

Determination of thermodynamic parameters of antigen–antibody interaction from real-time kinetic studies

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Real-time kinetic parameters of the interaction of iodinated human chorionic gonadotropin (hCG) with one of its monoclonal antibodies are sought and are determined after an analysis of the dissociation profile of hCG–MAb (monoclonal antibody) complexes in the presence of excess unlabelled hCG at various temperatures. The equilibrium association constant (K_A) of the epitope–paratope reaction (the first interaction) was determined from this data. K_A values obtained at different temperatures were fitted to the nonlinear form of the vant Hoff equation. Values of $\ln K_{A0}$, ΔH_0 and ΔC_P were obtained from the fit. ΔG_0 and ΔS_0 were calculated using standard formulae. A change in heat capacity at constant pressure, ΔC_P of -3300 cal/degree/mol indicated that a large surface area is buried upon complex formation. This method can be an alternative to the micro-calorimetric determination of thermodynamic parameters of ligand–ligate interaction. Our results also raise new questions about the interpretations of micro-calorimetry results from large-molecular weight ligand–ligate interactions.

MACROMOLECULAR interactions involve cooperative, independent and contiguous binding regions. The complexity caused by this is increased by the dynamic structural changes that the interaction engenders. The rate of the reaction and the equilibrium condition are influenced by the algebraic sum of the energies involved in reversible macromolecular interactions. The interplay of the two major interacting energy components, namely electrostatic and hydrophobic, is best understood by a study of the thermodynamic parameters of the interaction. The method most widely used for such investigations is isothermal titration calorimetry^{1–3}, which directly determines the heat changes during the binding process, and derives the thermodynamic parameters, enthalpy (ΔH), free energy (ΔG), entropy (ΔS) and the heat capacity (ΔC_P) changes. In the last two decades the method has been used to unravel the intricacies of the interaction between several ligand–ligate pairs, like protease–protease inhibitor⁴, receptor–ligand⁵ and antigen–antibody².

However, use of this method is limited to such systems where the available quantity of the ligand/ligate is large (100–200 μg). Besides, sophistication of this method requires an excellent infrastructure for its execution. An alternative approach, viz. the BIAcore⁶, has failed to make an impressive impact on the study of thermodynamics of macromolecular interaction for several reasons⁵. Studies using fluorescent probes are also only a few, because of their poor sensitivity and requirement of large quantities of the ligand/ligate⁷.

Thus, there is a need for a laboratory method for the study of macromolecular interactions. The potential of the classical approach based on variation of rate constants with temperature has not been properly exploited, probably because of the non-availability of quantitative methods for real-time monitoring of the reaction, and the assumption that radio-labelling alters the binding properties of the ligand. Our previous investigations have shown that single-step solid-phase radioimmunoassay (SS-SPRIA) monitors the real-time kinetics of the interaction accurately^{8,9} and that radio-labelling does not alter the binding properties of the ligand¹⁰. In this article we present an extension of the kinetic approach to obtain the thermodynamic parameters of the interaction using radio-labelled human chorionic gonadotropin (hCG) as a quantitative probe.

Materials and methods

hCG was prepared from early-pregnancy urine through affinity chromatography. The material was found to be homogeneous by SDS PAGE¹¹. VM4 was raised in the laboratory against the native heterodimer and was specific to the β -subunit of hCG.

Determination of the capacity of ascites

Varying volumes (10–100 μl) of 1/4000 diluted ascites were added to tubes containing 100 ng of hCG in 50 μl of RIA buffer (0.1% BSA in 0.05 M phosphate buffer having 0.05 M EDTA, pH 7.4). Volumes were made up to 250 μl by addition of RIA buffer, and the reaction was

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allowed to occur overnight. Fifty μl of this incubation mixture and 100 μl of iodinated human chorionic gonadotropin (IhCG: 50,000 cpm) were added to microtiter wells coated with VM4 monoclonal antibody (MAB). Radioactivity bound to the microtiter well was measured at the end of 2 h. The profile of binding was a typical bell-shaped curve, as in a precipitin reaction. The capacity of the ascites was calculated from these data.

