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THEORETICAL AND EXPERIMENTAL STUDIES ON ZONE-INTERFERENCE CHROMATOGRAPHY AS A NEW METHOD FOR DETERMINING MACROMOLECULAR KINETIC CONSTANTS

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Zone-interference chromatography is a new method for studying macromolecular interactions (S. Endo and A. Wada, Anal. Biochem. 124 (1982) 372). This method is a new style of affinity chromatography which requires no preparation of affinity-column materials but utilizes the velocity difference in a column between interacting molecular species. Using the stochastic theory on the behavior of solute molecules, both the association and the dissociation rate constants can be analytically obtained from the degree of deformation of elution patterns, i.e., the change of the first and second moments. In order to verify the present theory, computer simulation of elution profiles by the extended plate theory and a binding experiment between glutamate dehydrogenase and ADP have been carried out.

1. Introduction

Detailed studies of molecular constants of the interaction between biological macromolecules (for example, DNA-protein complex formation) are a prerequisite to understanding the molecular mechanism of biological functions. Considering that high interaction specificity is one of the distinctive properties prevailing in biological systems, affinity chromatography should be the most appropriate tool to obtain quantitative information on the subject [1,2]. Unfortunately, even though conventional high-performance liquid chromatography (HPLC) has demonstrated a high resolving power in separating molecular species, it is technically difficult to prepare affinity-column materials which fulfill the precision requirements of HPLC. In other cases, intermolecular interactions have been studied by such mass transport systems as sedimentation, electrophoresis and chromatography [3–7]. However, versatile methods which give quantitative results using only a small amount of sample are few in number.

In a previous report we presented a new method, 'zone-interference chromatography', for the study of macromolecular interactions as well as macromolecule-small molecule interactions [8]. The principle is based on affinity chromatography, but it is not necessary to prepare a column with immobilized affinity ligands because its affinity elution is carried out utilizing the velocity difference in a column between interacting molecular species. It was proved with this method that the binding constant can be quantitatively obtained from the shift of the elution volume of a slow migrating zone when the fast migrating zone does not permeate into a gel and its amount is sufficiently higher than that of the slow zone. The usage of high-performance gel permeation chromatography enabled versatile and convenient measurement with high precision and reproducibility.

In this report we extend the previous theory to

show (1) that the association and dissociation rate constants can be given analytically when the time scale of interactions between two molecular species is too slow to attain the equilibrium mass distribution of these molecules in a column, (2) the case where fast zone permeation into a gel can be formulated, and (3) that the corrected binding constant can be calculated even if the amount of the fast zone is not much more than that of the slow zone. Thus, the theoretical aspects of zone-interference chromatography have been accomplished. In order to verify the usefulness of the present theory, the computer simulations of various elution profiles are carried out by the plate theory considering the effect of nonequilibrium mass transfer, and the simulated profiles are compared with the results of the binding experiment between glutamate dehydrogenase and ADP. The kinetic constants obtained by the present method using mass transport are compared with those by the chemical relaxation method, and the differences between the two methods are discussed.

2. Theory

2.1. Physical picture

Let us consider two molecular species, A and B, which migrate at different velocity in a gel permeation column. The fast migrating zone, (A-zone), which is injected later into a column, passes the slow migrating zone (B-zone), which is injected earlier. If A and B interact such that A + B = AB, the A-zone and B-zone interfere with each other at that passing point in the column. The interference between the two zones deforms the elution profiles of both A and B because the AB complex has a migration velocity different from that of others. A time course of the movement of the A-zone and B-zone is shown in fig. 1a.

When a gel permeation column is chosen to permeate B but not A, AB complex, whose size should be larger than A, moves at the same velocity as A. Therefore, the elution of B will be accelerated by the formation of AB complex. The reaction schemes are assumed in the same way as in our previous report [8] as.

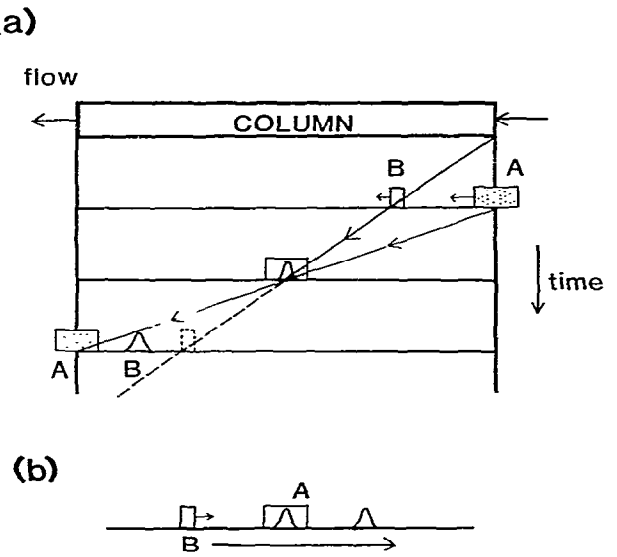

Fig. 1. (a) Time course of zone-interference chromatography in a laboratory fixed coordinate. The dark and the dotted areas represent slow (B) and fast (A) zones, respectively. (b) Time course of zone-interference chromatography in a fast-zone fixed coordinate where it is equivalent to conventional affinity chromatography.

$$B^g \overset{K_B}{\rightleftharpoons} B \tag{1a}$$

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB, \tag{1b}$$

where B^g represents the B permeated into a gel and K_B the mass distribution coefficient of B. B^g does not migrate, while free B, AB complex and A migrate in the column with the same velocity of eluant, v. Since reaction 1a is more rapid than 1b it can be generally considered that the equilibrium between the amount of B and B^g,

$$B^g = K_B B, \tag{2}$$

is attained at every instant.

