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Determination of the association constant of monovalent mode protein–sugar interaction by capillary zone electrophoresis

Susumu Honda*, Atsushi Taga, Kenji Suzuki, Shigeo Suzuki and Kazuaki Kakehi

Faculty of Pharmaceutical Sciences, Kinki University, Kowakae, Higashi-osaka (Japan)

ABSTRACT

Protein–sugar interaction was observed by capillary zone electrophoresis, using a few β-galactose-specific lectins and lactobionic acid as protein and sugar models, respectively. The lectin peaks were retarded in a concentration-dependent manner by addition of lactobionic acid in a carrier, and association constants of monovalent mode interactions could be obtained from t_1 (migration time of protein), t_2 (migration time of complex, obtainable as the migration time at the plateau) and the slope of the $(t - t_1)^{-1}$ *vs.* $[S]^{-1}$ plots, where t and [S] are the migration of protein in the presence of lactobionic acid and the concentration of lactobionic acid, respectively. The values for *Ricinus communis* agglutinin, peanut agglutinin and soy bean agglutinin at pH 6.8 were $3.3 \cdot 10^3$, $9.1 \cdot 10^2$ and $1.1 \cdot 10^2$ l mol^{-1}, respectively. This method required only small amounts of protein samples and was reproducible. The amount of the sugar could be minimized under the conditions that the carrier was a buffer containing the sugar whereas the electrode solutions consisted only of the buffer.

INTRODUCTION

Specific recognition of a protein by a sugar chain is an important biological phenomenon. As the specificity of protein–sugar interaction is highly dependent on the structures of the compounds involved, elucidation of structure–affinity correlations is of primary importance.

The evaluation of affinity is usually performed by calculating the association constant between the components or the dissociation constant of the resultant complex. They can be obtained by determining either a free or bound protein in the reaction solution, after these two states of protein have been separated by an appropriate means such as equilibrium dialysis [1]. They can be also determined, without separation, from the change in UV absorption (*e.g.*, [2]), fluorescence intensity (*e.g.*, [3]), nuclear magnetic resonance [4] or the transition temperature in differential scanning calorimetry [5]. The latter methods are convenient but generally lack accuracy. On the other hand, chromatographic (*e.g.*, [6]) and electrophoretic (*e.g.*, [7]) methods have been developed with the use of sugar-immobilized solid supports. These methods are also convenient but require the sugar-immobilization process.

High-performance capillary electrophoresis is attractive for studies of such molecular interactions, as it allows efficient separation and reproducible quantification by on-tube detection. This paper describes the determination of the association constants of protein–sugar interactions by zone electrophoresis using some lectins and an acidic disaccharide as model couples.

EXPERIMENTAL

Materials

Ricinus communis agglutinin (RCA_{60}), peanut agglutinin (PNA) and soy bean agglutinin (SBA) were obtained from Honen Seiyu (Tokyo, Japan) and used as received. Lactose and lactobionic acid were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals were of the highest grade available. Water was deionized and doubly distilled.

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Inhibitory effects of saccharides on haemagglutination induced by RCA_{60}

These effects were studied by the method of Nicolson and Blaustein [8] by using rabbit erythrocytes.

Capillary zone electrophoresis

A Model 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) was used. It was equipped with a fused-silica capillary tube (75 cm × 50 μm I.D.) and a UV detector. The tube length between the anodic end and the detector was 60 cm. Solutions of RCA_{60} in 50 m*M* phosphate buffer (pH 6.8) containing or not containing various concentrations of lactobionic acid were used as the carrier. In the former instance aqueous solutions of lactobionic acid were neutralized with 0.1 *M* sodium hydroxide and the resulting solutions were evaporated to dryness. The residues were dissolved in 50 m*M* phosphate buffer to give the desired concentrations of lactobionic acid. Solutions of lectins (1 ng nl^{-1}) in the same buffer as that for the carrier were automatically introduced by suction to the anodic end of the tube for programmed periods, and zone electrophoresis was performed at 30 ± 0.1°C.

