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Semi-Micro-Scale Frontal Gel Chromatography of Interacting Systems of a Protein and Small Molecules: Binding of Warfarin, Tryptophan, or FMN to Albumin, and of *o*-Nitrophenol to Catechol 2,3-Dioxygenase[1]

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Frontal gel chromatography is a convenient and accurate method to obtain the free ligand concentration of a protein-ligand mixture. Because a large amount of sample (more than 6 ml) is required for the method, it has been rarely used for binding experiments. We have developed a system to carry out frontal gel chromatography on a semi-micro scale using short gel filtration columns (4.6 mm×50-100 mm); frontal chromatograms could be obtained with small amounts of samples (1-2.5 ml) within 20 min. We used this technique to examine the binding of warfarin, L-tryptophan, or FMN to human serum albumin, the binding of warfarin to bovine serum albumin, and the interaction of catechol 2,3-dioxygenase with *o*-nitrophenol. The data fitted to a binding model in which a protein has one or several independent binding sites. Both human and bovine serum albumin showed the high-affinity bindings of two warfarin molecules. The binding number for L-tryptophan on human serum albumin was confirmed to be one, whereas maximal binding of FMN was 0.6 molecule per albumin molecule. *o*-Nitrophenol showed high-affinity binding only to holo-catechol 2,3-dioxygenase. The absorption spectrum of the bound *o*-nitrophenol resembled that of anionic *o*-nitrophenol. These results demonstrated that frontal gel chromatography on a semi-micro scale is useful for the study of binding systems; the method is rapid and would be easy to automate fully.

Key words: binding system, catechol 2,3-dioxygenase, frontal gel chromatography, ligand, serum albumin.

The basic processes of virtually all biological phenomena are the binding of specific ligands to proteins such as enzymes, receptors, and carrier proteins. It is therefore of central importance in understanding a biological process to determine both the stoichiometry and equilibrium constants of ligand interactions with macromolecules involved in the process.

In the present study, we focused on interactions of small ligands with proteins. Numerous methods have been developed to measure the concentration of the free ligand or that of the bound ligand of a protein-ligand mixture. To determine accurately the stoichiometry and related binding constants of the binding system, it is important to collect binding data over a wide range of total concentrations of the protein and ligand (*1-3*). To do such extensive binding experiments the method should be speedy, easy to automate, and accurate with small amounts of samples.

Three methods, equilibrium dialysis, ultrafiltration, and frontal gel chromatography, are universally applicable to any binding system to obtain the free ligand concentration. Equilibrium dialysis is most often used, and a relatively small amount of sample (0.2-1 ml) is needed for one experiment (*4, 5*). The method is generally laborious and time-consuming because it takes long time (4-24 h) to establish equilibrium between the internal solution in a dialysis bag and the exterior solution. It is also difficult to automate. Ultrafiltration is theoretically equivalent to equilibrium dialysis, and easy to carry out rapidly (*6*). In this method the amount of the filtrate should be small compared to the sample volume to lessen the disturbance of the initial concentrations of both the protein and ligands. Therefore non-specific binding of ligands to a semipermeable membrane may significantly affect the ligand concentration of the filtrate. Thus, it is generally difficult to obtain accurate data with small amounts of sample.

Frontal gel chromatography is one of the frontal analyses of differential migration such as ultracentrifugation and free-boundary electrophoresis, and therefore has a firm theoretical basis (*7-9*). When a sufficient volume of a protein-ligand mixture (usually more than 1.5 times the total gel volume) is applied to a column of a small-pore gel filtration medium which completely excludes the protein, the resulting elution profile contains a plateau region of the original mixture and another plateau region of free ligands (see Figs. 2 and 3). The concentration of the ligand in the latter plateau region is equal to the free ligand concentration in the original mixture. Because soft molecular-sieve gel columns such as Sephadex G-25 are necessarily large in

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Abbreviation: *I*, ionic strength.

