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Variable path length and counter-flow continuous variation methods for the study of the formation of high-affinity complexes by absorbance spectroscopy. An application to the studies of substrate binding in cytochrome *P*450

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Abstract

Studies of the equilibrium of protein–ligand interactions and determination of the stoichiometry of protein complexes constitute an important element of routine biochemical practice. In this paper we describe two innovative modifications of Job's method of continuous variation, which allow us to analyze tight interactions and determine stoichiometry in multi-site binding systems, including cases where the absorbance of the ligand overlaps with that of the enzyme–ligand complex. Our results on the interactions of cytochromes *P*450 3A4 and *P*450eryF with substrates illustrate the applicability of these approaches to the studies of substrate binding in enzymes that exhibit homotropic cooperativity.

Keywords: Ligand binding; Stoichiometry; Job's titration; Cooperativity; Cytochrome *P*450 3A4; Cytochrome *P*450eryF

1. Introduction

Studies of the equilibrium of protein–ligand interactions and the determination of the stoichiometry of protein complexes constitute an important element of routine biochemical practice. In many cases the concentration of the complex may be assessed from the changes in the absorbance spectra of the ligand and/or protein so that a series of spectra recorded at a constant protein concentration (E_0) and increasing ligand concentration (L_0) may be employed to judge the mechanism of the interaction and to determine the dissociation constant(s). The applicability of this titration approach in its classical implementation is limited by the fact that the Michaelis-Menten, Hill, or Adair equations, which are routinely used to interpret the binding isotherms, are valid only when $L_0 \gg E_0$. However, for this approach to be reliable the enzyme concentration should be kept far below the value of the dissociation constant (K_D), a condition that is difficult to maintain experimentally if the binding is tight. Although in the case of simple bimolecular interaction the use of a so-called "tight binding equation"[1,2] may suffice, there is no appropriate common approach to the initial exploratory analysis of tight interactions in multi-site binding systems with unknown stoichiometry of complex formation. Another important impediment to studies of ligand binding by absorbance spectroscopy often arises from the overlap of the absorbance band of the ligand with that specific for the complex. In this communication, we describe two innovative modifications of Job's method of continuous variation [3] for determining the stoichiometry of formation of molecular complexes of two compounds. These modifications overcome the above mentioned difficulties and provide a versatile methodological background for studies of the mechanisms of formation of high-affinity complexes of proteins. Job's approach is based on measurement of the concentration of the complex in a series of mixtures composed in such a way that the molar ratio of the interacting compounds varies, while the sum of their concentrations is kept constant. For an enzyme

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or receptor E that binds simultaneously n molecules of a ligand L to form complex X we can write:

$$E + nL \underset{}{\overset{K_D}{\rightleftharpoons}} X \quad (1)$$

Defining the sum of the total concentrations of E and L as $C_0=E_0+L_0$ and the molar fraction of L in the mixture as $F=L_0/C_0$ we obtain the following equation:

$$K_D X = (C_0 F - X)[C_0(1-F) - nX]^n \quad (2)$$

In the case of bimolecular association ($n=1$) the solution of this equation with respect to X is given by the relationship

$$2X = C_0 + K_D - \left\{(C_0 + K_D)^2 - 4C_0^2 F(1-F)\right\}^{1/2} \quad (3)$$

which is analogous to a "tight binding" equation. Although at $n>1$ Eq. (2) may be solved only by numerical means, the plot of X versus F is always represented by a bell-shaped curve with the maximum located at $F=n/(n+1)$ [4]. This method is now widely applied in biochemistry to determine the stoichiometry of protein–ligand complexes [4–6]. It should be noted that the experimental data usually provide a value that is proportional to X, such as absorbance or reaction rate, so that the fitting of the experimental data requires a coefficient of proportionality, which is usually unknown and treated as an additional parameter of the fitting [7]. Although the formalism above is strictly valid only in the case of "all-or-none" binding to n identical binding sites, its applicability may be extended to more complex cases by studying the dependency of the shape of Job's plot on C_0 [4]. In addition to offering a simple way to determine the stoichiometry of complex formation, continuous variation titration provides an excellent experimental strategy for determination of the parameters of tight ligand binding, which is the most appropriate when the values of the dissociation constant(s) are comparable in magnitude with the minimal enzyme concentration required for positive monitoring of the interactions [4,8]. The most common implementation of Job's method in enzymology is based on activity measurements, whereas use of this method with absorbance spectroscopy is quite rare due to decreased sensitivity at low enzyme concentrations. To overcome this limitation we introduce here a variable path length variant of Job's titration, where the light path of the sample increases simultaneously with the dilution, making the amplitude of the spectra virtually invariable. This method provides a simple means to overcome the loss of sensitivity at low enzyme concentration. However, this technique is limited when the absorbance band of the ligand overlaps with that specific for the complex. The traditional approach to overcome this difficulty in double-beam absorbance measurements is based on the addition of the ligand to both the sample and reference cells. However, this simple stratagem is of limited applicability due to inevitable differences in the path lengths of the cells and uncertainties in the volume of the aliquots. In the case of Job's titration these difficulties are of special importance due to the high impact of points obtained at excess substrate. To overcome these difficulties we developed a novel counter-flow titration technique. The idea is to "double up" the Job's titration, thus having the branches corresponding to excess ligand and excess substrate taking place simultaneously in two adjacent compartments of a tandem cell. Applicability of these novel approaches are illustrated by our experimental results on the interactions of cytochrome *P*450 3A4 (CYP3A4) and cytochrome *P*450eryF (*P*450eryF) with their ligands – bromocriptine (BCT) and pyrenemethylamine (PMA).

