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Effects of Cations on Affinity of Calmodulin for Calcium: Ordered Binding of Calcium Ions Allows the Specific Activation of Calmodulin-Stimulated Enzymes†

Jacques Haiech,* Claude B. Klee, and Jacques G. Demaille

Appendix: Theoretical Approach to Study of Multiple Ligand Binding to a Macromolecule

Jacques Haiech

ABSTRACT: The acid stability of calmodulin has been used to devise a rapid and efficient method of decalcification based on trichloroacetic acid precipitation. Study of the competitive binding of K^+, Mg^{2+}, and Ca^{2+} to the Ca^{2+}-binding sites of calmodulin has allowed determination of the intrinsic binding constants of each of the three cations for the four Ca^{2+}-binding sites. The data are compatible with an ordered binding of Ca^{2+}. If the Ca^{2+} sites are labeled A, B, C, and D starting at the NH_2 terminus, the order of binding is postulated to be B, A, C, and D. The ordered binding properties support the suggestion that calmodulin translates quantitative Ca^{2+} signals into qualitatively different cellular responses.

Calmodulin, a heat-stable Ca^{2+}-binding protein responsible for the Ca^{2+} stimulation of cyclic nucleotide phosphodiesterase (Cheung, 1970; Kakiuchi, 1970), is now known to play a central role in the Ca^{2+}-dependent regulation of eukaryotic cells. [For reviews, see Wolff & Brostrom (1979), Wang & Waisman (1979), Cheung (1980), Means & Dedman (1980), and Klee et al. (1980).] A basic scheme for the stimulation of enzyme by calmodulin was initially proposed for cAMP[1] phosphodiesterase

step 1

$$CaM + nCa^{2+} \rightleftharpoons CaM{\cdot}Ca_n^{2+} \rightleftharpoons CaM^*{\cdot}Ca_n^{2+}$$

step 2

$$CaM^*{\cdot}Ca_n^{2+} + E \rightleftharpoons E{\cdot}CaM^*{\cdot}Ca_n^{2+} \rightleftharpoons E^*{\cdot}CaM^*{\cdot}Ca_n^{2+}$$

where the asterisk indicates the active species. As a first step toward the understanding of the overall mechanism of calmodulin-mediated stimulation of enzymatic reactions, a study of step 1 was undertaken. The Ca^{2+}-binding properties of the purified proteins were first reported by Teo & Wang (1973) and subsequently examined by others (Lin et al., 1974; Watterson et al., 1976; Klee, 1977; Wolff et al., 1977; Jarrett & Kyte, 1979). Although all studies indicate the presence of four specific Ca^{2+}-binding sites, there are some discrepancies between different reports about the affinities of the different sites for Ca^{2+} and, more particularly, about the effect of Mg^{2+} on the binding constants. Therefore, Ca^{2+} binding to calmodulin was studied in the presence of various concentrations of H^+, K^+, and Mg^{2+} ions.

Materials and Methods

Trichloroacetic acid (Cl_3CCOOH) was obtained from Eastman-Kodak. All other reagents were as previously described (Klee, 1977; Haiech et al., 1979; Crouch & Klee, 1980). Calmodulin was purified from ram testes according to Autric et al. (1980). The calmodulin concentration was measured as previously described (Crouch & Klee, 1980).

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[1] Abbreviations used: cAMP, cyclic adenosine monophosphoric acid; CaM, calmodulin; Cl_3CCOOH, trichloroacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane; UV, ultraviolet.

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Removal of contaminating metals from buffer solutions was as previously described (Haiech et al., 1979; Crouch & Klee, 1980).

Determination of divalent cations was performed with a Perkin-Elmer Model 5000 atomic absorption spectrophotometer equipped with a graphic furnace Model 500 and an auto-sampler Model AS40. Pyrocoated rods were used when high sensitivity was necessary with the following program: step 1, temperature 120 °C, hold time 30 s, ramp time 1 s; step 2, temperature 1100 °C, hold time 20 s, ramp time 1 s; step 3, temperature 2500 °C, hold time 5 s, ramp time 2 s. Recording was started 2 s before step 3 and reading with the high peak method 1 s after the beginning of step 3.