For a clear physical picture of zone-interference chromatography, we introduce a virtual species B* which represents the total B which is not bound to A in a column, and whose amount is

$$B^* = (1 + K_B) B. \tag{3}$$

B* molecules migrate with mean velocity $v_B =$

$v/(1+K_B)$ and interact with the A-zone. When the concentration of A-zone injected into a column is much higher than that of B-zone, i.e., $[A^t] \gg [B^t]$, the bimolecular reaction in eq. 1b can be approximated by the pseudo-first-order reaction:

$$B^* \underset{k'}{\overset{k}{\rightleftharpoons}} AB, \tag{4}$$

where

$$k' = k_d, \tag{5}$$

and

$$k = \frac{k_a[A^t]}{1+K_B}. \tag{6}$$

2.2. Determination of the rate constants

It is shown in this section that the rate constants of the interaction between A and B can be obtained from the profile of deformation of the elution pattern of the B-zone.

Zone-interference chromatography may be analyzed as an affinity chromatography when it is referred to the coordinate that is fixed in A-zone as shown in fig. 1b. The time necessary for the B-zone to pass through the A-zone, i.e., the virtual 'affinity column' is

$$t_P = \frac{V_A}{v - v_B}. \tag{7}$$

where V_A is the volume of the A-zone injected into a column. The 'elution' of B* from the A-zone is obtained when the total lifetime of B* in the free state is equivalent to a definite time t_P. The total lifetime of AB complex may be considered as a random variable during the time interval t_P, so that the binding of B* to A and the dissociation from AB are Poisson processes. Thus, the probability that B* has a chance to bind n times during the time t_P, dissociates $n-1$ times during the time t, and finally dissociates between t and $t+dt$ is

$$P(n,t)dt = \frac{e^{-kt_P}(kt_P)^n}{n!}\frac{e^{-k't}(k't)^{n-1}}{(n-1)!}k'dt. \tag{8}$$

The probability density $P(t)$, that the total lifetime of AB complex is t for any n, is obtained by summation of eq. 8 with respect to n,

$$P(t) = \sum_{n=1}^{\infty}\frac{e^{-kt_P}(kt_P)^n e^{-k't}(k't)^{n-1}k'}{n!(n-1)!} + \delta(t)e^{-kt_P} \tag{9}$$

where $\delta(t)$ is Dirac delta function. The second term is necessary for the normalization of eq. 9, because it expresses the probability that a B* molecule passes through the A zone without binding to A molecules; but it does not contribute moments above the first order. The r-th moment of the distribution $P(t)$ is given for conventional affinity chromatography by the formula of Denizot and Delaage [9],

$$E(t^r) = \int_0^\infty t^r P(t)dt \quad (r>0)$$
$$= \frac{r!(r-1)!}{k'^r}\sum_{j=0}^{r-1}\frac{(kt_P)^{r-j}}{j!(r-j)!(r-j-1)!} \tag{10}$$

On the other hand, gel permeation of B is itself a stochastic process. Thus, the velocity of B* molecule, β, is another random variable and the elution profile of B alone exhibits dispersion. Incidentally, the v_B mentioned above is the ensemble average of β. Since B migrates with velocity v in the AB complex during time t, its elution time is

$$t_e = t_B - \frac{v-\beta}{\beta}t, \tag{11}$$

where $t_B = V_0/\beta$ is a random variable and V_0 the void volume of a column.

Let us next consider the time course of the state of a B* molecule, when t_P in eq. 7 is not a constant but is a random variable as follows

$$t_P = \frac{V_A t_B}{V_0 \kappa}, \tag{12}$$

where, for simplicity of calculation, a random variable corresponding to K_B is set to be,

$$\kappa = \frac{t_B}{t_0} - 1 = \frac{v-\beta}{\beta}, \tag{13}$$

and $t_0 = V_0/v$ is the mobile phase hold-up time of a column. It is not necessary to consider the detailed properties of the t_B distribution, $P'(t_B)$, but it is sufficient to assume that the first two moments do not diverge. Eq. 9 is considered as the conditional probability of t for fixed t_P. The q-th moment of t_e can be calculated using eqs. 11 and 13 as

$$E(t_e^q) = \int\int P(t)P'(t_B)t_e^q dt dt_B$$
$$= \int\int P(t)P'(t_B)\sum_{r=0}^{q}\frac{q!}{r!(q-r)!}(-\kappa t)^r t_B^{q-r}dt dt_B. \tag{14}$$