Adsorption of MAB on nitrocellulose disc

Adsorption of the MAB on nitrocellulose (NC) disc was carried out as described by Srilatha and Murthy¹². In brief, about 60–80 fresh NC discs (6 mm in diameter) were taken in a 6 ml plastic tube and coated with 3 ml of MAB solution at the required dilution in RIA buffer on a rotator. After coating, the discs were washed thoroughly with RIA buffer and blocked with 1% BSA solution. MAB-coated discs were found to be stable for up to 3 months at 4°C. MAB-coated NC disc is referred to as NC_{MAB} .

Hormone-binding capacity of NC_{MAB}

Hundred ng of hCG in 100 μl of RIA buffer was added to three discs taken in separate test tubes. The discs were incubated for 10 h. The hCG that remained unadsorbed after incubation was measured by radioimmunoassay. The difference between the original amount of hCG (i.e. 100 ng) and that remaining unbound was taken to be the capacity of NC_{MAB} .

Binding of IhCG to immobilized MAB

The NC_{MAB} was taken in a test tube and 0.3 ml of IhCG (1–3 lakh cpm) was added. Binding was allowed to occur for 16–20 h at the required temperature using a heating block procured from Neolabs, Mumbai. At the end of the incubation period, the disc so obtained (referred to as NC_{Com} for IhCG–MAB complex on NC disc) was washed with 3 ml of buffer at the same temperature. The quantity of IhCG bound was measured before the start of the dissociation. The apparent concentration of MAB in the reaction was obtained by assuming that the MAB on the NC disc is distributed over the incubation volume of 0.3 ml. The concentration of IhCG was obtained from specific-activity measurements.

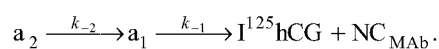
Dissociation of the IhCG- NC_{MAB}

NC_{Com} was taken in a glass tube as shown in Figure 1 and it was slipped into the bottom of the column. The column was then placed into a glass tube containing 20 $\mu\text{g}/\text{ml}$ hCG in RIA buffer at the required temperature. At speci-

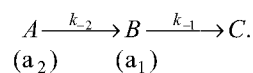
fied time intervals the glass column was taken out (NC_{Com} separated from the solution) of the test tube and the radioactivity in the test tube (released into the buffer) was counted for 30 s. Subsequently, the column was placed back in the glass tube at the appropriate temperature and dissociation was allowed to continue. Dissociation was monitored for about 14 h and the dissociation profile was obtained at different temperatures.

Mathematical treatment of data

The scheme of reaction used in the analysis of the dissociation profile according to the two-step model is as follows¹³:



The hypothesis:



At equilibrium, the complex is assumed to consist of two forms, namely *A* (conc. *a*₂) and *B* (conc. *a*₁) in a fixed proportion. The release of radioactivity takes place by way of the dissociation of *a*₁ directly, and the dissociation of *a*₂ to the free radiolabel with *a*₁ as an intermediate. Thus, the radioactivity released into the medium at any time is the sum of the radioactivity released from *a*₁ and *a*₂. The radioactivity released from *a*₁ is calculated sepa-

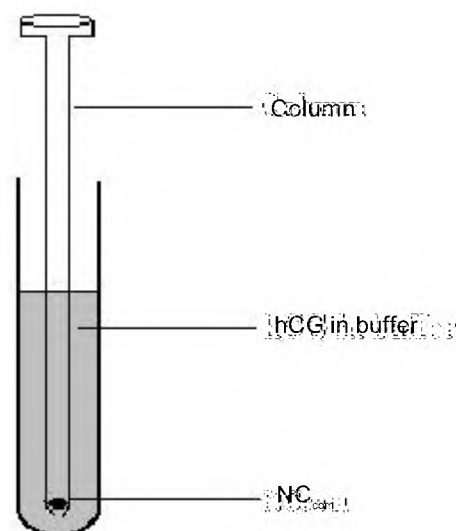


Figure 1. Schematic presentation of the method used for the study of dissociation of NC_{Com} . To measure the extent of dissociation, the column was taken out of the tube and radioactivity in the tube was measured. The column was put back into the same tube for continuation of dissociation.

rately from that of a_2 . The sum of the two is considered to be the radioactivity released.