Flow dialysis experiments were performed as described previously (Haiech et al., 1979, 1980). The dialysis cell described by Feldman (1978) was built with Teflon upper and lower compartments and was thermostated at 25 ± 1 °C. The lower chamber (0.1–0.2 mL) was perfused at a flow rate of 240 mL/h with a Pharmacia P3 peristaltic pump. A hydraulic buffer was used to minimize variations in flow rate. Fractions were collected every 36 s. The response time of the cell was within 72 s. Five fractions were collected after each addition of ligand, and 1 mL from each of the three last fractions was counted in 10 mL of Aquasol (New England Nuclear) or 10 mL of Ultrafluor (National Diagnostic). The average of these three values was used. The experiment was completed within <50 min. Less than 1–2% of the radioactivity added to the upper chamber at the start of the experiment (5 μCi of $^{45}CaCl_2$) was lost during the experiment; no correction for ligand depletion was therefore needed.

The data were analyzed as described by Colowick & Womack (1969). One given experiment gives a set of data (ν, x) where ν is the average number of moles of ligand bound per mole of protein and the free concentration of ligand is x. Each set of data was then fitted to the general Adair–Klotz equation by using the approach of Fletcher et al. (1970)

$$\nu = \frac{\beta_1 x + \ldots + n\beta_n x^n}{1 + \beta_1 x + \ldots + \beta_n x^n} \quad (1)$$

where $\beta_1, \ldots, \beta_n$ are the apparent macroscopic binding constants. For an analysis of the effect of various cations on these constants, see Appendix.

Results

Calcium Removal from Calmodulin. Calmodulin can be renatured after trichloroacetic acid precipitation (Yazawa et al., 1980). Since low pH decreases the affinity of calmodulin for Ca^{2+} (Figure 1), the following method was used to free calmodulin of calcium. Calmodulin (20 mg) was dissolved in 1 mL of deionized water, and 30 μL of a fresh solution of 100% Cl_3CCOOH (w/v) was added. The suspension was left on ice 5 min and centrifuged at 3000 rpm for 2 min in a table-top centrifuge. The pellet was resuspended in 160 μL of fresh solution of 1 M Tris and diluted to a final volume of 5 mL with deionized water. The protein was then precipitated 3 times by addition of 160 μL of 100% Cl_3CCOOH as described above. After the last cycle, the pellet was resuspended in 1 mL of 100 mM Hepes–KOH[1] buffer, pH 7.55, containing 0.15 M KCl. Alternatively, resuspension was made in 100 μL of 1 M Tris, the volume was adjusted to 1 mL with water, and the pH was adjusted to 7.55 with HCl.

The protein recovery varied between 80 and 95%, and the amount of residual Ca^{2+} per mole of protein was <0.04 mol. During the experiment great care must be taken to remove Cl_3CCOOH since its decomposition products can alter the spectroscopic properties of the protein. The pellet must be gently washed with water at the expense of protein recovery if spectroscopic studies have to be performed. The UV-absorption spectra of native calmodulin and of Cl_3CCOOH-treated calmodulin supplemented with Ca^{2+} were indistinguishable. The isoelectric points of both samples are identical when measured by isoelectric focusing (data not shown). Circular dichroic and fluorescence spectra of both samples were also similar (M. C. Kilhoffer, personal communication). The Ca^{2+}-free protein solution was stored at −20 °C and used within 3–4 days. Storage for 2 months at −20 °C resulted in a decrease in the number of calcium binding sites.

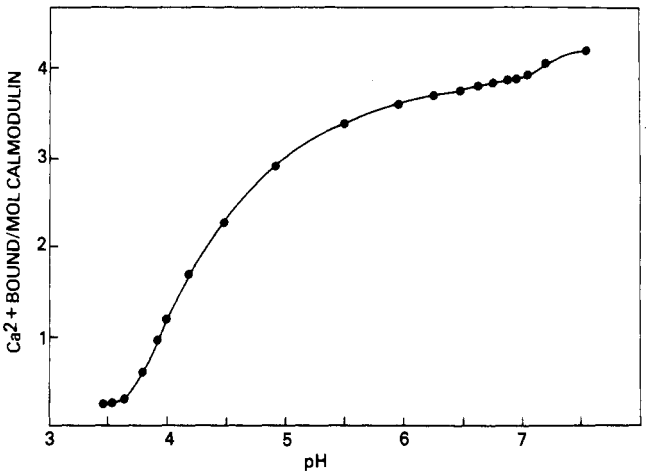

FIGURE 1: Effect of pH on affinity of calmodulin for Ca^{2+}. The number of moles of calcium bound per mole of protein was monitored by flow dialysis. The pH was adjusted by successive additions of HCl. The total increase in volume due to addition of HCl was <5%, and therefore the negligible decrease in calmodulin concentration was not corrected. The calmodulin concentration was 3.9×10^{-5} M in 10 mM Hepes–KOH buffer, pH 7.55, and 15 mM KCl. T = 25 °C. The Ca^{2+} concentration was 2.15×10^{-4} M.

