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Mini-Review

NMR titration for studying isomer-specific supramolecular complexation of biogenic substances

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ABSTRACT

The conformational isomers of a biogenic substance may present isomer-specific bioactivities when the isomers have different binding forces towards a particular receptor because of differences in geometrical relations so as to form the supramolecular complexes individually with distinct thermodynamic stabilities. The correct determination of the individual stabilities is the first step to study the isomer-specific effects. It can be performed by NMR titration, the application of which has demonstrated the isomer-specific supramolecular complexation of D-glucosamine. The NMR studies extended to a variety of biogenic isomers would establish the isomer-specific molecular interaction and the consequent isomer effects on the bioactivities. This review article outlines the principles and experiments of the NMR-titrimetric method, together with a convenient data-analysis process using Excel® spreadsheets, to facilitate its use in biochemical research.

KEYWORDS: isomerism, conformational isomer, supramolecular complex, stability constant, NMR titration, glucosamine.

1. Introduction

The anomeric isomers of D-glucosamine (2-amino-2-deoxy-D-glucose) have been demonstrated to form the supramolecular complexes of capsaicin individually with different stabilities: the α-form has a stronger molecular interaction with the counter reactant than does the β-form [1]. If such isomer-specific molecular interaction generally occurs for biogenic substances, it may characterize the bioactivity of each isomer. To examine this possible isomer-specific effect, the individual thermodynamic stabilities should be determined for the supramolecular complexes of the isomers of a target substrate with a selected receptor.

Thermodynamic stability constants are determined mostly by spectrometric titration [2]. If the isomers of a substrate are isolated individually, the stability constants of their complexes can be determined independently by ordinary titration methods. In common conformational isomerism, the isomers cannot be resolved even when they are detected separately by NMR. In some cases such as monosaccharides, even an isolated isomer undergoes isomerization equilibrium in solution. The complexation of such "unresolvable" isomers proceeds individually under the concurrent isomerism, as illustrated in Figure 1: isomers **Aa** and **Ab**, which are equilibrated with each other, form individual complexes with reactant **B**. The equilibrium constants K_a and K_b of the complexations present the thermodynamic stabilities of the resulting complexes **AaB** and **AbB**, respectively. Basic NMR-titrimetric methods for determining K_a and K_b have been proposed, and the accuracy as well as difference from the ordinary titration methods has been examined [3]. Later, the methodology has been generalized to isomer complexes having stoichiometry other than 1:1,

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and it has been applied to a study of the isomer complexes of cationic glucosamine with aromatic carboxylates [4]. Since many biogenic substances undergo conformational isomerism, the extended studies of isomer-specific complexation by NMR titration would prove the isomer effects on the bioactivities. This review article outlines the principle and method, and presents convenient data analysis processes using Excel® spreadsheets, to prompt the application to biochemical systems.

2. Principle

When the isomers of a target substance individually show NMR signals characteristic of their conformations, the complexation can be studied by NMR titration, which is performed by monitoring the signals of the isomers (*i.e.*, titrates) with successive addition of a counter reactant (titrant). Common supramolecular complexations of organic substances proceed so rapidly that the frequency of chemical exchange between the free and complexed molecules is sufficiently greater than difference between their chemical shifts δ in Hz. In such a rapid exchange case of NMR, the complexation is detected as a change in δ. Figure 2 presents the NMR spectra observed for the anomeric CH(1) protons of D-glucosamine at different concentrations (C_B) of capsaicin added as the titrant – the molecular structures are shown in Figure 3. The δ-changes referenced to the value of free glucosamine, $\Delta = \delta - \delta(C_B{=}0)$, show asymptotic curves against the capsaicin concentration C_B, as plotted in Figure 4; similar monotonic curves are observed for N-CH(2) proton as well [1]. The observed titration curves suggest that the isomers individually form capsaicin complexes. The individual stabilities are determined by least-squares analyses, as explained below.

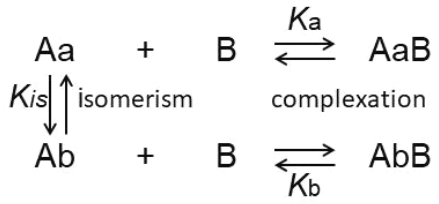

Figure 1. Reaction scheme of complexation of isomers **Aa** and **Ab** with reactant **B** under concurrent isomerism: the overall reaction is controlled by three equilibrium constants, K_{is}, K_a and K_b. The latter two present the individual thermodynamic stabilities of the isomer complexes.