The equations used to determine the contribution from components A through B (C_1) are as follows:

$$\frac{dA}{dt} = -k_{-2} \cdot A, \quad (1)$$

$$\frac{dB}{dt} = k_{-2}A - k_{-1}B, \quad (2)$$

$$\frac{dC}{dt} = k_{-1}B. \quad (3)$$

Solving the equations using Laplace transformation, we obtain

$$C_1 = \left[\frac{a_2 k_{-1} k_{-2}}{k_{-1} - k_{-2}} \right] \left[\frac{(1 - e^{-k_{-2}t})}{k_{-2}} - \frac{(1 - e^{-k_{-1}t})}{k_{-1}} \right]. \quad (4)$$

The equations used to determine the contribution by the B component (C_2) are as follows

$$\frac{dC}{dt} = k_{-1}B, \quad (5)$$

$$\frac{dB}{dt} = -k_{-1}B. \quad (6)$$

Solving the equations using Laplace transformation yields

$$C_2 = a_1(1 - e^{-k_{-1}t}). \quad (7)$$

Therefore, the total C component dissociated from A and B at any given time is given by their sum, i.e. eq. (4) + eq. (7) or $C_1 + C_2$:

$$C = a_1(1 - e^{-k_{-1}t}) + \left\{ \frac{a_2 k_{-1} k_{-2}}{k_{-1} - k_{-2}} \right\} \left\{ \left(\frac{1 - e^{-k_{-2}t}}{k_{-2}} \right) - \left(\frac{1 - e^{-k_{-1}t}}{k_{-1}} \right) \right\}. \quad (8)$$

Since the dissociation is started by adding 10,000-fold excess of unlabelled hCG, the release of radioactivity is a measure of the dissociation constant. It is assumed that the conversion of a_1 to a_2 favoured by unlabelled hCG does not contribute to the overall kinetics of dissociation, because the conversion rate of a_1 to a_2 ($k_2 < 10\%$ of k_{-1}) is very low. Furthermore, the binding of the dissociated hCG to MAb is insignificant in the presence 10,000-fold excess of unlabelled hCG.

Analysis of dissociation profile

The amount of radioactivity released at different time points was monitored and fitted to a two-step reaction model using the formula shown in eq. (8). Curve-fitting was done with a computer program to obtain the parameters k_{-1} , a_1 , a_2 and k_{-2} . Effective association constant of hCG and MAb at equilibrium (initial interaction of the epitope and paratope) was obtained by the following equation using the a_1 value as bound at equilibrium.

$$K_A = \frac{a_1}{[Ag][MAb]}, \quad (9)$$

where $[Ag]$ and $[MAb]$ represent the equilibrium concentration of antigen and antibody respectively.

Thermodynamic parameters from kinetic data

van't Hoff analysis: Affinity constants (K_A) for the first step of the interaction, obtained at several temperatures were plotted as a function of inverse absolute temperature ($1/T$) and fitted to the nonlinear form of the van't Hoff equation⁷ as given below. The GNU plot in the LINUX platform was used for this purpose.

$$\ln K_A = -\frac{\Delta H_0}{R} \left(\frac{1}{T} - \frac{1}{T_0} \right) + \frac{\Delta C_p T_0}{R} \left(\frac{1}{T} - \frac{1}{T_0} \right) + \frac{\Delta C_p}{R} \ln \left(\frac{T}{T_0} \right) + \ln K_{A0}, \quad (10)$$

where K_{A0} is the equilibrium constant at 25°C (T_0), which is an arbitrarily chosen reference temperature and K_A is the same at any other temperature (T). Values of K_{A0} , ΔH_0 and ΔC_p were obtained from the fit, where ΔH_0 is the enthalpy change at the reference temperature.