After integrating eq. 14 with respect to t by using eq. 10, we have

$$E(t_e^q) = \int P'(t_B) t_B^q dt_B + \sum_{r=1}^{q} \frac{(-1)^r q!(r-1)!}{(q-r)!k'^r!} \times \int \sum_{j=0}^{r-1} \frac{\kappa^r (kt_B)^{r-j} t_B^{q-r}}{j!(r-j)!(r-j-1)!} P'(t_B) dt_B. \quad (15)$$

By substituting eqs. 12 and 13 into eq. 15, then

$$E(t_e^q) = E(t_B^q) + \sum_{r=1}^{q} \frac{(-1)^r q!(r-1)!}{(q-r)!k'^r!} \times \sum_{j=0}^{r-1} \frac{(kV_A/V_0)^{r-j}}{j!(r-j)!(r-j-1)!} \times \int \left(\frac{t_B}{t_0} - 1\right)^j t_B^{q-j} P'(t_B) dt_B. \quad (16)$$

From eq. 16 the first moment, i.e., the mean elution time of B in zone-interference chromatography, is

$$\bar{t}_e = \bar{t}_B \left(1 - \frac{kV_A}{k'V_0}\right). \quad (17)$$

where $\bar{t}_B$ expresses the mean elution time of B alone:

$$\bar{t}_B = (1 + K_B) t_0. \quad (18)$$

The second moment can also be calculated as

$$E(t_e^2) = E(t_B^2)\left(1 - \frac{kV_A}{k'V_0}\right)^2 + \frac{2kV_A}{k'^2V_0}\left[\frac{E(t_B^2)}{t_0} - \bar{t}_B\right]. \quad (19)$$

and the variance is

$$\sigma_e^2 = \left(1 - \frac{kV_A}{k'V_0}\right)^2 \sigma_B^2 + \frac{2kV_A}{k'^2V_0}\left(\frac{\sigma_B^2 + \bar{t}_B^2}{t_0} - \bar{t}_B\right). \quad (20)$$

where

$$\sigma_B^2 = E(t_B^2) - \bar{t}_B^2. \quad (21)$$

Thus, eqs. 17 and 20 are solved with respect to k/k' and k', and finally k_a and k_b are obtained using eqs. 5, 6 and 18 as

$$k_d = \frac{2\bar{t}_B(\bar{t}_B - \bar{t}_e)}{\sigma_e^2 \bar{t}_B^2 - \sigma_B^2 \bar{t}_e^2}\left(\frac{\sigma_B^2 + \bar{t}_B^2}{t_0} - \bar{t}_B\right). \quad (22)$$

$$k_a = \frac{(\bar{t}_B - \bar{t}_e)v}{m_A} k_d. \quad (23)$$

where m_A ($= [A^t]V_A$) is the total amount of A injected into a column. From eqs. 22 and 23 the equilibrium binding constant between A and B is obtained

$$K = \frac{k_a}{k_d} = \frac{V_B - V_e}{m_A}. \quad (24)$$

where V_B ($= \bar{t}_B v$) is the elution volume of B without A and V_e ($= \bar{t}_e v$) the elution volume in zone-interference chromatography. This expression agrees with the equation derived from the equilibrium conditions for reaction 1a as well as 1b in our previous report [8].

In conclusion, using zone-interference chromatography we can obtain the binding constant from the shift of elution volume, i.e., the change of the first moment of the elution pattern and the dissociation constant from the deformation of the elution pattern, i.e., the change of the first and second moments.

2.3. The case where A permeates into a gel

For a column in which the A-zone does not elute at the void volume, A and AB complex as well as B permeate into a gel. But their reactions are rapid and the equilibrium conditions are held.

$$A^g = K_A A \quad (25)$$

$$AB^g = K_{AB} AB. \quad (26)$$

where the superscript g denotes the B and AB complex in a gel, and K_A and K_{AB} the mass distribution coefficients. In the case of $[A^t] \gg [B^t]$, the total reaction scheme is

$$\begin{array}{ccc} B & \overset{k_a[A]}{\underset{k_d}{\rightleftharpoons}} & AB \\ \updownarrow & & \updownarrow \\ B^g & \overset{k'_a[A^g]}{\underset{k_d}{\rightleftharpoons}} & AB^g. \end{array} \quad (27)$$

where k'_a denotes the association rate constant in the stationary phase, i.e., gel, and the concentration of A in a gel is

$$[A^g] = \xi K_A [A] \quad (28)$$

where ξ is the ratio of the volume of the mobile

phase to that of the stationary phase. k_d is assumed to be invariable in both phases, because the dissoaction is a unimolecular reaction which is not greatly affected by an ambient state. From the equilibrium condition for the reaction scheme (eq. 27), k'_a is given as

$$k'_a = \frac{K_{AB}}{\xi K_A K_B} k_a. \tag{29}$$

Consequently, using eqs. 2, 26, 28 and 29, the reaction scheme corresponding to eq. 4 is obtained

$$B^* \underset{k'}{\overset{k}{\rightleftharpoons}} AB^*, \tag{30}$$

where

$$k' = k_d. \tag{31}$$

$$k = \frac{1 + K_{AB}}{1 + K_B}[A] k_a. \tag{32}$$

Since the virtual concentration of A in the case that all A molecules exist in the mobile phase is equal to the concentration of A injected into a column,