For the concurrent isomerization and complexation of isomers (illustrated in Figure 1), the overall reaction consists of three component reactions whose equilibrium constants are defined as follows.

$$K_{is} = [\mathrm{Ab}]/[\mathrm{Aa}] \quad (1)$$

$$K_a = [\mathrm{AaB}]/[\mathrm{Aa}][\mathrm{B}] \quad (2)$$

$$K_b = [\mathrm{AbB}]/[\mathrm{Ab}][\mathrm{B}] \quad (3)$$

Here, [AB], [A] and [B] are the molar concentrations (M = mol dm^{-3}) of the bracketed species. The isomerization constant K_{is} is determined from the integrated intensities of the NMR signals of the isomers in the absence of the counter reactant. The equilibrium constant of the complexation presents the thermodynamic stability of complex **AxB** (x = a or b). The mass balances are held throughout the reaction.

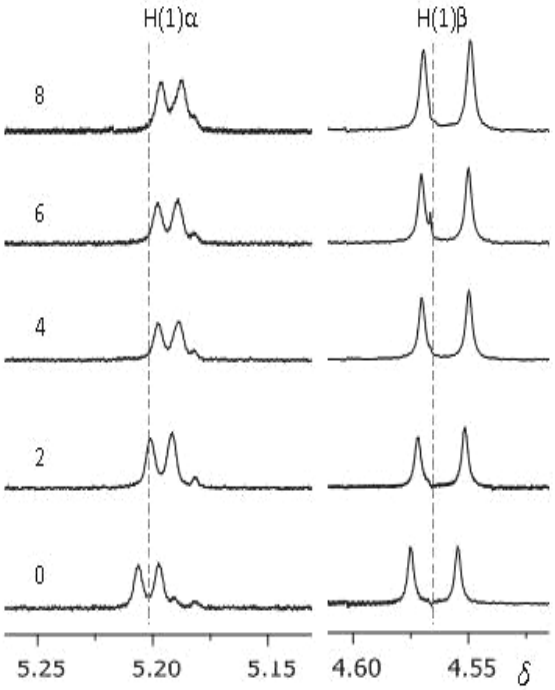

Figure 2. ^{1}H NMR spectra of anomeric CH(1) proton in α- and β-glucosamine isomers (2×10^{-3} M) at different concentrations of capsaicin (0 to 8×10^{-3} M) in D_2O at pD = 9.55: the doublet of each isomer shifts up-field with increasing concentration of capsaicin [Ref. 1].

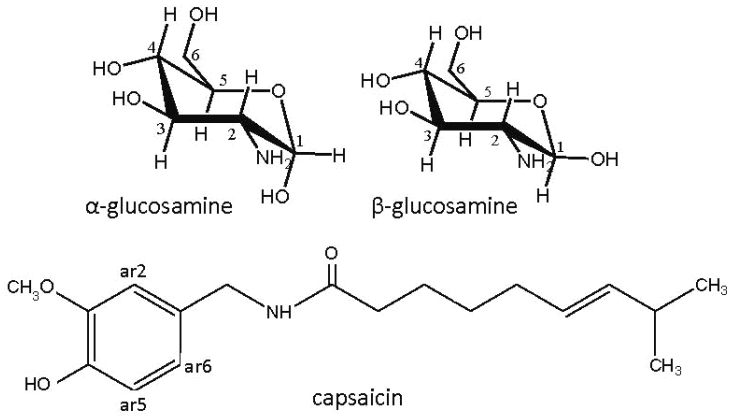

Figure 3. Molecular structures of D-glucosamine isomers and capsaicin: their complexation is presented as an example for studying isomer-specific complexation.

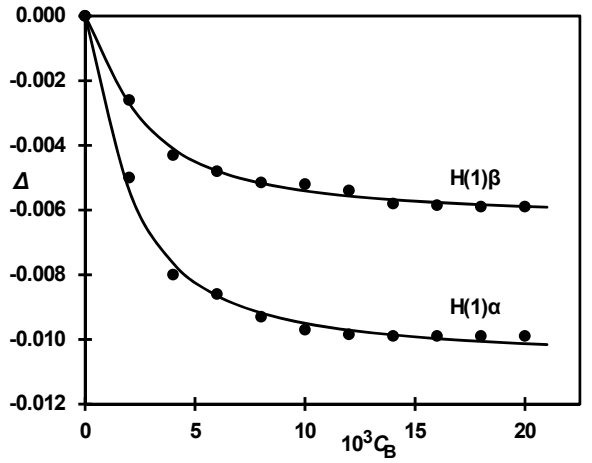

Figure 4. Change in ^{1}H NMR shift δ, $\Delta = \delta(C_B) - \delta(0)$, observed for anomeric CH(1) of α- and β-glucosamine isomers as a function of concentration of capsaicin C_B (in 10^{-3} M) in D_2O: the concentration of glucosamine, $C_A = 2 \times 10^{-3}$ M; pD = 9.55; T = 25 °C. The solid lines show the best fits obtained by the simultaneous fitting of the two curves on the basis of the reaction scheme in Figure 1: the stability constants (with standard deviations) of the isomer complexes, K_α = 950 (80) M^{-1} and K_β = 720 (100) M^{-1} [Ref. 3].