Determination of free energy and entropy changes: ΔG_0 and ΔS_0 , which are free energy change and the entropy change at the reference temperature (298 K) were calculated using the following relations:

$$\Delta G = \Delta H - T\Delta S = -RT \ln K_A, \quad (11)$$

where R is the gas constant.

$$\Delta C_p = \frac{\partial \Delta H}{\partial T}. \quad (12)$$

Equation (10) was derived using eqs (11) and (12).

Results

The hCG-binding capacity of the VM4 MAb ascites is 16 mg/ml, as calculated from Figure 2 (see legend).

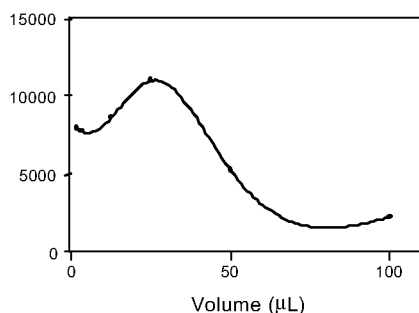


Figure 2. Capacity of VM4 ascites: 25 μL of 1/4000 antisera (equivalence point from the graph) neutralized 100 ng hCG. Thus the capacity of the antisera was calculated to be 16 mg/ml.

Table 1. Capacity of antibody on nitrocellulose disc

Ascites dilution used for coating	Capacity (ng/disc)
1 : 1000	73
1 : 2000	50
1 : 2000	59

The capacity per disc determined according to the methods described earlier gave ranges from 50 to 70 ng/disc (Table 1). In an alternative method, 24 μg of MAb (3 ml of 1/2000 dilution) was used for coating 60 discs. Quantitative adsorption of the antibody occurred at the end of coating. Though 400 ng/disc was adsorbed, the functional capacity of the disc was just 60–70 ng/disc, indicating inactivation of 75% of the MAb on/during adsorption. The discs were coated in batches of 60–70 and the capacity determined in each batch was used for analysis of the data obtained using those discs.

Curve-fitting of the dissociation data at different temperatures was carried out as described earlier. Figure 3 shows the fit of the dissociation profile of IhCG from IhCG–MAb complex at 30°C–45°C to the two-step mechanism. The parameters k_{-1} and a_1 were obtained with sufficient accuracy. The extent of non-dissociating component of the complex varied from 30 to 50% at 45°C and 25°C respectively (data not shown). The non-dissociable component was unaffected by the radiolabel used and the batch of the antibody-coated discs, and indicated clearly that it is not an artifact. This matches well with earlier data, which showed a total non-dissociable component of 40% in the hCG–MAb complex adsorbed on plastic at room temperature¹⁰.

Table 2 shows the experimental parameters k_{-1} and a_1 obtained by curve-fitting, and K_A calculated by eq. (9) from equilibrium concentration of a_1 , IhCG and MAb at each temperature. The free energy change ΔG at each temperature calculated from the K_A values, are also enlisted. The fact that ΔG remains unaltered indicates that there is complete enthalpy–entropy compensation.

Figure 4 shows the van't Hoff plot where the points are the experimental values of $\ln K_A$ and the solid line represents the theoretical values obtained using eq. (10). Values of $\ln K_{A0} \pm 0.1326$, $\Delta H_0 \pm 4.638$ and $\Delta C_P \pm 366$ obtained from curve fitting are given in Table 3. The value of ΔC_P which is -3300 cal/deg/mol, is higher than the values obtained for several systems in the literature¹⁴.

Discussion

The dissociation profile fits to a two-step mechanism, and both k_{-1} and K_A can be measured with 10% precision (intra-experiment variation). The heat capacity change obtained is consistent in several determinations, and is within $\pm 15\%$ (inter-experiment). The ΔC_P is independent of specific activity of IhCG. It is also unlikely to be affected by the errors in measurement, since it is determined from the temperature-dependent rather than the absolute values of ΔH . However, when different batches of the IhCG and MAb-coated discs were used, greater errors (25%, inter-experiment) occurred in the absolute values of the changes in enthalpy, entropy and free energy. Nonetheless, conclusions on the nature of the interaction and the value of ΔC_P were independent of the batch of the antibody discs and the hormone used for iodination. Automation of the system would improve the precision of measurements and accuracy of data in the evaluation of the thermodynamic parameters in such systems.