$$[A^t] = [A] + [A^g]/\xi \tag{33}$$

and using eq. 28, we have

$$[A^t] = (1 + K_A)[A]. \tag{34}$$

When B is a small molecule ($K_B = 1/\xi$), A and AB complex give almost the same elution properties, i.e., $K_A = K_{AB}$. In such a case, eq. 32 agrees with eq. 6, with eq. 34 being taken into account, and both A and AB complex migrate with the same velocity:

$$v_A = \frac{v}{1 + K_A}, \tag{35}$$

and their elution time is

$$t_A = (1 + K_A) t_0. \tag{36}$$

It is easily understood that eqs. 11–17, 19 and 20 in which v and t_0 are substituted by v_A and t_A, respectively, are similarly valid. Thus we obtain,

$$k_d = \frac{2\bar{t}_B(\bar{t}_B - \bar{t}_e)}{\sigma_e^2 \bar{t}_B^2 - \sigma_B^2 \bar{t}_e^2} \left(\frac{\sigma_B^2 + \bar{t}_B^2}{t_A} - \bar{t}_B \right), \tag{37}$$

$$k_a = k_d \frac{V_B - V_e}{m_A}. \tag{38}$$

Eq. 25 on the equilibrium binding constant holds as it stands under the condition of $K_A = K_{AB}$. Considering the conclusion of the preceding section, the condition that the rate constants are measurable is that A and AB complex migrate with the same velocity in a column.

2.4. The effect of B-zone concentration

When it is difficult to obtain enough A to satisfy $[A^t] \gg [B^t]$, which is the situation that always happens for precious biological samples, k in eq. 6 is no longer constant. Therefore, the rate constants, k_a and k_d, are not determined analytically, but the approximate equilibrium binding constant can be obtained when A and AB complex do not permeate into a gel.

The mass conservation laws corresponding to reaction scheme, eq. 1, are

$$[A^t] = [A] + [AB] \tag{39}$$

$$[B^t] = [B] + [AB] + [B^q]/\xi. \tag{40}$$

And the equilibrium conditions are

$$[AB] = K[A][B] \tag{41}$$

$$[B^g] = \xi K_B [B] \tag{42}$$

In such a case the elution time of zone-interference chromatography is (see ref. 8)

$$V_e = V_B - V_A K [A], \tag{43}$$

provided that [A] is a constant, i.e., that $[B^t]$ in the A-zone is assumed to be a constant by neglecting the dilution of B-zone with gel permeation. Using the following relation derived from eqs. 40 and 42:

$$[AB] = [B^t] - (1 + K_B)[B], \tag{44}$$

and eq. 41, eq. 43 is rearranged as

$$\frac{V_B - V_e}{V_A} + 1 + K_B = \frac{[B^t]}{[B]}. \tag{45}$$

By eliminating [AB], [A] and [B] in eqs. 39, 44 and 45, the corrected K^{-1} (the dissociation constant) is obtained

$$K^{-1} = \frac{m_A}{V_B - V_e} - \frac{[B^t] V_A}{V_B - V_e + (1 + K_B) V_A}. \tag{46}$$

The second term is a correcting term, which can be negligible under the condition $[A^t] \gg [B^t]$.

3. Materials and Methods

A block diagram of the present system is shown in fig. 2. The permeation column used was a TSK G2000SW from the Toyo Soda Manufacturing Co., Tokyo. A detailed description of the HPLC apparatus and the data input into the microcomputer was given in our previous report [8]. The broad solid line is an eluant flow path and thin lines are electric signals. The temperature at which zone-interference chromatography was carried out is controlled by circulating water with a constant temperature (± 0.1°C) in the water jacket around the column. The flow rate was 1 ml/min.

Eluant was 0.1 M phosphate buffer, pH 7.5, with 3 mM glutarate. Glutamate dehydrogenase (EC 1.4.1.3) from beef liver was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan), and used without further purification. ADP (sodium salt) was purchased from P.L. Biochemicals, Inc. Glutamate dehydrogenase (GLDH) was dissolved and dialyzed in eluant. The concentrations of GLDH and ADP were spectrometrically determined from the extinction coefficient at 279 nm (54400 M^{-1} cm^{-1}) and 259 nm (15400 M^{-1} cm^{-1}), respectively. GLDH (A-zone) was injected from injector A 5 s after the injection of ADP (B-zone) from injector B. The injected volumes of both GLDH and ADP were 100 μl.

The simulations of elution profiles were performed on an M-200H computer system at the Tokyo University Computer Center. Details of the computer algorithm are described in the appendix.

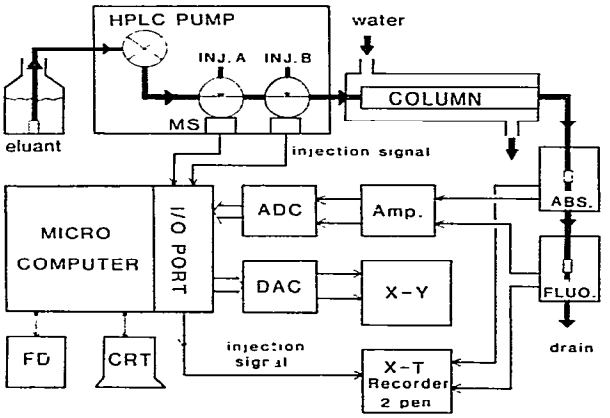

Fig. 2. Block diagram of zone-interference chromatography. INJ.A and INJ.B, sample injectors of A-zone and B-zone, respectively; MS, micro switch; ABS., spectrophotometer; FLUO., spectrofluorometer; Amp., amplifier; X-Y, XY recorder or monitor scope; FD, floppy disk; CRT, display terminal; ADC and DAC, analog-to-digital and digital-to-analog converter, respectively; I/O, input/output.