$$C_A = [Aa] + [AaB] + [Ab] + [AbB] \quad (4)$$

$$C_B = [B] + [AaB] + [AbB] \quad (5)$$

Here, C_A and C_B are the total molar concentrations of the reactants added to the reaction system. When the isomerization equilibrium is established throughout the titration run, [Ab] is equal to K_{is}[Aa] according to Equation 1. The combination of the above equations leads to the following quadratic equation of [Aa].

$$S{\cdot}P[Aa]^2 + \{S + (C_B - C_A){\cdot}P\}[Aa] - C_A = 0 \quad (6)$$

$$S = 1 + K_{is} \quad (6a)$$

$$P = K_a + K_{is}{\cdot}K_b \quad (6b)$$

For certain K_a and K_b values, the solution of Equation 6 gives [Aa], from which [B] is calculated by the following equation.

$$[B] = C_B/(1 + P[Aa]) \quad (7)$$

Successively, the concentrations of complexes [AaB] and [AbB] are calculated from the definitions of the stability constants (Equations 2 and 3), respectively. The δ-change, Δ, observed for each isomer is proportional to the mole fraction of the complex in the rapid exchange case of NMR.

$$\Delta_a = \Delta_{ca}[AaB]/([Aa] + [AaB]) \quad (8)$$

$$\Delta_b = \Delta_{cb}[AbB]/([Ab] + [AbB]) \quad (9)$$

Here, Δ_{ca} and Δ_{cb} are Δ values intrinsic of **Aa** and **Ab** in their complexes, respectively. The denominator in each equation (*i.e.*, the total concentration of each isomer) is changed upon complexation while the ratio [Ab]/[Aa] is kept constant. Plots of Δ_a and Δ_b versus C_B yield a pair of titration curves. The simultaneous fittings of the observed curves with the theoretical curves will determine the individual stability constants K_a and K_b of the isomer complexes **AaB** and **AbB**; the solid lines in Figure 4

present the best fits for the α-glucosamine (**Aa**) and β-glucosamine (**Ab**), giving the individual stability constants described in the figure caption.

3. Experimental process

The NMR spectrum of isomeric reactant **A** is monitored by changing the concentration of counter reactant **B** as titrant. Basically, there are two methods for sample preparation.

3.1. C_A-constant method

A series of sample solutions are prepared by the following procedure: (1) ten or more NMR tubes are loaded with the same quantity of a stock solution of isomeric reactant **A**; (2) into one of the tubes, "solvent" is added to adjust the total volume (normally 0.5 mL), this solution being used for determining the isomerization constant K_{is} as well as the δ values of free **Aa** and **Ab**; (3) into the other tubes is added a stock solution of **B** in different volumes so that the C_B/C_A ratios are in a desired range; (4) all solutions are adjusted to the same total volume. The same "solvent" should be used for preparing all solutions so that the conditions are identical including ionic strength and pD (pH in D_2O). Alternatively, sample solutions are prepared by mixing the following two stock solutions in desired ratios: (1) solution containing **A** only; (2) solution containing **B** at the highest concentration planned in the titration run in addition to **A** at the same concentration in solution 1. After a sufficient waiting time to ensure the equilibrated conditions, the NMR spectra of the sample solutions are recoded in turn.

3.2. Aliquot-addition method

The spectral change of **A** is traced by adding **B** successively: (1) the NMR spectra of **Aa** and **Ab** are recorded at a suitable concentration; (2) into the solution is added an aliquot of a stock solution of **B**; (3) after a sufficient waiting time, the NMR spectrum is recorded, and the spectrum recording is repeated to ensure the establishment of equilibrated conditions; (4) operations 2 and 3 are continued until a sufficient number of data are collected. The concentration C_A is no longer constant due to the volume change. To minimize possible concentration-dependent phenomena of **A** (such as self-association), the volume change of the final solution should be less than 10 % of the initial volume – this requirement needs a highly concentrated stock solution of **B**.

3.3. Note

The chemical-shift changes, $\Delta = \delta - \delta(C_B{=}0)$, are determined individually for the isomers. Plots of observed Δ_a and Δ_b versus C_B give a pair of titration curves (*e.g.*, Figure 4). Generally, the C_A-constant method provides better titration data with smaller fluctuation; the aliquot-addition method consumes less chemicals although the collection of a larger number of data is necessary to reduce an error caused by data fluctuation. The use of an analytical balance for sample preparation yields more reliable data; in this case, the stability constants are expressed by reciprocal molality m^{-1} ($m = mol\ kg^{-1}$) in place of reciprocal molarity M^{-1}.

4. Data analysis

Microsoft Office Excel® includes an optimization package called Solver, which works for non-linear least-squares calculations. Solver, which is an Excel® Add-in, should be installed from Options/ Add-ins if not located on Data tab. By using this popular software, the computational analyses of titration curves can be programed readily.

