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DETERMINATION OF BINDING CONSTANTS FROM CONTINUOUS CIRCULAR DICHROISM TITRATION DATA BY NUMERICAL ANALYSIS*

NIEK ROOSDORP and INGVAR SJÖHOLM†
Department of Pharmaceutical Biochemistry, Biomedical Center, University of Uppsala, Box 578, S-751 23 Uppsala, Sweden

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The extrinsic Cotton effects created by a ligand, when bound to a macromolecule, together with any changed intrinsic Cotton effect induced during the ligand–macromolecule interaction can be used to obtain both qualitative and quantitative information about the interaction. In the mathematical treatment of such data, as well as from other spectroscopic studies, a proportionality constant has to be used for each stoichiometric equilibrium, in addition to the binding constant. Thus, in the case of interactions with N reversible equilibria, $2N$ unknowns are involved. For interactions with two and more equilibria the large number of unknowns necessitates the use of a computer and high precision of the experimental data. It was then necessary to improve the precision of the experimental procedure by constructing a continuous titration system with controlled temperature and pH for the spectropolarimeter. The method has proved to be simple and rapid and gives precise data suitable for a computerized analysis.

Three different computer programs have been designed based on a one-, two- and three-site binding model, respectively. Conclusions about the number of binding sites and the binding constants are drawn by comparing the constants obtained in the three programs and from the discrepancies between the experimental and theoretical ellipticities of the data points. The procedure is illustrated by a study of the binding of bromdiazepoxide—a benzodiazepineoxide derivative, Ro 5-0991—to human serum albumin (HSA) taken from the accompanying paper.

EXPERIMENTAL METHOD

The circular dichroism was recorded with an automatic spectropolarimeter, JASCO J-20, as described in the earlier paper. Figure 1 shows the principle of the experimental set-up. Two pumps were used, a peristaltic one with a capacity of 175 ml/hr, which pumps the solution through the cell and a thermostated mixing vessel, and a calibrated infusion pump (Perfusor IV, B. Braun, Melsungen, W. Germany), by which a protein–ligand solution is added to the mixing vessel at constant rate (1.9 ml/hr). As the protein concentration in the infusion pump is the same as in the mixing vessel, the protein concentration will be constant in the whole system during the titration. The pH was measured in the mixing vessel and a range of 7.40 ± 0.04 units was tolerated.

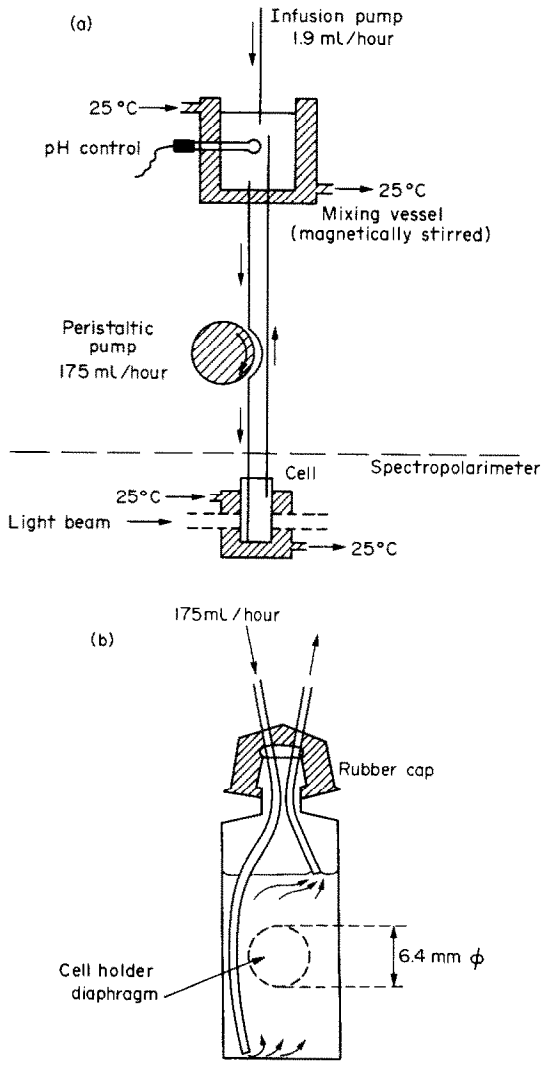

Fig. 1. (a) Block scheme of the experimental arrangement for a continuous circular dichroism titration of a macromolecule with a ligand. See text for further explanations. (b) Detail of the measuring cell for continuous circular dichroism titration.