2. Experimental procedures

2.1. Materials

1-Pyrenemethylamine hydrochloride (PMA) was from Aldrich (Milwaukee, WI), and bromocriptine mesylate was from Sigma Chemicals (St. Louis, MO). All other chemicals used were of the highest grade available from commercial sources and were used without further purification.

2.2. Expression and purification of CYP3A4 and P450eryF

The enzymes were expressed as His-tagged proteins in *Escherichia coli* and purified as described previously [9,10].

2.3. Design of the custom spectrometer setup

The absorbance spectra were recorded with an S2000 CCD rapid scanning spectrometer from Ocean Optics, Inc. (Dunedin, FL). We used an L7893 UV–VIS fiber-optics light source from Hamamatsu Photonics K. K. (Hamamatsu City, Shizuoka, Japan) in our tandem cell experiments and an LS-1102-1 pulsed xenon FlashPack light source from Perkin-Elmer Optoelectronics (Salem, MA) in our variable path length setup. For the variable path length titrations the S2000 spectrometer was equipped with a custom-designed fiber optic adapter for a 10-cm-long cylindrical cell with a total volume of 5.1 mL (Cell Type-521) from NSG Precision Cells (Farmingdale, NY) as shown in Fig. 1. This adapter, which is designed on the basis of a CUV-ALL-UV 4-way cuvette holder from Ocean Optics, Inc., contains four optical windows, allowing two collimated light beams to pass through the cell. The primary measuring beam is directed from the top to the bottom of the cell, and the auxiliary beam is allowed to pass through the normal cross section, 4 mm from the cell bottom. The spectrometer is connected to the cell adapter with a 100-nm core bifurcated optical fiber (Ocean Optics Inc.). To measure both spectra with the same single-channel S2000 spectrometer we used a FOS-2-inline fiber optic switch from Avantes (Eerbeek, The Netherlands), which was employed to alternate the direction of the light between the principal and the auxiliary paths. Two inputs of this device receive light from the lamp via a 600-nm core bifurcated fiber (Ocean Optics Inc.), and its outputs are connected to the cell adapter via 3-mm core liquid light guides from Newport (Stratford, CT). The control line for FOS-2 is provided by a digital output line of NUDAM Nu-6080 Counter module from ADLink Technology (Taipei, Taiwan). The counter of this module was connected to the integration strobe of S2000 and used to ensure synchronization of the beam switching with the

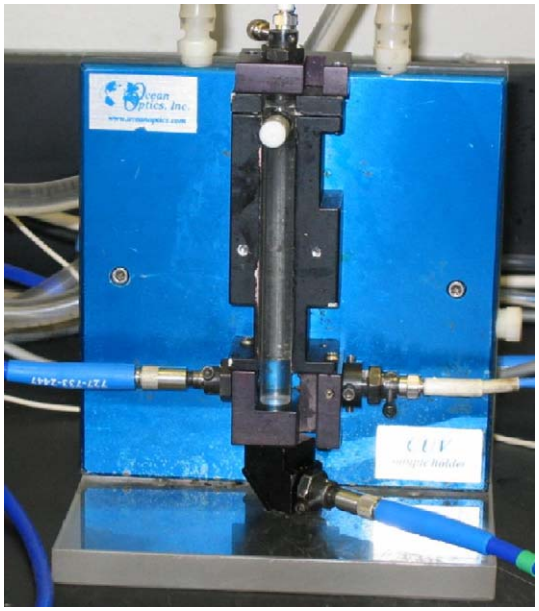

Fig. 1. The vertical cell for variable path length titrations in a custom-designed cell holder with light guides connected.

spectrometer scanning. The data acquisition and the control of both S2000 and FOS-2 were performed with a custom-designed software written in Borland Delphi 7 from Borland Corporation (Scotts Valley, CA) and Win32Forth Public Domain Forth interpreter (www.win32forth.org) using a High-Speed Driver Library (HDL) from Ocean Optics, Inc. for data acquisition with S2000.