Table I: Effect of K^+ on Macroscopic Constants Describing Ca^{2+} Binding to Calmodulin[a]

KCl (mM)	K_1 (μM)	K_2 (μM)	K_3 (μM)	K_4 (μM)	K_5 (μM)
0	0.13	0.14	0.60	1.3	80
20	0.37	0.46	1.6	8.5	460
40	0.73	0.79	3.8	31	320
100	2	1.9	7.3	61	ND[b]
200	3.7	3.3	22[c]	580[c]	ND[b]
k' (mM):[d]	4	11	9	1.5	
r^2:	1.0	1.0	0.99	0.98	

[a] Experimental conditions were as described in legend to Figure 2. [b] At these K^+ concentrations, we were unable to detect more than four sites. [c] These numbers were not taken into account in the determination of k' and r^2. [d] k' is the intrinsic binding constant of K^+ for each Ca^{2+} site.

Effect of K^+ on Ca^{2+} Binding to Calmodulin. Effects of K^+ on calcium binding to calmodulin were studied by using the flow dialysis technique as described under Materials and Methods (Figure 2). The macroscopic constants giving the best fit for each set of data are given in Table I. K^+ ions decrease the affinity of calmodulin for Ca^{2+}, even at concentrations as low as 20 mM. The data for the four Ca^{2+} binding sites were fitted with the eq 2. A linear regression analysis $1 \leq i \leq 4$

$$\frac{\beta_{i-1}^{app}}{\beta_i^{app}} = \frac{\beta_{i-1}}{\beta_i}(1 + [K^+]k_i') \quad (2)$$

of the data $(\beta_{i-1}^{app}/\beta_i^{app}, [K^+])$ gave values for k_i' (see Ap-

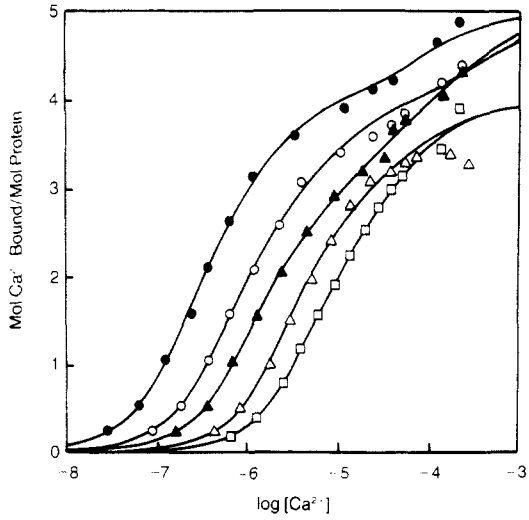

FIGURE 2: Effect of KCl on affinity of calmodulin for Ca^{2+}. The average number of Ca^{2+} ions (moles bound per mole of protein) is represented as a function of the negative logarithm of the free ligand concentration. The calmodulin concentration was 3.8×10^{-5} M. The Ca^{2+} content of the Cl_3CCOOH-treated calmodulin was $<10^{-6}$ M and was not taken into account. The buffer solution was 10 mM Tris-HCl, pH 7.55. T = 25 °C. KCl concentrations were as follows: 0 mM KCl (●); 20 mM KCl (○); 40 mM KCl (▲); 100 mM KCl (△); 200 mM KCl (□). The solid lines were drawn by using the general Adair–Klotz equation and the constants reported in Table I.

Table II: Effects of Mg^{2+} on Macroscopic Constants Describing Ca^{2+} Binding to Calmodulin[a]

$MgCl_2$ (mM)	K_1 (μM)	K_2 (μM)	K_3 (μM)	K_4 (μM)
0	0.38	0.47	1.85	10.4
1	1.6	0.66	30	4.3
5	4.3	5.1	79	36
10	9.2	8.9	170	51
25	28	16	560[b]	70[b]
50	47	38	480[b]	230[b]
k' (mM):[c]	0.6	0.6	0.4	1.5
r^2:	0.99	0.99	0.99	0.93

[a] Experimental conditions are described in legend to Figure 3. K_5 was not determined with sufficient certainty to be used in the determination of K_1, K_2, K_3, and K_4. Moreover, the additions of a fifth term to the eq 1 would not significantly alter the values of K_1, K_2, K_3, and K_4. [b] These values were not used for the determination of k' and r^2. [c] k' is the apparent binding constant of Mg^{2+} for each Ca^{2+} site in presence of 20 mM K^+.

pendix) and the coefficient of correlation r^2 which permit an estimation of the fitness of the model used for calculation of the binding constants. The values of k_i' and r^2 are reported in Table I. Nonspecific Ca^{2+} binding was also detected but disappeared upon addition of K^+. Such binding has also been reported by Jarrett & Kyte (1979). The competition experiments indicate that the Ca^{2+} sites bind K^+ competitively. The lower value for k_1' (4×10^{-3} M) compared to the k_2' (11×10^{-3} M) indicates that an increase in K^+ concentration results in an increase of the apparent positive cooperativity between the two first sites, shown to be present at 0.15 M K^+ (Crouch & Klee, 1980).

