

## An Integral Method to Analyze Sedimentation Equilibrium

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Experimental data of the sedimentation equilibrium on ideal or non-ideal associating systems are analyzed successfully by an integral method, which gives optimum values of parameters at the minimum of standard deviation. According to this procedure, the concentration at meniscus,  $c_a$ , the weight average molecular weight,  $M_w$ , and the second virial coefficient, B, can be obtained simultaneously, without calculating the derivatives of the standard deviation. This procedure was applied to the sedimentation equilibrium data on tropomyosin, one of muscle proteins, in 1 M NaCl, and on maleylated tropomyosin in the absence of salts. The analyses based on monomer-dimer equilibrium for tropomyosin, and on subunit-monomer equilibrium for maleylated tropomyosin, gave the good fit for the data. The applicability of the integral method is discussed in relation to the usual gradient method.

KEY WORDS: Sedimentation equilibrium / Non-linear equation / Integral method / Tropomyosin /

### INTRODUCTION

The experimental technique based on sedimentation equilibrium has been one of the most excellent methods up to the present to investigate a system of interacting biopolymers such as protein; from the experimental point of view, it has an advantage that the concentration and amount required for measurements are low, of the order of 1 mg/ml (or less than 1  $\mu$ g/ml if an absorption optics is used in the ultracentrifuge) and of a few tenths of ml, and from the theoretical point of view, the theoretical basis is founded rigorously on the realm of thermodynamics. The molecular weights of components and some thermodynamic information (*e.g.*, an equilibrium constant of interacting system and the second virial coefficient as a measure of molecular interaction) can be obtained from the analysis of the data.

The data obtained by a sedimentation equilibrium experiment on the ideal or non-ideal system are analyzed usually by a least-squares determination of parameters which link the data and the theory with a computer.<sup>1,2)</sup> A matrix inversion program or some iterative programs are used to obtain the optimum parameters to describe the system under investigation so as to make the standard deviation minimum. The minimum obtained by such programs, however, does not necessarily reach the global minimum, but might fall in a local minimum. Recently, a numerical method to search the global maximum of a function of many variables has been developed by Tsuda.<sup>3)</sup> In the present paper, we shall present an application of this procedure to the analysis of sedimentation equilibrium experiments on non-ideal

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associating systems and an attempt to calculate the concentration at meniscus,  $c_a$ , the weight average molecular weight,  $M_w$ , and the second virial coefficient,  $B$ , simultaneously.

For that purpose, it is preferable to have data of sedimentation equilibrium on monodisperse macromolecules which associate reversibly. We select one of muscle proteins, tropomyosin, as the material, since the molecule constituted of two identical subunits associates into polymer in a low salt concentration and dissociates in a high salt concentration.<sup>4)</sup> Furthermore, when positive charges on the molecule are substituted into negative charges by maleylation, the molecule dissociates to subunits in a low salt concentration due to electrostatic repulsion.<sup>5)</sup> Therefore, tropomyosin may be a good system to examine the method.

### THEORETICAL SECTION

Let us consider a sedimentation equilibrium of a solution which contains  $n$  species of solutes. The fundamental differential equation to describe the sedimentation equilibrium for  $i$ -th species is given by a following equation,<sup>1)</sup>

$$\frac{dc_i(r)}{dr^2} \left( 1 + c_i(r) \frac{\partial \ln \gamma_i}{\partial c_i(r)} \right) = A_i M_i c_i(r) \quad (1)$$

where  $c_i(r)$ , and  $\gamma_i$  are a concentration at radius  $r$  (a distance from the center) expressed in grams per milliliter of solution, and the activity coefficient of  $i$ -th species, respectively.  $A_i$  is the quantity,  $((1 - \bar{v}_i \rho) \omega^2 / 2RT)$ , where  $\rho$  is the density of the solution,  $\omega$  is the angular velocity,  $\bar{v}_i$  is the partial specific volume of  $i$ -th species, and  $R$  is the gas constant. For an associating system of single solute ( $i$ -th species in this case is an  $i$ -mer of monomers), the total concentration of the system ( $c(r)$ ) is expressed as follows, if we assume that