Two least-squares fitting methods have been proposed for calculating stability constants from a pair of titration curves observed for isomers: (1) simultaneous fitting method, which searches for the global minimum of the overall residual factor, $R = \{\Sigma[\Delta_x(\mathrm{obs}) - \Delta_x(\mathrm{cal})]^2\}^{1/2}$; (2) alternate fitting method, which alternately minimizes the residual factor of each curve [3]. The effects of the isomerization rate have been examined by comparing two extreme cases: (1) established isomerism case in which K_{is} is kept identical at every data point; (2) sluggish isomerism case in which the isomerism does not reach the equilibrium but the total concentration of each isomer is unchanged during NMR titration [3]. The stability constants determined by both fitting methods in the two cases of isomerism agree with each other within the standard deviations, and the ratio K_a:K_b is practically identical, unless paired titration curves involve large data fluctuations at very different degrees. In practical data analyses much more convenient is the simultaneous fitting method based on the assumption of the established isomerism case. Excel® spreadsheets specified to this choice are appended to reference 4: others are available in reference 3.

Figure 5 shows an Excel® worksheet for the simultaneous fitting method [4]. The observed shifts

Δ_a and Δ_b are input at C_A and C_B, which are denoted by [A]t and [B]t, respectively, in the worksheet. The value of K_{is} is obtained from the integrated intensities of selected signals of the isomers at $C_B = 0$. For given initial values of the variables (K_a, Δ_{ca}, K_b, and Δ_{cb}), [Aa] is obtained from Equation 6, yielding Δ_a(cal) and Δ_b(cal). These calculation results are displayed in corresponding shaded cells. The residual factor is calculated for the pair of titration data, and is minimized by Solver with refinement of the variables, the final values of which are presented for the best fit. The standard deviations are calculated in a separate sheet. In Figure 5, curve fitting is executed for the titration curves obtained for the complexation of α- and β-glucosamine isomers with capsaicin (Figure 4). The stability constants are determined individually as $K_\alpha = 950$ (80) and $K_\beta = 720$ (100) on the basis of the NMR shifts of CH(1) protons (the standard deviations are given in the parentheses). The alternate fitting method yields $K_\alpha = 940$ (100) and $K_\beta = 690$ (60). The good agreement guarantees the usefulness and convenience of the simultaneous fitting method because of its much simpler operation. When two pairs of titration curves are obtained, the simultaneous fitting of four titration curves will give more reliable stability constants [3, 4].

5. Stoichiometry other than 1:1

5.1. Formulation

Most organic supramolecular complexes have 1:1-stoichiometry, but other stoichiometry is found for some complexes. The formation of isomer complexes with compositions $\mathbf{Aa}_m\mathbf{B}_p$ and $\mathbf{Ab}_n\mathbf{B}_q$ proceeds in the following equilibria in addition to the isomerization equilibrium between **Aa** and **Ab**.

$$m\cdot\mathbf{Aa} + p\cdot\mathbf{B} \rightleftarrows \mathbf{Aa}_m\mathbf{B}_p \quad (10)$$

$$n\cdot\mathbf{Ab} + q\cdot\mathbf{B} \rightleftarrows \mathbf{Ab}_n\mathbf{B}_q \quad (11)$$

On the assumption of the established isomerism, the titration curves are formulated in the same way as for the 1:1-complexation. When the complexes are formed in a single step with the stoichiometry of either $p = q = 1$ or $m = n = 1$, the least-squares fittings can be performed readily with Excel® spreadsheets [4].

$\mathbf{Aa}_m\mathbf{B}$, $\mathbf{Ab}_n\mathbf{B}$ ($p = q = 1$): The equilibrium constants (or the overall stability constants) are defined as follows.

$$\beta_{m1a} = [\mathrm{Aa}_m\mathrm{B}]/[\mathrm{Aa}]^m[\mathrm{B}] \quad (12)$$

$$\beta_{n1b} = [\mathrm{Ab}_n\mathrm{B}]/[\mathrm{Ab}]^n[\mathrm{B}] \quad (13)$$

Obviously, β_{11x} is K_x (x = a or b). The combination with the mass balances leads to the following polynomial.

$$P_a[\mathrm{Aa}]^{m+1} + P_b[\mathrm{Aa}]^{n+1} + Q_a[\mathrm{Aa}]^m + Q_b[\mathrm{Aa}]^n + S[\mathrm{Aa}] - C_A = 0 \quad (14)$$

$$P_a = (1 + K_{is})\cdot\beta_{m1a} \quad (14a)$$

$$P_b = (1 + K_{is})\cdot K_{is}^{\,n}\cdot\beta_{n1b} \quad (14b)$$

$$Q_a = (m\cdot C_B - C_A)\cdot\beta_{m1a} \quad (14c)$$

$$Q_b = (n\cdot C_B - C_A)\cdot K_{is}^{\,n}\cdot\beta_{n1b} \quad (14d)$$

$$S = 1 + K_{is} \quad (14e)$$

The concentration of **B** is calculated from [Aa] with the following relation.