Equilibrium dialysis

A Sanplatic (Osaka, Japan) multiple microdialyser was used, which permits the simultaneous dialysis of multiple solutions under the same conditions. Each dialysis chamber was divided into two compartments of equal volume (*ca.* 500 μl) by a sheet of cellophane membrane. A $1.6 \cdot 10^{-5}$ *M* RCA_{60} solution in 50 m*M* phosphate buffer (pH 6.8) was placed in one compartment of each of five chambers, and solutions containing various concentrations of lactobionic acid in the same buffer were placed in the other compartments. Dialysis was performed for 84 h at 30°C; subsequently, the concentration of unbound lactobionic acid in each compartment was determined by using a Dionex high-performance liquid chromatographic apparatus equipped with an HPIC-AS6 column (25 cm × 4.6 mm I.D.) and a pulse amperometric detector, under similar conditions to those described previously [9]. The peak of lactobionic acid appeared at 12.3 min by isocratic elution with 0.3 *M* sodium hydroxide. The dissociation constant of the RCA_{60}–lactobionic acid complex was obtained from a Scatchard plot [10]. The association constant was obtained as the reciprocal dissociation constant.

THEORY

Generally, the binding of a protein to a sugar is an equilibrium reaction, and the association constant (K_a) of the protein–sugar interaciton can be written as [PS]/[P] [S], provided that the reaction proceeds in monovalent mode, where [P], [S] and [PS] are the concentrations of the protein, the sugar and the resulting complex, respectively. The molar fraction (α) of the complex can be expressed as

$$\alpha = [PS]/([P] + [PS]) \tag{1}$$

from which can be derived

$$\frac{1}{K_a[S]} + 1 = \frac{1}{\alpha} \tag{2}$$

When a protein sample is introduced to the anodic end of a fused-silica capillary tube filled with carrier not containing the sugar, the observed velocity of the protein sample (V_P) is given by

$$V_P = V_{eo} - U_P \tag{3}$$

where V_{eo} and U_P are the velocities of electroosmotic flow and electrophoretic migration of the protein, respectively. On the other hand, when the protein sample is introduced to a carrier containing the sugar, the observed velocity of the protein sample (V'_P) is given by

$$V'_P = V_{eo} - u'_P \tag{4}$$

where u'_P is the apparent velocity of electrophoretic migration of the protein sample in the presence of the sugar. The term u'_P can be written as the sum of the velocities of the protein (U_P) and the complex (U_{PS}), multiplied by their molar fractions:

$$u'_P = (1 - \alpha)U_P + U_{PS} \tag{5}$$

From eqns. 3–5, α can be expressed as follows:

$$\alpha = (V_P - V'_P)/(U_{PS} - U_P) \tag{6}$$

Substitution of α in eqn. 2 by that in eqn. 6 gives

$$\frac{1}{K_a[S]} + 1 = \frac{\Delta U}{V_P - V'_P} \tag{7}$$

where $\Delta U = U_{PS} - U_P$. As $V'_P = l/t$ and $V_P = l/t_1$, where t and t_1 are the migration times of the protein

sample in the presence and absence of the sugar, respectively, and l is the length of the capillary tube between the anodic end and the detector, eqn. 7 can be converted into

$$\frac{1}{t - t_1} = \frac{l}{\Delta U t_1^2 K_a} \cdot \frac{1}{[S]} + \frac{l - \Delta U t_1}{\Delta U t_1^2} \quad (8)$$

Therefore, a plot of $(t - t_1)^{-1}$ *vs.* $[S]^{-1}$ gives a straight line of slope $l\Delta U^{-1} t_1^{-2} K_a^{-1}$. ΔU can be expressed as

$$\Delta U = U_{PS} - U_P = l\left[\left(\frac{1}{t_0} - \frac{1}{t_2}\right) - \left(\frac{1}{t_0} - \frac{1}{t_1}\right)\right]$$

$$= l \cdot \frac{(t_2 - t_1)}{t_1 t_2} \quad (9)$$

where t_0 is the time required by electroosmotic flow to travel through the tube length between the anodic end and the detector l and t_2 is the migration time of the complex. Hence,

$$K_a = \frac{t_2}{t_1} \cdot \frac{1}{(t_2 - t_1)} \cdot \frac{1}{A} \quad (10)$$

where A is the slope.

Therefore, association constants can be calculated by using migration times, t_1 and t_2, together with the slope of the $(t - t_1)^{-1}$ *vs.* $[S]^{-1}$ plots. The term t_2 can be regarded as the apparent migration time when a plateau is reached.