volume to get sufficient resolution, a large amount of sample (10–20 ml) is required to obtain frontal chromatograms. This has hampered application of this method to the study of the interaction of proteins with small molecules. Recently, Nenortas and Beckett investigated protein association equilibria by frontal gel chromatography using a small Sephacryl S-200 HR column (Pharmacia; 6.6×68 mm, bed volume of 2.3 ml), and obtained accurate frontal chromatograms with 4.0 ml of samples (*10*). Because in the case of protein-ligand interactions not only the protein, but also the ligand show accurate leading and trailing boundaries, further reduction in column size is necessary to reduce the amount of sample required for the chromatography.

Porous silica- or polymer-based supports developed for high-performance gel chromatography show much higher resolution compared to soft gel columns (*11*). We tried to do frontal gel chromatography using short high-resolution gel filtration columns with an ordinary HPLC system, but failed to obtain frontal chromatograms. The main cause of the failure was that the trailing boundary of a sample was significantly disrupted during the passage through the long sample loop of the injection unit attached to the HPLC system. In this study, we developed a system to carry out frontal gel chromatography on a semi-micro scale using short high-resolution gel filtration columns (4.6×50–100 mm, bed volumes of 0.83–1.7 ml). We tested the usefulness of the frontal gel filtration system by applying the instrument to the study of the following binding systems: human serum albumin binding to warfarin, L-tryptophan, or FMN; bovine serum albumin binding to warfarin; the interaction of catechol 2,3-dioxygenase with *o*-nitrophenol.

EXPERIMENTAL PROCEDURES

Materials—Human and bovine serum albumins were purified from the respective Fraction V albumin (Sigma) according to the method of Janatova *et al.* (*12*) using a DEAE-Sepharose CL-6B column (Pharmacia). Catechol 2,3-dioxygenase was prepared as described previously (*13*). The concentrations of human serum albumin, bovine serum albumin, and catechol 2,3-dioxygenase were determined spectrophotometrically using molar absorption coefficients at 280 nm of 35,200, 44,300, and 43,830 $M^{-1} \cdot cm^{-1}$, respectively. FMN was obtained from Nacalai Tesque, and purified by HPLC on a Cosmosil 10C18 column (Nacalai Tesque; 2×25 cm) according to the method of Nielsen *et al.* (*14*). Warfarin was obtained from Sigma; L-tryptophan and *o*-nitrophenol from Nacalai Tesque. All other chemicals used were of analytical grade.

Frontal Gel Chromatography—Protein stock solutions in 0.1 M Tris-HCl (pH 9.0, $I=0.16$) or 50 mM HEPES (pH 7.5 or 6.5, $I=0.15$) were prepared by subjecting the purified proteins to gel chromatography on a Sephadex G-25 column (Pharmacia, 1×15 cm) equilibrated with the respective buffers.

Frontal gel chromatography was performed at 25°C on a Bio-Gel P-10 column (Bio-Rad; 4.6×100 mm) equilibrated with 0.1 M Tris-HCl (pH 9.0, $I=0.16$) or on a TSK-GEL G2000SW_{XL} column (Tosoh; 4.6×50 mm, or 4.6×75 mm) equilibrated with 50 mM HEPES (pH 7.5 or 6.5, $I=0.15$). A protein-ligand mixture (1.5–3 ml) was prepared in the same buffer. The mixture was applied to the column using the frontal gel chromatography system illustrated in Fig. 1, using the following procedures. The flow rate was 0.2 ml/min. (1) The column was connected to a Shimadzu LC-10AS HPLC pump through the valve V2, and the equilibration buffer was delivered to the column by the pump. (2) To avoid dilution of the sample in the dead spaces of both the tubing and syringe head, an aliquot of the mixture (0.3–0.5 ml) was first loaded into the syringe through the tube P, then the loaded mixture was discarded through the tube D1. (3) After priming the spaces of the syringe head and tubing as described above, the mixture (1.0–2.5 ml) was loaded into the syringe through the tube P. Then the loaded mixture was injected into the column. (4) Immediately after the injection of the mixture was completed, the column was connected to the HPLC pump through the valve V2, and elution was carried out with the equilibration buffer. (5) To wash the syringe and the tubing, the buffer (2.5 ml) was loaded into the syringe through the tube D1, and then the loaded buffer was discarded *via* the tube P.

