A BIOPHYSICAL MODEL OF LYSOZYME SELF-ASSOCIATION

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ABSTRACT The concentration dependence of the self-association of hen egg-white lysozyme was studied spectrophotometrically at pH 6, 25°C, and low ionic strength within a concentration range of 2.5–50 μg/ml. Of several possible mathematical models, an ideal or nearly ideal two-stage model representing an equilibrium between monomers and dimers and between dimers and trimers best describes the data. The dimerization and trimerization constants were found to be 2.5×10^{-2} and 38×10^{-2}. Dialysis experiments confirmed that the mechanism involves three associating species. A "head-to-tail" contact between the associating sites was inferred from dialysis studies of the effect of indole and imidazole derivatives on lysozyme self-association.

INTRODUCTION

It has long been recognized that lysozyme is a protein that self-associates in aqueous solution (1–8). The various methods used for studying this phenomenon, have resulted in the proposal of different association mechanisms. The sedimentation equilibrium technique, has led to the proposal of a monomer-dimer equilibrium (1, 4), although some evidence for indefinite association has been found. The use of other methods of analysis, such as light scattering (3), electron microscopy (7), and gel filtration at low ionic strength (8), have shown the presence of associated forms larger than dimers, and a "head-to-tail" mechanism of contact between the associating species has been proposed (9–11). Calorimetric studies of the native and chemically modified Trp-62, Trp-108, and Glu-35 residues from the active site of the enzyme have shown that Trp-62 and Glu-35 residues are involved in the "head" site of contact (12). Additional evidence for this mechanism has come from nuclear magnetic resonance studies of the effect of *N*-acetyl-D-glucosamine on the self-association of this macromolecule (13, 14). These studies have shown that the portion of the lysozyme surface involved in the head-to-tail association includes His-15 and possibly one or more adjacent positively charged residues; the other spatially distinct region involved consists of Glu-35, Trp-108, and Trp-63 from the active cleft of the enzyme.

Our goal in the present study was to find a model that better represents the mechanism of self-association of lysozyme at pH 6 and at low ionic strength by employing equilibrium absorption spectrophotometry and dialysis kinetic methods.

MATERIALS AND METHODS

Hen egg-white lysozyme from Nutritional Biochemicals Co., Cleveland, OH (lot 6575, three-times crystallized) was further purified by ion-exchange chromatography in an Amberlite CG-50 (lot 3341 from Mallinckrodt Chemical Works, St. Louis, MO) resin column with 0.2 M phosphate buffer, pH 6.9 (15). The protein was then exhaustively dialyzed against deionized water with acetylated cellulose membranes and lyophilized. Standard protein concentrations of 1 mg/ml were checked spectrophotometrically using a molar absorptivity, ϵ, of 39,000 mol^{-1} cm^{-1} at 280 nm (16) and subsequently diluted to the desired concentration.

The lytic activity of the lysozyme was assayed on *Micrococcus lysodeikticus* cell suspensions at pH 6.4 and $\mu = 10^{-2}$ according to the procedure described (17). The dried cells of *M. lysodeikticus* were from Sigma Chemical Co., St. Louis, Mo.

Kinetic dialysis studies were performed using an apparatus similar to that previously reported (18). A 0.05-ml sample of a 1 mg/ml solution of lysozyme was placed in the inner dialysis chamber, and the time dependence of the protein concentration diffusing into the outer dialysis chamber, containing 2.2 ml of the solvent, was monitored at 240 nm in a spectrophotometer (model 46 BCD, Perkin-Elmer Corp., Instruments Div., Norwalk, Conn.). The experimental data were plotted according to the first-order kinetic expression

$$\log (A_f - A) = \log A_f + 0.4343.k_1.t, \quad (1)$$

where A corresponds to the recorded absorbance; A_f is the absorbance at dialysis equilibrium; and k_1 is the first-order velocity constant (1).

The porosity of the cellulose membrane was increased, when necessary, by stretching it in a 64% zinc chloride solution (19).

The far ultraviolet spectra of protein at different concentrations were recorded in a DMR 21 double-beam spectrophotometer (Carl Zeiss, FRG) flushed with nitrogen and equipped with a cell holder with a thermostat. All spectrophotometric measurements were made at 25°C,

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with an optical path length of 10 mm. The slit width in no case exceeded 0.2 mm.

