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Affinity Capillary Electrophoresis Using a Low-Concentration Additive with the Consideration of Relative Mobilities

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Most affinity studies in capillary electrophoresis assume that the analyte concentration is much smaller than the additive concentration so that the migration of the analyte has no effect on the concentration of the additive in the capillary. However, in most medium- to high-affinity interactions, the additive concentration has to be kept rather low to observe the changes in analyte mobility before saturation is reached. In this paper, a mathematical model is developed to describe the migration behavior of the analyte in a system where the complex formed becomes concentrated to levels much greater than the original concentration of the additive due to the differences in the mobilities of the analyte, additive, and complex. The analyte is flurbiprofen, the additive is transthyretin, and the stoichiometry of the reaction between the two is 1:2. This study also provides a new algorithm to determine medium- to high-affinity binding interaction constants by CE.

Protein−ligand binding studies play an important role in many aspects of protein biochemistry and enzymology. The binding of the ligand to its specific receptor triggers a cascade of biochemical reactions that eventually leads to a physiological or pathological response. The qualitative and quantitative information obtained from binding studies can play an important role in elucidating the mechanism involved in a particular biological system.

The techniques used to measure binding affinity can be generally classified into two groups. The measurements can be based either on the separation and the quantification of the different species in which the ligand is present at equilibrium (free and complex) or on monitoring the change in specific physicochemical properties of the complexed ligand or of the binding protein.[1] The advantage of nonseparating procedures is the possibility to detect the presence of both high- and low-affinity binding interactions. Conventional methods such as equilibrium dialysis and ultrafiltration, belonging to the first group, are widely used because of their simplicity and general applicability. Many problems have been reported for both methods.[2−5] Ultracentrifugation is a slow technique, and sedimentation, back diffusion, and viscosity effects influence the free ligand concentration.[3] The major controversy surrounding ultrafiltration concerns the stability of the equilibrium during the separation process.[4] Furthermore, all these techniques require an additional analysis step such as the measurement of the free ligand concentration. To the second group belong the spectroscopic methods (UV, fluorescence, NMR) that give complementary information about the binding mechanism and the structural and conformational properties of the complex formed. However, it has been reported that these methods are unable to provide quantitative binding parameters in the presence of multiple equilibria.[3] During the past few years, isothermal titration calorimetry, surface plasmon resonance methods, affinity chromatography, and affinity capillary electrophoresis (CE) have been used to measure binding parameters because of their ability to provide more reasonable estimates when compared with the methods listed above and due to their feasibility of automation. Calorimetry[5] and SPR methods can be classified as part of the second group because they monitor the change in specific physiochemical properties of the receptor. Calorimetry is based on measurements of the heat released or absorbed by the stepwise addition of a ligand molecule to a solution containing the receptor. Surface plasmon resonance methods are based on the measurement of signal changes when a solution of ligand is passed over the surface of a sensor chip where the receptor is immobilized.[6,7] Affinity chromatography and affinity CE[3,8−16]

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encompass many different methods that are based either on the separation of the interacting species or on the detection of a specific physicochemical property of the complexed ligand or binding protein and, therefore, can belong to both of the two groups previously described. The principal advantages of affinity CE compared to the other techniques are the small-volume consumption, the speed of analysis, and the fact that the interaction takes place in free solution. All methods belonging to affinity CE are based on the differences between the migration velocities of the interacting species. Some of the methods use this difference to separate and quantify the free and complexed ligand present in a preequilibrated mixture using non equilibrium electrophoresis,[17] while others exploit the difference in mobility between the free and complexed ligand in order to observe a gradual analyte (ligand or receptor) migration shift as a function of different additive (receptor or ligand, respectively) concentrations present in the capillary.[10] The kinetics of the interaction between protein and ligand determines the choice between the two affinity CE methods.[18−20] For the second group of affinity CE methods, an important condition is that the analyte injected into the capillary must be much less than the additive present in the whole capillary so that the equilibrium is maintained during the analyte migration. In most cases, the experimental conditions are chosen to maintain this assumption. However, there are many cases in which it is also important to consider situations where the concentration of the additive is smaller than that of the analyte injected.[21] To observe a significant change in the mobility for medium- to high-affinity interaction and to accurately determine the effect of binding on the analyte migration, the additive concentration has to be kept low to allow enough time before the analyte plug is saturated.[22]

Furthermore, in many cases, the availability of protein is low (mg or less) due to difficulties in purification from biological matrixes, low availability of these matrixes (i.e., blood of patients with a particular mutant form of protein), or high cost. For this reason, most dynamic complexation capillary electrophoresis experiments are carried out using an experimental setup in which the capillary is filled with the ligand and the protein is injected as the analyte, allowing the use of very small amounts of protein.[8,9,13,14] Under such conditions, small errors in migration time determination may lead to large discrepancies in the determination of binding constants.[23] Thus, it is often better, especially when the differences in the mobilities of the interacting species are small, to use a second experimental setup in which the ligand is used as the analyte and the protein is used as the additive.[24] In this way, the errors in the analyte migration time measurements are lower due to a generally better shape of the ligand peak, a higher reproducibility of the ligand migration time, and a wider shift in the mobility of the analyte, according to the changes in both mass and charge of the complexed form. In this case, it is important to use a low concentration of protein so that even though the volume used is high the amount required still remains low. In addition, the use of a very high concentration of protein in the capillary can decrease the sensitivity when the ligand is detected by UV absorbance (which already suffers from a low sensitivity).[25]

In this paper, we describe a new method for the determination of medium- to high-affinity binding constants. The drug flurbiprofen was used as the analyte and was injected into a capillary, filled with low-concentration transthyretin (TTR). When the electric field was applied, the migration of the drug and the protein, as well as the complex formed, presented an interesting phenomenon that can be described by the proposed mathematical model. The binding constant and the complex mobility can be obtained by solving the differential equation presented in this paper.

THEORY

The equilibrium describing the interaction can be written as follows:

$$2A + P \rightleftarrows A_2P \qquad (1)$$

where A is the analyte (flurbiprofen), P is the protein (TTR), and A_2P is the complex formed. Previous work has shown the two binding sites to be identical in nature, with the same affinity for the two molecules of flurbiprofen.[26] The mobility of the analyte in the presence of the additive, μ_{ep}^{A}, depends on two factors: (1) the fractions of the species in which the analyte is present and (2) the mobility of these species, and is given by

$$\mu_{ep}^{A} = f_A\mu_{ep,A} + f_{A_2P}\mu_{ep,A_2P} \qquad (2)$$

where f_A is fraction of free analyte, f_{A_2P} is the fraction of analyte in a complexed form, $\mu_{ep,A}$ is the mobility of free analyte, and μ_{ep,A_2P} is the mobility of complexed analyte.

The fractions in eq 2 are defined as

$$f_{A_2P} = 2[A_2P]/[A]_0 \qquad (3)$$

$$f_A = 1 - f_{A_2P} \qquad (4)$$

where $[A_2P]$ is the concentration of the complex and $[A]_0$ is the concentration of the injected analyte.

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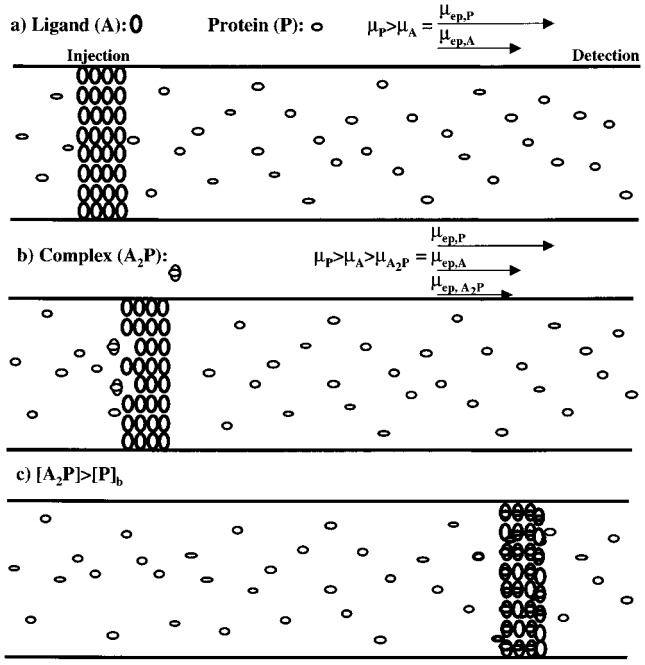

Figure 1. Migration of the analyte plug (a) before the start of the CE process, (b) shortly after the start of the CE process, and (c) at the end of the CE process. The relationship between the relative effective mobilities (in which the EOF has already been considered) is expressed with arrows, which represent the magnitude and the direction of the molecular velocity.

