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Determination of the Affinity Constants of Pea Lectin for Neutral Sugars by Capillary Affinophoresis with a Monoligand Affinophore

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Affinophoresis is a type of affinity electrophoresis in which an affinophore, a conjugate of an affinity ligand and a multiply charged soluble matrix, causes a change in migration velocity of proteins which have a specific affinity for the ligand. A monoligand affinophore bearing a mannoside was prepared by coupling iodoacetylated *p*-aminophenyl α-D-mannoside to the free thiol group of *N*-succinylated glutathione, and used for the affinophoresis of pea lectin in a capillary. The electrophoretic mobility of pea lectin towards the anode increased in the presence of the affinophore as a function of its concentration in a manner that is described by the equation for affinity electrophoresis. Analysis of the suppression of the affinophoresis on the addition of neutral sugars to the system allowed the determination of the dissociation constants of the lectin for these neutral sugars. The dissociation constants obtained on affinophoresis agreed well with the values in the literature. The preparation of a monoligand affinophore for ligands bearing an amino group should facilitate the application of this type of microscale analysis (0.14 ng of protein for each run) to protein ligand interactions.

Key words: affinity electrophoresis, capillary electrophoresis, lectin, ligand binding, molecular recognition.

As a type of affinity electrophoresis, we introduced affinophoresis in which an affinity ligand is attached to an ionic molecule to form an affinophore (*1, 2*). In the presence of an affinophore, the electrophoretic migration of a protein having an affinity for the ligand is specifically accelerated. The distinctive feature of an affinophore is the structural separation of its functions into the portion which exerts affinity and the portion which causes migration in an electric field, *i.e.*, an affinity ligand and a charged affinophore matrix, respectively (*3*). Thus, once a suitable ionic molecule is obtained as an affinophore matrix, the method can be generally applied to many substances by simply changing the affinity ligand. Proteases, lectins, and an antibody were specifically separated by affinophoresis, and the specific interactions between the affinophore and proteins were analyzed in agarose gel slabs.

On the other hand, the use of capillary electrophoresis for the analysis of binding equilibria between molecules of biochemical interest has been reported by several groups (*4-9*). One approach for this type of investigation, which is referred to as "affinity capillary electrophoresis" (*10*), consists of the zonal migration of a binding protein by free-solution capillary electrophoresis and the analysis of the change in its mobility caused by a charged ligand in the electrophoresis buffer using the equation for affinity electrophoresis (*2, 11-13*). The major advantages of affinity capillary electrophoresis are the small sample required, the feasibility of automation using commercially available instruments, the high precision in the measurement of mobility, the ability of analysis in a free solution, and the superior temperature control. When the ligand of interest is not originally charged or the number of the charges on the ligand is not sufficient to induce the necessary mobility change of the binding protein, a charge or charges have to be introduced to the ligand.

Considering the many favorable features of capillary electrophoresis for the analysis of molecular interactions, application of the principle of affinophoresis should be important for further development of such microscale analysis. This paper reports a procedure for the preparation of a triply charged monoligand affinophore involving the coupling of an iodoacetylated affinity ligand to succinylated glutathione as an affinophore matrix. With *p*-aminophenyl α-D-mannoside as an affinity ligand, the affinophore induced a sufficient mobility change of a divalent lectin, pea lectin, to permit the analysis of its specific interaction with the affinophore. Competition experiments involving the affinophoresis with a series of neutral sugars yielded dissociation constants for each sugar.

MATERIALS AND METHODS

The following chemicals were obtained from commercial sources: *p*-aminophenyl α-D-mannoside (Sigma, St. Louis,

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Abbreviations: GSSG, glutathione, oxidized form; GSH, glutathione, reduced form; sGSSGs, succinylated glutathione, oxidized form; sGSH, succinylated glutathione, reduced form; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; AP-Man, *p*-aminophenyl α-D-mannoside; IAc, iodoacetyl-; APCE, affinity probe capillary electrophoresis.

MO), glutathione (oxidized form) (GSSG) (Wako Pure Chemicals, Osaka), and fused silica capillaries (GL Sciences, Tokyo). *N*-Iodoacetoxysuccinimide was prepared as described previously (*14*).

Preparation of Pea Lectin—Pea lectin was extracted from pea seeds obtained at a local seed shop and purified according to a published procedure involving affinity chromatography on Sephadex G100 (*15*). The purified lectin (2 mg) dissolved in 0.4 ml of 4 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl buffer (pH 8.8) was applied on a Mono Q HR 5/5 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl buffer (pH 8.8), and eluted with a gradient of NaCl from 0 to 0.5 M in the buffer over a period of 15 min. The first major peak was collected and used as pea lectin. The protein preparation was nearly homogeneous, as evidenced by isoelectric focusing in a polyacrylamide-gel slab, with a pI value of 7.0.