RESULTS

The effect of lysozyme concentration from 0.07 to 12.5 μg/ml on lysozyme lytic activity on *M. lysodeikticus* at pH 6.4 and $\mu = 0.01$ is represented by a biphasic curve (Fig. 1). In the first phase of the curve, the activity shows a linear dependence on protein concentration up to 0.7 μg/ml, while in the second phase the activity is progressively reduced with the increase in protein concentration in such a way that at values higher than 12.5 μg/ml no activity can be detected. Similar activity-concentration behavior at pH 6.6 and $\mu = 0.1$ has been reported (20). This inhibitory effect of increasing the concentration might be interpreted as being due to the head-to-tail contact that occurs in the self-association of the protein. However, since the relationship between the decay of biological activity and the degree of association is unknown (for example, whether total or partial inhibition of activity occurs during dimerization), changes in activity cannot be taken as an indicator of self-association.

Dialysis at pH 6.0 shows a first-order kinetic behavior of three components (Fig. 2). The experimental dialysis data at times higher than 120 min show a slow dialysis component (C_S). Fig. 2 represents the dialysis behavior of components with higher dialysis velocity, C_{F1} and C_{F2}. They are calculated from the difference between the extrapolated straight line back to 0 time and the experimental points at $t < 140$ min. The first-order velocity constants are 1.1×10^{-3} min^{-1}, 17.4×10^{-3} min^{-1}, and 25.0×10^{-3} min^{-1} for C_S, C_{F1}, and C_{F2}, respectively. The component concentrations at zero dialysis time correspond to 83.9, 7.8, and 8.3%, respectively.

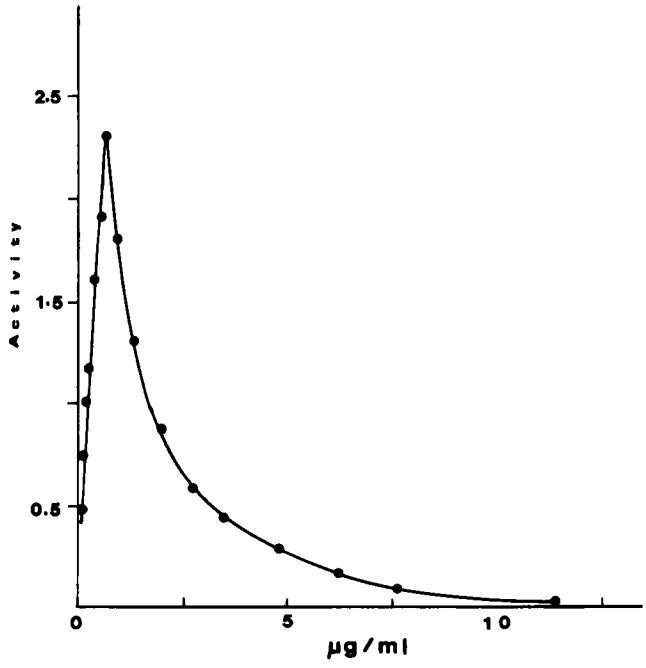

FIGURE 1 Effect of lysozyme concentration on lysosome lytic activity on *Micrococcus lysodeikticus*. The biological activity measurements were accompanied by a decrease in absorbance at 450 nm by bacteria cells at pH 6.4 and $\mu = 0.01$.