In the presence of a low concentration of additive, it is important to consider the relative mobilities of the interacting species when the binding constant is high. Under our experimental conditions, the mobility of the additive is higher than the mobility of the free analyte, which itself has a higher mobility than the complex. Figure 1a shows the situation after the injection and before the start of the CE process. The additive in the capillary, positioned ahead of the analyte, migrates more quickly and cannot be reached by the analyte. However, the additive that enters the capillary from behind the analyte can migrate into the analyte plug, as demonstrated in Figure 1b. Thus, the amount of the additive in the analyte plug with which the analyte can interact increases during the run. At the beginning of the run, the analyte-to-additive ratio is very high due to the low starting concentration of the additive and the high analyte concentration. As the run progresses, this ratio becomes smaller since the concentration of additive increases in the analyte plug, as demonstrated in Figure 2c, and eventually, the amount of protein going into the plug will be equal to that leaving the plug.

The mobility of the analyte plug is affected by the amount of additive that enters into this plug, making the change in the concentration of the additive time dependent. This is one of the cases where the mobility of the additive affects the net analyte mobility. The change in the net analyte mobility with time can be expressed by

$$\frac{d\mu_{ep}^{A}}{dt} = \frac{d\mu_{ep}^{A}}{d[P]_{total}} \frac{d[P]_{total}}{dt} \tag{5}$$

in which $[P]_{total}$ is the total additive concentration in the analyte plug. To define the relationship between $[P]_{total}$ and time, the distances that the analyte and the additive have moved need to be considered. The infinitely small distances that the analyte and the additive have moved (dx and dy, respectively) in an infinitely short time (dt) can be expressed as the product of the velocity of each species and the time ($\mu_{ep}^{A}E$ dt and $\mu_{ep,P}E$ dt, respectively). The change in the concentration of additive in the plug can be expressed in terms of additive added to, and removed from, the analyte plug:

$$d[P]_{total} = \frac{|dy - dx|}{a}([P]_b - [P]) \tag{6}$$

where $[P]_b$ is the concentration of additive behind the analyte plug, (equal to the concentration in the background electrolyte (BGE)), [P] is the concentration of free additive present in the plug and a is the length of the analyte plug.

Substituting the values for dx and dy, eq 6 can be rewritten as

$$\frac{d[P]_{total}}{dt} = \frac{|\mu_{ep,P} - \mu_{ep}^{A}|}{a}E([P]_b - [P]) \tag{7}$$

This equation describes the change in the total protein concentration within the analyte plug during the time of the separation process. It is a general equation and is valid for any kind of interaction because it is independent of the particular equilibria occurring in the analyte plug. In this description, it is assumed that the protein is uniformly distributed throughout the analyte plug and that only the free protein can migrate into or out of the plug. The complex can exist only in the analyte plug and thus can be assumed to be restricted to this plug.

From eq 7 it is evident that there are two key effects that can cause the total amount of protein to change in the analyte plug: a difference in concentration between the free protein and the protein present in the BGE and a difference between the mobilities of the protein and the analyte plug. There are two different conditions under which the mobility of the plug will become constant. The first case occurs when the concentration of the free protein that leaves the analyte plug is equal to the concentration of the protein present in the background electrolyte ($[P] = [P]_b$). The second case occurs when the mobility of the protein is equal to the mobility of the analyte plug, that is $\mu_{ep,P} = \mu_{ep}^{A}$. In either case, a steady-state condition is reached and the mobility of the plug becomes constant.

Using eqs 2−4, the change in the analyte mobility as the protein migrates into the analyte plug is given by (detailed derivation is given in Appendix 1)

$$\frac{d\mu_{ep}^{A}}{d[P]_{total}} = \frac{2(\mu_{ep,A_2P} - \mu_{ep,A})}{[A]_0} \frac{d[A_2P]}{d[P]_{total}} \tag{8}$$

The derivative on the right-hand side of eq 8 is obtained from the equilibrium expression

$$K = [A_2P]/[A]^2[P] \tag{9}$$

Because we have defined the initial concentrations of the protein

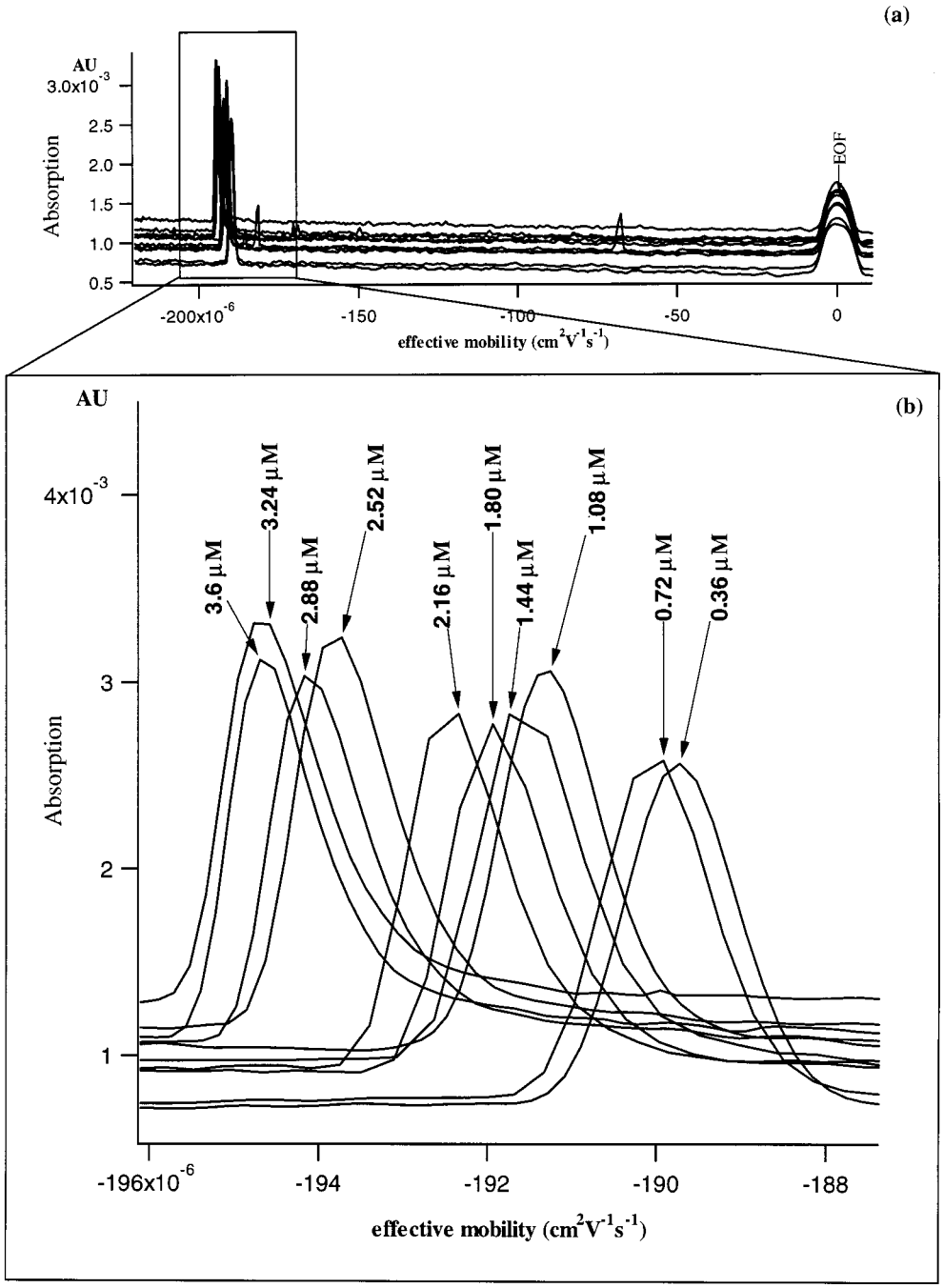

Figure 2. Electropherograms of flurbiprofen obtained using setup 1, for a concentration of analyte equal to 50 μM. The electropherograms are presented with a transformed mobility scale (transformation from the EOF peak). Part b is an expansion on the analyte peaks from part a.