4. Results

4.1. Computer simulation

Fig. 3 shows typical simulated elution patterns of B-zone in zone-interference chromatography with nonequilibrium mass transfer. The A-zone elutes at the void volume of a column, i.e., $K_A = 0$. Numerical values for the simulation are described in the figure legend. Each zone-interference chromatogram is calculated for varying k_a and k_d for a fixed value of K, and the furthest to the right is the chromatogram for B-zone alone. The larger variance of the elution pattern and the smaller peak shift result from the smaller k_a and k_d. However, these elution patterns have the same mean elution time, i.e., the first moment, because

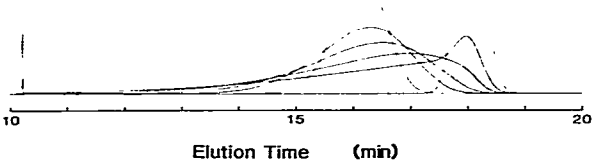

Fig. 3. Computer-simulated elution profiles of B-zone in zone-interference chromatography. Further to the right is the chromatogram of B-zone alone: $[B^t] = 10^{-7}$ M. For all elution profiles, $K = 2 \times 10^6$ M^{-1}, and $k_d = \infty$, 0.1, 0.05, 0.025, 0.013 s^{-1} from left to right on the peak position, i.e., the trace further to the left is calculated using eq. A11′ instead of eq. A11. Other constants used are $K_A = K_{AB} = 0$, $K_B = 0.8$, $t_0 = 10$ min, $j_M = 3000$, $i_1 = 30$, $i_2 = 40$ and $i_3 = 70$.

the binding constant, K, only contributes to the difference of the mean elution time between the zone-interference chromatogram and the chromatogram of B. A narrow peak at about 10.2 min represents the B which elutes with A.

The results of the quantitative analyses of these elution patterns by eqs. 22 and 24 are summarized in table 1. The constants used for the simulation are the same as those in fig. 3. Calculated values of the binding constant K agree closely with the true values under all conditions shown here, and those of k_d also agree well with the true ones except those at the upper left in the table. The reason for this slight disagreement is that under such conditions, $k_a[A^t]$ and k_d are not much smaller than the rate of gel permeation (more than 25 s^{-1}; see section 5), so that the effect of nonequilibrium mass transfer in a column does not sufficiently contribute to the deformation of the elution profile in zone-interference chromatography. At the upper right in the table, where K is large and k_d small, a great deal of B elutes with A in the simulated elution profiles. Under such conditions, eqs. 22 and 24 are not applicable, so that K and k_d cannot be obtained precisely.

Fig. 4 shows the degree of correction of the binding constant for the concentration of A-zone, which elutes at the void volume of a column. The ordinate represents the ratio of the binding constant K_{obs} calculated from the simulated elution profiles to the real binding constant K. The simulations of these elution profiles were carried out by using eq. A11′ for the case where the reaction $A + B = AB$ as well as $B^g = B$ equilibrates in a column. The abscissa represents the change of the A-zone concentration and the binding constant under the condition that $[A^t]K = 10$ (fixed), i.e., the simulated elution profiles have almost the same mean elution time and the concentration of B zone is 10^{-7} M. The K_{obs} of solid line III was calcu-

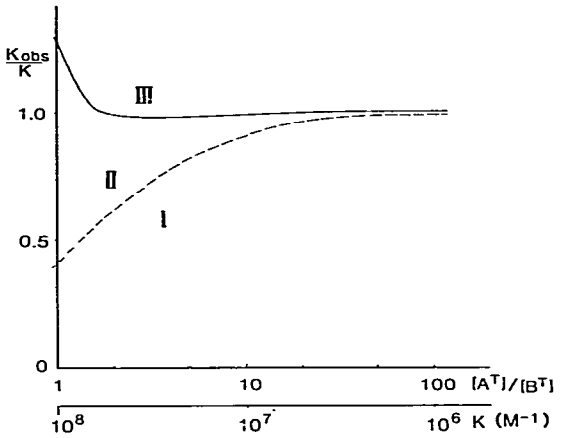

Fig. 4. The degree of correction of the binding constant for the A-zone concentration. The K_{obs} of solid line III is the corrected binding constant using eq. 46. See the text for a detailed description. The constants used are the same as those in fig. 3 except that $[A^t]K = 10$ and $j_M = 1000$.