Effects of Mg^{2+} on Ca^{2+} Binding to Calmodulin at Low K^+ Concentration. The effect of Mg^{2+} on the macroscopic constants characterizing the binding of Ca^{2+} to calmodulin was first studied at low K^+ concentration (10 mM Hepes–KOH buffer, pH 7.55, 15 mM KCl, i.e., 20 mM K^+). The results are shown in Figure 3. The effect of Mg^{2+} is similar to that of K^+. The binding constants for Mg^{2+}, calculated as discussed above, are summarized in Table II. Both K^+ and Mg^{2+}

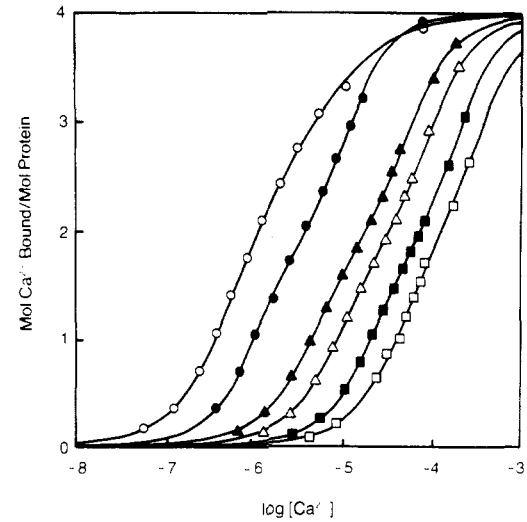

FIGURE 3: Effect of Mg^{2+} on affinity of calmodulin for Ca^{2+}. The same representation is used as in Figure 2. The calmodulin concentration was 5.7×10^{-5} M. The buffer was 10 mM Hepes–KOH, pH 7.55, and 15 mM KCl. T = 25 °C. The Mg^{2+} concentration was adjusted with $MgCl_2$ in the protein solution and in the flow dialysis buffer as mentioned for each curve: 0 mM Mg^{2+} (○); 1 mM Mg^{2+} (●); 5 mM Mg^{2+} (▲); 10 mM Mg^{2+} (△); 25 mM Mg^{2+} (■); 50 mM Mg^{2+} (□).

Table III: Effects of Mg^{2+} on Macroscopic Constants Describing Ca^{2+} Binding to Calmodulin[a]

$MgCl_2$ (mM)	K_1 (μM)	K_2 (μM)	K_3 (μM)
0	3.7	12	33
5	7.0	56	67
10	10	40	160
25	18	67	630[b]
50	42	190	170[b]
100	91[b]	110[b]	
k' (mM):[c]	3.0	5.0	2.0
r^2:	0.98	0.91	0.94

[a] Experimental conditions are described in legend to Figure 4. [b] These values were not used for the determination of k' and r^2. [c] k' is the apparent binding constant of Mg^{2+} for each Ca^{2+} site in presence of 200 mM K^+.

appear to compete with Ca^{2+} at the specific Ca^{2+}-binding sites. To confirm this conclusion, we studied further the effects of Mg^{2+} on Ca^{2+} binding to calmodulin at high K^+ concentration.

Effects of Mg^{2+} on Ca^{2+} Binding to Calmodulin at High K^+ Concentrations. The effects of Mg^{2+} at high K^+ concentrations are shown in Figure 4 and Table III (100 mM Hepes–KOH buffer, pH 7.55, 150 mM KCl, i.e., 200 mM K^+). Under these conditions, we were unable to titrate more than three specific Ca^{2+} sites. We also noticed a disappearance of the apparent positive cooperativity between the two first sites. Differences observed between the values of K_1, K_2, K_3, and K_4 shown in Table I (200 mM KCl) and Table III (0 mM $MgCl_2$) could be explained by a difference in the buffer used for the two experiments (100 mM Hepes as opposed to 10 mM Tris). Although Mg^{2+} ions under these conditions decrease the affinity of calmodulin for Ca^{2+}, the apparent dissociation constants for Mg^{2+} are higher than those obtained at low K^+ concentrations. This result can be predicted on the basis of a simple competition between K^+ and Mg^{2+} for the same sites.