2.4. Data processing

All data treatment and curve fitting procedures were performed using our custom designed SPECTRALAB software [11]. Prior to the analysis of the series of the spectra taken in the variable path length setup in the vertical direction (termed as primary spectra) they were corrected for non-linearity of the path length increase and for the distortions of the baseline caused by the displacement of the meniscus. This correction was done using the spectra taken with the horizontal light beam (termed as auxiliary spectra). In the beginning of titration the cell was completely filled with the buffer, and the baseline spectra were recorded for each of two channels. We also measured three reference spectra taken in the vertical direction with the cell filled with 2.5, 1 and 0.5 mL of buffer. These baseline spectra were used for correction of the baseline distortion. For each pair of spectra (primary and auxiliary) we used a linear least square surface fit procedure [12] to fit the primary spectrum with a linear combination of the auxiliary spectrum with three baseline spectra. The combination of the baseline spectra found in this way was subtracted from the primary spectrum to correct it for the baseline distortion. The coefficient of proportionality between the variable path length (primary) and the fixed path length (auxiliary) spectra found in this procedure was used to calculate the actual path length and to correct for the non-linearity of path length increase. The path length correction did not exceed 15% of the amplitude of the spectra, and was more important for the first (small volume) data points and negligible for the measurements with the sample volume > 1 mL. The series of absorbance values obtained in our experiments were interpreted in terms of the changes in the concentrations of the *P*450 high spin, substrate-free ferric low-spin, and Type II-substrate-bound ferric low-spin states using a combination of principal component analysis with the least square surface fit [12] to the set of the appropriate spectral standards, as described earlier [13,14]. The fitting of the titration curves to the modeling equations was performed by a