The elutions of the protein and ligand were continuously monitored with a UV-8000 UV-VIS detector (Tosoh). The dead space between the column outlet and the inlet of the flow cell of the detector was made as small as possible to minimize possible disturbance of the chromatogram. To determine accurately the concentrations of both the protein and ligand in the plateau regions, an aliquot of the eluate (50–130 μl) was fractionated, and the absorption spectrum was obtained with a Shimadzu UV-2200 spectrophotometer equipped with a Shimadzu ultra micro cell holder.

Analysis of Binding Data—The binding data were analyzed in terms of the following model. A protein has one or two independent and identical binding sites which show high affinity to a ligand, and also has several independent binding sites which show much lower affinity to the ligand compared to the primary sites. In the present study, we focused on the primary binding sites of albumin and catechol 2,3-dioxygenase, and obtained the binding data in the ligand concentration range where the secondary sites were only partly saturated with the ligand. If a binding system follows the above model, the binding data can be expressed by the following equation:

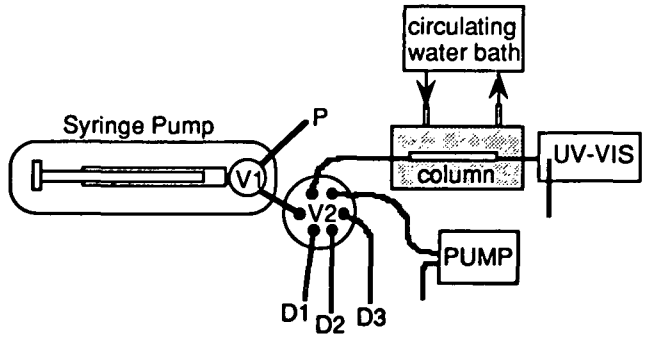

Fig. 1. **Diagram of the frontal gel chromatography system.** V1 is a two-way valve, and V2 a six-way valve. The plunger of the gas-tight syringe (2.5 ml) and the valves (V1 and V2) are computer-controlled (Kyoto Chromato). The column was attached to the valve V2 and detector (UV-8000 UV-VIS detector, Tosoh) using Teflon or Peek tubing with minimum dead volumes. A Shimadzu HPLC liquid delivery module (LC-10AS) was used to deliver buffers. Samples were loaded into the syringe through the tube P.

$$r=\frac{nkL_f}{1+kL_f}+aL_f \quad (1)$$

where r is the molar ratio of bound ligand per protein, n the number of the primary binding sites, k the primary binding constant, L_f the free ligand concentration, and a a constant, $a=\sum_i n_i k_i$, where n_i is the number of the ith secondary binding sites and k_i the binding constant for the ith sites. The value of r is given by the following equation:

$$r=\frac{L_t-L_f}{P_t} \quad (2)$$

where L_t is the total ligand concentration and P_t the total protein concentration. We fitted the binding data to Eq. 1 with a nonlinear least-squares algorithm.

Enzyme Assay—The activity of catechol 2,3-dioxygenase was assayed by measuring the increase in absorbance at 375 nm due to the formation of 2-hydroxymuconate semialdehyde (*13*) at 25°C in air-saturated buffer of 50 mM HEPES, pH 7.5, $I=0.15$. The reaction mixture (3.0 ml) contained 0.11 mM catechol, and the reaction was initiated by the addition of 1 μl of the enzyme solution.