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† To whom correspondence should be addressed.

With the experimental arrangement described, the change of the ellipticity at a constant wavelength is followed vs time when the ligand concentration is increased. With the scale and paper speed generally used, 1 cm on the vertical axis corresponds to one millidegree and on the horizontal axis to 4.834 min (see Fig. 2). Normally, the initial volume of the protein solution in the system was 10 ml, to which 5 ml of the protein–ligand solution with the same protein concentration were added by the infusion pump. After the titration, 30 cm of the time axis was divided into 30 points. The coordinates, expressed in cm, of the corresponding points on the manually smoothed curve, were fed into a computer. The computer calculated the difference molar ellipticities at the different ligand concentrations, which can be calculated at each time from the pumping speed of the infusion pump. The concentrations were so chosen as to give a reasonable excess of ligand and change of ellipticity.

NUMERICAL ANALYSIS

The one-, two- and three-site binding models, which formed the basis for the three computer programs, are based on the following principles: there is one binding site per macromolecule for each type of complex formed; each site is independent, i.e. the binding to one site will not in any way affect the binding to an adjacent site and the proportionality constant is the same for a certain complex regardless the ligand is bound or not to other sites.

In the following the theory of the program for the two-site binding model is presented. According to the law of mass action, we can write

$$P'_f + D_f \rightleftarrows PD1$$

and

$$P''_f + D_f \rightleftarrows PD2.$$

As the binding to the two sites, P' and P'', is assumed to be independent, the association constants are

$$K_1 = \frac{PD1}{P'_f \cdot D_f} = \frac{PD1}{(PT - PD1)\cdot(DT - PD1 - PD2)} \quad (1)$$

and

$$K_2 = \frac{PD2}{P''_f \cdot D_f} = \frac{PD2}{(PT - PD2)\cdot(DT - PD1 - PD2)}, \quad (2)$$

where PT is the total concentration of the protein, DT, the total concentration of the ligand. $PD1$ and $PD2$ are the *total* concentrations of the macromolecules containing ligand bound to the first and second site, respectively*. Thus,

$$DT = D_f + PD1 + PD2.$$

The change in ellipticity, expressed as $\Delta\theta$, during a titration is due to perturbation of the chromophores when the drugs are bound to the protein. The difference in ellipticity is directly proportional to the concentration of the protein–drug complexes, whether the chromophores belong to the macromolecule (internal Cotton effect) or to the ligand (external Cotton effect) or to both the ligand *and* the macromolecule.

When PT is constant and DT is varied, the following equations are valid

$$\Delta\theta = \Delta\theta 1 + \Delta\theta 2 \quad (3)$$

$$\Delta\theta 1 = e_{PD1} \cdot PD1 \quad (4)$$

$$\Delta\theta 2 = e_{PD2} \cdot PD2 \quad (5)$$

$$\Delta\theta\text{max}1 = e_{PD1} \cdot PD1\,\text{max} = e_{PD1} \cdot PT \quad (6)$$

$$\Delta\theta\text{max}2 = e_{PD2} \cdot PD2\,\text{max} = e_{PD2} \cdot PT \quad (7)$$

