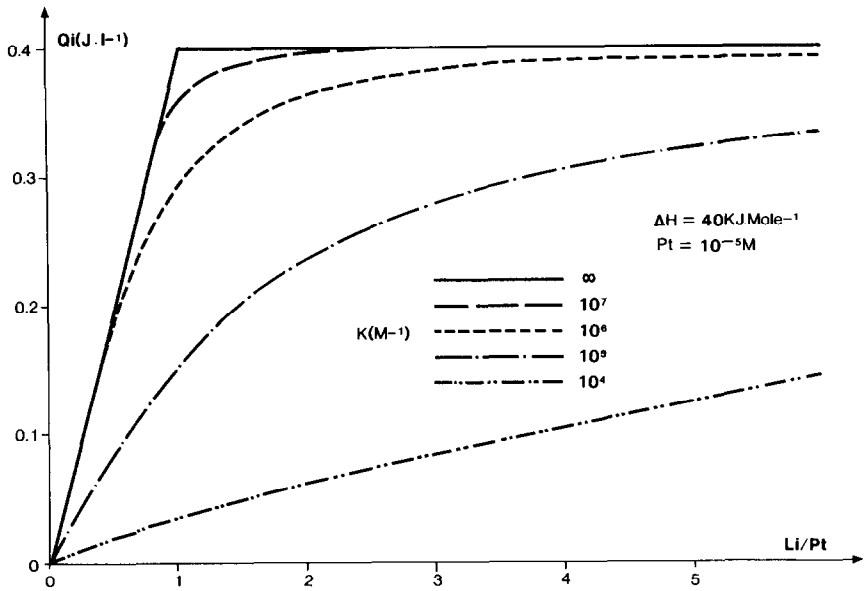


Fig. 1. Theoretical enthalpic titration curves.

Theoretical enthalpic titration curves $Q_i = f(L_i/P_t)$ can be drawn for different K values and constant P_t and ΔH values (Fig. 1).

EXPERIMENTAL PROCEDURE

Materials and methods

An LKB Bioactivity Monitor (BAM) flow apparatus equipped with a double circuit was used. A sensitivity of 10 μW/full scale was generally used. Mixing was effected by constant reactant flow into the "Y" mixing junction. The volume of the calorimetric cell was about 0.6 ml. The total volume of one circuit, including pump tube and UV spectroscopic cell, was 2 ml. Reactants were equilibrated to the temperature of the thermostated bath during their passage in thermic exchangers. Maximum flow rate for each pump (V_1 and V_2) was 20 ml h^{-1} to obtain a perfect temperature equilibrium of reactants before their mixing. When mixing time is longer than 6 min, a steady state thermogram was recorded. The heat flux (Δ) is proportional to the heat of reaction (Q) and to the total flow rate: $Q = \Delta/(V_1 + V_2)$.

First phase

Ligand was continuously pumped in circuit 1 at a flow rate of 20 ml h^{-1}. A buffered electrolyte support solution identical with those containing ligand or protein was pumped in circuit 2 at the same flow rate. The heat flux of this base line corresponded to the heat of dilution of ligand solution.

Second phase

The buffered electrolyte support solution in circuit 2 was replaced by a protein solution. The initial protein solution was in a range between 10^{-5} and 5×10^{-5} M, depending on the ΔH value. It was necessary to pump protein solution (2 ml) for at least 6 min to obtain a constant heat flux (Δ). The heat of dilution of protein solution must be determined in a separate experiment without ligand in circuit 1.

Third phase

The buffered electrolyte support solution was again pumped into circuit 2 until the initial base line was obtained. About 5 ml of ligand solution was used for each ligand concentration.

To calculate ΔH, K and, consequently, ΔG and ΔS for all binding reactions it is sufficient to determine 4 or 5 Q_i experimental values with a constant P_t and a different L_i in the range $L_i = P_t$ to $L_i = 5\ P_t$. The experimentation time required to obtain an experimental enthalpic titration curve was about 100 min.

Mathematical treatment

K, ΔH and, if needed, stoichiometry values were calculated by an iterative mathematical treatment of this experimental enthalpic titration curve [8]. The interest of the iterative enthalpic titration method lies in the fact that only the K value is optimized, the ΔH and stoichiometry values being deduced from the best K value.

The following procedure is used: assuming a stoichiometry value for the complex, a first K value is chosen arbitrarily. Using relationship (1), a complex concentration (PL_i) can be calculated for each ligand concentration value L_i. Then, it is possible to express a linear regression

$$Q_i = A(PL_i) + R \tag{4}$$

The Q_i value for $(PL_i) = 0$ is called the residue R. The correlation coefficient r and the R value for such a straight line are generally very different from 1 and 0 respectively.

The K values are computer-iterated until the correlation coefficient and the residue value tend toward 1 and 0 respectively. The K value corresponding to the best correlation coefficient and the lowest residue is retained. As the residue value is lower than 10^{-10} J l^{-1}, relationships (2) and (4) can be identified, and the slope of the straight line is used to calculate the ΔH value.