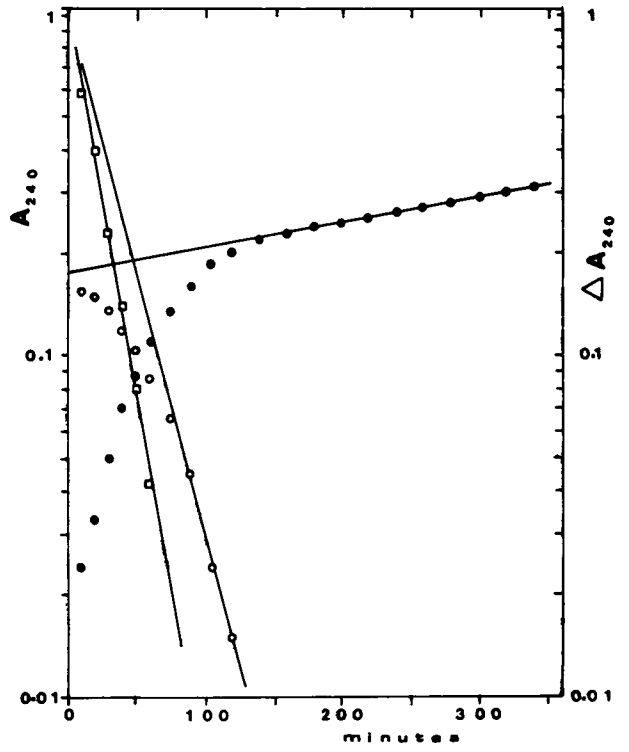

FIGURE 2 Kinetics of dialysis of lysozyme (1 mg/ml) at pH 6. The filled circles represent the experimental data monitored at 240 nm. A slow dialysis component C_S, with $k_1 = 1.1 \times 10^{-3}$ min^{-1} and a concentration at zero dialysis time of 83.9% is characteristic. Two faster dialysis components C_{F1} (○) and C_{F2} (□), are obtained by the ΔA_{230} between the extrapolated strength of C_S and the corresponding points of the experimental data.

To test whether the three protein components are molecular forms of lysozyme at different association degrees, the dialysis of this protein at 1 mg/ml was performed in the presence of 10^{-3} M and 10^{-2} M GlcNAc, an inhibitor of the self-association process (13, 14) (Fig. 3). The correspondence between the decrease in C_{F1} and C_{F2} and the increase in C_S indicates that the three components separated by dialysis are indeed lysozyme species of different degrees of association. Thus, the slow dialysis component might correspond to the monomeric form of lysozyme, while the fast dialysis components may be associated products. The apparently contradictory dialysis behavior of monomers (C_S), which have smaller Stokes radii than the associated species, can be explained by the adsorption of monomers on the cellulose membrane surface, as has been reported for several basic peptides and proteins (8, 17, 19–23).

The effect of other inhibitors of the lytic activity of this macromolecule (24) on the equilibrium among C_S, C_{F1}, and C_{F2} was also tested by dialysis studies (Table I). The rapid decrease in the concentration of the fast dialysis compo-

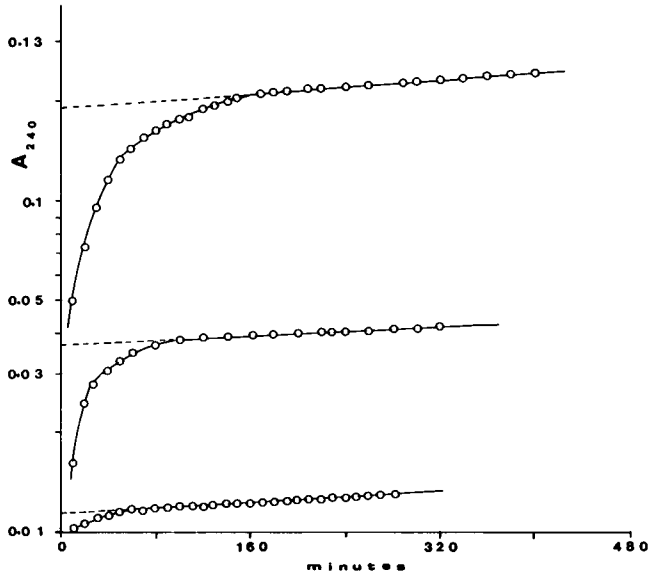

FIGURE 3 Effect of GlcNAc on the kinetics of dialysis of 1 mg of lysozyme at pH 6. The upper, middle, and lower curves represent the dialysis of lysozyme through a stretched cellulose membrane in water, in the presence of 10^{-3} M, and in 10^{-2} M GlcNAc, respectively. Their corresponding k_1 values of C_S are 30×10^{-5} min^{-1}, 3.2×10^{-5} min^{-1}, and 1.6×10^{-5} min^{-1}, respectively, and the concentrations of C_S and C_{FI} plus C_{F2} at zero dialysis time are 82.6 and 17.4%, 96.5 and 3.5%, and 99 and 1%, respectively.

nent caused by these compounds supports the validity of previously reported head-to-tail mechanism (13, 14).

On the other hand, the dependence of maximum absorbance (A_{max}) of the far ultraviolet absorption bands of lysozyme at pH 6 on the protein concentration is shown in Fig. 4. From 2.5 to 9.5 μg/ml the plot is linear, whereas at higher protein concentrations it is nonlinear. The hypochromic effect causing the nonlinear region of the plot reflects the masking of chromophores in the self-associated species and also represents the self-association degree. However, this hypochromicity can be abolished if the spectra in the same protein concentration range are recorded in the presence of 0.4 M urea.