The reaction effected through the above method is heterogeneous. So the validity of the results may not be the same as in the case of a homogeneous reaction. Nevertheless, it suits us in the absence of an alternative method to study the antigen–antibody interaction through dissociation in the homogeneous phase. However, the results presented in the paper are internally consistent and conform to the hypothesis. Hence they can be used in comparative study with no uncertainty. The BIAcore method for thermodynamics in limited cases^{4,5} has used the data from solid phase interactions. Other methods derive the thermodynamic parameters from the association mode in homogeneous solution. Though they have the advantage of reaction in homogeneous solution phase, they may not satisfy the criteria of a completely reversible equilibrium, important for analysing thermodynamic parameters.

The van't Hoff plot is concave towards the x -axis, which is characteristic of the binding processes involving a large negative heat capacity change. The value of ΔC_P obtained from this plot was -3300 cal/deg/mol. This indicates that a very large surface area is buried upon complex formation. In our experience most of the hCG–MAb systems involve a large favourable entropic contribution (158 cal/deg/mol in this case). In other words, the interaction involves extensive hydrophobic interaction. Hence a large value of ΔC_P is not surprising.

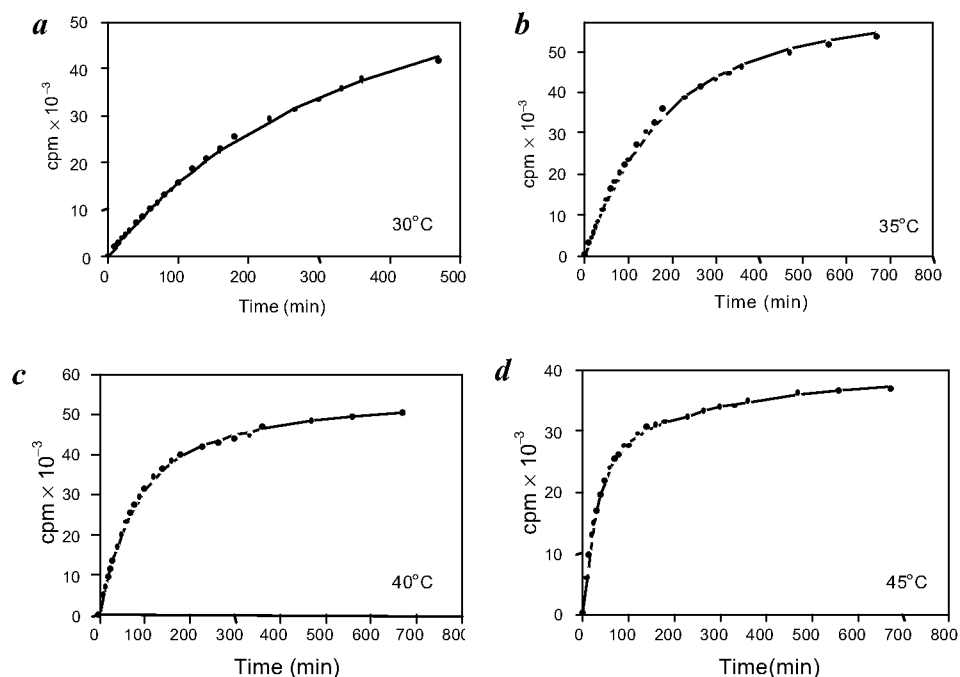


Figure 3. *a–d*, Dissociation profile of NC_{Com} at varying temperatures. Incubation of the complex as well as dissociation were carried out at respective temperatures. Points present the experimental data and lines the best fit for the two-step analysis.