and the analyte as

$$[P]_{total} = [P] + [A_2P] \quad (10)$$

and

$$[A]_0 = [A] + 2[A_2P] \quad (11)$$

Solving eqs 10 and 11 for the concentration of the free protein and of the free analyte, respectively, substituting into eq 9, and differentiating with respect to $[P]_{total}$ then gives (detailed derivation is given in appendix 2)

$$\frac{d[A_2P]}{d[P]_{total}} = \frac{K[A]_0^2\left(\frac{(\mu_{ep}^A - \mu_{ep,A_2P})}{(\mu_{ep,A} - \mu_{ep,A_2P})}\right)^2}{1 + K[A]_0^2\left(\frac{(\mu_{ep}^A - \mu_{ep,A_2P})}{(\mu_{ep,A} - \mu_{ep,A_2P})}\right)^2 - 2\left(\frac{(\mu_{ep}^A - \mu_{ep,A})}{(\mu_{ep}^A - \mu_{ep,A_2P})}\right)} \quad (12)$$

where the mobility terms are obtained from eq 2 by expressing $[A_2P]$ in eq 3 in terms of mobilities.

Table 1. Definitions of the Reduced Quantities in Eq 13

reduced quantities	definitions
z	$\frac{(\mu_{ep,A} - \mu_{ep}^{A})}{(\mu_{ep,A} - \mu_{ep,A_2P})} = f_{A_2P}$
δ	$2K[A]_0[P]_b$
γ	$K[A]_0^2$
β	$\frac{(\mu_{ep,A} - \mu_{ep,A_2P})}{(\mu_{ep,P} - \mu_{ep,A})}$
ω	$\frac{\lvert\mu_{ep,P} - \mu_{ep,A}\rvert E}{a}$
z_1	$1 + \frac{1 - \sqrt{1 + 4\delta}}{2\delta}$
z_2	$1 + \frac{1 + \sqrt{1 + 4\delta}}{2\delta}$

Sustituting eq 12 into eq 8 and combining with eq 7 substituted into eq 5 then gives (detailed derivation is given in Appendix 3)

$$\frac{dz}{dt} = \omega\delta\frac{(1 - z)(1 + \beta z)(z - z_1)(z - z_2)}{1 + z + \gamma(1 - z)^3} \qquad (13)$$

where the reduced quantities are defined in Table 1.

It can be shown that z_1 is less than 1 and z_2 is greater than 1. Also, in general, β can take on negative or positive values. Equation 13 shows that $dz/dt = 0$ when $z = z_1$ or $z = -1/\beta$. In fact, if $z = 0$ at $t = 0$, then the mobility of the analyte plug will reach a steady state when z is equal to the smaller of z_1 or $-1/\beta$. Since the fraction of the complex, z, satisfies $0 \le z \le 1$, $z = -1/\beta$ can only be attained if $\beta < -1$. Thus, two scenarios arise. In scenario one, $\beta < -1$, which corresponds to the following restrictions on the mobilities:

$$\mu_{ep,A} < \mu_{ep,P} < \mu_{ep,A_2P} \qquad (14)$$

or

$$\mu_{ep,A} > \mu_{ep,P} > \mu_{ep,A_2P} \qquad (15)$$

If either of the conditions in eqs 14 and 15 apply, and the value of $-1/\beta < z_1$, then the mobility of the analyte plug will reach a steady state when $z = -1/\beta$. Physically, this corresponds to the point where $\mu_{ep}^{A} = \mu_{ep,P}$, since the $(\mu_{ep,P} - \mu_{ep}^{A})$ term in eq 7 transforms to the $(1 + \beta z)$ term in the numerator of eq 13. This is clear from eqs 14 and 15 since the mobility of the plug starts at $\mu_{ep,A}$ and moves toward μ_{ep,A_2P}. If eqs 14 and 15 hold, then at some point the mobility of the plug must attain the same value as that of the protein. At this point the analyte plug and the protein move at the same velocity so no further change in μ_{ep}^{A} can occur. Note though that in this case, $[P] \neq [P]_b$. If it so happens that $z_1 < -1/\beta$, then the steady state will be reached first when $z = z_1$. In this case, it is true that $[P] = [P]_b$, since z_1 corresponds to the fraction of the complex when the free protein in the plug is the same as that behind the plug. This is the steady state discussed with reference to eq 7.

In scenario two, the mobility of the protein does not fall between the mobilities of the analyte and the mobility of the complex and eqs 14 and 15 are no longer valid. In this case, a steady state can be reached only when $z = z_1$, that is, $[P] = [P]_b$. This is the scenario that is relevant in the experiment described in this paper.

When the steady-state $z = z_1$ is reached, the total concentration of the protein in the analyte plug can be expressed, from the equilibrium expression (eq 10) at $[P] = [P]_b$, as

$$[P]_{total} = [P]_b + ([A]_0/2)z_1 \qquad (16)$$

Detailed derivation is given in Appendix 4.

If the interaction is very weak, the value of the binding constant is small, and for a fixed value of $[A]_0[P]_b$, the value of z_1 is close to zero and the protein does not bind to the analyte. In this case, the total concentration of protein in the plug should be equal to the concentration of the protein in the bulk solution, as is predicted by eq 16 when $z_1 = 0$.

Equation 16 can also be used to describe high-affinity interactions. In this case, the value of the binding constant is very high and thus, for a fixed value of $[A]_0[P]_b$, the value of δ is very high, the value of z_1 is close to 1, and the protein is expected to be completely complexed with the analyte. In this case, one expects $[P]_{total} = [P]_b + [A]_0/2$ as predicted by eq 16 when $z_1 = 1$.

Although eq 13 is quite general, the discussions above suggest that the experimental conditions should be chosen to keep the value of $[A]_0[P]_b$ constant throughout a set of trials. In this way, because z_1 depends only on δ, each point on the binding curve will have the same limiting value, z_1, once the steady state is reached.

The overall time scale of the experiments is determined by ω, in eq 13, which has units of reciprocal seconds. If ω has a large value, for example, when the mobilities of the interacting species are very different, the applied voltage is high, or the width of the analyte plug is very small, the limiting value of $z = z_1$ is reached sooner. Since ω is a multiplicative parameter that appears only on the right side of eq 13, ωt is a natural reduced time parameter for the system.

For a given physical system, β is a constant. In addition, if δ is kept constant, then eq 13 shows that the mobility depends only on the parameter γ, since ω is simply a factor that scales the time.

Using the method of partial fractions, eq 13 can be integrated analytically (detailed derivation is given in Appendix 5), assuming $z(t=0) = 0$ to give

$$e^{\delta\omega t} = \left(1 - \frac{z}{z_1}\right)^c\left(1 - \frac{z}{z_2}\right)^d(1 + \beta z)^{b/\beta}(1 - z)^{-a} \qquad (17)$$

in which the coefficients a, b, c, and d satisfy the following set of equations:

$$\beta a - b - \beta c - \beta d = -\gamma \qquad (18a)$$

$$a + b - z_2 c - z_1 d = 1 + \gamma \qquad (18b)$$

$$[1 - \beta(z_1 + z_2)]a + [1 + z_1 + z_2]b + [\beta z_2 + \beta - 1]c + [\beta z_1 + \beta - 1]d = 3\gamma \qquad (18c)$$

$$[1 - \beta(z_1 + z_2)]a - [1 + z_1 + z_2]b + [1 + z_2 - \beta z_2]c + [1 + z_1 - \beta z_1]d = 1 - 3\gamma \qquad (18d)$$

Solving eq 18 for the coefficients, and simplifying the expressions where possible, then gives

$$a = -\frac{2\delta}{\beta + 1},$$

$$b = \gamma + \frac{\beta}{(\beta + 1)(\beta + z_1)(\beta + z_2)}\left(\beta(\beta - 1) - \frac{\gamma}{\delta}(\beta + 1)\right),$$

$$c = -\frac{\gamma + \delta^2(z_2^{\,2} - 1)}{(\beta + z_2)\sqrt{1 + 4\delta}},$$

$$d = \frac{\gamma + \delta^2(z_1^{\,2} - 1)}{(\beta + z_1)\sqrt{1 + 4\delta}}$$

Note that c is always less than zero.