$$\ln \gamma_i = iBM_1c + O(c^2)$$

and  $\bar{v}_i$  is the same value for any  $i$ ,

$$c(r) = \sum_i c_{i_a} \exp (AM_i(r^2 - a^2) - BM_i(c(r) - c_a)) \quad (2)$$

where  $a$  means the position of the meniscus of the solution in the cell.<sup>2)</sup> Here, each term in the right hand side of Eq. (2) satisfies Eq. (1). The present problem is to find the unknown parameters ( $c_{i_a}$ ,  $M_i$ , and  $B$ ) in Eq. (2) which minimize the function STD ( $c_{1_a}$ ,  $c_{2_a}$ ,  $\dots$ ,  $M_{1_a}$ ,  $M_{2_a}$ ,  $\dots$ ;  $B$ ) defined by the following relation,

$$\text{STD} = \left[ \sum_{r=b}^{r=a} (c(r) - \bar{c}(r))^2 \right]^{1/2} \quad (3)$$

where  $b$  is the position of the bottom of the solution in the cell. The notation  $\bar{c}(r)$  denotes the total concentration distribution obtained experimentally in the cell.

Now, we shall consider such a hypercube,  $D$ , in an  $n$ -dimensional Euclidean space,  $R^n$ , that

$$D = D_1 \times D_2 \cdots \times D_n$$

$$D_i = x_i; \quad 0 \leq x_i \leq 1, \quad (i=1, 2, \dots, n)$$

and  $F(\mathbf{x}) = F(x_1, x_2, \dots, x_n)$  defined in  $D$ . Further,  $F(\mathbf{x})$  is assumed to satisfy the following conditions: (a)  $F(\mathbf{x})$  is a continuous function, (b)  $0 \leq F(\mathbf{x}) < \infty$ , (c)  $F(\mathbf{x})$  has one and only one global maximum at  $\mathbf{x}_M \in D$ . When  $f(\mathbf{x}) = \exp(F(\mathbf{x}))$ , we have the following relation,

$$\lim_{\alpha \rightarrow \infty} (f(\mathbf{x}))^\alpha / \int_D (f(\mathbf{x}))^\alpha d\mathbf{x} = \delta(\mathbf{x} - \mathbf{x}_M) \quad (4)$$

where  $\delta(\mathbf{x}) = \prod_i \delta(x_i)$ . Hence,  $\mathbf{x}_M$  may be obtained as

$$\lim_{\alpha \rightarrow \infty} \int_D \mathbf{x} (f(\mathbf{x}))^\alpha d\mathbf{x} / \int_D (f(\mathbf{x}))^\alpha d\mathbf{x} = \mathbf{x}_M \quad (5)$$

The proof of Eq. (4) and a numerical method to calculate the Eq. (5) are described in detail by Tsuda.<sup>3)</sup>

In order to solve the problem in obtaining the unknown parameters which minimize the function STD, a hypercubic domain of search must be set first. We have some preliminary informations about the region where the optimum values of the parameters can exist, *i.e.*, (a)  $0 \leq c_{i0} \leq c_0$  ( $c_0$  is the initial concentration) for any  $i$ , (b) a possible range of molecular weight of the monomer may be  $A \leq M_i \leq B$  ( $A$  and  $B$  are estimated by the technique of SDS gel-electrophoresis or gel chromatography), and (c) the second virial coefficient must be in the range of  $-10^{-4} \leq B \leq 10^{-2}$ . According to the informations described above, we set the hypercubic domain of search  $D$  in  $R^n$  ( $n$  is the number of the parameters). Of course, it is advantageous that these domains are selected as small as possible. Now let be  $F(\mathbf{x}) = 1/\text{STD}(\mathbf{x})$  ( $\mathbf{x} = (c_{1a}, c_{2a}, \dots; M_{1a}, M_{2a}, \dots; B)$ ). When  $F(\mathbf{x})$  satisfies the above conditions, we have  $\mathbf{x}_M \in D$  by maximizing  $F(\mathbf{x})$  (or minimizing  $\text{STD}(\mathbf{x})$ ) in  $D$  performing numerical integrations of Eq. (5) at large values of integrands ( $10^{59} - 10^{70}$  which are the large numbers conventionally available in the computer).

All the computations were performed by FACOM 230-48 at the computing center of the Institute for Chemical Research, Kyoto University.

## EXPERIMENTAL SECTION

**Materials** - Tropomyosin extracted from rabbit skeletal muscle was purified according to the procedure of Ebashi *et al.*<sup>5)</sup> The purity of the material was examined by the ultraviolet absorption spectrum and SDS-gel electrophoresis to detect the absence of any other contaminations.