RESULTS AND DISCUSSION

Elution Profiles of Frontal Gel Chromatography—To test the validity of the present frontal gel chromatography procedure we injected 1.4 ml of 20 μM catechol 2,3-dioxygenase, or 50 μM *o*-nitrophenol, or a mixture containing 20 μM enzyme and 50 μM *o*-nitrophenol into a small TSK-GEL G2000SW$_{XL}$ column (4.6×50 mm, bed volume of 0.83 ml). The chromatograms are shown in Fig. 2A. When the enzyme alone was injected, the leading and trailing boundaries clearly appeared at positions of 0.64 and 1.94 ml, respectively. The elution profile of *o*-nitrophenol alone showed the leading boundary at 1.34 ml and the trailing boundary at 2.54 ml, and the *o*-nitrophenol concentration in the plateau region was identical to that of the original solution.

When the mixture of the enzyme and *o*-nitrophenol was injected into the column, the elution of *o*-nitrophenol showed a new boundary at the same position where the trailing boundary of the enzyme alone appeared. The leading boundary of *o*-nitrophenol appeared significantly sooner compared to the elution of the ligand alone, and the shape of the boundary changed. The trailing boundary of the ligand appeared at the same position as in the elution of the pure ligand. The *o*-nitrophenol concentration of the first plateau region between the leading and the new middle boundaries was identical to that of the original mixture. These results showed that the bound ligand migrated at the same velocity as the enzyme, and that the stationary phase of the gel (inner volume) was rapidly equilibrated with the free ligand in the moving phase (outer volume). After the passage of a sufficient amount of the enzyme-ligand mixture both the stationary and moving phases of the gel were equilibrated with the free ligand concentration of the original mixture, so the *o*-nitrophenol concentration in the second plateau region, which corresponded to the elution of the free ligand, gave that in the original mixture.

We further tested the usefulness of the present method by examining the interaction between bovine serum albumin and warfarin (Fig. 2B). The profile of the frontal chromatogram of 1.4 ml of bovine serum albumin alone (20 μM) was essentially the same as that of catechol 2,3-dioxygenase. On the other hand, warfarin (50 μM, 1.4 ml) eluted much more slowly than *o*-nitrophenol. The leading boundary appeared at 1.66 ml and the trailing boundary at 2.94 ml. The concentration of warfarin was first raised slightly above the original concentration, and then a plateau region appeared. The warfarin concentration in the plateau region was identical to that of the original solution. The result indicated the interaction of warfarin with the gel matrix. When the albumin-warfarin mixture (1.4 ml) was injected into the column, the elution of the warfarin showed first a near-plateau region and then a plateau region of free warfarin (Fig. 2B). Like the elution profile of warfarin alone, the warfarin concentration in the initial part of the first near-plateau region overshot the warfarin concentration in the original mixture. The warfarin concentration in the middle part of the first near-plateau region was identical to that in the original mixture. The second plateau appeared between the trailing boundary of albumin alone and that of warfarin alone, and the warfarin concentration in the plateau gave the free ligand concentration in the original mixture. Of all binding systems examined in the present study the overshooting in the first plateau region (or near plateau region) was clearly detected only in frontal analyses of bovine serum albumin-warfarin mixtures containing relatively low concentrations of warfarin (less than 100 μM). Osmotic perturbation of the bead size of the gel matrix can induce anomalous frontal patterns (*15, 16*). If