While eq 17 cannot be formally inverted to give a simple function of $z(t)$, however, it does define $z(t)$ implicitly. At each time, a value of z can be found numerically with a root-finding algorithm applied to eq 17.

While eq 17 provides the instantaneous fraction of the complex, $z(t)$, and hence the instantaneous mobility of the analyte plug at time t, the experiment measures only the time, T, it takes the analyte plug to move a fixed distance, l, along the capillary (i.e., detector length). This distance, including the effect of the electroosmotic flow, is calculated according to

$$\int_0^T (\mu_{\mathrm{ep}}^{\mathrm{A}}(t) + \mu_{\mathrm{eof}})E \, \mathrm{d}t = \int_0^{\mathrm{l}} \mathrm{d}x \tag{19}$$

If $\mu_{\mathrm{eof}}ET_{\mathrm{eof}} = \mathrm{l}$ and $\bar{\mu} = 1/T\int_0^T \mu_{\mathrm{ep}}^{\mathrm{A}}(t)\,\mathrm{d}t$, then eq 19 can be rewritten as

$$\bar{\mu} = \frac{l}{E}\left(\frac{1}{T} - \frac{1}{T_{\mathrm{eof}}}\right) \tag{20}$$

In terms of z, we have $\bar{\mu} = \mu_{\mathrm{ep,A}} + (\mu_{\mathrm{ep,A_2P}} - \mu_{\mathrm{ep,A}})\,\bar{z}$ where

$$\bar{z} = \frac{1}{T}\int_0^T z(t)\,\mathrm{d}t = \frac{1}{\omega T}\int_0^{\omega T} z(\omega t)\,\mathrm{d}(\omega t) \tag{21}$$

Numerically, the integration in eq 21 can be performed by taking small values of ωt and inverting eq 17 at each time slice to obtain the values of z (ωt). Thus, given measured values of T, one approach for comparison with the experiment is to use eq 21 to predict values of the average mobility, which can be compared to those determined from eq 20 using measured values of T and T_{eof}.

Alternatively, eq 19 can be expressed in terms of reduced quantities as

$$\frac{\mu_{\mathrm{epA}} + \mu_{\mathrm{eof}}}{\mu_{\mathrm{epA}} - \mu_{\mathrm{epA_2P}}} - \bar{z} = \frac{l}{\beta a \omega T} \tag{22}$$

Thus, given a length l, eq 22 can be used to predict the time T needed for the plug to travel this distance, since there is only one value of T for which the left- and right-hand sides of eq 22 are equal. This predicted time T then fixes the values of $\bar{z}$ and $\bar{\mu}$. In this case, the predicted values of T, $\bar{\mu}$, or both can be compared with the experimentally measured ones. Note that the values of $\bar{\mu}$ predicted from eq 22 will not be necessarily the same as those predicted by eq 21 using measured values of T (although in practice the predicted mobilities are quite close).

EXPERIMENTAL SECTION

Materials. Transthyretin was purified from human plasma as described by Malpeli et al.[27] The homogeneity of the protein preparation was confirmed by SDS PAGE, N-terminal amino acid sequence, and mass spectrometry, and the final product was lyophilized.

A stock solution (7.2 μM) was prepared by dissolving known amounts of TTR in 50 mM sodium phosphate buffer (pH 7.4), filtering the resultant mixture through a 0.45-μm Millipore membrane and diluting appropriately with the same buffer.

Flurbiprofen was purchased from Sigma (St. Louis, MO). Sample preparation was carried out by dissolving known amounts of flurbiprofen in a suitable volume of 10 mM sodium phosphate buffer to reach the desired concentration. All buffer solutions were prepared fresh daily using doubly distilled water. Phosphate buffer solutions were prepared by mixing analytical grade dibasic sodium hydrogen phosphate and sodium dihydrogen phosphate (Sigma) solutions to give a pH of 7.4. All solutions were filtered through a 0.2-μm Millipore (Bedford, MA) membrane filter and degassed by sonication prior to use.

Equipment. CE experiments were performed using a Beckman P/ACE System 5000 (Beckman Instruments Inc., Mississauga, ON, Canada) with a built-in UV detector. The capillary tubing (Polymicro Technologies, Phonix, AZ) was of uncoated fused silica with an internal diameter of 50 μm, a total length of 47 cm, and a length from inlet to detector of 40 cm.

Procedure for CE. The capillary was rinsed before each run with NaOH (SDS for the analyses with protein additive[28]) and H_2O (1000 mbar, 2 min each) and conditioned with the BGE (1000 mbar, 3 min).

The dynamic complexation CE experiments were carried out following two different procedures:

(1) The mobility of the analyte (ligand) at various concentrations (100, 50, or 25 μM) in buffer was evaluated at different concentrations of additive (TTR) (0.00, 0.36, 0.72, 1.08, 1.44, 1.80, 2.52, 2.88, 3.24, 3.6 μM). The electropherograms obtained are shown in Figure 2 (note that the horizontal axis is a mobility scale as opposed to the standard time scale[29]).

(2) The mobility of the analyte was evaluated by holding the product of the concentrations of analyte and additive, $[A]_0[P]_b$, fixed at one of the three values 3.6×10^{-11}, 1.08×10^{-10}, and 1.8×10^{-10}. To obtain these values, the concentration of the analyte was adjusted to give the appropriate product with the concentration of TTR (0.36, 0.72, 1.08, 1.44, 1.80, 2.52, 2.88, 3.24, 3.6 μM).

Electrophoresis was carried out at 20 kV and 25 °C; UV signal channels were set at 254 for flurbiprofen. The measurements of the ligand mobility in neat buffer ($\mu_{\mathrm{ep,A}}$) were repeated before carrying out a new protein concentration level in order to test the actual conditions of the capillary wall within a working day and provide a suitably adjusted $\mu_{\mathrm{ep,A}}$ parameter.

The concentrations of TTR solutions used as additives in the BGE were estimated by using a calibration curve and measuring

(27) Malpeli, G.; Folli, C.; Berni, R. *Biochim. Biophys. Acta* **1996**, *1294*, 48−54.
(28) Lloyd, D. K.; Watzig, H. *J. Chromatogr., B* **1995**, *663*, 400−405.
(29) Schmitt-Kopplin, P.; Garmash, A. V.; Kudryavtsev, A. V.; Menzinger, F.; Perminova, I. V.; Hertkorn, N.; Freitag, D.; Petrosyan, V. S.; Kettrup, A. *Electrophoresis* **2001**, *22*, 77−87.

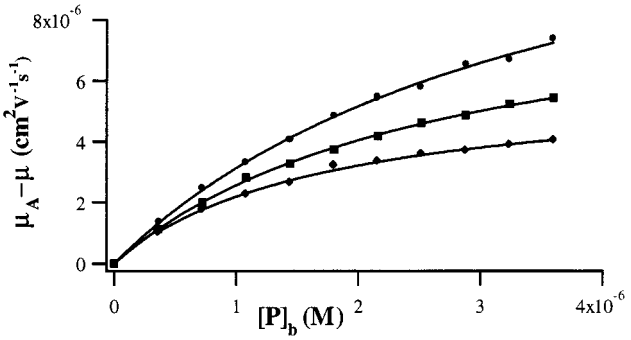

Figure 3. Binding isotherm for flurbiprofen at different concentrations: (◆) 25, (■) 50, (●) and 100 μM).

the absorbance at 280 nm. The change in buffer viscosity upon addition of TTR was measured by filling the capillary with solutions containing different concentrations of TTR and measuring the time necessary for a water marker to be pushed through the capillary past the detector when a negative pressure was applied to the outlet end.[30] The sample volume introduced into the capillary by applying a pressure of 0.5 psi for 3 s was calculated to be 3.6 nL.

Data were collected and processed using System Gold software (Beckman) and a Pentium II PC computer. All regressions were performed using a form of nonlinear least-squares fitting (Levemberg−Marquardt algorithm) with Igor Professional 3.1 software.

RESULTS AND DISCUSSION

Figure 3 plots the values of ($\mu_{ep,A} - \mu_{ep}^{A}$) versus $[P]_b$ obtained from the experiments carried out under the first set of conditions (see the Procedure for CE section) for three different concentrations of analyte.