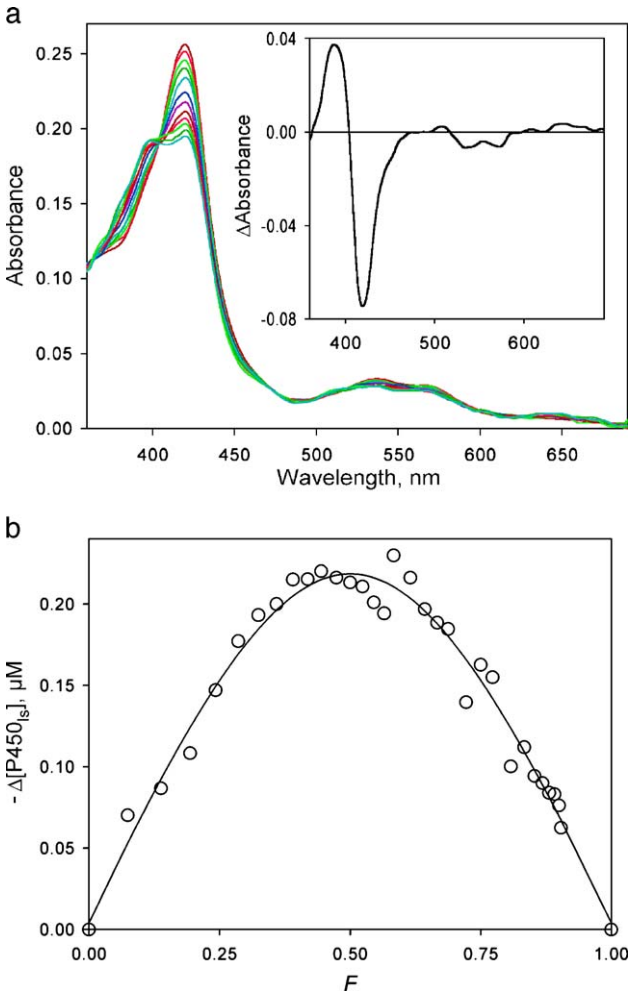

Fig. 2. Interactions of CYP3A4 with bromocriptine studied by the variable path length continuous variation technique at C_0=3 μM. Conditions: 0.1 M Na–HEPES buffer, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 25 °C. (a) A series of spectra corresponding to *F* values of 0, 0.074, 0.14, 0.24, 0.32, 0.39, 0.5, 0.58, 0.64, 0.72, 0.83, and 0.9. The series was corrected for non-linearity of the path length increase and fluctuations of the baseline as described. Inset shows the first principal component of the spectral changes observed in this experiment scaled to represent a transition of 1 μM heme protein. (b) Results of the same experiment interpreted in terms of the changes in the concentration of the low-spin state of CYP3A4 ($P450_{ls}$) [13], which are proportional to the changes in the concentration of the substrate-free enzyme. The solid line shows the results of data fitting to Eq. (3) with K_D=0.69±0.06 μM.

combination of Marquardt and Nelder-Mead non-linear least squares algorithms [11]. In this fitting procedure and in the calculation of the modeling curves shown in Figs. 2 and 3 of the manuscript we used a numerical golden section algorithm to find the roots of Eq. (2) and the sequential binding equation [14].

2.5. Experimental

All experiments were carried out at 25 °C in 100 mM HEPES buffer (pH 7.4), containing 1 mM DTT and 1 mM EDTA (HEPES + buffer). A 300 μM stock solution of bromocriptine mesylate was made in 20 mM Na–acetate buffer, pH 4.0. The stock solution of PMA (2–2.5 mM) was prepared in warm HEPES + buffer and kept warm during the experiments.

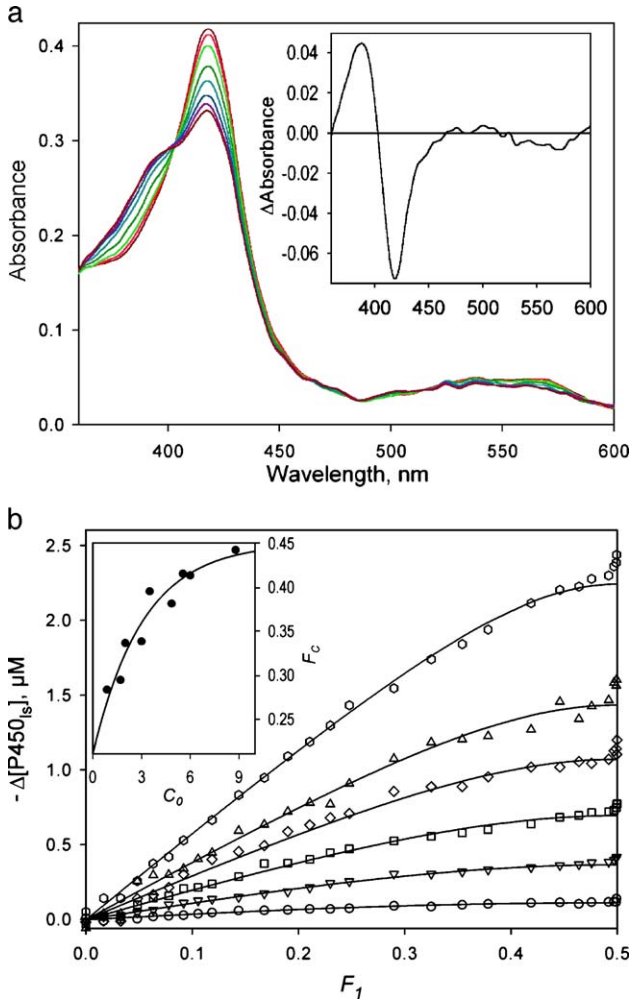

Fig. 3. Interactions of CYP3A4 with bromocriptine studied by the counter-flow continuous variation technique at various values of C_0. Conditions as indicated in Fig. 2. (a) A series of spectra obtained in the experiment at $C_0 = 8.8$ μM. The spectra shown correspond to F_1 values of 0, 0.048, 0.092, 0.19, 0.29, 0.38, 0.46, and 0.5. Inset shows the first principal component of the spectral changes observed in this experiment scaled to represent a transition of 1 μM heme protein. (b) Titration curves shown were obtained at C_0 of 0.9, 2, 3.3, 4.7, 6 and 8.8 μM. The solid lines show the results of the fitting of the data set to Eq. (3) with $K_D = 0.41 \pm 0.33$ μM. Inset shows the plot of F_c vs. C_0. To find F_c values we approximated the initial sections ($F < 0.2$) of the binding curves by a second order polynomial and interpreted the coefficient of the linear term as a slope of the tangential line plotted to the binding curves at $F = 0$. The solid line in the inset approximates the data points by an exponential function.