The difference in ellipticity, $\Delta\theta$, is the sum of the contribution of the first and second sites (eq. 3). In eqs (4)–(7), e_{PD1} and e_{PD2} are proportionality constants. $\Delta\theta$max1 and $\Delta\theta$max2 are the differences in ellipticity when the first and second site, respectively, is saturated. The proportionality constants, e_{PD1} and e_{PD2}, can be eliminated, yielding for varying $(DT)\,(i)$

$$\Delta\theta = \frac{PD1\,(i)}{PT}\cdot\Delta\theta\text{max}1 + \frac{PD2\,(i)}{PT}\cdot\Delta\theta\text{max}2. \quad (8)$$

From eq. (1) and eq. (2)

$$DT\,(i) - PD1\,(i) - PD2\,(i)$$

can be eliminated, which gives

$$\frac{PD1\,(i)}{(PT - PD1\,(i))\cdot K1} = \frac{PD2\,(i)}{(PT - PD2\,(i))\cdot K2}. \quad (9)$$

Express $PD2\,(i)$ in $PD1\,(i)$, PT, $K1$ and $K2$:

$$PD2\,(i) = \frac{PD1\,(i)\cdot K2\cdot PT}{(PT - PD1\,(i))\cdot K1 + K2\cdot PT}, \quad (10)$$

or, in general

$$PDn\,(i) = \frac{PD(n-1)\,(i)\cdot Kn\cdot PT}{(PT - PD(n-1)(i))\cdot K(n-1) + Kn\cdot PT}.$$

When we substitute eq. (10) in eq. (1), rearrange and multiply with the denominator, which is $\neq$ zero for $0 < PD1\,(i) < PT$, we obtain a third degree polynomial:

$$A\cdot PD1^3\,(i) + B\cdot PD1^2\,(i) + C\cdot PD1\,(i) + D = 0. \quad (11)$$

with coefficients

$$A = K1\cdot(K2 - K1),$$
$$B = K1^2\cdot DT\,(i) + 2\,K1^2\cdot PT - K1\cdot K2\cdot DT\,(i) + K1 - K2,$$
$$C = K1\cdot K2\cdot PT\cdot DT\,(i) - 2\,K1^2\cdot PT\cdot DT(i) - K1^2\cdot PT^2 - K1\cdot K2\cdot PT^2 - K1\cdot PT,$$
$$D = K1^2\cdot PT^2\cdot DT\,(i).$$

With assumed values for $K1$ and $K2$, we can solve eq. (11) with Newton–Raphson's iterative method [1]. With $PD1(i) = x^{(i)}$ and $f(x^{(i)}) = A\cdot PD1^3(i) + B\cdot PD1^2(i) + C\cdot PD1\,(i) + D$, we can write:

$$x^{(i)}_{k+1} = x^{(i)}_k - H,$$

with

$$H = f(x^{(i)}_k)\frac{\mathrm{d}f(x^{(i)}_k)}{\mathrm{d}\,x^{(i)}_k};\ k = \text{the number of iterations.}$$

As starting value, $x^{(i)}_0$, we used: $x^{(1)}_0 = 0$ and $x^{(i)}_0 = x^{(i-1)}_k$.

* If the two sites on the macromolecule are denoted $\sim P$ and $P-$, respectively, this means that $PD1 = D \sim P$, $+ D \sim P - D$ and $PD2 = \sim P - D + D \sim P - D$.