Preparation of a Monoligand Affinophore—Succinylated glutathione (sGSH) was used as an affinophore matrix and iodoacetylated *p*-aminophenyl α-D-mannoside was coupled to the thiol group of the matrix as an affinity ligand for the lectin (Fig. 1).

Preparation of succinylated glutathione (oxidized form) (sGSSGs): GSSG (100 mg, 0.163 mmol) was dissolved in 1 ml of 0.2 M sodium borate buffer (pH 9.0) and the pH was readjusted to 9 by the addition of 0.32 ml of 1 M NaOH. Succinic anhydride (66 mg, 0.66 mmol) was added in two portions at 10 min intervals and the pH was maintained at 7 to 9 by the addition of 6 M NaOH. Disappearance of the amino group to less than 1/100 of the original level was verified by the application of a ninhydrin spraying reagent to a spot of the reaction mixture on a filter paper. The reaction mixture was stored in a freezer (−20°C) and used in the following step without purification.

Preparation of reduced succinylated glutathione (sGSH): To the crude succinylation product of glutathione (oxidized form) (sGSSGs) (about 2 μmol in 20 μl), 4 μmol (1 M, 4 μl) of dithiothreitol (DTT) was added to cleave the disulfide, and then the solution incubated for 10 min at room temperature. The reaction was stopped by the addition of 40 μl of 1 M HCl. sGSH was purified by HPLC on a reversed-phase chromatographic column (TSK-Gel ODS-80TS, 4.6 mm I.D. × 25 cm) with a cartridge guard column (TSK guard gel ODS-80TS, 3.2 mm I.D. × 1.5 cm) (Tosoh, Tokyo). Half of the reaction mixture, which contained approximately 2 μmol of sGSH, was applied to the column, which had been equilibrated with 0.1% trifluoroacetic acid, and eluted with a gradient of 0 to 25% acetonitrile in 0.1% trifluoroacetic acid over a period of 25 min, with detection by absorption measurement at 240 nm. Three major peaks appeared, and were identified in order of elution as DTT (13.3 min), sGSH (13.8 min), and oxidized DTT (16.5 min), respectively, on separate chromatographic runs with each compound. The second peak fraction was collected and combined with the same fraction of the second half of the reaction mixture. Rechromatography of a small portion of the combined fraction revealed slight contamination by DTT (5% of peak area). The fraction was evaporated to dryness and used in the following coupling step without further purification.

Iodoacetylation of p-aminophenyl α-D-mannoside: p-Aminophenyl α-D-mannoside (AP-Man) (3 μmol, 0.81 mg) was dissolved in 0.15 ml of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH buffer (pH 6.0), and then *N*-iodoacetoxysuccinimide (6 μmol, 1.7 mg) in 30 μl of dimethylformamide was added to the solution. After 1 h reaction at room temperature in the dark, the reaction mixture was stored in an ice bath before further purification.

Each half of the reaction mixture was applied to a HPLC column and separation was achieved under the same conditions as described above except for detection at 280 nm. The largest peak fraction at 22.9 min, which constituted 95% of the combined peak area for all the peaks on the chromatogram, was collected and combined with the same fraction of the other half. The combined fraction was evaporated to dryness and used as iodoacetyl-*p*-aminophenyl α-D-mannoside (IAc-AP-Man) in the following coupling step. We previously reported the synthesis of this compound using iodoacetic anhydride (*9*), but the yield was better with the present method.

Coupling of IAc-AP-Man to sGSH: IAc-AP-Man (*ca.* 3 μmol) was dissolved in 0.1 ml of 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, and then the solution was transferred to a tube containing sGSH (*ca.* 4 μmol). The pH of the solution was readjusted to 7-8 by the addition of 1 M NaOH, and then the solution was allowed to stand overnight at room temperature under nitrogen in the dark. The coupling product, sGS-AP-Man, was separated by two HPLC runs under the same conditions as described for the separation of IAc-AP-Man. The single peak which appeared at 19-20 min was collected. The fractions from two runs were combined and evaporated to dryness. The residue was dissolved in 1.25 ml of water, and then the solution was neutralized by the addition of 1 M NaOH. It was stored in a freezer (−20°C) and used as a stock solution of the affinophore. The concentration of sGS-AP-Man was determined by the phenol-sulfuric acid reaction method with methyl α-D-mannoside as a standard. The final yield from AP-Man was 80%. Mass spectrometry with fast atom bombardment ionization revealed a peak at *m/e* 741, corresponding to $(M+Na)^+$.