Tropomyosin was maleylated by the addition of maleic anhydride according to the procedure of Woods *et al.*<sup>6)</sup> The extent of maleylation determined spectrophotometrically using the extinction coefficient at 250 nm,  $\epsilon_{250} = 3360$  for the maleyl groups given by Butler *et al.*<sup>7)</sup> showed that all the lysyl residues in the present preparation were maleylated.

## An Integral Method to Analyze Sedimentation Equilibrium

Protein concentration were determined by ultraviolet absorption ( $A_{277\text{ nm}}^{1\text{ cm}}=0.25$  for 1 mg/ml) for tropomyosin and by the biuret method for maleylated tropomyosin.

*Methods* - Sedimentation equilibrium experiments were performed in a Spinco Model *E* analytical ultracentrifuge equipped with a Rayleigh interference optical system. All runs were carried out at 20°C and the pH was maintained using phosphate buffer of pH 6.9. The partial specific volume,  $v$ , of tropomyosin and that of maleylated tropomyosin were taken as 0.739 and 0.733, respectively.<sup>6)</sup>

Distances between fringes were measured by a Nikon profile projector.

### RESULTS AND DISCUSSION

Figure 1 shows the plot of  $\ln c(r)$  vs.  $r^2$  for a tropomyosin solution at the initial concentration of 1.24 mg/ml in 1.1 M NaCl at 20°C. As shown in Fig. 1,  $\ln c(r)$

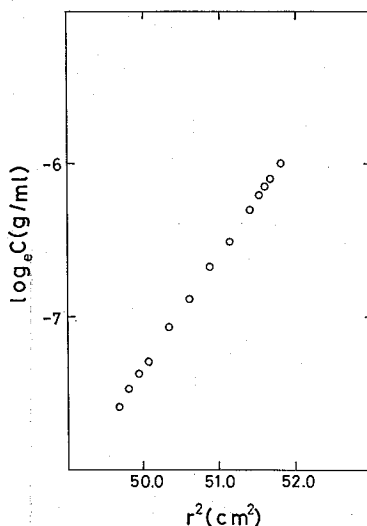


Fig. 1. Plots of  $\log_e c$  vs.  $r^2$  for tropomyosin in 1.1 M NaCl, pH 6.9 at 20°C. 14,290 rpm. The initial protein concentration is 1.24 mg/ml.

depends linearly on  $r^2$ , and the apparent molecular weight ( $M_{app}$ ) of the molecule is estimated to be 72,300 from the conventional equation,

$$M_{app} = (c_b - c_a) / c_0 (r_b^2 - r_a^2) A.$$

This value seems to be somewhat larger than the calculated molecular weight of tropomyosin according to the amino acid sequence, 66,000. Therefore, the plots are analyzed by the present integral method described previously on the basis of the monomer-dimer equilibrium in solution.

The hypercubic domain of search was set such that  $0 \leq c_{1a}$  (monomer in mg/ml)  $\leq 0.8$ ,  $0 \leq c_{2a}$  (dimer in mg/ml)  $\leq 0.1$ ,  $6 \times 10^4 \leq M_1$  (monomer)  $\leq 8 \times 10^4$ ,  $1.2 \times 10^4 \leq M_2$  (dimer)  $\leq 1.6 \times 10^4$ , and  $0 \leq B \leq 0.1 \times 10^{-2}$ . Each integration was performed numerically taking  $10^4$  points in the domain randomly, and this number was large enough

as judged from the convergence of the parameter values.

Since Eq. (1) becomes a linear differential equation when  $B=0$ , some gradient method may be applied to the present system.<sup>8)</sup> The results of the analyses using a gradient method and the integral method on assuming that the system is ideal are listed in Table I. In applying the gradient method, the molecular weight of dimer was taken as twice that of monomer, while we put it as a variable for the integral method. On the other hand, the inclusion of a finite value of  $B$  makes difficult to solve Eq. (1) by gradient method because of non-linearity of the equation, and therefore, we analyzed only by the integral method as listed in Table I. The results show that the value of  $B$  is small, an order of  $10^{-3}$ , *i.e.*, the results are all the same regardless the method employed. The analyses yield around 66,000 for the molecular weight of monomer, and 130,000 for that of dimer with the standard deviation of  $0.8 \times 10^{-5}$ . Therefore, the data in Fig. 1 is better to be interpreted as a mixture of monomers and dimer of tropomyosin with ideal mixing state than a system of a single component of molecular weight of 77,000. The results obtained by the use of the present method, give concentrations of monomer and dimer at meniscus, from which we can estimate apparent dissociation constant of tropomyosin in 1.1 M NaCl as  $3.0 \times 10^{-4}$  M. This corresponds to the free energy of dissociation at 20°C of approximately 4.8 Kcal/mol.