Table 2. Kinetic/equilibrium parameters of VM4–IgCG interaction based on the dissociation profile of NC_{Com} at varying temperatures

Temperature (°C)	k_{-1} (min^{-1})	a_1 cpm	K_A ($\times 10^{-7} \text{ M}^{-1}$)	ΔG kcal/mol
25	0.002583 ± 0.000161	10821 ± 2621	7.90 ± 3.57	-10.804 ± 0.247
30	0.005583 ± 0.001258	17165 ± 2924	19.57 ± 2.28	-11.567 ± 0.069
35	0.009033 ± 0.000782	23237 ± 4394	27.17 ± 4.57	-11.957 ± 0.010
40	0.016683 ± 0.000939	22602 ± 6285	22.80 ± 3.99	-12.041 ± 0.115
45	0.036650 ± 0.002232	12557 ± 1286	10.79 ± 2.17	-11.756 ± 0.123
50	0.046217 ± 0.001077	8010 ± 236	5.82 ± 0.21	-11.550 ± 0.024

Table 3. Thermodynamic parameters obtained for VM4A–hCG interaction

K_{A0} ($\times 10^{-7} \text{ M}^{-1}$)	ΔG_0 kcal/mol	ΔH_0 kcal/mol	ΔS_0 cal/deg/mol	ΔC_p cal/deg/mol
8.39	-10.875	36.631	159	-3300

Our method has several advantages. Reactants are required in very small quantities in comparison to any other method. However, the ligand or ligate should be of iodination grade. One should find a suitable solid support for adsorption of the ligate, which in this case happens to be NC. Since NC has the capacity to bind several types of ligands like proteins, RNA and DNA, this may not pose any problem in extending the method to other ligand–ligate systems. The infrastructure required in a laboratory to adopt this method of analysis is very simple, essen-

tially an iodination facility and a gamma counter. The effect of salt concentration/pH/polarity of the reaction medium can be studied more easily than in a microcalorimeter, as experiments can be carried out simultaneously. The calculations do not involve the heat changes of salt dissolution/dilution, etc. which are very important in the microcalorimeter. The method provides all the thermodynamic parameters, including the heat capacity change.

The temperature ranges that can be studied are relatively small, i.e. 20–25°C above the ambient in most hCG–MAB interactions. In this particular case, a temperature below ambient is not possible as the dissociation is extremely small (despite good specific binding), as already reported¹³. At temperatures above 50°C, the binding decreased rapidly or the dissociation was so fast that accurate measurement of k_{-1} was difficult, and resulted in an apparent temperature-dependent ΔC_p .

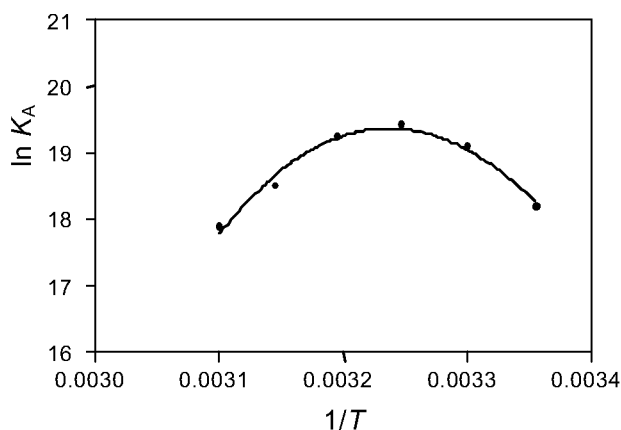


Figure 4. van't Hoff plot ($\ln K_A$ vs $1/T$) for VM4-IhCG interaction. Curve represents the theoretical fit to the data points (●) obtained experimentally.

One parameter that raised some concern is the quantity of the complex that failed to dissociate (ND, for non-dissociable) even after overnight dissociation. This apparent non-dissociation is observed with unlabelled complexes as well, to the same extent of 40% (ref. 13), thus ruling out the possibility of this being an artifact of iodination of hCG. This was not an artifact of adsorption of MAb on NC, as it is observed with MAb adsorbed via the immunochemical bridge⁹. Experiments have indicated that even in the liquid phase, the ND form is much like in the solid phase (data not shown). The relevance of this ND form of the complex is not known at present, though its existence is unequivocally demonstrated. Whether this form represents a chemical entity that is biologically significant will have to be investigated.