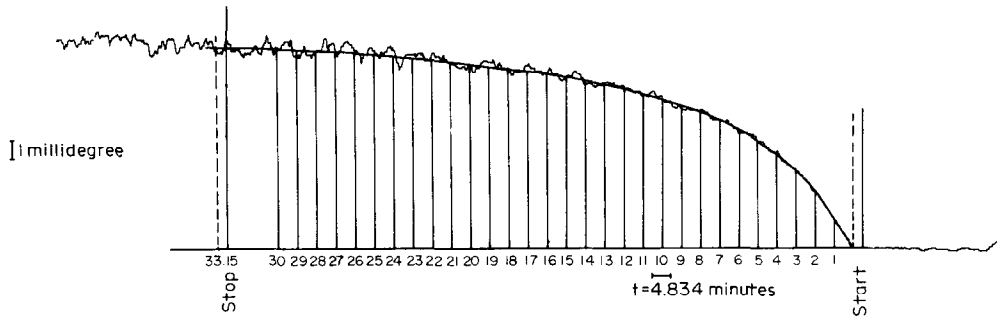

Fig. 2. The change of ellipticity recorded at 262 nm, when a bromdiazepoxide–albumin solution (5 ml, 1.62×10^{-4} M and 9.48×10^{-6} M, respectively) was added continuously to human serum albumin (10 ml, 9.48×10^{-6} M) in 0.1 M KCl and 0.005 M phosphate buffer at 25° and pH 7.4.

The iteration was continued until:

$$\left|\frac{H}{i}\right| = \left|\frac{x_{k+1}^{(i)} - x_k^{(i)}}{x_{k+1}^{(i)}}\right| < 0.5 \times 10^{-7}.$$

$PD2\,(i)$ can be calculated from eq. (10). In order to calculate $\Delta\theta$max1 and $\Delta\theta$max2 with the least-squares method, we defined a new function:
FMIN ($\Delta\theta$max1, $\Delta\theta$max2):

$$\text{FMIN} = \sum^{N}\left(\Delta\theta_{(i)} - \frac{PD1\,(i)}{PT}\cdot\Delta\theta\text{max1} - \frac{PD2\,(i)}{PT}\cdot\Delta\theta\text{max2}\right)^2, \quad (12)$$

with N = the total number of experimental points.

FMIN will reach a minimum for

$$\frac{\partial\text{FMIN}}{\partial\Delta\theta\text{max1}} = \frac{\partial\text{FMIN}}{\partial\Delta\theta\text{max2}} = 0. \quad (13)$$

Taking these first derivatives of eq. (12) with respect to $\Delta\theta$max1 and $\Delta\theta$max2, two linear equations in $\Delta\theta$max1 and $\Delta\theta$max2 are created, from which $\Delta\theta$max1 and $\Delta\theta$max2 can be solved. Now FMIN can be calculated with eq. (12) and be compared with the FMIN-values obtained with other $K1$ and $K2$ values.

Figure 3 shows a flow diagram of the two binding sites program. The program generates values for $K1$ and $K2$, beginning at 0.2×10^3 and increasing with 0.2×10^p, with $p = 3, 4 \ldots 10$, up to 0.1×10^{11}. For every combination of the $K1$ and $K2$ values (with $K1 \geq K2$), $\Delta\theta$max1 and $\Delta\theta$max2 are calculated from eq. (13) and then FMIN from eq. (12). The $K1$ and $K2$ of the lowest found value of FMIN are then varied by $\pm 50\%$. The created intervals are divided into 10 new values of $K1$ and $K2$, for which the same procedure is repeated. Another subroutine (BIBL) can finally be used. This subroutine varies the hitherto found values of $\Delta\theta$max1 and $\Delta\theta$max2 and calculates according to the same principles as above $K1$, $K2$ and FMIN. In the 'three-site' computer program, a subroutine from the Harwell Subroutine Library [2], called VAO4AD is used to find the best combination of $K1$, $K2$ and $K3$. Furthermore, $\Delta\theta$max1, $\Delta\theta$max2 and $\Delta\theta$max3 are calculated with the least-squares method as above with an IMSL-subroutine [3], called LINV3F.

All three programs are written in FORTRAN IV and developed on an IBM 370/155 computer.

RESULTS AND DISCUSSION

Precise primary data is a condition for a successful numerical analysis of the type described. The experimental arrangement (Fig. 1) used with continuous addition of the ligand has been shown to yield such data. The rate of the peristaltic pump is high enough (175 ml/hr) to attain a rapid mixing of the ligand in the system, without causing turbulence in the measuring cell which might disturb the CD measurements.