Discussion

In order to study the binding of Ca^{2+} to calmodulin, we have analyzed the binding of different cations, Ca^{2+}, Mg^{2+}, and K^+, to the protein. If these ions interact differently with the different Ca^{2+}-binding sites, information can be obtained about

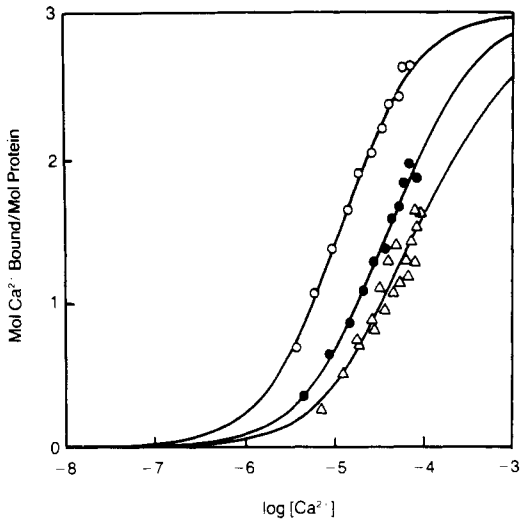

FIGURE 4: Effect of Mg^{2+} on affinity of calmodulin for Ca^{2+}. The same representation is used as in Figure 2. The calmodulin concentration was 2.87×10^{-5} M. The buffer was 100 mM Hepes–KOH, pH 7.55, and 150 mM KCl. T = 25 °C. The Mg^{2+} concentration was adjusted with $MgCl_2$ in the protein solution and in the flow dialysis buffer as indicated for each curve: 0 mM Mg^{2+} (○); 10 mM Mg^{2+} (●); 25 mM Mg^{2+} (△).

the specific Ca^{2+}-binding characteristics of each of the four sites.

To analyze the experimental data, it was found necessary to develop general equations describing the interaction of one macromolecule with r ligands L^1, ..., L^r on n_1, ..., n_r sites, respectively. Specific models applicable to Ca^{2+} binding to calmodulin are presented under Appendix.

The experimental data were found to be in good agreement with the following model. First, calmodulin presents four sites, each of them binds Ca^{2+}, Mg^{2+}, and K^+. The three cations bind competitively on the same sites. Second, in the Ca^{2+}-free state, one of the four sites exhibits a significantly higher affinity for Ca^{2+}, as compared with the other three sites. Third, the binding of Ca^{2+} but not of Mg^{2+} and K^+ follows a sequential pathway. A similar scheme was recently proposed for all calcium-binding proteins (Reid & Hodges, 1980).

Even though all theoretical schemes and experimental data are in agreement with the model proposed herein, it is impossible to exclude entirely the possibility that other mechanisms may actually be true. For instance, the presence of additional low-affinity K^+ binding sites cannot be excluded, pending a direct determination of K^+ and Mg^{2+} binding properties, now under way.

On the basis of this model, the intrinsic binding constants of each site for each ion were calculated (Table IV). These data show that discrepancies between the different affinity constants reported in the literature can be easily explained on the basis of the different experimental conditions used in various laboratories. As previously reported (Lin et al., 1974; Watterson et al., 1976; Klee, 1977; Wolff et al., 1977; Crouch & Klee, 1980), we observed four Ca^{2+}-specific sites and under specific conditions additional nonspecific sites (Jarrett & Kytes, 1979) which are probably not physiologically relevant. The four specific Ca^{2+}-binding sites exhibit site–site interaction and bind K^+ with affinities from 10^2 to 10^3 M^{-1}. This is in contrast to the Ca^{2+}-binding sites of parvalbumin, another calcium-binding protein of the same class, which binds monovalent cations weakly ($K_d > 10^{-2}$ M) (Haiech et al., 1979). In the case of calmodulin, the binding of K^+ is responsible for the apparent positive cooperativity between the two first sites which was reported previously (Crouch & Klee, 1980). K^+ indeed binds more strongly to the first than to the second site (Table IV). Therefore, an increase of K^+ concentration results in an increase of the apparent positive cooperativity. In a physiological medium (0.15 M KCl), this apparent positive cooperativity may allow a steeper activation of the calmodulin-dependent enzymes when the Ca^{2+} levels increase upon cell stimulation (Crouch & Klee, 1980).