The initial points of the binding isotherm when very low concentrations of the protein are used represent a non-steady-state system; the protein enters into the analyte plug from behind and binds the analyte following the equilibrium, and the free protein that is left exits the plug. The steady state is reached only when the amount of protein that enters into the analyte plug is equal to the amount exiting from this plug, i.e., only when the concentration of the free protein is equal to the protein added to the buffer. Starting with a very low concentration of protein added to the running buffer, the equilibrium is either reached slowly or not at all when the concentration of the analyte is high. At the beginning (first points when the protein concentration is 0.36, 0.72, and 1.08 μM in Figure 3), the binding isotherms obtained for the three concentrations of flurbiprofen are similar, indicating that in the three cases a non-steady-state condition is present in the analyte plug. When the concentration of the protein is increased, the binding isotherms start to diverge, as shown most clearly in the last points (2.88, 3.24, and 3.6 μM in Figure 3). The steady state in the analyte plug is reached at different times for each of the three starting concentrations of additive because the amount of analyte bound by the protein is different. If the same concentration of protein is used, the steady state is reached more slowly for a higher concentration of injected analyte than it would be if a lower concentration of injected analyte is used.

These experiments are performed by keeping the drift distance from the injection end to the detection window) constant and measuring only one time for a set of parameters (i.e., the concentration of protein in solution and the starting concentration of analyte).

To use the new theory to calculate the binding parameters (binding constant and mobility of the complex), for the reasons explained in the Theory section, the product $[A]_0[P]_b$ ($= \epsilon$) was fixed by using the second set of conditions (see the Procedure for CE section) to carry out the experiments, with three different values of ϵ selected.

Since the limiting value of the fraction of the complex (z) at the steady state, z_1, is dependent on δ, when $[A]_0[P]_b = 1/K$, z_1 is equal to 0.5 and hence the fraction of the analyte bound would be 25%. When $[A]_0[P]_b > 1/K$, $z_1 > 0.5$ so that the fraction of the analyte bound would be more than 25%. Based on a previous study,[26] the value of the binding constant is known to be within 1 order of magnitude of 1×10^{10}. Assuming a value of this order for the binding constant and choosing a value of ϵ higher than 3.6×10^{-11}, the fraction of the analyte bound would be higher than 25%, and for a value of ϵ equal to 1.8×10^{-10}, the fraction of the analyte bound would be approximately 60%. These values of ϵ were chosen in order to collect the data over a large portion of the binding isotherm.

The data obtained for a value of $\epsilon_1 = [P]_b[A]_0 = 3.6 \times 10^{-11}$ are reported in Figure 4a. With the decrease in the analyte concentration and increase in the protein concentration, the steady state is reached and the fraction of the complex no longer changes. At this point, a value for the difference between the free analyte mobility and the steady-state analyte mobility ($\mu_1 = (\mu_{ep,A} - \mu_{ep}^{A})_1$) of the analyte can be extrapolated. Since eq 13 contains two unknown parameters, two sets of experiments are needed. The same type of experiment was carried out for $\epsilon_2 = [P]_b[A]_0 = 1.8 \times 10^{-10}$, and the value of the migration behavior ($\mu_2 = (\mu_{ep,A} - \mu_{ep}^{A})_2$) was extrapolated at the steady state (as shown in Figure 4b). At the steady state $z = z_1$, so it follows using the definition for z_1 and δ_1 in eq 14, that

$$z_{1,1} = \frac{(\mu_{ep,A} - \mu_{ep}^{A})_1}{(\mu_{ep,A} - \mu_{ep,A_2P})} = 1 + \frac{1 - \sqrt{1 + 8K\epsilon_1}}{4K\epsilon_1} \quad (23a)$$

and

$$z_{1,2} = \frac{(\mu_{ep,A} - \mu_{ep}^{A})_2}{(\mu_{ep,A} - \mu_{ep,A_2P})} = 1 + \frac{1 - \sqrt{1 + 8K\epsilon_2}}{4K\epsilon_2} \quad (23b)$$

The ratio $z_{1,1}/z_{1,2}$ gives an equation that can be solved for the value of K. Substituting this value of K into the expression for $z_{1,1}$ or $z_{1,2}$ then allows $\mu_{ep,A} - \mu_{ep,A_2P}$ and hence μ_{epA_2P} to be determined.

The value obtained for the binding constant ($K = K_1K_2$) in our system was $(2.0 \pm 0.08) \times 10^{10}$ M^{-1}, and the value for the complex mobility was $(-2.19 \pm 0.03) \times 10^{-4}$ cm^2 V^{-1} s^{-1}. Substituting these values into the definition of z_1 the value of $\mu_{ep,A} - \mu_{ep}^{A}$ at the steady state for $\epsilon_3 = [P]_b[A]_0 = 1.08 \times 10^{-10}$ was predicted to be 1.02×10^{-5}. A set of experiments was carried out using this value of ϵ_3, and the migration times were measured. The measured mobility difference at steady state was 9.91×10^{-6}, giving a difference of 2.8% when compared to the predicted value, which

(30) Shibukawa, A.; Lloyd, D. K.; Wainer, I. W. *Chromatographia* **1993**, *35*, 419−429.

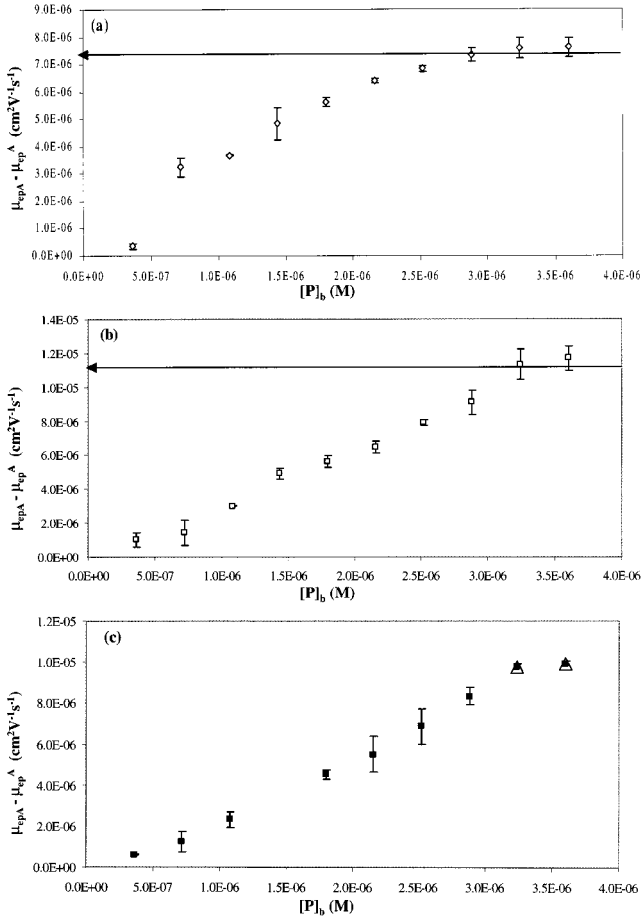

Figure 4. Binding of flurbiprofen to TTR using $\epsilon = 3.6 \times 10^{-11}$ (a), 1.8×10^{-10} (b), and 1.08×10^{-10} (c). Plots a and b yield the individuation of the values of μ_1 and μ_2 (see text) at the steady state, respectively. Plot c shows the difference between the predicted (△) and measured (■) value of μ_3 at the steady state.