RESULTS

The theoretical treatment mentioned above indicates that the association constant for protein–sugar interaction can be obtained as the product of three terms, the t_2/t_1 ratio, the reciprocal of the difference $t_1 - t_2$ and the reciprocal of the slope of the $(t - t_1)^{-1}$ *vs.* $[S]^{-1}$ plot (eqn. 10). A greater delay of the protein peak allows a more accurate calculation of these terms, hence an acidic sugar giving an acidic complex, which is more strongly held back by electrophoresis, is preferable to neutral sugars as the ligand. In this work RCA_{60}, a β-galactose-specific lectin, and lactobionic acid (4-O-β-galactopyranosylgluconic acid) were selected as models of the protein and the acidic sugar, respectively, based on this consideration. Capillary zone electrophoresis of RCA_{60} in carriers of pH 6.8, containing and not containing lactobionic acid, gave electropherograms as shown in Fig. 1.

In the absence of lactobionic acid, this lectin gave its peak almost superimposed on that of mesityl oxide, an internal neutral marker. When lactobionic acid was added to the carrier at a concentration of 0.2 mM, the lectin peak was retarded slightly and its

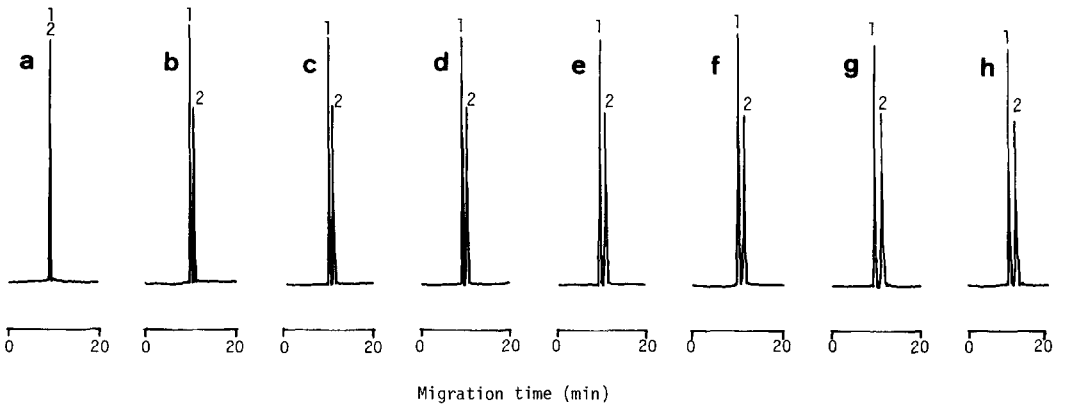

Fig. 1. Concentration-dependent retardation of the RCA_{60} peak in carriers containing lactobionic acid. Capillary, fused silica (85 cm × 50 μm I.D.); carrier, 50 mM phosphate buffer (pH 6.8) containing lactobionic acid at concentrations of (a) 0, (b) 0.2, (c) 0.3, (d) 0.4, (e) 0.5, (f) 1.0, (g) 5.0 and (h) 10.0 mM; applied voltage, 20 kV; detection, UV absorption at 220 nm; sample, 50 mM phosphate buffer (pH 6.8) containing RCA_{60} and mesityl oxide at concentrations of 1 ng ml^{-1} and 100 mM, respectively. Peaks: 1 = mesityl oxide; 2 = RCA_{60} in equilibrium with the RCA_{60}–lactobionic acid complex.

peak was resolved from that of mesityl oxide. Retardation became more prominent with higher concentrations of lactobionic acid.

Incidentally, the apparent mobility (μ_{ep}) of a lectin can be written as

$$\mu_{ep} = \frac{Ll}{V}\left(\frac{1}{t_0} - \frac{1}{t}\right) \tag{11}$$

where L is the total length of the capillary tube and V is the applied voltage. Manipulation of eqn. 11 leads to

$$t = \frac{Llt_0}{Ll - Vt_0\mu_{ep}} \tag{12}$$

In this work, L, l and V were maintained constant. The term μ_{ep} varies with the concentration of lactobionic acid, but is considered to be constant at every given concentration. However, t_0 varies, although only slightly, on addition of lactobionic acid, owing to small changes in the viscosity, permittivity and other properties of the carrier, and t, t_1 and t_2 are also shifted slightly in response to this variation. Therefore, t was corrected by eqn. 12 using the t_0 value obtained in the absence of sugar. In this equation, μ_{ep} values were those obtained from eqn. 11 for given concentrations of lactobionic acid. The t_1 *and* t_2 values were also corrected in a similar manner.