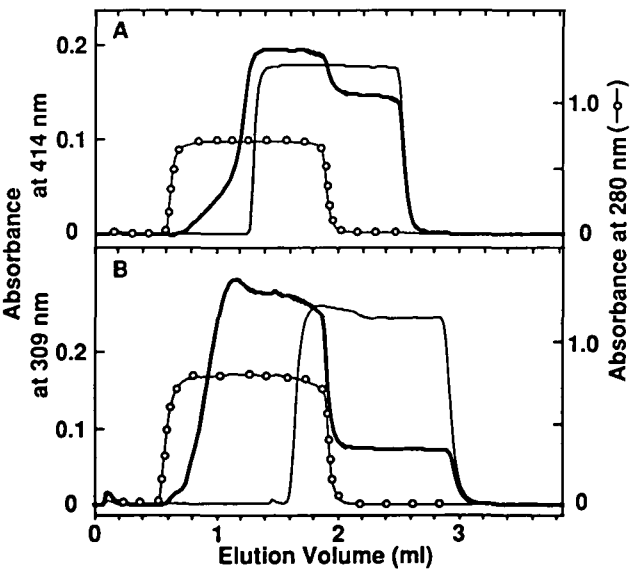

Fig. 2. **Elution profiles of frontal gel chromatography.** Samples (1.4 ml) were injected into a TSK-GEL G2000SW$_{XL}$ column (4.6×50 mm) at 25°C. The buffer was 50 mM HEPES (pH 7.5, $I=0.15$), and the flow rate was 0.2 ml/min. A: Catechol 2,3-dioxygenase (20 μM, open circles), *o*-nitrophenol (50 μM, thin line), and the mixture (thick line) containing the enzyme (20 μM) and *o*-nitrophenol (50 μM) were subjected to frontal gel chromatography. The elutions of the enzyme alone and *o*-nitrophenol alone were detected by measuring the absorbance at 280 and at 414 nm, respectively. The elution of the mixture was monitored only at 414 nm. B: Bovine serum albumin (20 μM, open circles), warfarin (50 μM, thin line), and the mixture (thick line) containing the albumin (20 μM) and warfarin (50 μM) were subjected to frontal gel chromatography. The elutions of warfarin alone and the mixture were detected by measuring the absorbance at 309 nm.

this is the sole cause, then the same kind of overshooting should be observed in the frontal profiles of other binding systems examined. The interaction of the ligand with the gel matrix may be the cause of the overshooting. However, no overshooting in the ligand elution profile was observed in the case of FMN binding to human serum albumin at pH 9, where FMN showed a substantial interaction with the gel matrix used (Bio-Gel P-10). Thus, the exact cause of the overshooting is not known, and further investigation is necessary to improve the reliability and accuracy of the present method of frontal gel chromatography.

Effect of Sample Volume on Frontal Chromatogram—To assess the minimum volume of a protein-ligand mixture required to obtain the free ligand concentration, we injected various amounts of the bovine serum albumin-warfarin mixture into a TSK-GEL G2000SW$_{XL}$ (4.6×50 mm), and detected the elution of warfarin (Fig. 3). The chromatograms obtained by using 2.0 or 1.4 ml of the sample showed two plateau regions. The first plateau region, where the original mixture eluted as itself, was wider by 0.6 ml in the case of 2.0 ml injection than in the case of 1.4 ml injection. On the other hand, the second plateau region, where the free ligand eluted, was the same size in both cases. When 1.0 ml of the mixture was injected, only the second plateau region was detected, and the free warfarin concentration in this region was the same as that obtained from the chromatograms using 2.0 or 1.4 ml of the mixture. These results suggested that more than about 1.5 volumes of the total gel bed volume is the sample volume required to obtain an unambiguous frontal chromatogram. If the injected sample cannot saturate completely the total available column space including stationary and moving phase, the first plateau does not appear. However, a plateau region corresponding to the elution of the free ligand can be obtained when the total available column space is nearly but not completely saturated with the sample.

Application to Binding Systems of Albumin—Human serum albumin is in an equilibrium between two conformations (N and B conformers) at physiological pH, and the B conformer is predominant at pH 9.0 (for reviews, see Refs. *17* and *18*). Although numerous binding experiments have been done to study the interaction of albumin with various ligands, few experiments have focused on the binding of ligands to the B conformer. We therefore examined the