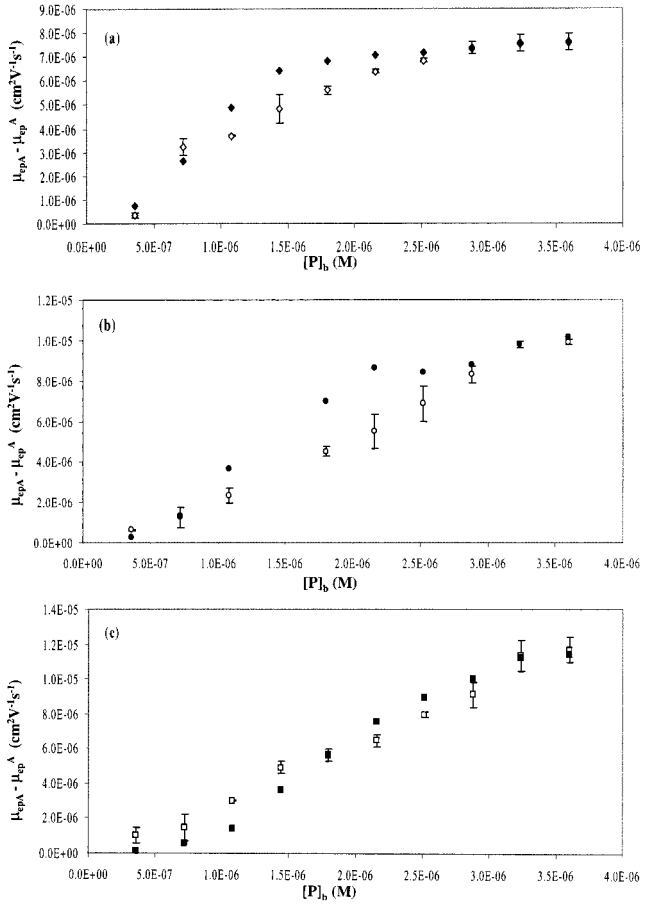

Figure 5. Binding of flurbiprofen to TTR using $\epsilon = 3.6 \times 10^{-11}$ (a), 1.08×10^{-10} (b), and 1.8×10^{-10} (c). The plots report the predicted and the measured values using solid and open points, respectively.

is within the standard deviation of the three measurements made for the single point.

Using these values of μ_{ep,A_2P} and K, as well as the other experimental parameters, eq 22 was used to predict values of $\bar{z}$ and, hence, $\mu_{ep,A} - \mu_{ep}^{A}$ as a function of $[P]_b$ for the three data sets reported in Figure 5. Figure 5 shows the comparison between the values predicted using eq 22 plotted as solid points, compared with the experimental data, shown as empty points. The predicted values agree very well with the general trend of experimental results, proving that the solution proposed to solve the differential equation accurately describes the migration behavior of the analyte.

Steady-State Equations. When the steady state is reached, so that the total amount of the protein in the analyte plug is no longer changing, there are three different scenarios to consider.

If the concentrations of the interacting species in the analyte plug at the equilibrium are equal to the starting concentrations, the migration behavior can be described by the following equation.[30]

$$\mu_{ep,A} - \mu_{ep}^{A} = \frac{2K[P]_b[A]_0}{1 + 2K[P]_b[A]_0}(\mu_{ep,A} - \mu_{ep,A_2P}) \quad (23)$$

If the concentration of the additive (protein) is not sufficiently high and when the binding constant has a high value, the additive concentration ([P]) present in the analyte plug at equilibrium cannot simply be approximated as the added protein concentration ($[P]_b$), but rather a depletion of the protein during the equilibrium must be considered in the expression of the binding constant:

$$K = \frac{[A_2P]}{[A]_0^2([P]_b - [A_2P])} \quad (24)$$

The mobility of the analyte can then be expressed as

$$\mu_{ep,A} - \mu_{ep}^{A} = \frac{2K[P]_b[A]_0}{1 + K[A]_0^2 + 2K[P]_b[A]_0}(\mu_{ep,A} - \mu_{ep,A_2P}) \quad (25)$$

A similar case can also be considered where the injected analyte is depleted by its interaction with the additive. Again, the concentration at equilibrium (this time of the analyte rather than the additive) cannot be considered equal to that of the injection. The binding constant can be expressed as

$$K = \frac{[A_2P]}{([A]_0 - [A_2P])^2[P]_b} \quad (26)$$

Table 2. Binding Constant, Mobility of the Complex, and Concentration of the Complex

flurbiprofen, μM	$K_1 = K_2$ (10^5) M^{-1}	μ_{A_2P}, (10^{-4}) $cm^2/V\cdot s$	$[A_2P]$ at $[P]_b = 3.6 \times 10^{-6}$, ($10^{-5}$) M
	(a) According to Eq 23		
25	1.068 ± 0.036	−1.940 ± 0.002	2.565
50	0.607 ± 0.013	−1.970 ± 0.002	0.830
100	0.363 ± 0.014	−2.030 ± 0.006	0.296
	(b) According to Eq 25		
25	2.013 ± 0.039	−1.940 ± 0.002	0.542
50	1.172 ± 0.046	−2.067 ± 0.002	0.613
100	0.909 ± 0.051	−2.125 ± 0.006	0.641
	(c) According to Eq 27		
25	0.925 ± 0.044	−1.970 ± 0.004	1.43
50	0.501 ± 0.011	−2.029 ± 0.004	0.92
100	0.295 ± 0.012	−2.118 ± 0.011	0.50

allowing the mobility of the analyte to be rewritten as

$$\mu_{ep,A} - \mu_{ep}^{A} = \left(1 + \frac{1 - \sqrt{1 + 8K[P]_b[A]_0}}{4K[P]_b[A]_0}\right)(\mu_{ep,A} - \mu_{ep,A_2P}) \quad (27)$$

This equation can be obtained from the general equation presented in the Theory section when the steady state is reached and $z = z_1$.

The measured mobility of the free analyte ($\mu_{ep,A}$) allows the binding constant and the mobility of the complex in eq 23 (2:1 binding stoichiometry) to be calculated using a nonlinear regression of ($\mu_{ep,A} - \mu_{ep}^{A}$) versus [P]. Table 2a lists the equilibrium constant, the mobilities of the complex obtained from the curve fitting, and the concentration of the complex calculated from eq 23. The equation seems to describe the experimental data very well, illustrated by the very low errors obtained from regression.

It is interesting to note that different values of the binding constants are obtained when different concentrations of flurbiprofen are used. Furthermore, for all the concentrations of flurbiprofen tested in these experiments, the concentrations of the complex calculated are higher or similar to the concentration of the protein added to the running buffer. When a concentration of flurbiprofen of 25 μM is used, the concentration of the complex is greater than the starting concentration of the analyte. The same results are obtained when eqs 25 and 27 (2:1 stoichiometry considering the depletion of the additive and the analyte, respectively) are used to fit the experimental data. The value of the binding constants are different when three different concentrations of Flurbiprofen are used, and the complex concentration is still found to be higher than the initial protein concentration (Table 2b and c).

This proves that all these equations, even the one that considers a depletion of one of the interacting species, cannot be used to fit the experimental data obtained when the steady state has not yet been reached. In this case, the mobilities of the interacting species are the important parameter to consider.

The results obtained by applying eqs 23, 25, and 27 suggest that the protein concentration available for binding in the analyte plug is much higher than the concentration of the protein in the rest of the capillary. There is a higher amount of protein in the analyte plug at the end of the run than is initially present at the beginning, allowing for the formation of a higher concentration of complex. As a result, the concentration of the complex is limited by the concentration of analyte.

CONCLUSIONS

The theory presented in this paper accurately describes the migration behavior of a ligand in a CE system when a very low concentration of protein is used as an additive and when a 1:2 stoichiometry is present. The method presented has the advantage of using a very small amount of protein as an additive in the background electrolyte, which often cannot be achieved in other methods.

Finally, the theory presented here underlines the role of the relative mobilities of the interacting species and the role of the analyte concentration in the equilibrium. When the steady state is reached, so that the additive entering the analyte plug is equal to the additive exiting, the mobility of the analyte plug is unaffected by the mobility of the additive. However, when the steady state is not reached, the mobility of the analyte plug may be affected by the additive mobility through the amount of additive entering the plug and the time required to reach the steady state. An accurate value for the binding constant can be obtained only when the analyte migration behavior is properly understood.

The experiment performed with constant values at $[A]_0[P]_b$ allows for the single extraction of K and the mobility of the complex from the data, since the theory predicts that in the saturation limit $z = z_1$. However, the theory should also describe the data taken with constant $[A]_0$ values, shown in Figure 3. In this case, the limit of $z = z_1$ is not necessarily attained, so that the extraction of K and $\mu_{ep,\ A_2P}$ from the data is more complex. A nonlinear least-squares fit is required which uses predictions from eq 22 for differing values of K and μ_{A_2P} until values are found that lie most closely to the experimental data.