3. Results and discussion

3.1. Variable path length Job's titration

We introduced this novel modification of continuous variation technique in order to eliminate a decrease in sensitivity of traditional Job's titration at low enzyme concentrations, which makes it poorly applicable in combination with absorbance spectroscopy. In our implementation we utilized a variable path length spectrophotometric cell, specifically a 10-cm-long cylindrical cell with vertical direction of the light beam and total volume of 5.1 mL. At the beginning of the experiment we placed into the cell 0.5 mL of the enzyme solution with a concentration equal to the desired C_0 value. The titration was carried out by progressive addition of the substrate solution at the same concentration, as suggested by Facchiano and Ragone [6]. As the measuring light beam was directed from the top to the bottom of the cell, the light path of the sample increased simultaneously with the dilution, making the amplitude of the spectra virtually invariable. However, the meniscus at the surface of the sample, which introduces some non-linearity in the increase in path length, complicates the analysis of the spectra. Furthermore, the displacement of the meniscus along the cell results in some distortion of the baseline. We therefore introduced a second, horizontal measuring beam in order to use the constant-path-length auxiliary spectra to correct the amplitude and the baseline of the variable path length measurements. This correction was made by a linear least-squares procedure, as described in Materials and Methods. An image of our variable path length setup is shown in Fig. 1.

The application of variable path length technique is illustrated by our results obtained upon binding of bromocriptine (BCT) to CYP3A4. A series of absorbance spectra recorded in the variable path length Job's titration is shown in Fig. 2a. The titration curve obtained in this experiment (Fig. 2b) clearly exhibits the formation of CYP3A4–BCT complexes with 1 : 1 stoichiometry, which is consistent with earlier results [13,15].

3.2. Counter-flow Job's titration

We developed this approach to make Job's titration technique applicable when the absorbance band of the ligand overlaps considerably with that specific for the complex. However, due to its simplicity and versatility, this method may provide a convenient approach to study tight binding interactions even in the cases when the above overlapping is not critical. The idea of the method is to "double up" the Job's titration, thus having the branches corresponding to excess ligand and excess substrate taking place simultaneously in two

adjacent compartments of a tandem cell. At the beginning of the titration equal volumes of the enzyme and the substrate each at the desired concentration of C_0 are placed into two separate compartments of a tandem cell that is placed in the spectrophotometer in such a way that the light beam goes through both compartments consecutively. Titration is made by reciprocal displacement of aliquots of the solutions between the compartments. The fraction of the ligand in the first compartment at i-th step of the titration ($F_{1,i}$) is given by

$$F_{1,i} = F_{1,i-1}(1-v_i/V) + (1-F_{1,i-1})(v_i/V); \qquad F_{1,1} = 0 \qquad (4)$$

where v_i and V are the volume of the aliquot displaced at the i-th step ($i>1$) and the total volume in each of the compartments, respectively. At an infinite number of titration steps the value of F in each of the compartments approaches 0.5. As the spectra recorded with the tandem cell represent the sum of the absorbances in two compartments, the specific absorbance of the complex obtained at each $F_{1,i}$ corresponds to the sum of the values that would be obtained at $F=F_{1,i}$ and $F=1-F_{1,i}$ in the titration by the traditional Job's method in one of the compartments of the same cell. In the case of bimolecular association, inasmuch as the plot of X versus F obeys Eq. (3), which is symmetric with regard to the interacting species, the concentrations of the complex in two compartments will be similar. This titration curve, which is equivalent to the ascending branch of the traditional Job's titration, may be used to determine the K_D of the complex by fitting to the "tight binding" equation. To ensure the best accuracy obtainable when C_0 is chosen close to the value of K_D, it is desirable to repeat the titration at several different C_0 values. Such a series obtained for the interactions of CYP3A4 with BCT is illustrated in Fig. 3.