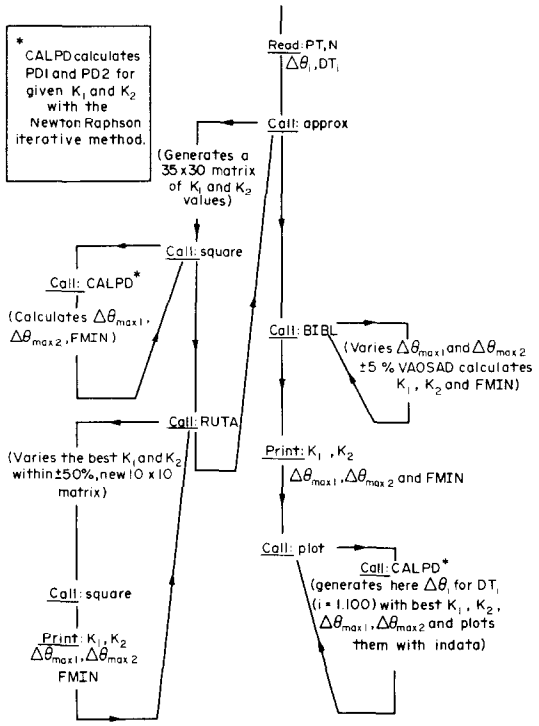

Fig. 3. Flow diagram of the computer program used for the calculation of association constants ($K1$ and $K2$) and maximal ellipticity values ($\Delta\theta$max1 and $\Delta\theta$max2) for a two-site equilibrium between a macromolecule (P) and a small molecule (D). APPROX, SQUARE, RUTA, CALPD, PLOT and BIBL are subroutines, with the last one containing the Harwell subroutine library program VAO5AD.

With 1.9 ml added per hr, the concentration gradient in the light beam (maximally 2-mm diameter with the slit program used) is negligible and a precise determination of the ligand concentration is possible. The procedure used will give higher precision as the instrumental variations will be smaller because the time used is shorter than in a stepwise titration, the cell is not touched during the whole experiment and no separate baseline run is needed. A larger number of experimental points can also be used. We have found that 30 data points will give the most favourable precision/cost relation for the computer analysis. Certainly, the system can be further developed by using a proper interface unit for a direct feeding of the signals into the computer.

The procedure is illustrated in Fig. 2 and Table 1, which show the results obtained when the binding of bromdiazepoxide (Ro5-0991) to HSA at pH 7.4 and 25° was studied. The figure shows the succesive change of ellipticity at 262 nm when increasing amounts of the drug is added to the protein. The table collects the experimental ellipticity of the data points and the theoretical values obtained, if the constants calculated by the respective computer programs are used. As is evident from the table, the theoretical fit to the experimental data is excellent in all the three cases. The relative deviation, however, can be large at the beginning of the titration, where the experimental errors both in $\Delta\theta$ and ligand concentration are largest, but in the example shown the deviation never corresponded to more than about 1.3 mm on the original graph. The largest deflection ($\Delta\theta$) was about 10 cm.

In the example given the titration was completed in about 2 1/2 hr. The pumping rate of the infusion pump delivering the ligand and the scanning speed of the recorder can, however, be varied within wide limits according to the needs and the properties of the equilibrium system under study. Thus, the equilibrium must have been attained before the added ligand reaches the measuring cell and the scanning speed must be high enough to allow a high precision in the determination of the time elapsed (= the concentration of ligand). The time required to reach the equilibrium can easily be tested in batch experiments. In the present case it was reached faster than could be measured with the spectropolarimeter, which is in accord with the experience from other protein–drug systems [4, 5].

Graphical methods have earlier been applied to calculate the association constants from CD-data [6, 7]. These are, however, applicable only in 1:1-systems and are in some cases based on approximations. In our computation method, we relate the changed ellipticity to the concentration of the complex with a proportionality constant. This is done by estimating the $\Delta\theta$max-values for every complex in question, i.e. the maximal change of the ellipticity brought about, when the site is saturated by the ligand. However, it is of the utmost importance in this context to stress that the computer can never make judgements about the "correctness" of the constants obtained, but can only apply a mathematical program, which in turn is based on an assumed model system.