Parameters			Observed Data					Calculated Values					Concentraions (M)				
Kis, [Ab]/[Aa]	1.06	2.06	No	[A]t/mM	[B]t/mM	Δa(ob)	Δb(ob)	[B]t/[A]t	Δa(d)	Δb(d)	Dev-a	Dev-b	[Aa]	[AaB]	[Ab]	[AbB]	[B]
(Ka+KisKb)	1.72E+03	3.54E+03	0	2.00	0.00	0.0000	0.0000	0.00	0.0000	0.0000	0.0000	0.0000	9.71E-04	0.00E+00	1.03E-03	0.00E+00	0.00E+00
Floating Variables		(Std. Dev.)	1	2.00	2.00	-0.0050	-0.0026	1.00	-0.0054	-0.0027	0.0004	-0.0001	5.15E-04	5.21E-04	5.46E-04	4.18E-04	1.06E-03
Δca	-0.0107	0.0001	2	2.00	4.00	-0.0080	-0.0043	2.00	-0.0077	-0.0041	-0.0						3
Ka	953	83	3	2.00	6.00	-0.0086	-0.0048	3.00	-0.0087	-0.0048	0.0						3
Δcb	-0.0063	0.0001	4	2.00	8.00	-0.0093	-0.0052	4.00	-0.0092	-0.0052	-0.0						3
Kb	721	96	5	2.00	10.00	-0.0097	-0.0052	5.00	-0.0095	-0.0054	-0.0						3
100*R	0.076		6	2.00	12.00	-0.0099	-0.0054	6.00	-0.0097	-0.0056	-0.0						2
100*Rh	2.286		7	2.00	14.00	-0.0099	-0.0058	7.00	-0.0099	-0.0057	0.0						2
100*Rh(a)	2.258		8	2.00	16.00	-0.0099	-0.0059	8.00	-0.0100	-0.0058	0.0						2
100*Rh(b)	2.371		9	2.00	18.00	-0.0099	-0.0059	9.00	-0.0101	-0.0058	0.0						2
100*SD(y)	0.019	4	10	2.00	20.00	-0.0099	-0.0059	10.00	-0.0101	-0.0059	0.0						2
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			12														
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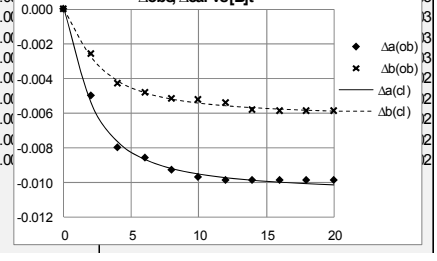

Figure 5. Example of Excel® worksheet for simultaneous least-squares fitting of a pair of titration curves observed for isomers in 1:1-complexation [Ref. 4].

$$[B] = C_B/(1 + \beta_{m1a}[Aa]^m + K_{is}{}^n\cdot\beta_{n1b}[Aa]^n) \quad (15)$$

Other concentrations are calculated successively from the definitions of the stability constants, yielding the titration curves, *i.e.*, Δ_x versus C_B plots.

$$\Delta_a = m\cdot\Delta_{ca}[Aa_mB]/([Aa] + m[Aa_mB]) \quad (16)$$

$$\Delta_b = n\cdot\Delta_{cb}[Ab_nB]/([Ab] + n[Ab_nB]) \quad (17)$$

AaB_p, AbB_q ($m = n = 1$): The titration curves are derived from the following definitions and equations.

$$\beta_{1pa} = [AaB_p]/[Aa][B]^p \quad (18)$$

$$\beta_{1qb} = [AbB_q]/[Ab][B]^q \quad (19)$$

$$\beta_{1pa}[B]^{p+1} + \beta_{1qb}\cdot K_{is}[B]^{q+1} + R_a[B]^p + R_b[B]^q + S[B] - S\cdot C_B = 0 \quad (20)$$

$$R_a = (p\cdot C_A - C_B)\cdot\beta_{1pa} \quad (20a)$$

$$R_b = (q\cdot C_A - C_B)\cdot K_{is}\cdot\beta_{1qb} \quad (20b)$$

$$S = 1 + K_{is} \quad (20c)$$

$$[Aa] = C_A/(S + \beta_{1pa}[B]^p + K_{is}\cdot\beta_{1qb}[B]^q) \quad (21)$$

$$\Delta_a = \Delta_{ca}[AaB_p]/([Aa] + [AaB_p]) \quad (22)$$

$$\Delta_b = \Delta_{cb}[AbB_q]/([Ab] + [AbB_q]) \quad (23)$$

The polynomials (Equations 14 and 20) are solved by a numerical method such as a Newton-Raphson routine [5]. The least-squares fitting of a pair of titration curves determines the stability constants. These calculations are programmable with Excel® worksheets using Solver tool [4].