In the case of multi-site binding ($n>1$) the situation is more complex. For infinite cooperativity (Eq. (1)), the titration curves always display a maximum at $F=0.5$. However, the shape of the curves is drastically affected by the stoichiometry of the complex. A series of curves calculated for $n=1$, $n=2$, and $n=3$ is shown in Fig. 4. It may be seen that the increase in n increases the initial slope of the curve, and the stoichiometry of complex may be determined by a technique similar to the method of initial tangents used with traditional Job's titrations [4,7] . Our calculations show that provided that $C_0 \gg K_D$, the tangential line plotted to the titration curve at $F_1=0$ reaches the level of the maximum obtained at $F=0.5$ at the point where the abscissa is equal to $1/(n+1)$. In practice it is desirable to perform a series of titrations at various values of C_0 and plot the dependence of the position of the above cross point (F_c) versus C_0. The limit that this dependence approaches at $C_0 \to \infty$ yields $F_c^{max}=1/(n+1)$. Applicability of this approach to the case of 1 : 1 interactions is illustrated by our results on the interactions of CYP3A4 with bromocriptine. As shown in Fig. 3, our approach clearly reveals here a simple bimolecular association mechanism ($n=1$).

Although the analysis of the counter-flow titration technique given above is based on the predicate of infinite cooperativity (Eq. (1)), it appears to be equally informative in more complex

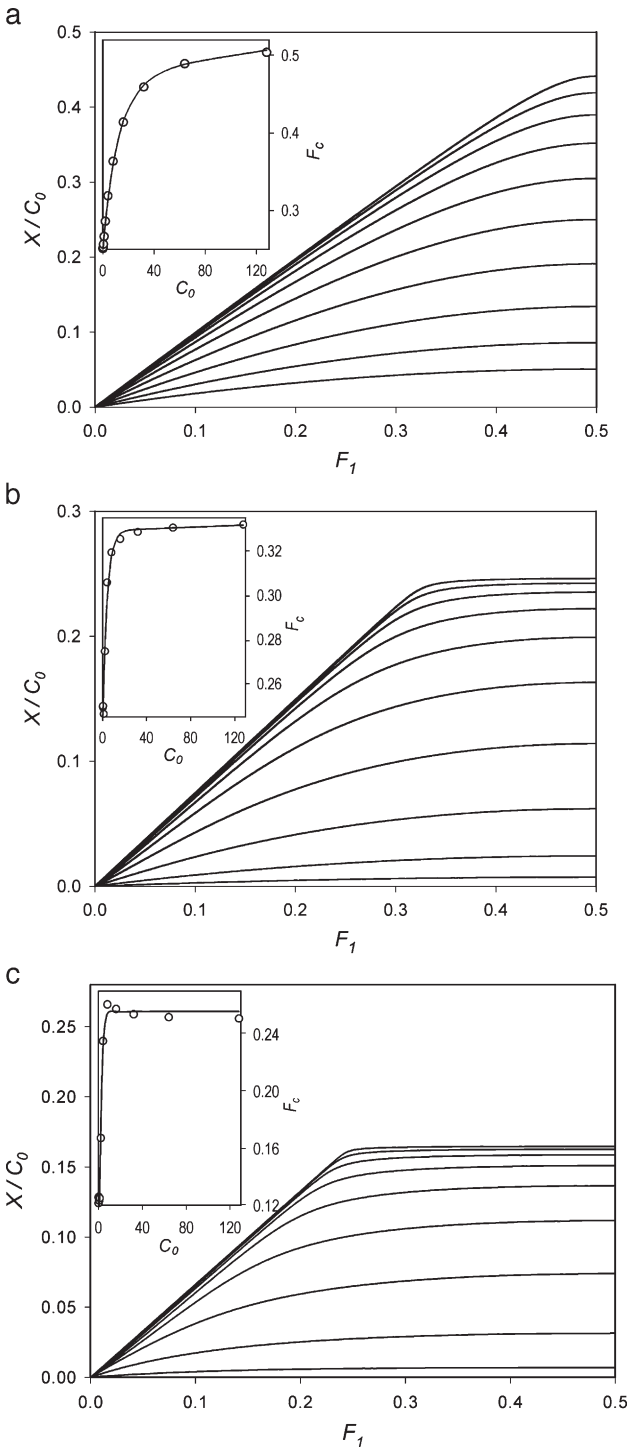

Fig. 4. Modeling curves for the counter-flow continuous variation titrations built according to Eq. (2) with $n=1$ (a), $n=2$ (b), and $n=3$ (**c**) and $K_D=1\,\mu M$. A series of curves corresponding to C_0 of 0.25, 0.5, 1, 2, 4, 8, 16, 64 and 128 μM is shown in each of the panels. The insets represent the plots of F_c vs. C_0. F_c values were found as described in Fig. 3. The solid lines in the insets approximate the plots by a biexponential function.