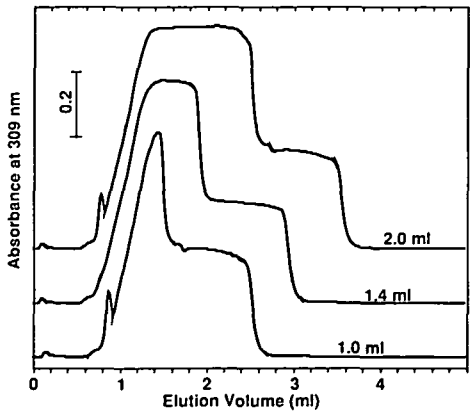

Fig. 3. **Effect of sample volume on frontal chromatogram.** Various amounts (2.0, 1.4, and 1.0 ml) of the bovine serum albumin-warfarin mixture containing 20 μM albumin and 20 μM warfarin were injected into a TSK-GEL G2000SW$_{XL}$ column (4.6×50 mm) at 25°C. The buffer was 50 mM HEPES (pH 7.5, I=0.15), and the flow rate was 0.2 ml/min. The elution of warfarin was detected by measuring the absorbance at 309 nm.

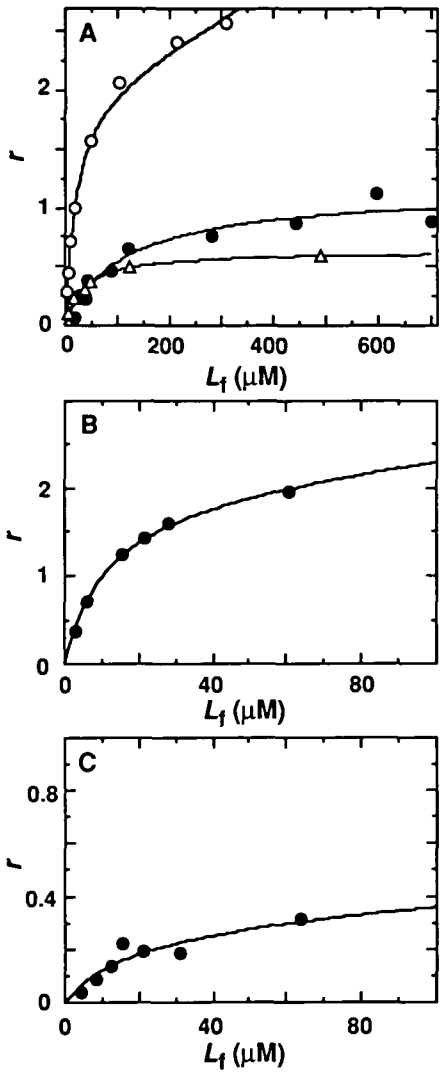

Fig. 4. **Binding curves for the interactions of human serum albumin with warfarin, L-tryptophan, and FMN (A), the interaction of bovine serum albumin with warfarin (B), and the interaction of catechol 2,3-dioxygenase with *o*-nitrophenol (C).** A: The binding of human serum albumin to warfarin (open circles), L-tryptophan (filled circles), and to FMN (open triangles) was examined at 25°C in 0.1 M Tris-HCl (pH 9.0, I=0.16). Samples (2.5 ml) were injected into a Bio-Gel P-10 column (4.6×100 mm). The best simulation for each binding curve was calculated with Eq. 1 using the following parameters: n=2.0, k=5.4×10^4 M^{-1}, and a=2.4×10^3 M^{-1} for the warfarin binding; n=1.2, k=8.4×10^3 M^{-1}, and a=0 for the L-tryptophan binding; n=0.62, k=2.7×10^4 M^{-1}, and a=0 for the FMN binding. B: The binding of bovine serum albumin to warfarin was examined in 50 mM HEPES (pH 7.5, I=0.15). The albumin-warfarin mixtures (1.4 ml each) were applied to a TSK-GEL G2000SW$_{XL}$ column (4.6×50 mm). The best simulation was calculated with Eq. 1 using the parameters of n=2.0, k=8.8×10^4 M^{-1}, and a=4.9×10^3 M^{-1}. C: The binding of catechol 2,3-dioxygenase to *o*-nitrophenol was examined in 50 mM HEPES (pH 7.5, I=0.15). The enzyme preparation contained 0.31 mol of ferrous iron per mol of enzyme subunit. The best simulation was calculated with Eq. 1 using the parameters of n=0.31, k=5.5×10^4 M^{-1}, and a=9.6×10^2 M^{-1}.