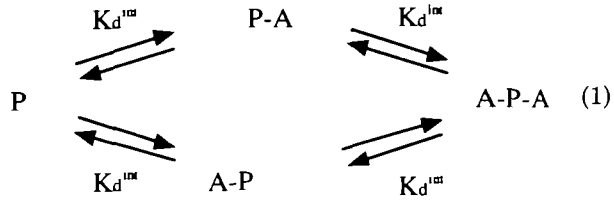
Capillary Affinophoresis—Electrophoresis was carried out with an automated capillary electrophoresis instrument (Beckman P/ACE 2210 with a UV detector). The inner wall of a fused silica capillary (25 μm i.d., 375 μm o.d.) was coated with succinylpolylysine as described previously (*9*), and then placed in a cartridge with a separation distance of 50 cm. The capillary was filled with an electrophoresis buffer [0.1 M Tris-acetic acid buffer (pH 7.9) containing 0.02% of NaN_3 as a preservative] with or without the affinophore (sGS-AP-Man) in the high-pressure-rinse mode for 3 min. The sample, which contained 0.2 mg/ml of pea lectin and 0.2 mM cytidine (as an electrophoresis marker) in the electrophoresis buffer, was injected at the positive end for 10 s under a pressure of 0.5 psi (injection volume of 0.67 nl, which corresponded to 0.14 ng of pea lectin). Electrophoresis was carried out at a field strength of 350 V/cm with an electric current of 6.7 μA. The cartridge temperature was set at 25°C and pea lectin was detected as the absorption at 214 nm. The electrophoresis buffer was used as an electrode solution in each electrode vessel. In the case of competition with neutral sugars, the solution of a sugar in the electrophoresis buffer was used as an positive-electrode solution (100 μl). Solutions of pea lectin (20 μl), the affinophore (50 μl), and neutral sugars (100 μl) were

placed in a polypropylene micro vial in a temperature-controlled rotating tray to minimize the change in concentration due to evaporation. After electrophoresis, the capillary was rinsed with 0.1 M sodium carbonate buffer (pH 10) containing 1 M NaCl and 10 mM EDTA in the high-pressure-rinse mode for 3 min and then with water for 3 min.

MATHEMATICAL BASIS

The following analysis for a divalent binding protein is only valid for a protein having identical and independent binding sites for ligands. Such interactions between a divalent protein and a charged ligand, that correspond to the first part of this section, have been analyzed previously (*4, 16-18*).

1. Determination of the Affinity-Electrophoresis Parameters of a Divalent Protein with a Monoligand Affinophore—The following two-step binding equilibrium is assumed for the interaction between a divalent protein (P) and a monoligand affinophore (A).



(1)

The dissociation constants for the complexes are defined as follows.

$$K_d^{int} = [P][A]/[P\text{-}A] = [P][A]/[A\text{-}P] = [P\text{-}A][A]/[A\text{-}P\text{-}A] = [A\text{-}P][A]/[A\text{-}P\text{-}A] \quad (2)$$

$$K_{d1} = [P][A]/([P\text{-}A]+[A\text{-}P]) = (1/2)\ K_d^{int} \quad (3)$$

$$K_{d2} = ([P\text{-}A]+[A\text{-}P])[A]/[A\text{-}P\text{-}A] = 2\ K_d^{int} \quad (4)$$

where K_d^{int} is the intrinsic dissociation constant of the complex for each binding site, and K_{d1} and K_{d2} are the overall dissociation constants of the complex for each step. The electrophoretic mobility (μ) of the protein in the course of the affinophoresis can be expressed by the following equation (*19*).

$$\mu = (\mu_0[P] + \mu_1([P\text{-}A]+[A\text{-}P]) + \mu_2[A\text{-}P\text{-}A])/[P]_t \quad (5)$$

$$[P]_t = [P] + ([P\text{-}A]+[A\text{-}P]) + [A\text{-}P\text{-}A] \quad (6)$$

where μ_0 is the mobility of the free protein, and μ_1 and μ_2 are those of the complexes with one and two affinophores, respectively.

When $\mu_2 - \mu_1 = \mu_1 - \mu_0$, *i.e.*, the mobility change is equal for the successive binding of two affinophores, Eq. 5 can be rearranged as follows.

$$\mu - \mu_0 = (\mu_2 - \mu_0)\{(1/2)([P\text{-}A]+[A\text{-}P]) + [A\text{-}P\text{-}A]\}/[P]_t \quad (7)$$

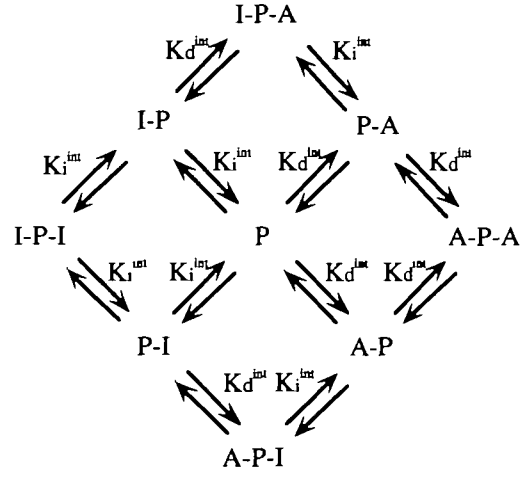
With Eqs. 2 and 6, and the substitution of $\mu - \mu_0$ and $\mu_2 - \mu_0$ with $\Delta\mu$ and $\Delta\mu_{max}$, respectively, Eq. 7 can be written as