cases. Analyzing the case of sequential substrate binding to two binding sites:

$$E + 2S \underset{}{\overset{K_{D1}}{\rightleftharpoons}} ES + S \underset{}{\overset{K_{D2}}{\rightleftharpoons}} SES$$

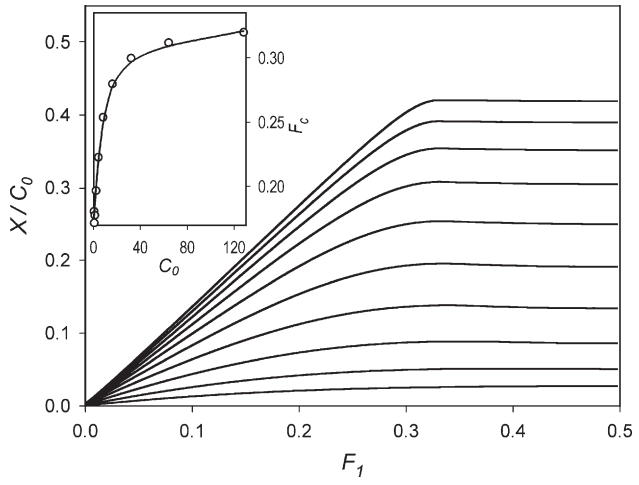

Fig. 5. Modeling curves for the counter-flow continuous variation titrations built with a sequential binding equation (K_{D1}=0.1 μM, and K_{D2}=1 μM) for C_0 values of 0.25, 0.5, 1, 2, 4, 8, 16, 64 and 128 μM. Inset represents the plots of F_c vs. C_0. F_c values were found as described in Fig. 1. The solid lines in the inset approximate the plot by a biexponential function.

we obtained the following relationship between the steady state concentration of the ternary complex, that of the complex ES ([ES]), and the total concentration of the enzyme and substrate [16]:

$$2[SES]^2-([E]_0-[SES]+K_{D1})[ES]-([S]_0+2[E]_0+K_{D2})[SES] + [S]_0[E]_0 = 0$$

where

$$[ES] = \frac{[S]_0}{2}-[SES]-\left([SES]^2-([S]_0+K_{D2})[SES]+\frac{[S]_0^2}{4}\right)^{1/2} \quad (5)$$

In contrast to the Adair equation, which describes the behavior of this system at $[S] \gg [E]$, the above relationship is valid at any concentrations of [S] and [E]. Although analytical solution of this equation for [SES] is intricate, it can be easily done by numerical means [14]. The modeling curves calculated with this equation are shown in Fig. 5. In this case the counter-flow titration curves exhibit a weak maximum at F_1 close to the value of $1/(n+1)$. Although this maximum is barely detectable in the experimental data, the abscissa of the intersection point F_c described above also approaches the value of $1/(n+1)$ at $C_0 \rightarrow \infty$ (Fig. 5, inset).

The application of this method in the case of multi-site binding is exemplified by our studies of the interactions of cytochrome *P*450eryF (*P*450eryF) with 1-pyrenemethylamine