ACKNOWLEDGMENT

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APPENDIX 1. DERIVATION OF EQ 8

Substituting eqs 3 and 4 into eq 2

$$\mu_{ep}^{A} = f_A\mu_{ep,A} + f_{A_2P}\,\mu_{ep,A_2P}$$

we obtain the following equation,

$$\mu_{ep}^{A} = \left(1 - \frac{2[A_2P]}{[A]_0}\right)\mu_{ep,A} + \frac{2[A_2P]}{[A]_0}\mu_{ep,A_2P} \quad (2a)$$

Rearranging eq 2a we obtain

$$\mu_{ep}^{A} = \mu_{ep,A} + \frac{2[A_2P]}{[A]_0}(\mu_{ep,A_2P} - \mu_{ep,A}) \quad (2b)$$

Differentiating μ_{ep}^{A} with respect to $[P]_{total}$ we obtain eq 8.

$$\frac{d\mu_{ep}^{A}}{d[P]_{total}} = \frac{2(\mu_{ep,A_2P} - \mu_{ep,A})}{[A]_0}\frac{d[A_2P]}{d[P]_{total}} \quad (8)$$

APPENDIX 2. DERIVATION OF EQ 12

Rearranging eq 10 we can obtain

$$[A_2P] = [P]_{total} - [P] \tag{10a}$$

Substituting this expression into eq 8 gives

$$\mu_{ep}^{A} = \mu_{ep,A} + \frac{2([P]_{total} - [P])}{[A]_0}(\mu_{ep,A_2P} - \mu_{ep,A}) \tag{8a}$$

Differentiating μ_{ep}^{A} with respect to $[P]_{total}$

$$\frac{d\mu_{ep}^{A}}{d[P]_{total}} = \frac{2(\mu_{ep,A_2P} - \mu_{ep,A})}{[A]_0}\frac{d([P]_{total} - [P])}{d[P]_{total}} \tag{8b}$$

By simplifying eq 8b, we obtain

$$\frac{d\mu_{ep}^{A}}{d[P]_{total}} = \frac{2(\mu_{ep,A_2P} - \mu_{ep,A})}{[A]_0}\left(1 - \frac{d[P]}{d[P]_{total}}\right) \tag{8c}$$

Comparing eq 8c with eq 8 we obtain the identity

$$\frac{d[A_2P]}{d[P]_{total}} = \left(1 - \frac{d[P]}{d[P]_{total}}\right)$$

Substituting eq 10a into eq 11 gives

$$[A] = [A]_0 - 2([P]_{total} - [P]) \tag{11a}$$

Substituting eq 10a and 11a into the equilibrium expression (eq 9) gives

$$K = \frac{([P]_{total} - [P])}{([A]_0 - 2([P]_{total} - [P]))^2[P]} \tag{9a}$$

Rearranging and differentiating [P] with respect to $[P]_{total}$ we ultimately obtain eq 9b

$$K[P]([A]_0 - 2[P]_{total} + 2[P])^2 = [P]_{total} - [P],$$

$$K\frac{d[P]}{d[P]_{total}}([A]_0 - 2[P]_{total} + 2[P])^2 + 2[P]K([A]_0 - 2[P]_{total} + 2[P])\left(-2 + 2\frac{d[P]}{dP}\right) = 1 - \frac{d[P]}{dP},$$

$$\frac{d[P]}{d[P]_{total}}\{1 + K([A]_0 - 2[P]_{total} + 2[P])^2 + 4[P]K([A]_0 - 2[P]_{total} + 2[P])\} = 1 + 4[P]K([A]_0 - 2[P]_{total} + 2[P]),$$

$$\frac{d[P]}{d[P]_{total}} = \{1 + 4[P]K([A]_0 - 2[P]_{total} + 2[P])\}/\{1 + K \times ([A]_0 - 2[P]_{total} + 2[P])^2 + 4[P]K([A]_0 - 2[P]_{total} + 2[P])\} \tag{9b}$$

Subtracting both sides of eq 9b from 1, the following is obtained

$$1 - \frac{d[P]}{d[P]_{total}} = 1 - \{[1 + 4[P]K([A]_0 - 2[P]_{total} + 2[P])]/[1 + K([A]_0 - 2[P]_{total} + 2[P])^2 + 4[P]K([A]_0 - 2[P]_{total} + 2[P])]\} \tag{9c}$$

Rearranging eq 8a gives

$$2[P]_{total} - 2[P] = \frac{\mu_{ep}^{A} - \mu_{ep,A}}{\mu_{ep,A_2P} - \mu_{ep,A}}[A]_0 \tag{8d}$$

Substituting eq 11a into eq 8d gives

$$[A] = [A]_0 - 2[P]_{total} + 2[P] = [A]_0 - \frac{\mu_{ep}^{A} - \mu_{ep,A}}{\mu_{ep,A_2P} - \mu_{ep,A}}[A]_0 \tag{8e}$$

Rearranging eq 9a gives

$$K[P]([A]_0 - 2[P]_{total} + 2[P]) = \frac{[P]_{total} - [P]}{([A]_0 - 2[P]_{total} + 2[P])} \tag{9d}$$

Substituting eq 8d and eq 8e into eq 9d ultimately gives eq 9e

$$K[P]([A]_0 - 2[P]_{total} + 2[P]) = \frac{[A]_0}{2}\frac{(\mu_{ep}^{A} - \mu_{ep,A})}{-(\mu_{ep}^{A} - \mu_{ep,A_2P})}\frac{(\mu_{ep}^{A} - \mu_{ep,A_2P})}{[A]_0(\mu_{ep}^{A} - \mu_{ep,A_2P})} = -\frac{1}{2}\frac{(\mu_{ep}^{A} - \mu_{ep,A})}{(\mu_{ep}^{A} - \mu_{ep,A_2P})},$$

$$K[P][A]_0\frac{\mu_{ep}^{A} - \mu_{ep,A_2P}}{\mu_{ep,A} - \mu_{ep,A_2P}} = \frac{[A]_0}{2}\frac{(\mu_{ep}^{A} - \mu_{ep,A})}{-(\mu_{ep}^{A} - \mu_{ep,A_2P})} \times \frac{(\mu_{ep}^{A} - \mu_{ep,A_2P})}{[A]_0(\mu_{ep}^{A} - \mu_{ep,A_2P})} = -\frac{1}{2}\frac{(\mu_{ep}^{A} - \mu_{ep,A})}{(\mu_{ep}^{A} - \mu_{ep,A_2P})},$$

$$[P] = -\frac{1}{2}\frac{(\mu_{ep}^{A} - \mu_{ep,A})}{(\mu_{ep}^{A} - \mu_{ep,A_2P})}\frac{(\mu_{ep,A} - \mu_{ep,A_2P})}{(\mu_{ep}^{A} - \mu_{ep,A_2P})}\frac{1}{K[A]_0} \tag{9e}$$

Substituting eq 8c and eq 9e into eq 9c gives eq 12

$$\frac{d[A_2P]}{d[P]_{total}} = \frac{K[A]_0^2\left(\frac{(\mu_{ep}^{A} - \mu_{ep,A_2P})}{(\mu_{ep,A} - \mu_{ep,A_2P})}\right)^2}{1 + K[A]_0^2\left(\frac{(\mu_{ep}^{A} - \mu_{ep,A_2P})}{(\mu_{ep,A} - \mu_{ep,A_2P})}\right)^2 - 2\left(\frac{(\mu_{ep}^{A} - \mu_{ep,A})}{(\mu_{ep}^{A} - \mu_{ep,A_2P})}\right)} \tag{12}$$

APPENDIX 3. DERIVATION OF EQ 13

Substituting eq 12 into eq 8 gives

$$\frac{d\mu_{ep}^{A}}{d[P]_{total}} = \frac{2(\mu_{ep,A_2P} - \mu_{ep,A})}{[A]_0} \times \frac{K[A]_0^2\left(\frac{(\mu_{ep}^{A} - \mu_{ep,A_2P})}{(\mu_{ep,A} - \mu_{ep,A_2P})}\right)^2}{1 + K[A]_0^2\left(\frac{(\mu_{ep}^{A} - \mu_{ep,A_2P})}{(\mu_{ep,A} - \mu_{ep,A_2P})}\right)^2 - 2\left(\frac{(\mu_{ep}^{A} - \mu_{ep,A})}{(\mu_{ep}^{A} - \mu_{ep,A_2P})}\right)} \quad (8f)$$