5.2. Example

Figure 6 presents titration curves observed for N-CH(2) protons of cationic glucosamine with the variation of the concentration of phthalate anion [4]. The paired curves are well interpreted by assuming the formation of $\mathbf{Ax_2B}$ for both isomers. The $\mathbf{Ax_2B}$ stoichiometry should be verified by testing the 1:1-stoichiometry, as the former is less common than the latter. The broken lines calculated for the 1:1-stoichiometry deviate systematically from the observed data, ruling out the assumed stoichiometry. The goodness of fit with the $\mathbf{Ax_2B}$ stoichiometry suggests that each complex is formed in a single step.

5.3. Note on two-step complexation

Commonly, organic supramolecular complexation yields a monotonic titration curve which is interpretable by the formation of a single species in a one-step reaction, as exemplified above. Probably, the stoichiometry is controlled by the chemical and structural features of the reactants rather than the simple mass action. When a stable $\mathbf{A_2B}$-type complex is formed with receptor **B** that has two equivalent binding sites, for example, it may no longer react with **B** even in the concentration range of $C_B >> C_A$ so that the titration curve is governed only by the major species. The presence of minor species is expected in the following cases: (1) the observed titration curve is non-monotonic; (2) the titration curve is not satisfactory interpreted by any one-step complexation model. The theoretical titration curves can be derived for the step-wise formation of two species.

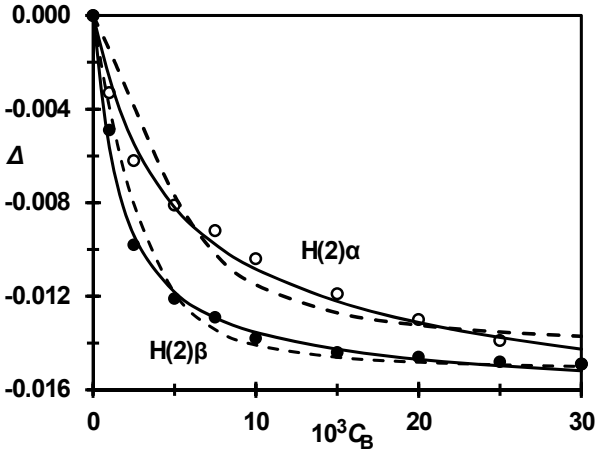

Figure 6. Change in chemical shifts, $\Delta = \delta - \delta(C_B{=}0)$, observed for N-CH(2) protons of α- and β-forms of cationic glucosamine (5×10^{-3} M) with the variation of phthalate concentration (C_B /10^{-3} M) in D_2O at pD = 6.30 and T = 25 °C. The solid lines present the best fits based on $\mathbf{Ax_2B}$ stoichiometry for both isomers: β_{21x} is 6.5 (1.5) × 10^4 M^{-2} for α-form; 35 (4) × 10^4 M^{-2} for β-form. The broken lines show the best fits for **AxB** stoichiometry with K_α = 680 (280) M^{-1} and K_β = 2080 (1040) M^{-1} [Ref. 4].

$\mathbf{Ax_jB}$ and $\mathbf{Ax_kB}$ (x = a or b): The overall stability constants are defined for the complexes of both isomers **Aa** and **Ab**, as follows.

$$\beta_{j1x} = [Ax_jB]/[Ax]^j[B] \text{ for } j\cdot\mathbf{Ax} + \mathbf{B} \rightleftarrows \mathbf{Ax_jB} \quad (24)$$

$$\beta_{k1x} = [Ax_kB]/[Ax]^k[B] \text{ for } k\cdot\mathbf{Ax} + \mathbf{B} \rightleftarrows \mathbf{Ax_kB} \quad (25)$$

The titration curves are derived from the definitions and the following equations.

$$S{\cdot}P_{1j}[\mathrm{Aa}]^{j+1} + S{\cdot}P_{1k}[\mathrm{Aa}]^{k+1} + P_{2j}[\mathrm{Aa}]^{j} + P_{2k}[\mathrm{Aa}]^{k} + S[\mathrm{Aa}] - C_\mathrm{A} = 0 \quad (26)$$

$$P_{1j} = \beta_{j1\mathrm{a}} + K_{is}^{\,j}{\cdot}\beta_{j1\mathrm{b}} \quad (26a)$$

$$P_{1k} = \beta_{k1\mathrm{a}} + K_{is}^{\,k}{\cdot}\beta_{k1\mathrm{b}} \quad (26b)$$

$$P_{2j} = (j{\cdot}C_\mathrm{B} - C_\mathrm{A})(\beta_{j1\mathrm{a}} + K_{is}^{\,j}{\cdot}\beta_{j1\mathrm{b}}) \quad (26c)$$

$$P_{2k} = (k{\cdot}C_\mathrm{B} - C_\mathrm{A})(\beta_{k1\mathrm{a}} + K_{is}^{\,k}{\cdot}\beta_{k1\mathrm{b}}) \quad (26d)$$

$$S = 1 + K_{is} \quad (26e)$$

$$[\mathrm{B}] = C_\mathrm{B}/(1 + P_{1j}[\mathrm{Aa}]^{j} + P_{1k}[\mathrm{Ab}]^{k}) \quad (27)$$

$$\Delta_\mathrm{x} = (j{\cdot}\Delta_{Cj\mathrm{x}}[\mathrm{Ax}_j\mathrm{B}] + k{\cdot}\Delta_{Ck\mathrm{x}}[\mathrm{Ax}_k\mathrm{B}])/([\mathrm{Ax}] + j[\mathrm{Ax}_j\mathrm{B}] + k[\mathrm{Ax}_k\mathrm{B}]) \quad (28)$$