Fig. 2 shows the change in corrected migration time (t) of RCA_{60} with the concentration of lactobionic acid in the carrier. The corrected t value rapidly increased to reach a plateau at *ca.* 5 mM, the maximum migration time (t_2) being 15.75 min.

Fig. 3 shows the plot of $(t - t_1)^{-1}$ *vs.* $[S]^{-1}$ obtained by using the corrected t and t_1 values. Good linearity was observed (correlation coefficient, 0.98), and the slope of the straight line was $8.1 \cdot 10^{-1}$ mM min^{-1}. The association constant calculated from eqn. 10 by using this value was $3.2 \cdot 10^3$ l mol^{-1}. This is in good agreement with the value obtained by equilibrium dialysis, followed by a Scatchard plot ($3.3 \cdot 10^3$ l mol^{-1}). The association constants of PNA and SBA to lactobionic acid were similarly determined using 50 mM phosphate buffer (pH 6.8) containing various concentrations of lactobionic acid as carrier and were $9.1 \cdot 10^2$ and $1.1 \cdot 10^2$ l mol^{-1}, respectively. The correlation coefficients of the $(t - t_1)^{-1}$ *vs.* $[S]^{-1}$ plots were *ca.* 0.99 for both lectins.

As mentioned above, the method based on the delay of protein peaks in zone electrophoresis was

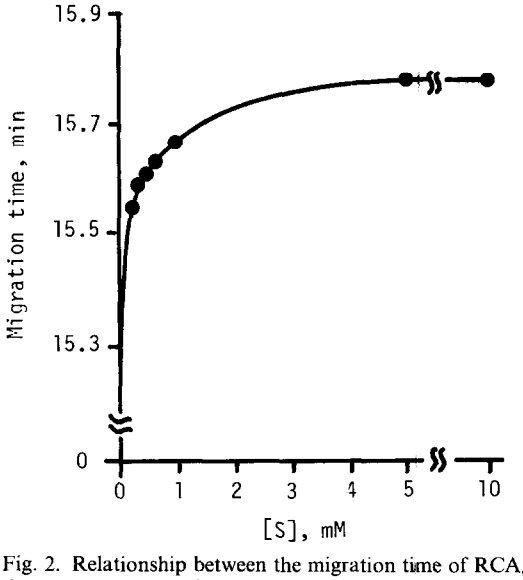

Fig. 2. Relationship between the migration time of RCA_{60} and the concentration of lactobionic acid. Each migration time (average of three measurements) was corrected as described in the text.

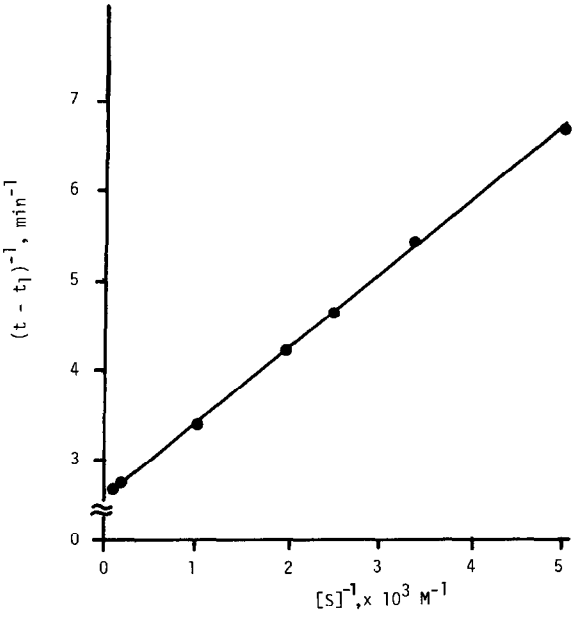

Fig. 3. Plots of $(t - t_1)^{-1}$ *vs.* $[S]^{-1}$ for the RCA_{60}–lactobionic acid couple.