Substituting eq 7 and eq 8f into eq 5 gives

$$\frac{d\mu_{ep}^{A}}{dt} = \{[-2K[A]_0(\mu_{ep}^{A} - \mu_{ep,A_2P})^2(\mu_{ep,P} - \mu_{ep}^{A})E]([P]_b - [P])\}/a(\mu_{ep,A} - \mu_{ep,A_2P})\left[1 + K[A]_0^2\left(\frac{\mu_{ep}^{A} - \mu_{ep,A_2P}}{\mu_{ep,A} - \mu_{ep,A_2P}}\right)^2 + 2\left(\frac{\mu_{ep,A} - \mu_{ep}^{A}}{\mu_{ep}^{A} - \mu_{ep,A_2P}}\right)\right] \quad (5a)$$

Replacing the appropriate quantity in eq 2b with the identity z as given in Table 1 yields

$$\mu_{ep}^{A} = \mu_{ep,A} + z(\mu_{ep,A_2P} - \mu_{ep,A}) \quad (2c)$$

Differentiating with respect to z gives

$$d\mu_{ep}^{A} = -(\mu_{ep,A} - \mu_{ep,A_2P})\, dz \quad (2d)$$

Substituting z into eq 9e and knowing that

$$\gamma = K[A]_0^2$$

as given in Table 1, we obtain

$$[P] = z[A]_0/2\gamma(1 - z) \quad (9f)$$

Substituting eq 2d into eq 5a and replacing the appropriate quantities with the identity γ given in Table 1 yields eq 5b

$$-(\mu_{ep,A} - \mu_{ep,A_2P})\frac{dz}{dt} = -\frac{2\gamma}{[A]_0}\{[(1 - z)^2(\mu_{ep,A} - \mu_{ep,A_2P})^2 \times (\mu_{ep,P} - \mu_{ep,A} + z(\mu_{ep,A} - \mu_{ep,A_2P}))E]([P]_b - [P])\}/\left\{a(\mu_{ep,A} - \mu_{ep,A_2P})\left[1 + \gamma(1 - z)^2 + 2\left(\frac{z(\mu_{ep,A} - \mu_{ep,A_2P})}{(\mu_{ep,A} - \mu_{ep,A_2P})(1 - z)}\right)\right]\right\} \quad (5b)$$

Substituting eq 9f into eq 5b, we obtain

$$\frac{dz}{dt} = -\frac{2\gamma}{[A]_0}\left([P]_b - \frac{z[A]_0}{2\gamma(1 - z)^2}\right)\left\{[(1 - z)^2(\mu_{ep,P} - \mu_{ep,A} + z(\mu_{ep,A} - \mu_{ep,A_2P}))E]/a\left[1 + \gamma(1 - z)^2 + \frac{2z}{(1 - z)}\right]\right\} \quad (5c)$$

Replacing the appropriate quantities in eq 5c with the identities ω and β given in Table 1 yields

$$\frac{dz}{dt} = \frac{(1 - z)^2(1 + \beta z)\omega}{\left[1 + \gamma(1 - z)^2 + \frac{2z}{(1 - z)}\right]}\left(\frac{2\gamma[P]_b}{[A]_0} - \frac{z}{(1 - z)^2}\right) \quad (5d)$$

Replacing the appropriate quantities in eq 5d with the identity δ given in Table 1 rearranged as follows

$$\delta = 2\gamma[P]_b/[A]_0 = 2K[A]_0[P]_b$$

yields

$$\frac{dz}{dt} = \omega\frac{(1 - z)(1 + \beta z)[\delta - (1 + 2\delta)z + \delta z^2]}{[1 + z + \gamma(1 - z)^3]} \quad (5e)$$

At the steady state, when z is no longer changing, we have

$$dz/dt = 0$$

thus, from eq 5e we obtain

$$[\delta - (1 + 2\delta)z + \delta z^2] = 0 \quad (5f)$$

solving eq 5f for z, denoted z^* to indicate the steady-state condition,

$$z^* = \frac{(1 + 2\delta) \pm \sqrt{(1 + 2\delta)^2 - 4\delta^2}}{2\delta}$$

which gives the solutions

$$z_1 = 1 + \frac{1 - \sqrt{1 + 4\delta}}{2\delta} \qquad z_2 = 1 + \frac{1 + \sqrt{1 + 4\delta}}{2\delta}$$

Rearranging eq 5e then gives eq 13

$$\frac{dz}{dt} = \omega\delta\frac{(1 - z)(1 + \beta z)(z - z_1)(z - z_2)}{[1 + z + \gamma(1 - z)^3]} \quad (13)$$

APPENDIX 4. DERIVATION OF EQ 16

Rearranging eq 9a gives

$$K[P]([A]_0 - 2[P]_{total} + 2[P])^2 = [P]_{total} - [P] \quad (9g)$$

if $[P] = [P]_b$, then

$$K[P]_b([A]_0 - 2[P]^*_{total} + 2[P]_b)^2 = [P]^*_{total} - [P]_b \quad (9h)$$

where $[P]^*_{total}$ is $[P]_{total}$ when the equality $[P] = [P]_b$ is true. A quantity y is defined as

$$y = [P]^*_{total} - [P]_b$$

Substituting y into eq 9h and rearranging the equation gives

$$4y^2 - \left(4[A]_0 + \frac{1}{K[P]_b}\right)y + [A]_0^{\,2} = 0 \qquad (9i)$$

Solving eq 9i for y, we obtain

$$y = \frac{(4K[A]_0[P]_b + 1) \pm \sqrt{8K[A]_0[P]_b + 1}}{8K[P]_b} = 0 \qquad (9j)$$

Replacing the appropriate quantities in eq 9j with the identity δ given in Table 1, and replacing y with its defined value, gives

$$[P]^*_{total} = [P]_b + (1/8K[P]_b)(2\delta + 1 \pm \sqrt{4\delta + 1}) \quad (9k)$$

Knowing

$$z^* = 1 + \frac{1 \pm \sqrt{1 + 4\delta}}{2\delta}$$

we can rearrange to find

$$\sqrt{1 + 4\delta} = 1 + (1 - z^*)2\delta$$

and substituting this into eq 9k gives

$$[P]^*_{total} = [P]_b + \frac{1}{8K[P]_b}(2\delta + 1 \pm (1 + 2\delta - 2z^*\delta)) \quad (9l)$$

Choosing the negative sign (limit verified), we obtain

$$[P]^*_{total} = [P]_b + z^*\delta/4K[P]_b \qquad (9m)$$

Replacing the identity δ with its defined quantity gives

$$[P]^*_{total} = [P]_b + [A]_0 z^*/2$$

Which, after verification of the limit as above, becomes eq 16

$$[P]_{total} = [P]_b + [A]_0 z_1/2 \qquad (16)$$

APPENDIX 5. DERIVATION OF EQ 17

Rearranging eq 13 gives

$$\frac{[1 + z + \gamma(1 - z)^3]}{(1 - z)(1 + \beta z)(z - z_1)(z - z_2)}\,dz = \omega\delta\,dt \qquad (13a)$$

The left-hand side of this equation can be transformed as follows

$$\frac{[1 + z + \gamma(1 - z)^3]}{(1 - z)(1 + \beta z)(z - z_1)(z - z_2)} = \frac{a}{1 - z} + \frac{b}{1 - \beta z} + \frac{c}{z - z_1} + \frac{d}{z - z_2}$$

where the coefficients a, b, c, and d are defined in the text (eqs 18a, b, c, and d). Integrating the rearranged eq 13a

$$\int_0^t \omega\delta\,dt = \int_0^z \frac{a}{1 - z} + \int_0^z \frac{b}{1 - \beta z}\,dz + \int_0^z \frac{c}{z - z_1}\,dz + \int_0^z \frac{d}{z - z_2}\,dz,$$

$$\omega\delta t = -\,a\ln(1 - z) + \frac{b}{\beta}\ln(1 + \beta z) + c\ln\left(1 - \frac{z}{z_1}\right) + d\ln\left(1 - \frac{z}{z_2}\right)$$

gives eq 17.

$$e^{\omega\delta t} = -\,(1 - z)^a(1 + \beta z)^{b/\beta}\left(1 - \frac{z}{z_1}\right)^c\left(1 - \frac{z}{z_2}\right)^d \qquad (17)$$

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