Table 1

Calculated binding constants and dissociation constants from the simulated zone-interference chromatogram

K (M^{-1})	k_d (s^{-1})				
	0.2	0.1	0.05	0.025	0.013
	0.12	0.077	0.046		
4.0×10^6	3.91 [a]	3.93	3.93		
	0.15	0.086	0.047	0.025	0.014
2.0×10^6	1.97	1.97	1.98	1.98	1.96
	0.17	0.092	0.049	0.025	0.013
10.0×10^5	9.87	9.90	9.93	9.95	9.92
	0.18	0.095	0.049	0.025	0.013
5.0×10^5	4.96	4.97	4.98	4.98	4.98
	0.19	0.097	0.049	0.025	0.013
2.5×10^5	2.49	2.49	2.49	2.50	2.49

[a] Mentissa of calculated binding constant; exponent is equal to the real value.

lated by eq. 46, that of dashed line II by eq. 24, and that of dotted line I also by eq. 24 where the elution volumes of the chromatographic peak were used instead of the mean elution volumes of the elution pattern.

From fig. 4 it is clear that the exact binding constant can be obtained from eq. 46 when the concentration of A-zone is more than 2-fold that of the B-zone and that the upper limit of the measurable binding constant extends to 10^8 M^{-1} when the volume of B-zone is 100 μl and the smallest amount of B for the quantitative measurement is 10^{-11} mol. This value is reasonable if B is a protein with a molecular weight of 10^4 and measured by fluorescence or far-ultraviolet absorption. Because the peak shift of B-zone of the order of 0.1 ml is quantitatively measured by the present device, the lower limit of measurable binding constant is $10^{-4}/A_{max}$ M^{-1} where A_{max} is the maximum amount of usable A.

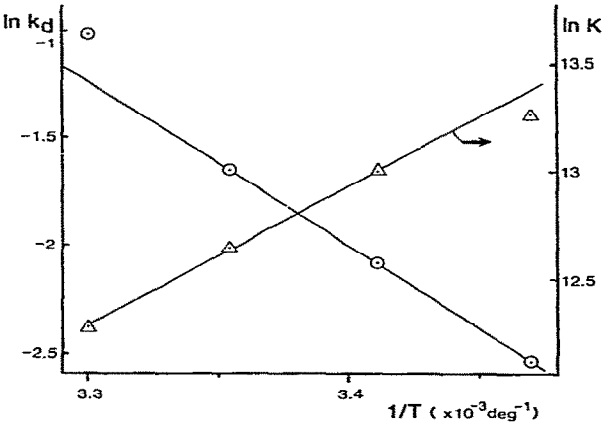

Fig. 6. Van't Hoff plot of the binding constant K and Arrhenius plot of the dissociation rate constant k_d on the interaction of GLDH and ADP. These values were determined from the zone-interference chromatograms in fig. 5 by eqs. 22 and 24 as $t_0 = 10.3$ min.

4.2. Experimental

A set of the measured elution profiles in fig. 5 demonstrates the interaction between GLDH (A-zone) and ADP (B-zone). These profiles represent only the elution profiles of ADP because those of GLDH alone are subtracted from the zone-interference chromatogram which has two peaks: a vast peak tailed backward from GLDH at the void volume and a small peak of ADP. The temperatures for the series of these elution profiles are 15, 20, 25 and 30°C in order from the left; the furthest to the right represents the chromatogram for ADP alone at 30°C. The position and shape of the ADP peak have slightly changed, reflecting the temperature (data not shown). Thus, for the calculation of K and k_d using eqs. 22 and 24, the values of $\bar{t}_B$ and σ_B for the elution profile of ADP at each temperature were used. The value of t_0 was set as 10.3 min from the mean elution time of the chromatogram of GLDH alone.

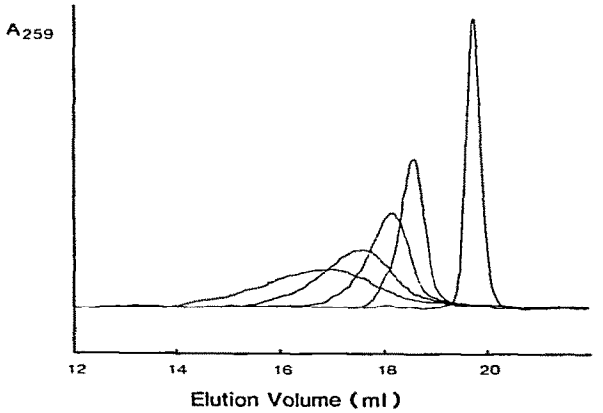

Fig. 5. Zone-interference chromatogram of ADP monitored by absorbance at 259 nm. The concentration of ADP (B-zone) is 2.5×10^{-6} M. A-zone is GLDH whose concentration is 5.7×10^{-5} M. The temperatures for the series of the chromatograms are 15, 20, 25 and 30°C in order from the left; the furthest right is the chromatogram of ADP alone at 30°C.

k_d and K thus obtained are plotted vs. $1/T$ in fig. 6. From the slopes of the lines, the activation energy of the dissociation reaction can be calculated as 15 kcal/mol and the enthalpy difference between the AB complex and the dissociated state as -1.3 kcal/mol. This is the first experiment to obtain kinetic constants analytically by the mass transport method without comparing the experimental patterns of elution, sedimentation or elec-

trophoresis with their computer-simulated patterns.