$$\Delta\mu = \Delta\mu_{max}[A]/(K_d^{int} + [A]) \quad (8)$$

Equation 8 has the same form as that applied to affinity electrophoresis through a monovalent interaction (*2, 11-13*). It should be noted that three assumptions have been made to reach Eq. 8, *i.e.*, the equivalency and the independency of the two binding sites, and approximately the same mobility change for the successive binding of the affinophores. Linear transformation of Eq. 8 to a form identical to a Woolf-Hofstee plot of enzyme kinetics, and a plot of $\Delta\mu$ *vs.* $\Delta\mu/[A]$ enables the determination of K_d^{int} and $\Delta\mu_{max}$ by linear least square analysis. These values are necessary for subsequent analysis of the results of competition experiments with neutral ligands.

$$\Delta\mu = -K_d^{int}\Delta\mu/[A] + \Delta\mu_{max} \quad (9)$$

2. Determination of the Intrinsic Dissociation Constant between a Divalent Protein and a Neutral Ligand—By assuming the equivalency and the independency of two binding sites, the following equilibrium should apply to the interaction between a divalent protein and an affinophore in the presence of a neutral ligand (I) that competes for the binding with the affinophore.



(10)

where

$$K_d^{int} = [P][A]/[A\text{-}P] = [P][A]/[P\text{-}A] = [P\text{-}A][A]/[A\text{-}P\text{-}A] = [A\text{-}P][A]/[A\text{-}P\text{-}A] = [P\text{-}I][A]/[A\text{-}P\text{-}I] = [I\text{-}P][A]/[I\text{-}P\text{-}A] \quad (11)$$

$$K_i^{int} = [P][I]/[I\text{-}P] = [P][I]/[P\text{-}I] = [P\text{-}I][I]/[I\text{-}P\text{-}I] = [I\text{-}P][I]/[I\text{-}P\text{-}I] = [A\text{-}P][I]/[A\text{-}P\text{-}I] = [P\text{-}A][I]/[I\text{-}P\text{-}A] \quad (12)$$

The total concentration of P ($[P]_t$) is written as

$$[P]_t = [P] + [I\text{-}P] + [P\text{-}I] + [I\text{-}P\text{-}I] + [A\text{-}P] + [P\text{-}A] + [I\text{-}P\text{-}A] + [A\text{-}P\text{-}I] + [A\text{-}P\text{-}A] \quad (13)$$

If the mobility change caused by the binding of I to P or PA complex is negligibly small, *i.e.*, P-I, I-P, and I-P-I, exhibit almost the same mobility as that of P (μ_0), and A-P-I and I-P-A have nearly the same mobility as that of A-P or P-A (μ_1), the observed mobility (μ_i) of the protein in the presence of the affinophore and a neutral ligand can be written as follows.

$$\mu_i = (([P] + [I\text{-}P] + [P\text{-}I] + [I\text{-}P\text{-}I])\mu_0 + ([A\text{-}P] + [P\text{-}A] + [A\text{-}P\text{-}I] + [I\text{-}P\text{-}A])\ \mu_1 + [A\text{-}P\text{-}A]\mu_2)/[P]_t \quad (14)$$

The equivalence of the two binding sites means $[P\text{-}I] = [I\text{-}P]$, $[A\text{-}P] = [P\text{-}A]$, and $[A\text{-}P\text{-}I] = [I\text{-}P\text{-}A]$, and if the

mobility changes are additive for the successive binding of two affinophores, namely $\mu_2 - \mu_1 = \mu_1 - \mu_0 = \Delta\mu_{max}/2$, Eq. 14 can be reformed as

$$\Delta\mu_l = \Delta\mu_{max}([P\text{-}A] + [I\text{-}P\text{-}A] + [A\text{-}P\text{-}A])/[P]_t \quad (15)$$

where $\Delta\mu_l$ is $\mu_l - \mu_0$, *i.e.*, the observed mobility change in the presence of the affinophore and the neutral ligand. Finally, from the relation of Eqs. 11 and 12, $\Delta\mu_l$ is rewritten as

$$\Delta\mu_l = \Delta\mu_{max}[A]/(K_d{}^{int}{}_{app} + [A]) \quad (16)$$

where

$$K_d{}^{int}{}_{app} = K_d{}^{int}\,(1 + [I]/K_l{}^{int}) \quad (17)$$

Since $\Delta\mu_{max}$ is already known, $K_d{}^{int}{}_{app}$ can be determined by measurement of $\Delta\mu_l$ at particular concentrations of A and I. Rearrangement of Eq. 17 gives

$$K_l{}^{int} = [I]\ K_d{}^{int}/(K_d{}^{int}{}_{app} - K_d{}^{int}) \quad (18)$$

where $K_d{}^{int}$ is also already known.