interaction of the albumin with warfarin, L-tryptophan, and FMN at pH 9.0 using the present method. Because silica-based supports are usually not suitable for use at alkaline pH, we used a Bio-Gel P-10 column (4.6×100 mm) and injected 2.5 ml of the albumin-ligand mixtures to obtain frontal gel chromatograms. All the chromatograms obtained clearly showed typical two-plateau profiles (data not shown).

First, we examined warfarin binding to the B conformer of albumin. Based on an X-ray crystallographic study of human albumin (*19*), the single binding site of warfarin is located in subdomain IIA. On the other hand, binding experiments using equilibrium dialysis have shown that in solution human serum albumin has two primary binding sites for the ligand with equally high affinity (*5*). As shown in Fig. 4A, the B conformer tightly bound two warfarin molecules, and the primary binding constant was obtained as $(5.4\pm0.4)\times10^4\ M^{-1}$. This result is consistent with that of a recent study done at pH 7.4 (*5*).

Secondly, we examined L-tryptophan binding to albumin (Fig. 4A). Intact albumin has a single L-tryptophan binding site (*20*). The binding data fitted well to a simple hyperbolic binding model with the parameters $n=1.2\pm0.1$ and $k=(8.4\pm2.1)\times10^3\ M^{-1}$ (Fig. 4A). The result indicated that the albumin preparation was intact and homogeneous in terms of L-tryptophan binding.

Thirdly, we examined the binding of FMN to albumin at pH 9.0 (Fig. 4A). Albumin is a carrier of riboflavin in blood (*21*). Because the binding of riboflavin to albumin is weak and the solubility of riboflavin in aqueous solution is low, it is difficult to obtain sufficient binding data to determine the binding stoichiometry. The solubility of FMN is high. As shown in Fig. 4A, FMN tightly bound to albumin, and the binding curve showed saturation at the level of $r=0.6$. The data fitted well to a hyperbolic binding with the parameters of $n=0.62\pm0.01$ and $k=(2.7\pm0.2)\times10^4\ M^{-1}$. The result indicated that the albumin preparation was heterogeneous in terms of FMN binding capacity, although it was homogeneous in terms of L-tryptophan binding.

Comparative study of human and bovine serum albumin is fruitful to understand the structural basis of their functions, although at present the three-dimensional structure of bovine serum albumin has not been determined. We examined warfarin binding to bovine serum albumin at pH 7.5 (Fig. 4B), and compared the result to the binding of warfarin to human serum albumin at pH 9.0 (Fig. 4A). As in the case of human serum albumin, two warfarin molecules bound tightly to bovine serum albumin with the binding constant of $k=(8.8\pm0.5)\times10^4\ M^{-1}$. Because a crystal of warfarin-human albumin complex revealed only a single location of bound warfarin, further investigation is required to elucidate the structural mechanism of warfarin binding to solution states of albumin.

o-Nitrophenol Binding to Catechol 2,3-Dioxygenase—Catechol 2,3-dioxygenase [EC 1.13.11.2] catalyzes an extradiol cleavage of catechol to form 2-hydroxymuconate semialdehyde. The enzyme needs nonheme ferrous ion for the activity, and the ferrous ion is easily lost in the absence of acetone or *o*-nitrophenol, which are competitive inhibitors of the enzyme (*13*). We prepared the enzyme containing 0.31 mol iron/mol enzyme subunit, and examined *o*-nitrophenol binding to it in order to investigate whether the active site iron is involved in the interaction with *o*-nitrophenol. As shown in Fig. 4C, the binding curve showed apparent saturation at the level of $r=0.35$, and fitted well to Eq. 1 with the parameters of $n=0.31$, $k=(5.5\pm1.6)\times10^4\ M^{-1}$, and $a=(9.6\pm6.9)\times10^2\ M^{-1}$. The value of the binding constant agreed fairly well with that ($10\times10^4\ M^{-1}$) estimated from the value of the inhibition constant (*13*). These results suggested that the active site iron is directly involved in the tight binding of *o*-nitrophenol.