The confidence intervals for ΔH and R values are calculated from the variances of the linear regression coefficients. The extremes of the K values are deduced from the confidence intervals for the R value.

Another complex stoichiometry is then tested to see if a better K optimization can be found. It protein and ligand purities have been verified, only integer stoichiometry values are considered.

If $\Delta H = 40$ kJ mol^{-1} and $P_t = 10^{-5}$ M, Fig. 1 shows that the upper K limit value which can be determined using microcalorimetry is about 10^7 M^{-1}. For a higher binding affinity, the ΔH and stoichiometry values can obviously be calculated with a great accuracy from the asymptote value and the abscissa of the broken line respectively.

To determine accurate K values higher than 10^7 M^{-1}, the protein concentration must be further decreased; unfortunately microcalorimetry is not yet sensitive enough to achieve this.

However, if two ligands compete for the same binding site, the constant ratio is calculable by microcalorimetric methods whatever the constant values are [6].

CLOSED CIRCUIT MICROCALORIMETRIC METHOD

Experimental procedure

Materials and methods

These are the same as above, but the flow rate in circuit 1 is slower (Fig. 2). By using the Gilson Micropulse peristaltic pump (F1) it is possible to decrease the flow rate to 50 μl h^{-1}, whereas the flow rate in circuit 2 (F2) remains 20 ml h^{-1}. At its exit, the solution is collected in a small overflow cone having numerous holes at its pointed extremity. The volume of liquid which is permanently in the cone must be lower than 20 μl to maintain homogeneous concentrations in the circuit (Fig. 2). Moreover, this cone is

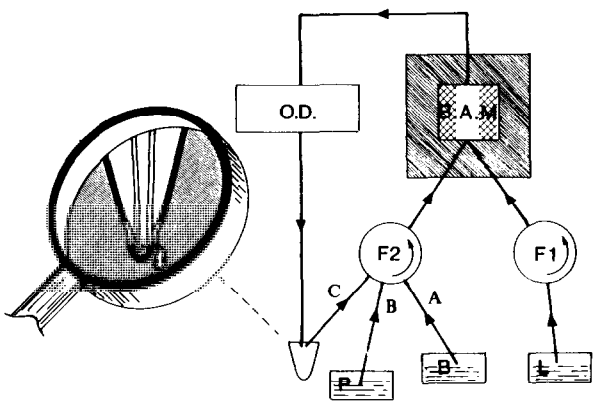
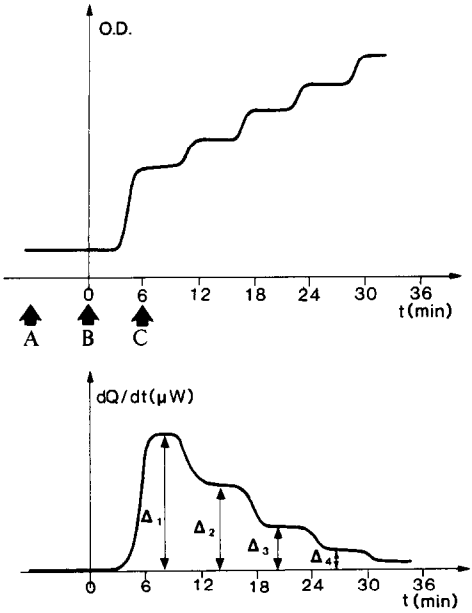


Fig. 2. The closed circuit method.



Figs. 3 and 4. Optical density variations and the corresponding heat fluxes versus time: A, ligand injection; B, protein injection; C, closed circuit.

vital in order to avoid excess pressure due to a direct junction between the entry and the exit of the circuit.

First phase

The ligand (L) is pumped into circuit 1 (flow rate V_1) and the buffered medium (B) into circuit 2 (flow rate V_2) (Fig. 2, position A). If L_0 is the initial ligand concentration value, the ligand concentration after dilution is $L_1 = L_0V_1/V_t$ with $V_t = V_1 + V_2$. The base line corresponds to heat of ligand dilution (Fig. 4).

Second phase

When a steady state of optical density (O.D.), which corresponds to the diluted ligand concentration (L_1), is recorded (Fig. 3), protein solution is pumped in circuit 2 (Fig. 2, position B). The initial protein concentration (P_0) is chosen to obtain $P_1 = P_0V_2/V_t = L_1$ after dilution. In these conditions the thermic flux is equal to Δ_1 (Fig. 4).

Third phase

After 6 min, when a new optical density steady state is obtained, circuit 2 is quickly closed at the bottom of the cone (Fig. 2, position C). In this closed circuit position, the ligand concentration increases at every loop (Fig. 3). The heat fluxes Δ_2, Δ_3, $\Delta_4, \ldots, \Delta_n$ recorded, which correspond to the 2nd, 3rd, 4th... nth loop (Fig. 4), are representative of the equilibrium displacements of the protein–ligand complex formation.