RESULTS AND DISCUSSION

Preparation of a Monoligand Affinophore—The thiol group of succinylated glutathione (sGSH) acts as a highly specific hook in the coupling reaction with an iodoacetylated affinity ligand (Fig. 1). Iodoacetylation of an affinity ligand having an amino group can be easily performed by reaction with *N*-iodoacetoxy succinimide. The intermediates and the affinophore were easily purified by HPLC on a reversed-phase column. Two runs of HPLC on an analytical scale column (4 mm I.D., 25 cm long) provided 2.4 μmol of the affinophore, that was sufficient for the experiments described in this report.

Affinophoresis of Pea Lectin—Electrophoresis was carried out in a fused silica capillary covalently coated with succinylpolylysine. The coating was required in order to suppress the adsorption of concanavalin A (*9*), and was also employed in the present experiments to minimize the adsorption of proteins. The electroosmotic mobility of the capillary was 2.8 to 2.9×10^{-4} $cm^2 \cdot V^{-1} \cdot s^{-1}$ toward the negative electrode under the experimental conditions. The electrophoretic mobility of pea lectin was very low in the electrophoresis buffer and it was detected slightly behind a neutral marker, cytidine or mesityl oxide. The electrophoretic mobility of the affinophore was found to be 2.2×10^{-4} $cm^2 \cdot V^{-1} \cdot s^{-1}$ toward the positive electrode. Thus the increase in the mobility of the lectin toward the positive electrode on the interaction with the affinophore should result in elongation of the detection time.

The temperature of the capillary was controlled by circulating a temperature-regulated fluid through the cartridge in which the capillary was housed. The cartridge was designed so that about 4 cm of each end of the capillary protrudes from the cartridge to reach the electrode vessels. To reduce the relative length of this part, which was out of temperature control, to less than 10% of the total separation distance, the length of the capillary from the positive end to the detection point was set at 50 cm, otherwise, a shorter separation distance was preferable for a shorter analysis time.