simple and required only small amounts of protein samples (*ca.* 10 ng as injected amount, to obtain six plots), but needed considerable amounts of the sugar when both electrode vessels were filled with buffer solutions containing the sugar; as much as 10 μmol were required for a series of experiments. However, when buffer solutions containing the sugar were loaded only in the capillary tube and the buffer solutions not containing the sugar was filled in both electrode vessels, the required amount of the sugar could be reduced to the 1-nmol level. There was no difference in the values using the normal and this special manner; the association constants of RCA_{60}–lactobionic acid interaction in both instances were $3.3 \cdot 10^3$ l mol^{-1} at pH 6.8.

It should be noticed that eqn. 10 does not contain a sample concentration term; association constants can be obtained by using any detectable concentration of the sample, provided that good separation of the peaks is ensured. In order to confirm this, the association constant of RCA_{60}–lactobionic acid interaction was determined using three different concentrations of RCA_{60}, namely 0.2, 1.0 and 5.0 ng nl^{-1}. The values obtained were $4.2 \cdot 10^3$, $4.3 \cdot 10^3$ and $4.1 \cdot 10^3$ l mol^{-1}, respectively, at pH 6.9 and the difference among sample concentrations could be neglected, at least for the 0.2–5.0 ng nl^{-1} range.

DISCUSSION

In the present mode of capillary electrophoresis (zone electrophoresis), lactobionic acid moved at a constant velocity from the anode to the cathode by the combined effects of electroosmosis and electrophoresis. When RCA_{60} was introduced to the carrier not containing lactobionic acid, it was transported almost only by electroosmotic flow, because under the operating conditions (pH 6.8) near the isoelectric point (7.1 [12]) the overall charge of this lectin was nearly zero. However, in the presence of lactobionic acid, it interacted with this acidic sugar to give a negatively charged complex, which was held back by electrophoresis. As a result, the RCA_{60} peak (peak 2) was delayed from the peak of the neutral marker (mesityl oxide, peak 1), and the magnitude of this delay was dependent on the concentration of lactobionic acid. The derivation of eqn. 10 was based on the assumption that the sugar is bound to a single site on the protein molecule and the apparent velocity of electrophoretic migration of the protein (u'_P), in equilibrium with the complex, is the sum of velocities of the bound and the free protein, multiplied by their molar fractions, as indicated by eqn. 5. The excellent linearity of the $(t - t_1)^{-1}$ *vs.* $[S]^{-1}$ plots supports this assumption. Podder *et al.* [13] concluded, based on equilibrium dialysis data, that the *Ricinus communis* agglutinin having a molecular mass of 120 000 had two binding sites. Olsnes *et al.* [14] also noted the same phenomen on, but stated that the ricin from the same source, having a smaller molecular mass, had only one binding site. The present work, probably dealing with the same lectin that Olsnes *et al.* studied, showed monovalent mode binding to lactobionic acid.

Lactobionic acid has a β-galactose residue linked to gluconic acid, and this β-galactose residue is considered to exert affinity to RCA_{60}, PNA and SBA. The association constants of these lectins with lactobionic acid have not been reported previously, but equilibrium dialysis, followed by the Scatchard plot, gave a value of $3.3 \cdot 10^3$ l mol^{-1} for the RCA_{60}–lactobionic acid interaction, which is consistent with the value obtained above by zone electrophoresis. Olsnes *et al.* [13] obtained a value of $1.5 \cdot 10^4$ l mol^{-1} for the association constant of *Ricinus communis* lectin to lactose by the equilibrium dialysis method. This value is slightly higher than that for the RCA_{60}–lactobionic acid interaction. The weaker affinity of lactobionic acid to RCA_{60} than that to lactose was also demonstrated by a haemagglutination inhibition experiment. Lactobionic acid showed 50% inhibition to haemagglutination of rabbit erythrocytes caused by RCA_{60} at $1.6 \cdot 10^{-4}$ *M*, whereas lactose showed 50% inhibition at $5.0 \cdot 10^{-5}$ *M*.