TABLE I
EFFECT OF SELF-ASSOCIATION INHIBITORS ON THE PERCENT OF SLOW AND FAST DIALYSIS COMPONENTS SEPARATED BY DIALYSIS

Inhibitor	$\%C_S$	$\%C_F$	$\%C_F$ disappearing
	82.6	17.4	0
0.01 M GlcNAc	99.0	1.0	93.5
0.001 M GlcNAc	96.5	3.5	80.0
0.005 M L-Trp	99.9	0.1	99.4
0.005 M indole-3-acetic acid	99.9	0.1	99.4
0.1 M L-His	99.9	0.1	99.4
0.1 M imidazole	99.9	0.1	99.4

All experiments were performed with 1 mg of lysozyme at room temperature and cellulose membrane stretched as described in Methods.

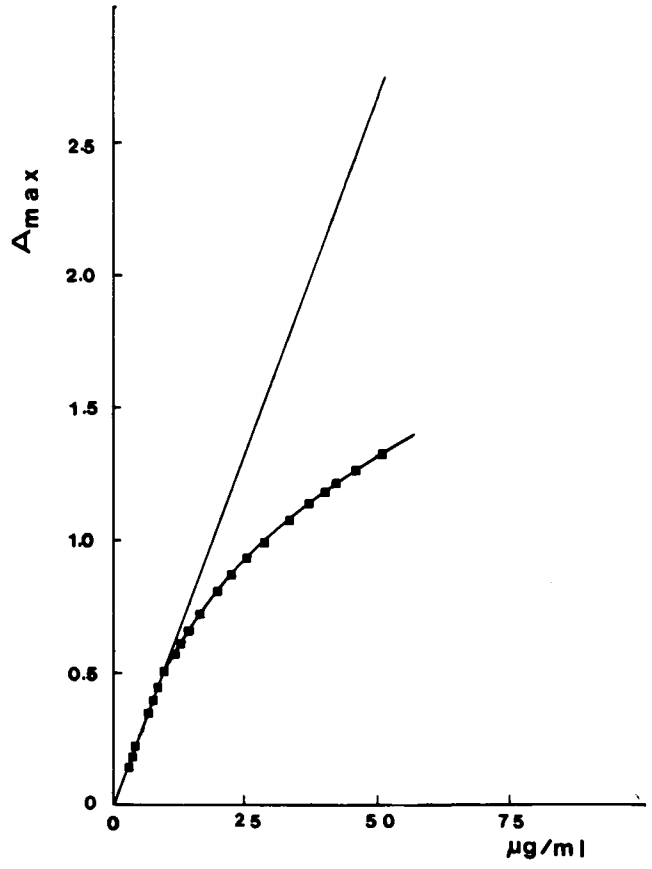

FIGURE 4 Effect of lysozyme concentration at pH 6, on the maximum absorbance values of the far UV absorption bands (196–205 nm). All protein spectra were recorded at 25°C.

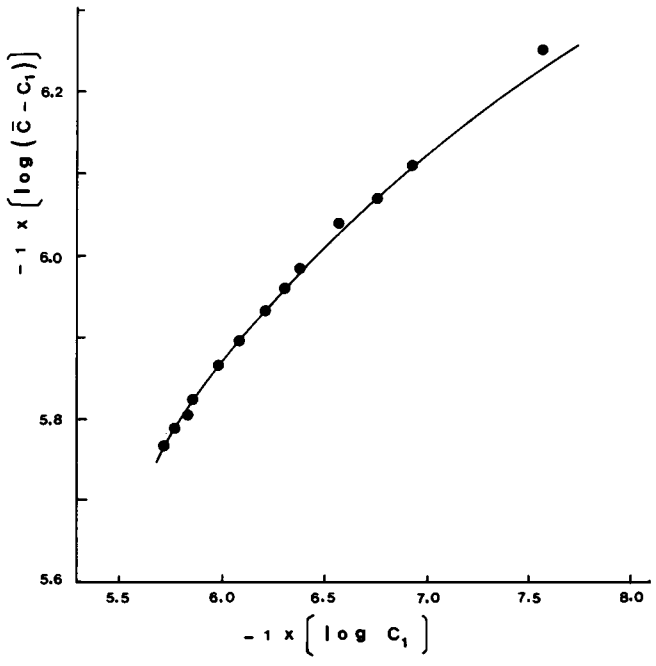

FIGURE 5 Double logarithmic plot of the molar concentration of the associated species of lysozyme ($\bar{c} - c_1$), against the monomer species c_1 with data from Fig. 4.