The condition to be satisfied in order to use the present method for thermodynamic study is that the dissociation profile should fit into the two-step mechanism. If the system is more complex, additional approaches need to be developed before the thermodynamic parameters can be evaluated.

Determination of thermodynamic parameters in microcalorimetry is based on direct measurement of ΔH and obtaining K_A by interpolation of the binding data. Such evaluation assumes the reaction to be completely reversible, but our studies have failed to demonstrate complete reversibility of the reaction. In addition, the product consists of multiple components of the complex, a significant contribution coming from the apparently nonreversible component (ND). The ND was found to be nearly 100% in a BIAcore study, where reactions are carried out under concentrations (100–200 $\mu\text{g/ml}$) comparable to microcalorimetry. Thus the K_A and obviously the ΔG evaluated may not represent true values, despite the directly measured ΔH . Thus the thermodynamic parameters obtained may have in-built errors. In contrast, our method measures ΔG directly for the epitope-paratope interaction

using the dissociation profile, where the determination of K_A is a direct experimental result. The analysis is able to discriminate between the ND and the two dissociable components a_1 and a_2 . K_A values reported here are those for the formation of the first complex (a_1). In other words, the analysis is focused on the first interaction between the epitope and the paratope. The concentrations of the ligand/ligate are comparable to physiological concentrations (100 ng/ml). Microcalorimetry has the advantage of adopting homogeneous reaction conditions, unlike the method described here. The kinetic approach has the advantage of minimal infrastructure and is independent of expensive equipment like the microcalorimeter. The only equipment required would be a gamma counter and iodination facility. In terms of material requirement, the kinetic approach requires few micrograms of reagents. The number of experiments that can be carried out in the kinetic approach is much more, since 5–10 dissociations can be carried out simultaneously. However, one of the reagents should be adsorbed on NC, while the other should be of iodination grade. In contrast, the microcalorimetric approach needs larger quantities of the reactant (at least 100–1000-fold), but does not need iodination/adsorption of the ligand/ligate as a prerequisite to carry out the investigations.

A laboratory method for the determination of the thermodynamic parameters of a macromolecular ligate–ligand interaction (hCG–MAb) is described. It is based on the classical approach to thermodynamics of equilibrium reactions. The method differs in its principle from microcalorimeter and BIAcore, and utilizes the kinetics of dissociation of the IhCG–MAb complex to arrive at the rate constants of the reaction. The dissociation approach has an in-built validation of the reversibility of the equilibrium, as the extent of maximum possible dissociation is measured automatically. In the event of partial dissociation, it is identified/quantified and eliminated in the analysis, such that even under circumstances where partial dissociation is observed, it is considered in the analysis of the result. This observation becomes all the more important in view of the fact that both BIAcore and microcalorimeter assume that all macromolecular ligand–ligate interactions are completely reversible equilibrium situations^{1–3}, an assumption proved wrong in several systems and in hCG–MAb system in the BIAcore¹⁵. The method is used to study the thermodynamics of the epitope–paratope interaction, and does not consider the inter-conversion dynamics of these two forms of the complex. It has an accuracy of $\pm 10\%$ in the determination of kinetic and thermodynamic parameters. So it is applicable to systems that have $\Delta C_p > 300$ cal/deg/mole. For systems with ΔC_p values less than this, the accuracy of the measurement of thermodynamic parameters will have to be increased markedly. Errors in our method can be significantly reduced if it is automated. In fact, preliminary studies employing automation have shown that

the accuracy of the kinetic constants increases markedly and the thermodynamic parameters can be obtained with greater precision. These results point to the possibility of extending this approach to systems which have much lower ΔC_p (< 300 cal/deg mole). Attempts are under way to materialize the automation component of the process.

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