The affinophoresis of the lectin was carried out using

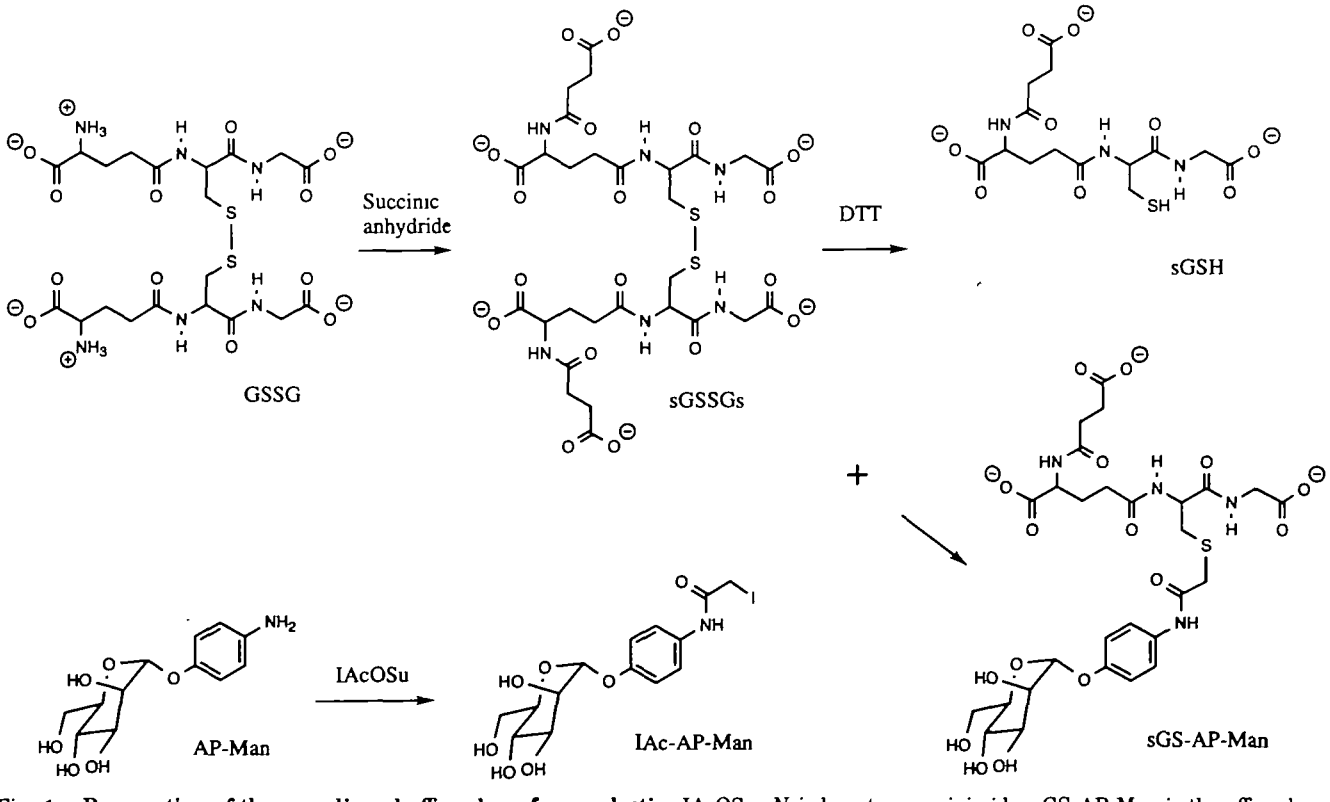


Fig. 1. **Preparation of the monoligand affinophore for pea lectin.** IAcOSu, *N*-iodoacetoxysuccinimide. sGS-AP-Man is the affinophore used.

different concentrations of the affinophore, from 0.05 to 0.5 mM, in 0.1 M Tris-acetic acid buffer (pH 7.9) (Fig. 2). Elongation of the detection time for pea lectin, corresponding to the increase in the mobility toward the positive electrode, was observed as the concentration of the affinophore increased. The baseline gap of unknown origin appearing just after the cytidine peak was proportional to the concentration of the affinophore.

Since the electrophoretic mobility is inversely related to the detection time, the mobility change ($\Delta\mu$) of the lectin had to be calculated with the following relation (*9, 20*).

$$\Delta\mu = \frac{L}{E}\left\{\left(\frac{1}{t}-\frac{1}{t_r}\right)-\left(\frac{1}{t_0'}-\frac{1}{t_r'}\right)\right\} \quad (19)$$

where L is the separation distance from the injection end to the detection point, E is the field strength, t and t_0' are the detection times for the lectin in the presence and absence of the affinophore, respectively, and t_r and t_r' are the detection times for an electrophoresis marker, cytidine, in the presence and absence of the affinophore, respectively. The prime symbols attached show possible variation of electroosmosis between two runs. Although the marker need not be electrically neutral (*9*), cytidine has almost no charge at the pH used in the electrophoresis experiments. A small change (a maximum of 1.4%) in the detection time for cytidine was observed in the presence of the affinophore, which is most likely due to the change in electroosmosis. Such fluctuations in electroosmosis can be completely canceled out by the use of Eq. 19, and the net mobility

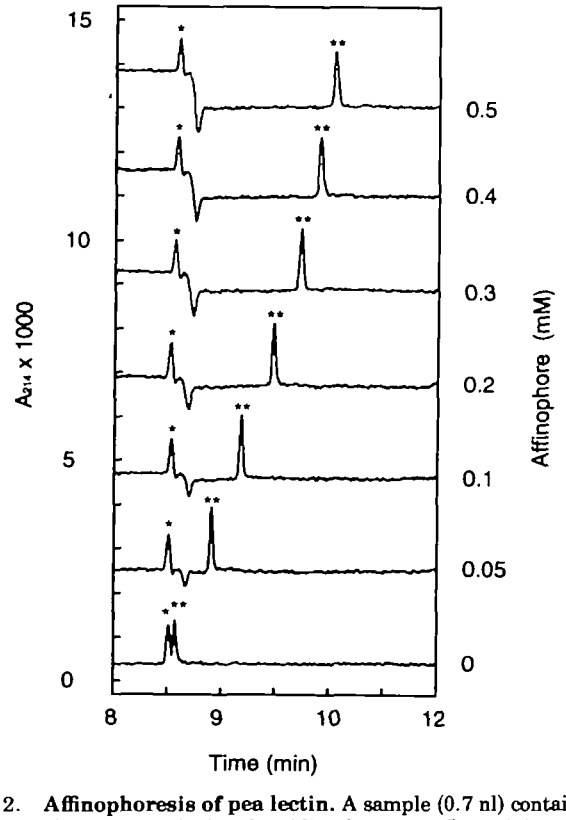


Fig. 2. **Affinophoresis of pea lectin.** A sample (0.7 nl) containing pea lectin (**, 0.2 mg/ml) and cytidine (*, 0.2 mM) was injected by means of pressure at the positive end of a capillary (25 μm I.D., 375 μm O.D., 57 cm total length, 50 cm separation distance, and inner-coated with succinylpolylysine) filled with a solution of the affinophore (sGS-AP-Man) at the concentrations shown on the right. Electrophoresis was carried out at a field strength of 350 V/cm (7 μA) at 25°C, with detection as A_{214}. Tris-acetate buffer (0.1 M, pH 7.9) was used throughout the system.