MODELS

The relative concentrations of monomeric lysozyme and of all its self-associated species can be obtained from the spectrophotometric experiments represented in Fig. 4. For this purpose we assume that the experimental A_{max} values (filled squares) correspond to the monomer concentration c_1; the extrapolated line of the curve represents the concentration of monomers plus all associated species of the protein $\bar{c}$; and the difference between $\bar{c}$ and $\bar{c}_1$ represents the concentration of the associated species in monomer concentration units ($\bar{c} - c_1$).

In the simplest case, where monomers are in equilibrium with a definite associated species P_n, ($nP \rightleftharpoons P_n$), a plot of log c_1 against log ($\bar{c} - c_1$) should be linear and this is found not to be the case (Fig. 5). Therefore, one can exclude such simple monomer-dimer, or monomer-n-mer equilibrium alone and instead search for models of an equilibrium that also contains larger associated species.

The indefinite self-association is considered a more realistic situation, especially for a head-to-tail mechanism of contact. The model in which all constants of association are equal, i.e., $K_2 = K_3 = K_4 = \ldots = K$, is studied by applying expression 2 to the experimental data of Fig. 4:

$$\bar{c} = 2Kc_1^2 + 3K^2c^3 + \ldots nK^{n-1}c_1^n + \ldots, \quad (2)$$

where $c_1, c_1^2, c_1^3, \ldots$ are the concentrations of the monomeric, dimeric, trimeric, ... protein species, and K is the ideal association constant.

Inasmuch as the relationship between c_1 and $\bar{c}$ obtained from the model for $K = 0.537$, $K = 0.233$, and $K = 0.300$ chosen arbitrarily, does not fit the experimental data well, we can exclude this mechanism too.

Another model involving a two-stage association represented by expression 3 is analyzed using Eq. 4:

$$c_1 \overset{K_n}{\rightleftharpoons} c_1^n \overset{K_m}{\rightleftharpoons} c_1^m \quad (3)$$

and

$$\bar{c} = c_1 + Kc_1^n + K'c_1^m, \quad (4)$$

where K is related to the n-merization constant by $K = nK_n$ and K' to the m-merization constant by $K' = mK_n^m.K_m$.

The plots of c_1 vs. $\bar{c}$ obtained with $n = 2$ and $m = 4$ with the K and K' values of 0.01 and 0.314, 0.07 and 0.2, and 0.01 and 0.24, respectively; with $n = 2$ and $m = 6$, $K = 0.05$ and $K' = 0.54$; and with $n = 2$ and $m = 8$, with $K = 0.02$ and $K' = 0.93$ also do not fit the experimental data well. In all these cases a great dependence of K and K' on the total protein concentration was found.

Several other n and m values were also tested in the model, and all of them have shown a dependence of K and K' on the total protein concentration. The curves of c_1 against $\bar{c}$ obtained with $n = 2$ and $m = 5$; $n = 3$ and $m = 4, 5, 6$; $n = 4$ and $m = 6, 8$; $n = 6$ and $m = 8$ for several K and K' values also are not adequately adjusted to the experimental data.

Nevertheless, the treatment of the experimental data with Eq. 4 using values of 2 and 3 for n and m, respectively, results in values of 2.5×10^{-2} and 0.38 for k_2 and k_3, respectively, which are not dependent on the total protein concentration (Fig. 6 *A* and *B*). In this case, the plot of c_1 against $\bar{c}$ shows that the curve which is representative of the model, fits the experimental data very well with a standard deviation of ±0.02 (Fig. 7).

Using a nonlinear regression method, the experimental data were fitted to a model represented by Eq. 5 to test the possibility of an additional self-associated species q in equilibrium with monomers, dimers, and trimers:

$$\bar{c} = c_1 + Kc_1^n + K'c_1^m + K''c_1^q. \quad (5)$$

Keeping values of 2 and 3 for n and m, respectively, and attributing to q values of 4, 5, 6, 7, and 8, in the plots of c_1 vs. $\bar{c}$ it appears that the additional term is not needed to represent the self-association of lysozyme.

The dimerization and trimerization constants were also obtained from the concentration of the three components separated on dialysis by applying expressions 6 and 7:

$$K_2 = [P_2]/[P_1]^2 \quad (6)$$

$$K_3 = [P_3]/[P_2][P_1]. \quad (7)$$

For this purpose, the concentrations of monomers, dimers, and trimers were taken as those of c_s, c_{F1}, and c_{F2}, respectively, and the values of 1.18×10^{-3} and 1.12×10^{-2} were found for K_2 and K_3, respectively.