Table IV: Intrinsic Binding Constants of Calmodulin for Ca^{2+}, K^+, and Mg^{2+} [a]

sites	1 [b]	2 [b]	3 [b]	4 [b]
Ca^{2+}	0.67×10^{-7}	1.7×10^{-7}	6.0×10^{-7}	9.0×10^{-7}
K^+	3.7×10^{-3}	10.6×10^{-3}	8.7×10^{-3}	1.5×10^{-3}
Mg^{2+}	0.70×10^{-4}	2.7×10^{-4}	1.0×10^{-4}	0.90×10^{-4}

[a] Intrinsic binding constants (M) derived from the theoretical model described under Appendix. $1/\beta, \beta_1/\beta_2, \beta_2/\beta_3, \beta_3/\beta_4$. The binding constants for Ca^{2+} were extrapolated from data to Table I. The binding constants from Mg^{2+} were derived from the data obtained at 20 and 200 mM K^+ by assuming a simple competition between K^+ and Mg^{2+}. [b] The numbers indicate the Ca^{2+}-binding sites according to the order in which they are saturated by Ca^{2+}.

The intrinsic dissociation constant of the four sites for Ca^{2+} is 10^{-7}–10^{-6} M. Such sites have been defined as triggering sites in a previous report (Haiech et al., 1979). Moreover, the fact that calmodulin has four Ca^{2+}-triggering sites was predicted on the basis of its primary structure (Goodman et al., 1979). In contrast, troponin C, which is structurally homologous to calmodulin, exhibits two triggering sites (Ca^{2+}-specific sites) and two relaxing sites (Ca^{2+}/Mg^{2+} sites) (Potter & Gergely, 1975). These physical differences could explain the functional differences between the two Ca^{2+}-binding proteins (Walsh et al., 1980).

The model reported herein is the simplest that is compatible with the experimental data. On this basis, the binding of Ca^{2+} to calmodulin is not only sequential but ordered, with a unique binding sequence. Similar conclusions were obtained from nuclear magnetic resonance studies of calmodulin (Seamon, 1980). However, nuclear magnetic resonance data do not allow identification of the sites which are sequentially saturated by Ca^{2+}. In our study, we have determined the affinity of each Ca^{2+}-binding site for K^+. If the affinity of a given Ca^{2+}-binding domain for K^+ is assumed to be proportional to the number of carboxyl groups in this domain, the sites can be assigned to primary structure domains on the basis of their affinity for K^+. If A, B, C, and D (from the NH_2 terminus to the COOH terminus) stand for the four Ca^{2+}-binding domains of calmodulin, the sequential pathway followed by Ca^{2+} binding can be either B → A → C → D or B → C → A → D. Domains A, B, C, and D indeed contain 4, 3, 3, and 5 COOH groups, respectively (Watterson et al., 1980). Recent experiments using Tb^{3+} as a Ca^{2+} probe show that the two first sites to bind Tb^{3+} are sites A and B (Kilhoffer et al., 1980a). If we consider that Tb^{3+} and Ca^{2+} behave similarly, the pathway followed by Ca^{2+} is B → A → C → D. In line with this scheme, Tb^{3+} binding to Octopus calmodulin was shown to occur at site C after saturation of sites A and B (Kilhoffer et al., 1980b).

Finally, the molecular mechanism of Ca^{2+} binding to calmodulin is described by a kinetic scheme with at least eight dissociation rate constants. Each calmodulin–Ca_n^{2+} complex (n = 1–4) exhibits a specific conformation (Klee, 1977; McCubbin et al., 1979; Walsh et al., 1979; Seamon, 1980). The different calmodulin-dependent enzymes may belong to different subsets E_0–E_{IV}, E_0 being the enzyme(s) that contain(s) calmodulin as a tightly bound subunit (for instance, phosphorylase kinase; Cohen et al., 1978). Therefore, upon

an increase of intracellular Ca^{2+}, the E_0 subset is activated before the E_I subset which recognizes CaM–Ca_I^{2+}, and E_I before E_{II}. A detailed description of the enzyme subsets E_0–E_{IV} and of their putative physiological behavior in the sequential activation–deactivation of Ca^{2+}-dependent regulations was presented elsewhere (Haiech & Demaille, 1981).

By this simple mechanism, calmodulin could transfer a quantitative signal into qualitatively different responses (Klee et al., 1980). Moreover, when the quantity of Ca^{2+} released in the cytoplasm saturates calmodulin, the different enzyme subsets may be sequentially activated (Haiech & Demaille, 1981). Similarly, deactivation could proceed sequentially with the help of calmodulin-binding proteins, such as calcineurin (Klee et al., 1979). This may provide the mechanistic basis for both sequential activation and deactivation of Ca^{2+}-dependent pathways in eukaryotic cells.