5. Discussion

Let us compare the actual chromatogram with the simulated elution profile. The j_M of the G2000SW column used is about 1.5×10^4 which is derived from the elution profile of ADP alone by the formula of the plate theory [8]:

$$j_M = \frac{\bar{r}_B(\bar{r}_B - r_0)}{\sigma_B^2}.$$

It is difficult to simulate the elution profiles on $j_M = 15000$ because the execution time of the algorithm described in the appendix is proportional to the square of j_M. Although table 1 shows that the upper limit of measurable dissociation rate constants is 0.2 s^{-1}, the simulation based on the data in table 1 was carried out for the case where the total number of theoretical plates j_M is 3000. Since the elution time per theoretical plate for the simulation was 0.2 s and that for the experiment with the G2000SW column was 0.04 s, the dissociation rate constant up to 1 s^{-1} can be exactly measured by the present device.

The reason the calculated binding constant K_{obs} in table 1 does not agree perfectly with the real binding constant K is that the values of $[A^t]$and $[B^t]$ used for the simulation do not wholly satisfy the condition of $[A^t] \gg [B^t]$. The simulations for $[A^t] = 10^{-4}$ M, $[B^t] = 10^{-7}$ M, and K whose value is 1/10 in table 1, give almost the same elution profiles as that based on table 1, and K_{obs} agrees with K to an accuracy of three significant figures. But, the degree of agreement for the dissociation rate constant is not improved; simulations with larger j_M are necessary to improve it.

Let us next compare the present result with that of the chemical relaxation method. The association rate constant between GLDH and ADP was measured by the stopped-flow method to be 8.4×10^4 M^{-1} s^{-1} at 20°C [10]. The measured value by zone-interference chromatagraphy at the same temperature is 5.5×10^4 M^{-1} s^{-1}, whose value is calculated from K and k_d as shown in table 2. Because the measured data points on the van't Hoff plot and the Arrhenius plot in fig. 6 line up closely in a straight line, it is clear that consistent values are measured by the present method. The reason for this slight disagreement may depend on the basic difference between the chemical relaxation method and the method using mass transfer. That is, in the former reactions proceed by diffusion in well stirred solution, while in the latter reactions are mediated by mass transport rather than diffusion, as noted by Hethcote and DeLisi [12]. The value of k_d by the present mass transport method agrees well with that calculated from K determined by difference spectroscopy [11] and k_a by the stopped-flow method [10] as shown in table 2. Thus, it seems that the difference between chemical relaxation and mass transport contributes mainly to the association rate constant and little to the dissociation rate constant, because the dissociation is a unimolecular reaction and is not greatly affected by diffusion.

Table 2

Measured binding constant and kinetic constant for the interaction of GLDH and ADP

All measured values are in 0.1 M phosphate buffer, pH 7.5–7.6, at 20°C.

	K (M^{-1})	k_a (M^{-1} s^{-1})	k_d (s^{-1})
Mass transport	4.4×10^5	5.5×10^4	0.12
Chemical relaxation	7.7×10^5	8.4×10^4	0.11

In the kinetic study of reaction mechanisms the advantages of zone-interference chromatography over chemical relaxation methods are that: (1) it is unnecessary to change optical or other types of signals between the free state A + B and the AB complex; (2) both K and k_d are obtained in a single experimental operation; and, therefore, (3) only a small quantity of sample is required. The disadvantage is that fast kinetic constants in the millisecond region are not measurable even if the latest HPLC is used. From the above-mentioned discussion, it is evident that zone-interference chromatography is suitable for the study of macromolecular interactions in biological systems whose time scale is of the order of seconds in most cases.

Appendix

Simulation of elution profiles of zone-interference chromatography can be carried out without the condition, $[A^t] \gg [B^t]$. The notation here is the same as in the main text. The general reaction scheme is

$$\begin{array}{ccc} A + B & \overset{k_a}{\underset{k_d}{\rightleftharpoons}} & AB \\ \downarrow\uparrow & & \downarrow\uparrow \\ A^g + B^g & \overset{k_a}{\underset{k_d}{\rightleftharpoons}} & AB^g. \end{array} \tag{A1}$$

The gel-permeation equilibria between the mobile phase (the upper law) and the stationary phase (the lower) are assumed to be attained at any moment within a plate as

$$[A^g] = \xi K_A [A]. \tag{A2-1}$$

$$[B^g] = \xi K_B [B]. \tag{A2-2}$$

and

$$[AB^g] = \xi K_{AB} [AB]. \tag{A2-3}$$

As in the text, k_a' is set to be

$$k_a' = \frac{K_{AB}}{\xi K_A K_B} k_a. \tag{A3}$$

Under these conditions, the reaction scheme, eq. A1, can be described by a time evolution equation:

$$\frac{d[AB]}{dt} = k_a [A][B] - k_d [AB]. \tag{A4}$$

On the other hand, from the mass conservation law, we have

$$[A^t] = [A] + [AB] + [A^g]/\xi + [AB^g]/\xi. \tag{A5-1}$$

and

$$[B^t] = [B] + [AB] + [B^g]/\xi + [AB^g]/\xi. \tag{A5-2}$$

For simplicity of calculation, we introduce the binding density in the mobile phase as

$$r(t) = \frac{[AB]}{[A^t]}. \tag{A6}$$

Then, using eqs. A2, A5 and A6, eq. A4 leads to

$$\frac{dr}{dt} = \frac{k_a}{(1+K_A)(1+K_B)} [1 - (1+K_{AB}) r] \times [[B^t] - (1+K_{AB}) r [A^t]] - k_d r. \tag{A7}$$