o-Nitrophenol is dominantly in protonated form at pH 6.5 and mainly in anionic form at pH 7.5. To determine whether the bound *o*-nitrophenol is anionic or protonated, we examined the interaction of the enzyme with *o*-nitrophenol at pH 6.5, and determined the absorption spectrum of the bound *o*-nitrophenol (Fig. 5) using the fractions of the first and second plateau regions. The absorption spectrum is similar to that of *o*-nitrophenol anion, although significant differences exist between the two spectra. The molar absorption coefficient of the complex with the ligand could be easily determined.

Conclusion—In the present study we showed that frontal gel chromatography can be done using a small amount of sample similar to that required to obtain reliable data in equilibrium dialysis. One of the main obstacles to the application of frontal gel chromatography to binding systems has been the large amount of sample required in the method. Because our system for semi-micro frontal gel chromatography developed in the present study is simple and would be easy to automate, the method is expected to contribute to studies of binding systems in the future. Techniques such as isothermal titration calorimetry (*22, 23*) and the plasmon resonance method (*24*) have also

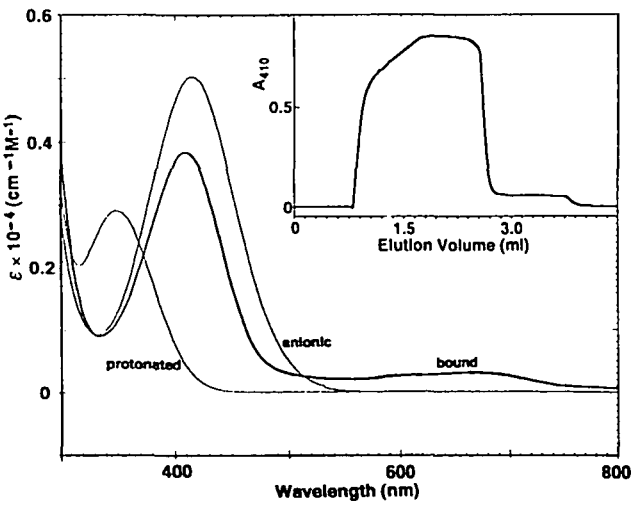

Fig. 5. **Absorption spectra of *o*-nitrophenol and its complex with catechol 2,3-dioxygenase.** The absorption spectrum of the bound *o*-nitrophenol was determined in 50 mM HEPES (pH 6.5, $I=0.15$). The mixture (1.9 ml) containing 0.274 mM enzyme and 0.223 mM *o*-nitrophenol was injected into a TSK-GEL G2000SW$_{XL}$ column (4.6×75 mm). The inset shows the resulting frontal chromatogram. Aliquots of the first and second plateau regions were collected, and the absorption spectrum of each fraction was measured. The spectrum of bound *o*-nitrophenol (0.183 mM, thick line) was obtained by subtraction of the absorption spectra of the enzyme (0.274 mM) and the free ligand (40.4 μM) from that of the original mixture or the fraction from the first plateau. The absorption spectra for protonated and anionic free *o*-nitrophenol were measured in 0.1 M HCl and 0.1 M NaOH, respectively.

advanced recently. The data obtained by these methods contains thermodynamic parameters or kinetic parameters in addition to stoichiometric parameters. In other words, because the binding models used for the analyses contain many parameters to be determined, it is generally difficult to analyze the data precisely. If stoichiometric parameters are independently determined by frontal gel chromatography, it would be much easier to analyze the data obtained by these other methods.

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