The present work is based on these fundamental principles. Three different programs are used in our procedure to study the binding of drugs to purified serum albumin and the procedure is applicable in other spectroscopic methods, as well as to any interaction system, in which the components are well-characterized. The choice of the applied programs has been based on a study* by H. Johansson and A. Sjöberg, Uppsala, who compared the usefulness of several methods for the minimization of the respective

* Unpublished work.

Table 1. Binding of bromdiazepoxide to human serum albumin at pH 7.4 and 25° in 0.1 M KCl and 0.005 M phosphate buffer

			One site program:		Two site program:		Three site program:	
			$K_1 = 0.11 \times 10^6$		$K_1 = 0.20 \times 10^6$		$K_1 = 0.18 \times 10^6$	$\Delta\theta$max1 = 0.10 × 10⁶
			$\Delta\theta$max1 = 0.13 × 10⁶		$\Delta\theta$max1 = 0.10 × 10⁶		$K_2 = 0.9 \times 10^4$	$\Delta\theta$max2 = 0.38 × 10⁵
					$K_2 = 0.4 \times 10^4$		$K_3 = 0.6 \times 10^4$	$\Delta\theta$max3 = 0.12 × 10⁶
Point no.	Molar ratio D_T/P_T	Found molar ellipticity $\Delta\theta_{obs}(\times 10^{-5})$	Theoretical molar ellipticity $\Delta\theta_{th}(\times 10^{-5})$	Deviation theoretical-found ellipticity (%)	Theoretical molar ellipticity $\Delta\theta_{th}(\times 10^{-5})$	Deviation theoretical-found ellipticity (%)	Theoretical molar ellipticity $\Delta\theta_{th}(\times 10^{-5})$	Deviation theoretical-found ellipticity (%)
1	0.26	0.156	0.160	2.4	0.163	4.5	0.164	4.8
3	0.75	0.425	0.407	−4.3	0.417	−2.0	0.418	−1.6
5	1.21	0.589	0.580	−1.5	0.588	−0.1	0.589	0.1
7	1.65	0.700	0.700	0.0	0.704	0.5	0.704	0.5
9	2.06	0.780	0.787	1.0	0.785	0.7	0.784	0.6
11	2.45	0.844	0.852	0.9	0.846	0.2	0.844	0.0
13	2.83	0.891	0.901	1.1	0.892	0.1	0.890	−0.1
15	3.18	0.931	0.939	0.8	0.930	−0.1	0.929	−0.3
17	3.52	0.965	0.970	0.5	0.962	−0.4	0.961	−0.4
19	3.84	0.985	0.995	1.0	0.988	0.3	0.989	0.4
21	4.14	1.013	1.016	0.3	1.012	−0.1	1.014	0.1
23	4.44	1.034	1.033	0.0	1.032	−0.2	1.035	0.1
25	4.72	1.055	1.048	−0.6	1.051	−0.4	1.053	−0.1
27	4.98	1.070	1.061	−0.8	1.067	−0.3	1.068	−0.1
29	5.24	1.078	1.072	−0.5	1.082	0.4	1.078	0.1
			FMIN = 20.9 × 10⁶		FMIN = 7.4 × 10⁶		FMIN = 5.8 × 10⁶	

The albumin concentration, PT, was 9.5×10^{-6} M^1. The experimentally obtained ellipticities, $\Delta\theta$, with different amounts of drug are compared with theoretically calculated ellipticities with association constants K, and maximal ellipticities, $\Delta\theta$max, obtained from the three different computer programs.

FMIN-functions obtained with the three programs. The results from the programs are compared and the constants obtained from the program with the smallest number of sites giving a reasonably good fit to the experimental points are considered to be "true". Generally, the fit will be better with programs involving more sites. (Compare the FMIN-values given in Table 1.) Therefore, a constant is considered to be true, when the same value is found also in the program that is based on a model with *one more* site.

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