MATHEMATICAL TREATMENT

The protein concentration remains constant after n *loops*

If the flow rate in circuit 2 (V_2) is much higher than in circuit 1 (V_1), after n loops ($n = 5$ or 6) the total protein concentration (P_n) remains equal to the initial protein concentration (P_0): $P_n = P_0 = P_t$. After n loops, ligand concentration (L_n) is equal to $L_0 V_1 n / V_2$.

The quantity of heat (Q_n) which corresponds to the equilibrium $P_0 + L_n$ is proportional to $\Delta_1 + \Delta_2 + \Delta_3 \ldots + \Delta_n$ (Fig. 4)

$$Q_n = \sum_{i=1}^{i=n} \frac{\Delta_i}{V_2}$$

Figure 5 shows a model of an experimental enthalpic titration curve obtained in these conditions. K, ΔH and, if needed, stoichiometry values of the complex are calculated by using the iterative method described earlier.

The protein concentration is a function of the number of loops

If ligand solubility is not high enough, the ligand concentration after dilution is so weak that the flow rate in circuit 1 must be increased. Thus, it is not possible to neglect V_1. After n loops one obtains

$$P_n = P_0 \left(\frac{V_2}{V_1 + V_2} \right)^n$$

$$L_n = L_0 \frac{V_1}{V_1 + V_2} \left(1 + \sum_{2}^{n} \left(\frac{V_1}{V_1 + V_2} \right)^{n-1} \right)$$

$$Q_n = \sum_{i=1}^{i=n} \frac{(\Delta_i)}{V_1 + V_2}$$

As the protein concentration (P_t) is a variable, it is not possible to use the iterative method previously described.

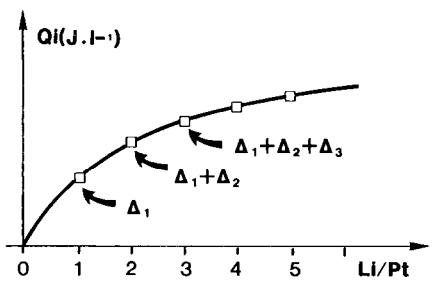


Fig. 5. Experimental enthalpic titration curve obtained with constant protein concentration.

However, K, ΔH and, if needed, stoichiometry values of the complex may be calculated from every pair of experimental values, e.g. P_1, L_1, (PL_1), Q_1 and P_2, L_2, (PL_2) and Q_2. One can write

$$K = \frac{(PL_1)}{[P_1 - (PL_1)][L_1 - (PL_1)]} = \frac{(PL_2)}{[P_2 - (PL_2)][L_2 - (PL_2)]} \tag{5}$$

$$Q_1 = (PL_1)\,\Delta H \qquad Q_2 = (PL_2)\,\Delta H \tag{6}$$

From relationships (5) and (6) one obtains

$$\Delta H = \{(S_2 - S_1 + P_2 - P_1)Q_1Q_2 - \sqrt{(S_1 - S_2 + P_1 - P_2)^2 Q_1^2 Q_2^2 - 4(P_1S_1Q_2 - P_2S_2Q_1)(Q_1Q_2^2 - Q_2Q_1^2)}\} \times \{2(P_1S_1Q_2 - P_2S_2Q_1)\}^{-1} \tag{7}$$

From relationships (5), (6) and (7) it is easy to calculate the ΔH and K values.

Using a permutation of the different pairs, one obtains $n!/(n-2)!\ 2$ K and ΔH values.

Statistical analysis of the results confirm or reject the assumed stoichiometry. If the standard deviation of the means is not adequate, another stoichiometry, i.e. another initial protein concentration value, is tested.

CONCLUSION

Twenty years ago it took 50 ml of 10^{-3} M adenosine triphosphate and one week of experimentation to determine the binding parameters of adenosine triphosphate–magnesium complex. To test the validity of the closed circuit microcalorimetry method, we carried out an identical experiment. To obtain similar results, with the same accuracy, we used only 2 ml of 10^{-5} M adenosine triphosphate and the experimentation time was only 40 min.

REFERENCES

1 J.P. Belaich and J.C. Sari, Proc. Natl. Acad. Sci. U.S., 64 (1969) 763.
2 E. Calvet and H. Prat, Microcalorimetry, Masson, Paris, 1956.
3 R.L. Biltonen and N. Langerman, Microcalorimetry for Biological Chemistry, Methods in Enzymology, Vol. 61, Academic Press, New York, 1979 p. 261.
4 J. Suurkuusk and I. Wadso, Chem. Scr., 20 (1982) 155.
5 R. Gilli, J.C. Sari, L.M. Sica and C.M. Briand, Biochem. Biophys. Acta, 964 (1988) 53.
6 R. Gilli, J.C. Sari, M. Chauvet, M. Bourdeaux and C. Briand, Thermochim. Acta, 85 (1985) 505.
7 F. Guerlesquin, J.C. Sari and M. Bruschi, Biochemistry, 26 (1987) 7438.
8 P. Coassolo, M. Sarrazin and J.C. Sari, Anal. Biochem., 104 (1980) 37.