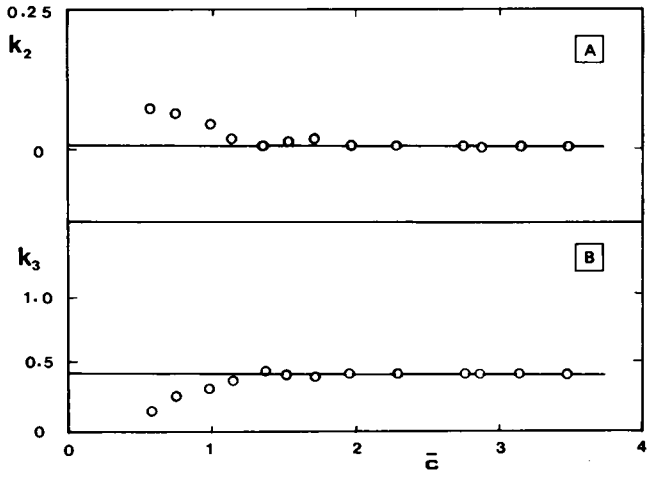

FIGURE 6 Ideality behavior of dimerization and trimerization constants on the total protein concentration for the model represented by expression 4 with $n = 2$ and $m = 3$. The k_2 and k_3 values found are of 2.5×10^{-2} and 0.38, respectively.

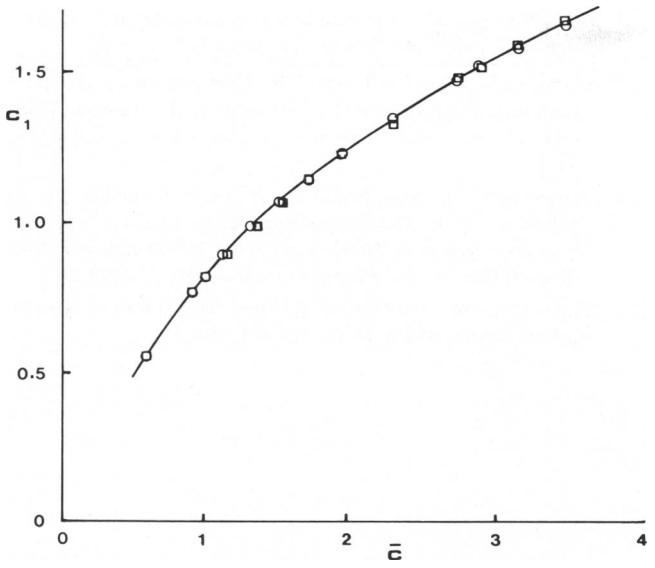

FIGURE 7 Plot of c_1 versus $\bar{c}$ for the model represented by expression 4 with $n = 2$ and $m = 3$. The curve representative of the model was calculated using values from Fig. 6 for k_2 and k_3. It shows a small standard deviation of ±0.02 using the experimental data of Fig. 4.

DISCUSSION

Using both the spectrophotometric and the dialysis data for lysozyme self-association studies at pH 6 and 25°C, a two-equilibrium association mechanism involving monomers, dimers, and trimers is proposed. However, the results may differ in dimerization and trimerization constants, which depend on whether the spectrophotometric data or the dialysis data are used. This can be explained by the nature of these two methods. Whereas a stable association equilibrium for each of several different protein concentrations is evaluated from the spectrophotometric measurements, the separation dialysis method is accompanied by a strong adsorption of monomers on cellulose membrane involving a re-equilibrium between the associating species. When the best fit of the experimental data is obtained for the model in which K and K' are not dependent on the total protein concentration (Fig. 6 *A* and *B*), the association is referred to as an ideal or nearly ideal monomer-dimer-trimer type (25). This mechanism is in accord with that proposed by Derechin (6) in which the sedimentation equilibrium data were analyzed using the multinomial theory. Our experimental data rule out the other mechanisms proposed to describe the self-association of lysozyme (1, 4, 8).

On the other hand, additional evidence for the contact mechanism of self-association is inferred from the dialysis studies performed in the presence of compounds that inhibit lytic activity of lysozyme (Table I). *N*-acetyl-D-glucosamine promotes a competitive inhibition at subsite C of the active site of the enzyme (head site) (26, 27). Indole and imidazole derivatives cause inhibition of self-association at both the head and tail sites, as was suggested by crystallographic studies of the diffusion of these compounds into lysozyme crystals (24).

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