Table I. Analyses of the Data in Fig. 1 by Gradient Method and by Integral Method. (concentration in g/ml)

	gradient method	integral method	
		ideal	non-ideal
$c_{12}$	$0.495 \times 10^{-3}$	$0.496 \times 10^{-3}$	$0.498 \times 10^{-3}$
$c_{22}$	$0.307 \times 10^{-4}$	$0.220 \times 10^{-4}$	$0.244 \times 10^{-3}$
$M_1$	$6.40 \times 10^4$	$6.76 \times 10^4$	$6.65 \times 10^4$
$M_2$	$1.28 \times 10^5$	$1.23 \times 10^5$	$1.53 \times 10^5$
$B$	—	—	$0.71 \times 10^{-3}$
$STD$	$0.83 \times 10^{-5}$	$0.81 \times 10^{-5}$	$0.86 \times 10^{-5}$

Table II. Analyses of the Data in Fig. 2 by Integral Method. (concentration in g/ml)

	1st	2nd
$c_{12}$	$1.284 \times 10^{-3}$	$1.261 \times 10^{-3}$
$c_{22}$	$0.098 \times 10^{-3}$	$0.099 \times 10^{-3}$
$M_1$	$3.63 \times 10^4$	$3.88 \times 10^4$
$M_2$	$6.81 \times 10^4$	$6.46 \times 10^4$
$B$	$0.84 \times 10^{-2}$	$0.89 \times 10^{-2}$
$STD$	$0.97 \times 10^{-5}$	$0.75 \times 10^{-5}$

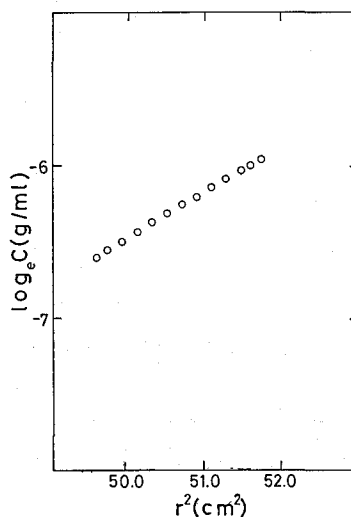


Fig. 2. Plots of  $\log_e c$  vs.  $r^2$  for maleylated tropomyosin in the absence of salts, pH 6.9 at 20°C. 14,290 rpm. The initial protein concentration is 1.93 mg/ml.

Figure 2 shows the plot of  $\ln c(r)$  vs.  $r^2$  for maleylated tropomyosin in the absence of salt. In contrast to Fig. 1, plots do not lie on a straight line but curve slightly downwards, showing some distribution of molecular weights due to interaction

## An Integral Method to Analyze Sedimentation Equilibrium

between the molecules. The results of the analysis using the integral method are listed in Table II. Apparently the molecule dissociates into two subunits of 34,000 and the virial coefficient is an order of  $10^{-2}$ , indicating the strong repulsion between the subunits. Since maleylated tropomyosin carries more negative charges than intact molecules,<sup>6)</sup> the result may reasonably indicate the presence of electrostatic repulsion due to the negative charges on the subunits. The apparent dissociation constant in this system estimated from the concentrations at meniscus is  $9.0 \times 10^{-3}$  M, corresponding to the free energy of dissociation at 20°C of 2.8 Kcal/mol. That is, the system dissociates more easily than the dimer of intact tropomyosin. These data could not be analyzed by the gradient method because of the finite value of B, or non-linearity of the equation.

The present results indicate the applicability of the integral method to estimate molecular weights of molecules in solution, concentrations at meniscus, and virial coefficients, simultaneously from Eq. (1). The method to solve Eq. (1) done so far has been to calculate standard deviation using iterative procedure as a function of B, since non-linear equation (1) could not be solved directly. Therefore, the integral method may be convenient in obtaining the values at once and useful for treatment of non-linear equations.

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