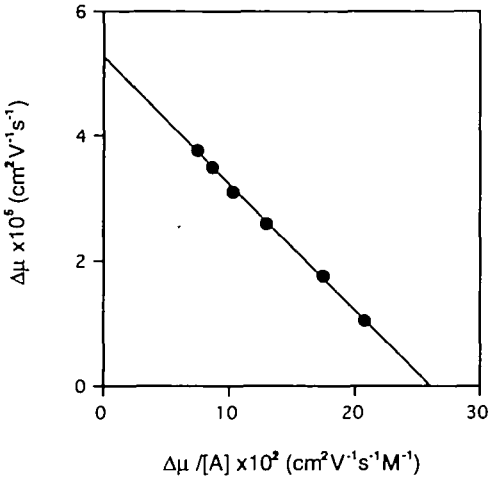


Fig. 3. **Determination of the dissociation constant of pea lectin for the affinophore (K_d^{int}), and the maximum mobility change of the lectin ($\Delta\mu_{max}$) by linear plotting.** The mobility change of the lectin was calculated by means of Eq. 19 from the detection-time data for the peaks in Fig. 2. The least squares method was used for the fitting of a line to the data points according to Eq. 9. The slope is the negative value of K_d^{int} and the intercept with the ordinate gives $\Delta\mu_{max}$.

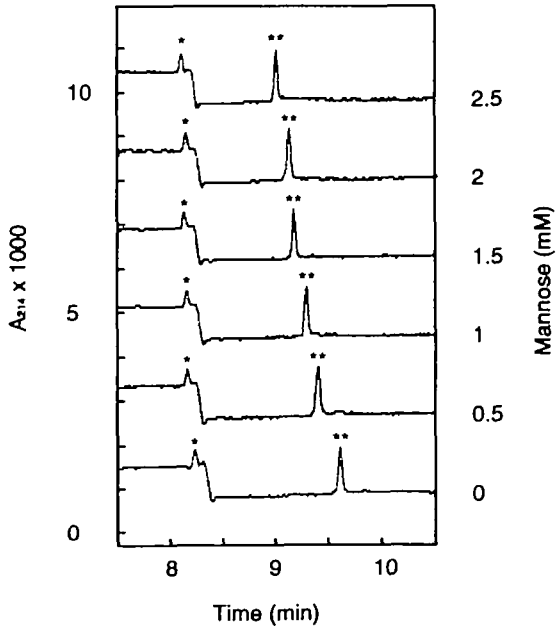


Fig. 4. **Suppression of the affinophoresis of pea lectin by mannose.** Affinophoresis of pea lectin was carried out using 0.5 mM affinophore (sGS-AP-Man) in the presence of mannose at the different concentrations shown on the right. Mannose was added to the positive-electrode buffer (100 μl) and it continuously moved into the capillary only by electroosmosis behind cytidine. Other conditions were the same as in the experiments in Fig. 2. **, pea lectin; *, cytidine.

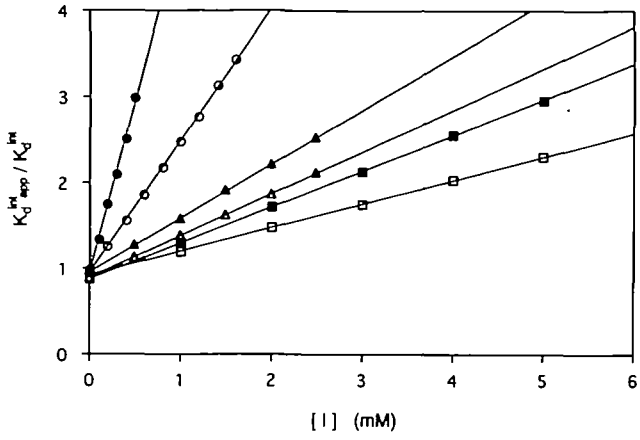


Fig. 5. **Determination of the dissociation constants of pea lectin for neutral sugars (I) through a competition experiment on the affinophoresis.** The results are plotted according to $K_d^{Int}{}_{app}/K_d^{Int} = [I]/K_I^{Int} + 1$ (see Eq. 17). ●, *p*-aminophenyl α-D-mannoside; ○, methyl α-D-mannoside; ▲, D-mannose; △, maltose; ■, methyl α-D-glucoside; □, D-glucose.

change of the lectin caused by the interaction with the affinophore can be calculated.

The plot of $\Delta\mu$ *vs.* $\Delta\mu/[A]$ according to Eq. 9 yielded a straight line with a K_d^{Int} of 0.202 mM and a $\Delta\mu_{max}$ of 5.25×10^{-5} cm²·V⁻¹·s⁻¹ (Fig. 3). The linearity of the plot should indicate the validity of the assumptions and approximations made for the analysis, *i.e.*, the equivalency and the independency of the two binding sites, and an approximately equal mobility change for the successive binding of two affinophores. Five runs of an identical experiment yielded an average value for K_d^{Int} of 0.199 mM (c.v. 6.0%) and $\Delta\mu_{max}$ of 5.35×10^{-5} cm²·V⁻¹·s⁻¹ (c.v. 1.6%). Once the two parameters were determined, we can use the affinophoresis system to analyze binding equilibria between the lectin and neutral sugars by inhibition of the affinophoresis.