Since the right-hand side of eq. A7 is a quadratic equation in r, it can be set as

$$\frac{dr}{dt} = \frac{k_a [A^t] (1+K_{AB})^2}{(1+K_A)(1+K_B)} (r-\alpha)(r-\beta) \quad (\beta > \alpha). \tag{A8}$$

It is clear that two real roots, α and β, exist. For the initial condition, $r(0) = r_0$, eq. A8 can be solved as

$$r(t) = \frac{\alpha(r_0-\beta) - \beta(r_0-\alpha)\exp(-k^*(\beta-\alpha)t)}{r_0 - \beta - (r_0-\alpha)\exp(-k^*(\beta-\alpha)t)}. \tag{A9}$$

where

$$k^* = \frac{k_a [A^t] (1+K_{AB})^2}{(1+K_A)(1+K_B)}. \tag{A10}$$

In the following calculation of elution profiles using the plate theory, we consider the volume of the mobile phase in a plate as a unit of volume and use the total amount of A and B within a plate, A^t and B^t, instead of the concentration of A and B. Since α and β depend on $[A^t]$ and $[B^t]$, $r(t)$ in each theoretical plate is a function of A^t, B^t and r_0. Thus, the final state of the binding between A and B is shown as

$$r(A^t, B^t, r_0) = \frac{\alpha(r_0-\beta) - \beta(r_0-\alpha)\exp(-k^*(\beta-\alpha)\Delta T)}{r_0 - \beta - (r_0-\alpha)\exp(-k^*(\beta-\alpha)\Delta T)}. \tag{A11}$$

where ΔT is the time of elution through one plate. When k_a and k_d are comparable to the rate of gel permeation of solutes and the equilibria of eq. A1 are attained within a plate, eq. A11 is reduced to

$$r(A^t, B^t) = \alpha = \frac{1}{2(1+K_{AB})} \left(D - \sqrt{D^2 - 4B^t/A^t} \right). \tag{A11'}$$

where $D = 1 + B^t/A^t + (1+K_{AB})k_d/k^*$ and which does not depend on r_0. In our previous report [8], the simulations by eq. A11' were carried out for the case of $K_A = K_{AB} = 0$.

According to the plate theory, the total amounts of A and B at the j-th plate after i steps of elution are obtained by the amount of A, B and AB complex in the mobile phase at the $(j-1)$-th and j-th plate after $(i-1)$ steps as

$$A^t = K_A A_j(i-1) + K_{AB} AB_j(i-1) + A_{j-1}(i-1) + AB_{j-1}(i-1). \quad (2 \leqq j \leqq j_M) \tag{A12-1}$$

and

$$B^t = K_B B_j(i-1) + K_{AB} AB_j(i-1) + B_{j-1}(i-1) + AB_{j-1}(i-1). \quad \text{(A12-2)}$$

where j_M is the total number of theoretical plates of a column. In eqs. A12, the first two terms represent the amount of A(B) and AB complex in the stationary phase, $A^g(B^g)$ and AB^g, respectively. Since the sum of the second and fourth terms in eq. A12 is the total amount of AB complex, the initial value of the binding density in the mobile phase is

$$r_0 = \frac{K_{AB} AB_j(i-1) + AB_{j-1}(i-1)}{(1+K_{AB})A^t}. \quad \text{(A13)}$$

Finally, using eqs. A11–A13, the amounts of A, B and AB complex at the j-th plate after i steps are obtained as

$$AB_j(i) = r(A^t, B^t, r_0)A^t, \quad \text{(A14-1)}$$

$$A_j(i) = \left(A^t - (1+K_{AB})AB_j(i)\right)/(1+K_A), \quad \text{(A14-2)}$$

and

$$B_j(i) = \left(B^t - (1+K_{AB})AB_j(i)\right)/(1+K_B). \quad \text{(A14-3)}$$

The boundary conditions for the first plate are

$$A^t = K_A A_1(i) + K_{AB} AB_1(i) + A_0 \quad (i_1 < i_2 < i \leqq i_3) \quad \text{(A15-1)}$$

$$A^t = K_A A_1(i) + K_{AB} AB_1(i) \quad \text{(otherwise } i\text{)} \quad \text{(A15-2)}$$

and

$$B^t = K_B B_1(i) + K_{AB} AB_1(i) + B_0 \quad (1 \leqq i \leqq i_1) \quad \text{(A15-3)}$$

$$B^t = K_B B_1(i) + K_{AB} AB_1(i) \quad \text{(otherwise } i\text{)}. \quad \text{(A15-4)}$$

where A_0 and B_0 are the concentrations of A and B injected into a column, and i_1 and $i_3 - i_2$ the number of theoretical plates corresponding to the volume of B-zone and A-zone, respectively. The amount of both B and AB complex in the mobile phase at the j_M-th plate represents the elution profile of B-zone.

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