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Appendix: Theoretical Approach to Study of Multiple Ligand Binding to a Macromolecule

The stepwise equilibrium model (Fletcher et al., 1970) has been extended to the case of a protein able to bind r different ligands L^1, L^2, ..., L^r on n_1, ..., n_r sites. If we consider a given complex $ML_{i_1}{}^1L_{i_2}{}^2...L_{i_r}{}^r$, where i_1 is the number of ligands L^1 bound to the complex and i_r is the number of ligands L^r, the formation of this complex is described as a one-step equilibrium:

$0 \leq i_1 \leq n_1$

.
.
.

$0 \leq i_r \leq n_r$

$$M + i_1L^1 + ... + i_rL^r \rightleftharpoons ML_{i_1}{}^1...L_{i_r}{}^r$$

$$\beta_{i_1,...,i_r} = \frac{[ML_{i_1}{}^1...L_{i_r}{}^r]}{[M][L^1]^{i_1}...[L^r]^{i_r}} \qquad (1)$$

$$\beta_{0,...,0} = \frac{[M]}{[M][L^1]^0...[L^r]^0} = 1$$

If M_T is the total concentration of macromolecule, let

$$\alpha_{i_1,...,i_r} = \frac{[ML_{i_1}{}^1...L_{i_r}{}^r]}{M_T} \qquad (2)$$

$\alpha_{i_1,...,i_r}$ represents the fraction of macromolecules present in the solution as the complex $ML_{i_1}{}^1...L_{i_r}{}^r$. As

$$\sum[ML_{i_1}{}^1...L_{i_r}{}^r] = M_T \qquad \sum\alpha_{i_1,...,i_r} = 1 \qquad (3)$$

If $x_1 = [L^1]$, ..., $x_r = [L^r]$, eq 1 can be written

$$\beta_{i_1,...,i_r} = \frac{\alpha_{i_1,...,i_r}}{\alpha_{0,0,...,0}} \frac{1}{x_1{}^{i_1}x_2{}^{i_2}...x_r{}^{i_r}} \qquad (4)$$

For a given ligand L^j, the average number of ligand L^j moles bound per mole of protein, ν_j, is defined as

$1 \leq j \leq r$

$$\nu_j = \sum_{\substack{0 \leq i_1 \leq n_1 \\ 0 \leq i_r \leq n_r}} i_j\alpha_{i_1,...,i_r}$$

From eq 3 and 4

$$\nu_j = \frac{\sum_{i_1,...,i_r} i_j\beta_{i_1,...,i_r}x_1{}^{i_1}...x_r{}^{i_r}}{\sum_{i_1,...,i_r} \beta_{i_1,...,i_r}x_1{}^{i_1}...x_r{}^{i_r}} \qquad (5)$$

The binding polynomial can be defined as

$$P(x_1,...,x_r) = \sum_{\substack{0\le i_1\le n_1 \\ 0\le i_r\le n_r}} \beta_{i_1,...,i_r} x_1^{i_1}...x_r^{i_r} \quad (6)$$

To each system defined by a stepwise equilibrium model, we are able to associate a binding polynomial. Finally, eq 5 becomes

$1 \le j \le r$

$$\nu_j = \frac{x_j \dfrac{\partial P(x_1,...,x_r)}{\partial x_j}}{P(x_1,...,x_r)} \quad (7)$$

Case of a Protein Able to Bind One Ligand L on n Sites. To this system is associated a binding polynomial $P(x)$. Experimental data yield the average number of ligand L molecules bound per mole of protein, ν, for a given concentration of free ligand L, x. Experimental data were fitted with the general Adair–Klotz equation derived from (7)

$$\nu = \frac{x\dfrac{\partial P(x)}{\partial x}}{P(x)} = \frac{\beta_1 x + 2\beta_2 x^2 + ... + n\beta_n x^n}{1 + \beta_1 x + ... + \beta_n x^n} \quad (8)$$

with

$$\beta_i = \frac{[ML_i]}{[M][L]^i}$$

β_i are the macroscopic association constants. On the other hand, we can theoretically suppose that it is possible to determine on which site a ligand L is bound. In other words, the protein M can be considered as a protein able to bind n ligands L^1, ..., L^n, each on one site. The term ν_j describes the binding of ligand L^j on the jth site. To this system, we associate a binding polynomial $Q(x_1,...,x_n)$ and with our previous notations

$$\nu = \sum_{1\le j\le n} \nu_j = \sum_{1\le j\le n} x_j \frac{\dfrac{\partial Q(x_1,...,x_n)}{\partial x_j}}{Q(x_1,...,x_n)}$$

and

$$Q(x_1,...,x_n) = \sum_{\substack{0\le i_1\le 1 \\ 0\le i_n\le 1}} \beta_{i_1,...,i_n} x_1^{i_1}...x_n^{i_n} \quad (9)$$