$\mathbf{AxB}_j$ and $\mathbf{AxB}_k$ (x = a or b): The key equations are shown below.

$$\beta_{1j\mathrm{x}} = [\mathrm{AxB}_j]/[\mathrm{Ax}][\mathrm{B}]^{j} \text{ for } \mathbf{Ax} + j{\cdot}\mathbf{B} \rightleftarrows \mathbf{AxB}_j \quad (29)$$

$$\beta_{1k\mathrm{x}} = [\mathrm{AxB}_k]/[\mathrm{Ax}][\mathrm{B}]^{k} \text{ for } \mathbf{Ax} + k{\cdot}\mathbf{B} \rightleftarrows \mathbf{AxB}_k \quad (30)$$

$$R_{1j}[\mathrm{B}]^{j+1} + R_{1k}[\mathrm{B}]^{k+1} + R_{2j}[\mathrm{B}]^{j} + R_{2k}[\mathrm{B}]^{k} + S[\mathrm{B}] - S{\cdot}C_\mathrm{B} = 0 \quad (31)$$

$$R_{1j} = \beta_{1j\mathrm{a}} + K_{is}{\cdot}\beta_{1j\mathrm{b}} \quad (31a)$$

$$R_{1k} = \beta_{1k\mathrm{a}} + K_{is}{\cdot}\beta_{1k\mathrm{b}} \quad (31b)$$

$$R_{2j} = (j{\cdot}C_\mathrm{A} - C_\mathrm{B})(\beta_{1j\mathrm{a}} + K_{is}{\cdot}\beta_{1j\mathrm{b}}) \quad (31c)$$

$$R_{2k} = (k{\cdot}C_\mathrm{A} - C_\mathrm{B})(\beta_{1k\mathrm{a}} + K_{is}{\cdot}\beta_{1k\mathrm{b}}) \quad (31d)$$

$$[\mathrm{Aa}] = C_\mathrm{A}/(S + R_{1j}[\mathrm{B}]^{j} + R_{1k}[\mathrm{B}]^{k}) \quad (32)$$

$$\Delta_x = (\Delta_{Cj\mathrm{x}}[\mathrm{AxB}_j] + \Delta_{Ck\mathrm{x}}[\mathrm{AxB}_k])/([\mathrm{Ax}] + [\mathrm{AxB}_j] + [\mathrm{AxB}_k]) \quad (33)$$

In least-squares curve fitting based on Equation 28 or 33, four variables have to be determined from each titration curve. Such a calculation is impossible to perform for a monotonic curve, and it is very tough even for a non-monotonic curve; stoichiometry (*i.e.*, the set of j and k) is presumable for expected species, but calculated stability constants are tentative because they are varied in a wide range depending on the initial values and accompanied by unacceptably large standard deviations. In summary, if the observed titration curves are monotonic and the least-squares calculation detects only a single species for each curve, the stoichiometry is definitely controlled by the chemical and structural features of the reactants, and the resulting stability constant describes essentially the true complexation scheme.

6. Additional remarks

6.1. Error of neglecting isomerism

The nature of monotonic curves shown in Figure 4 resembles that predicted for ordinary 1:1-complex formation. Even if the isomerization is neglected, the titration curves are apparently reproduced. However, the resulting apparent stability constants, $K_\alpha' = 670$ (80) M^{-1} and $K_\beta' = 470$ (50) M^{-1}, are much smaller than the proper values, $K_\alpha = 950$ (80) and $K_\beta = 720$ (100), determined by including the isomerization equilibrium. Such discrepancy becomes more serious as difference between K_a and K_b is increased: for a pair of titration curves simulated with $K_{is} = 2$, $K_\mathrm{a} = 100$ M^{-1}, $K_\mathrm{b} = 500$ M^{-1}, and $\Delta_\mathrm{ca} = \Delta_\mathrm{cb}$, the isomerization-neglected model gave $K_\mathrm{a}' = 59$ (7) M^{-1} and $K_\mathrm{b}' = 630$ (44) M^{-1}; even the ratio of the stability constants far deviates from the true value. The inclusion of the isomerism is indispensable for the proper evaluation of the individual stability constants of isomer complexes.