The presence of the gluconic acid residue in lactobionic acid seems to have a smaller effect on affinity to RCA_{60} compared with that of the glucose residue in lactose, but its negative charge is important in causing a long delay of the lectin peaks, from which the magnitude of the affinity could be accurately evaluated in the present method. This charged portion of the molecule is assumed to be almost indifferent to affinity.

The association constant can be obtained as the product of t_2/t_1, $(t_1 - t_2)^{-1}$ and A^{-1}, as mentioned above. The relative standard deviations ($n = 5$) of

t_2/t_1 and $(t_1 - t_2)^{-1}$ at a lactobionic acid concentration of 1 mM, for example, were as low as 2.7% and 0.17%, respectively. Although the relative standard deviation of A^{-1} could not be obtained directly, it is expected to be fairly low, because the relative standard deviation of $(t - t_1)^{-1}$ at a lactobionic acid concentration of 1 mM was also as low as 2.2%. Five repetitions of a series of experiments on five successive days gave an average value of $4.2 \cdot 10^3$ l mol^{-1} for K_a for the RCA_{60}–lactobionic acid interaction at pH 6.9. The relative standard deviation was as low as 3.6%. Therefore, the reproducibility of the determination of association constants is considered to be fairly high. This is comparable to the low reproducibility of the equilibrium dialysis method (more than 50% in our experiment).

In this work a synthetic acidic sugar was used as a model sugar, but this principle of determination of association constant is also applicable to neutral saccharides having affinity to proteins, although the accuracy cannot be ensured owing to the smaller delay of the peaks. However, chemical introduction of an acidic group to the inactive sites of such neutral saccharides will allow a more accurate determination of association constants by enhancement of the electrophoretic effect on the complex formed. Various attempts to achieve the rapid and easy conversion of neutral oligosaccharides to acidic derivatives are in progress.

It should also be noted that the theory in this paper can be applied only to the monovalent mode interaction. The multiple mode interaction needs other considerations. A theoretical treatment will be published elsewhere.

SYMBOLS

[P] molar concentration of protein
[S] molar concentration of sugar
[PS] molar concentration of protein–sugar complex
α molar fraction of complex
K_a association constant of protein–sugar interaction
V_P observed velocity of protein sample in the absence of sugar
V'_P observed velocity of protein sample in the presence of sugar
U_P velocity of electrophoretic migration of protein
U_{PS} velocity of electrophoretic migration of complex
u'_P apparent velocity of electrophoretic migration of protein sample in the presence of sugar
L total length of capillary tube
l length of capillary tube between the anodic end and detector cell

REFERENCES

1 R. N. Pinckard, *Handbook of Experimental Immunology*, Blackwell, Oxford, Vol. 1, 1978, Ch. 17.
2 R. J. Doyle, E. P. Pittz and E. E. Woodside, *Carbohydr. Res.*, 8 (1968) 89–100.
3 A. Jimbo, N. Seno and I. Matsumoto, *J. Biochem. (Tokyo)*, 95 (1984) 267–275.
4 K. A. Kronis and J. P. Carver, *Biochemistry*, 21 (1982) 3050–3057.
5 C. Borrenbasck and B. Mattiasson, *Eur. J. Biochem.*, 107 (1980) 67–71.
6 I. M. Chaiken, *Anal. Biochem.*, 97 (1979) 1–10.
7 K. Takeo and S. Nakamura, *Arch. Biochem. Biophys.*, 153 (1972) 1–7.
8 G. L. Nicolson and J. Blaustein, *Biochim. Biophys. Acta*, 266 1972) 543–547
9 S. Honda, S. Suzuki, S. Zaiki and K. Kakehi, *J. Chromatogr.*, 523 (1990) 189–200.
10 G. Scatchard, *Ann. NY Acad. Sci.*, 51 81949) 660–672.
11 S. Fujiwara and S. Honda, *Anal. Chem.*, 59 (1987) 487–490.
12 S. Olsnes, K. Refsnes and A. Pihl, *Nature (London)*, 249 (1974) 627–631.
13 S. K. Podder, A. Surolia and B. K. Bachhawat, *Eur. J. Biochem.*, 44 (1974) 151–160.
14 S. Olsnes, E. Saltvedt and A. Pihl, *J. Biol. Chem.*, 249 (1974) 803–810.