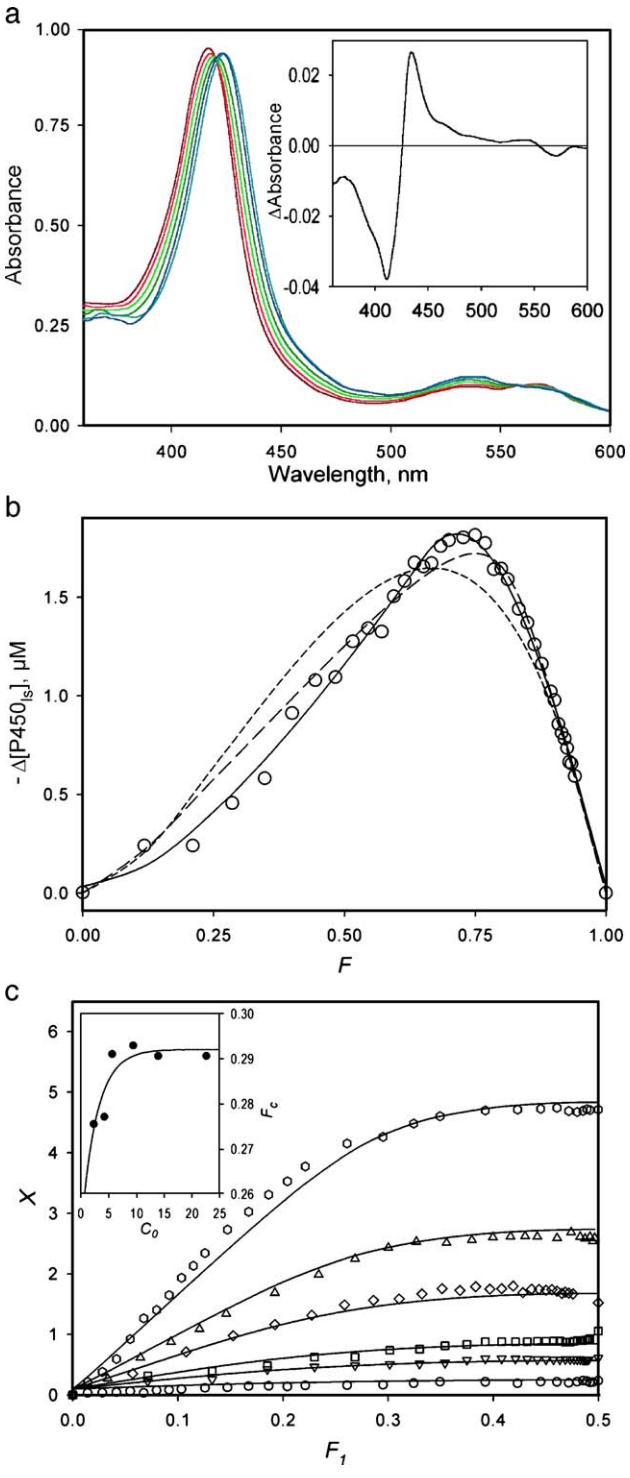

Fig. 6. Interactions of *P*450eryF with PMA studied by variable path length and counter-flow techniques. The interactions were monitored by the changes in the concentration of $P450_{ls}$ (see legend to Fig. 2). Conditions as indicated in Fig. 2. (a) A series of spectra obtained in the variable path length experiment at C_0=20 μM. Spectra shown correspond to *F* values of 0, 0.44, 0.63, 0.75, 0.85 and 0.94; the series was corrected for non-linearity of the path length increase and fluctuations of the baseline as described. The inset shows the first principal component of the spectral changes observed in this experiment scaled to represent a transition of 1 μM heme protein. (b) Results of the same experiment interpreted in terms of the changes in the concentration of the low-spin state of *P*450 ($P450_{ls}$), and the results of its fitting to Eq. (2) with n=2 and K_D=362±25 μM (short dash), Eq. (2) with n=3 and K_D=44±11 μM (long dash), and a sequential binding equation [14] with K_{D1}=0.5 μM (fixed during optimization) and K_{D2}=7.5±0.2 μM. (c) Titration curves obtained with counter-flow continuous variation technique at C_0 values of 2.3, 4.2, 5.6, 9.4, 14 and 23 μM. The lines show the results of the fitting to Eq. (3) with K_D=4.7±1.2 μM. Insets represent the plot of F_c vs. C_0. F_c values were found as described in Fig. 1. The solid line in the inset approximates the plot by a biexponential function.

(PMA), which is accompanied by a Type II spectral transition in the enzyme (Fig. 6a). Here the variable path length titration yields sharply asymmetric bell curve with the maximum around $F=0.7$, which is consistent with infinite cooperativity model with n ranging from 2 to 3. However, the fitting of the titration curve to Eq. (2) reveals significant systematic deviations of the experimental data from the fitting curves obtained with both n values (Fig. 6b, dashed lines). A series of titrations obtained in this case by a counter-flow titration technique is shown in Fig. 6c. Analysis of these data by the technique of initial tangents (Fig. 6c, inset) suggests the interactions of one enzyme molecule with two molecules of substrate. However, the fitting of the data set to Eq. (2) with $n=2$ reveals systematic deviation of the data points, which is particularly important at high C_0 values. Moreover, titration curves obtained at high C_0 values exhibit a weak maximum around $F=0.35–0.4$ suggesting that the mechanism of interactions is more complex than a simple "all-or-none" association (Eq. (1)). Indeed, a perfect fit of the data set (Fig. 6b, solid line) is obtained by a model suggesting that the spectral signal signifies the formation of 1 : 2 enzyme/substrate complex, where two molecules of substrate are bound sequentially to two non-equivalent binding sites [14]. This example shows that, in addition to its efficacy for determination of the stoichiometry of complex formation, the combination of the variable path length and counter-flow Job's titration techniques has high potential for discriminating among the mechanisms of substrate binding in allosteric enzymes.

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