Analysis of Binding Equilibria between Pea Lectin and Neutral Sugars—The affinophoresis of pea lectin with the affinophore at the concentration of 0.5 mM was carried out in the presence of different concentrations of mannose. Mannose was added only to the positive-electrode solution and it moved continuously into the capillary only by electroosmosis behind cytidine. As the concentration of mannose increased, the accelerated migration of the lectin caused by the affinophore was gradually suppressed (Fig. 4). The $K_d^{Int}{}_{app}$ values were determined by the measurement of the mobility changes ($\Delta\mu_i$) at particular concentrations of A and I according to Eq. 16. A plot of $K_d^{Int}{}_{app}/K_d^{Int}$ *vs.* [I] yielded a straight line (from Eq. 17), and the K_I^{Int} value for mannose was determined to be 1.6 mM from the slope of the line (Fig. 5).

The same plot was made for a series of neutral sugars, *i.e.*, D-glucose, methyl α-D-glucoside, maltose, methyl α-D-mannoside, and *p*-aminophenyl α-D-mannoside (Fig. 5). The preference of the lectin for mannose over glucose, and for mannosides over free mannose is clearly evident (Table I). The contribution of the additional glucose residue in maltose was very small compared with in the case of methyl α-D-glucoside. On the other hand, the *p*-aminophenyl group of *p*-aminophenyl α-D-mannoside contributes significantly to the affinity to the lectin.

TABLE I. **Intrinsic dissociation constants for neutral sugars.**

	Affinophoresis[a]	Calorimetry[b]
p-Aminophenyl α-D-mannoside	0.25 mM	Not determined
Methyl α-D-mannoside	0.65 mM	0.61 mM[c]
D-Mannose	1.6 mM	1.3 mM[d]
Maltose	2.1 mM	Not determined
Methyl α-D-glucoside	2.4 mM	1.3 mM[e]
D-Glucose	3.6 mM	2.8 mM[f]

[a]0.1 M Tris-acetate buffer (pH 7.9) containing 0.02% NaN_3, 25°C. [b]0.02 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. [c]25.1°C. [d]24.3°C. [e]16°C. [f]14.2°C.

The dissociation constants, determined by affinophoresis, were compared with the reported values determined by calorimetry (*21*) and are shown in Table I. Agreement of the values determined by the two methods was especially good for D-mannose and methyl α-D-mannoside, for which the determinations were performed at an identical temperature (approximately 25°C). For D-glucose and methyl α-D-glucoside, the dissociation constants reported in the literature are smaller than those determined in this study. The discrepancy can be largely explained by differences in the temperature used in the two determinations, and the positive ΔH° values of the dissociation reaction of pea lectin with the sugars, *i.e.*, 10 and 13.6 kJ/mol for D-glucose and methyl α-D-glucoside, respectively (*21*).

Comparison with APCE—We previously reported the determination of the affinity constants of lectin-neutral sugar interactions by affinity probe capillary electrophoresis (APCE) (*9*). In APCE, the zonal electrophoresis of a fluorescence-labeled sugar, an affinity probe, was carried out in the presence of a lectin, concanavalin A. In this earlier study, the mobility change of the affinity probe caused by the interaction with the lectin was analyzed. Since the mobility change is dependent on the concentration of the lectin binding site, as with other affinity electrophoresis systems, APCE can be used to monitor the concentration of the unoccupied binding sites of the lectin under binding equilibrium with a neutral sugar and, hence, it allows the determination of the equilibrium constant for the neutral sugar.

A fluorescent affinity probe can be detected at very low concentration (10^{-9} M) by the use of laser-induced fluorescence detection. When the supply of an affinity ligand is limited, APCE would clearly be an excellent method because it would involve significantly lower consumption of the ligand in the system, *i.e.*, 10^{-18} mol for each run. On the other hand, affinophoresis can be carried out with a smaller amount of lectin than for APCE, in which the zone electrophoresis of an affinity probe is carried out in a solution of the lectin. Observation of the mobility change may be easier by APCE, since a larger mobility change would be expected for a smaller molecule.

Capillary affinophoresis should be useful for the determination of intrinsic dissociation constants of divalent lectins for neutral sugars on a micro-scale. This system should be suitable for the determination of dissociation constants greater than about 10^{-6} M. For systems with smaller dissociation constants than this, its applicability will be restricted by the peak broadening due to slow dissociation kinetics (*22*). The small sample required, the feasibility of automation, the accuracy of measurement, and the tolerance to impurities in protein samples, which are features of

this type of analysis involving capillary electrophoresis, are very attractive for the analysis of protein-ligand interactions.

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