6.2. Spectrum of counter reactant B

In the reaction scheme in Figure 1, the NMR spectrum of reactant **B** also responds to the complexation, but it provides little information about the individual isomer complexes in the rapid exchange case of NMR. For instance, Figure 7 shows the aromatic proton signals of capsaicin at different concentrations of glucosamine. The signals exhibit up-field shifts accompanied by line-broadening with increasing concentration of glucosamine. This spectral change supports the complexation between the reactants, and indicates that the OH group works as the binding site in the complexes. However, signals specific of **AaB** or **AbB** complex do not appear. Plots of the Δ of the capsaicin signals versus glucosamine concentration present asymptotic titration curves, but such reverse titration cannot determine K_a or K_b; even the average of the constants is not obtained [3]. In a titrimetric study of supramolecular complexation of an artificial receptor, usually a signal of the receptor is monitored by changing the concentration of a target substrate, partly because the availability of the receptor is limited. For the complexation of the conformational isomers of a substrate, however, the NMR of the isomeric substrate should be monitored for the correct evaluation of the

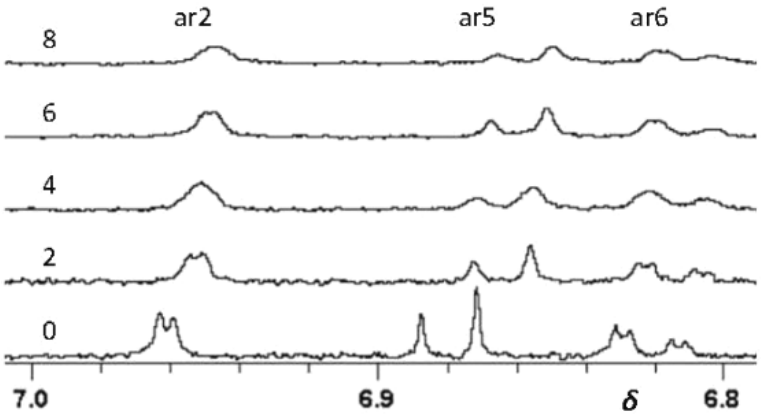

Figure 7. NMR spectra of aromatic protons of capsaicin (2×10^{-3} M) at different concentrations of glucosamine ($0–8 \times 10^{-3}$ M) in D_2O at pD = 9.55 (for the labels of the protons, see Figure 3); the signals are broadened and shifted upon complexation, but a new signal is not detected [Ref. 1].

thermodynamic stabilities, although the spectrum of the receptor still provides information about the binding sites and the interaction modes.

7. Conclusion

The conformational isomers of an organic substance form supramolecular complexes individually with a receptor, and the complexation can be studied by the NMR titration in which the spectra of the isomers are traced by changing the concentration of the receptor. The least-squares analysis of a pair of observed titration curves is programmable with Excel® spreadsheets to determine the individual stability constants of the isomer complexes. The ratio of the constants indicates the degree of the selective complexation of the isomers. The extended application of this NMR method to the complexation of biogenic isomers with particular receptors would prove the isomer-specific molecular interaction that may result in isomer effects on the bioactivities.

CONFLICT OF INTEREST STATEMENT

There is no conflict of interest.

ABBREVIATIONS

Aa, **Ab**	:	isomers of **A**
B	:	counter reactant
AaB, **AbB**	:	complexes of isomers
C_A, C_B	:	total concentrations of reactants
K_{is}	:	isomerization constant, [Ab]/[Aa]
K_a, K_b	:	stability constants of **AaB** and **AbB**
β_{mpa}, β_{nqb}	:	stability constants of $\mathbf{Aa}_m\mathbf{B}_p$ and $\mathbf{Ab}_n\mathbf{B}_q$
δ	:	chemical shift in NMR
Δ_a, Δ_b	:	δ-changes of isomers referenced to $\delta(C_B=0)$
Δ_{ca}, Δ_{cb}	:	Δ_a, Δ_b intrinsic of isomers in the complexes

REFERENCES

1. Higuera-Ciapara, I., Virués, C., Jiménez-Chávez, M., Martínez-Benavidez, E., Hernández, J., Domínguez, Z., López-Rendón, R., Velázquez, E. F., and Inoue, M. 2017, Carbohydr. Res. 452, 6-16; doi.org/10.1016/j.carres.2017.09.014.
2. Thordarson, P. 2011, Chem. Soc. Rev., 40, 1305-1323; doi.org/10.1039/c0cs00062k.
3. Inoue, M., Virués, C. and Higuera-Ciapara, I. 2019, Supramol. Chem., 31, 89-94; doi.org/10.1080/10610278.2018.
4. D-Yanez, S., Soberanes, Y., Navarro, R. E., Virués, C., and Inoue, M. 2020, Supramol. Chem., 32, 527-534; doi.org/10.1080/10610278.2020.1813293.
5. Billo, E. J. 2007, Excel® for Scientists and Engineers, Numerical Methods, John Wiley & Sons, Hoboken NJ.