Definition of Cooperative Factor and Intrinsic Association Constant. Let us write

$$\beta_{i_1,...,i_n} = \frac{[ML_{i_1}^1...L_{i_n}^n]}{[M]x_1^{i_1}...x_n^{i_n}} = \frac{[ML_{i_1}^1...L_{i_n}^n]}{[ML_{i_1}^1]...[ML_{i_n}^n]}[M]^{n-1}\frac{[ML_{i_1}^1]}{[M]x_1^{i_1}}...\frac{[ML_{i_n}^n]}{[M]^i x_n^n} \quad (10)$$

We define the cooperative factor associated to the complex $ML_{i_1}^1...L_{i_n}^n$ as

$$\delta_{i_1,...,i_n} = \frac{[ML_{i_1}^1...L_{i_n}^n][M]^{n-1}}{[ML_{i_1}^1]...[ML_{i_n}^n]} \quad (11)$$

Therefore, we can write (10) as

$$\beta_{i_1,...,i_n} = \delta_{i_1,...,i_n}\beta_{i_1,0,...,0}...\beta_{0,...,0,i_n}$$

Let $\beta_{1,0,...,0} = k_1$, $\beta_{0,1,0,...,0} = k_2$, and $\beta_{0,...,0,1} = k_n$, where k_i is the intrinsic association constant associated to the ith site of the protein

$$\beta_{i_1,...,i_n} = \delta_{i_1,...,i_n}k_1^{i_1}...k_n^{i_n} \quad (12)$$

Arbitrarily, the following notions were defined. Intrinsic positive cooperativity, intrinsic negative cooperativity, or no intrinsic cooperativity will be associated with the complex $ML_{i_1}^1...L_{i_n}^n$ if and only if $\beta_{i_1,...,i_n}$ is greater than 1, smaller than 1, and equal to 1, respectively. Using the previous notations, the binding polynomial Q is written

$$Q(x_1,...,x_n) = \sum_{\substack{0\le i_1\le 1 \\ 0\le i_n\le 1}} \delta_{i_1,...,i_n}k_1^{i_1}...k_n^{i_n}x_1^{i_n}...x_n^{i_n}$$

Moreover, since the ligands L^1, ..., L^n are identical, we have

$$x_1 = x_2 = ... = x_n = x$$

and

$$Q(x,...,x) = \sum_{\substack{0\le i_1\le 1 \\ 0\le i_n\le 1}} \beta_{i_1,...,i_n}k_1^{i_1}...k_n^{i_n}x^{i_1+...+i_n} \quad (13)$$

We can combine eq 8 and 13 and

$$1 + \beta_1 x + ... + \beta_n x^n = \sum_{i=0}^{n} x^i \sum_{\substack{i_1+...+i_n=i \\ 0\le i_1\le 1 \\ 0\le i_n\le 1}} \delta_{i_1,...,i_n}k_1^{i_1}...k_n^{i_n}$$

From that, we derive the equations giving the relationships between the macroscopic association constants β_i and the intrinsic association constants k_i

$1 \le i \le n$

$$\beta_i = \sum_{\substack{i_1+...i_n=i \\ 0\le i_1\le i \\ 0\le i_n\le 1}} \delta_{i_1,...,i_n}k_1^{i_1}...k_n^{i_n} \quad (14)$$

In the case where all the $\beta_{i_1,...,i_n}$ are equal to 1 (no intrinsic cooperativity)

$$\beta_i = \sum_{\substack{i_1+...+i_n=i \\ 0\le i_1\le 1 \\ 0\le i_n\le 1}} k_1^{i_1}...k_1^{i_n}$$

and the $1/k_i$'s appear as the roots of the polynomial

$$1 + \beta_1 x + ... + \beta_n x^n$$

In this case, the general Adair–Klotz equation is equivalent to

$$\nu = \frac{k_1 x}{1 + k_1 x} + ... + \frac{k_n x}{1 + k_n x} \quad (15)$$

which is the general Scatchard equation. However, it is possible that the polynomial $1 + \beta_1 x + ... + \beta_n x^n$ has n real negative roots and all the $\delta_{i_1,...,i_n}$ are not necessarily equal to 1. Therefore, the roots of this polynomial are not directly related to the intrinsic constants.

If $k = k_1 = ... = k_n$, we have

$$\beta_i = k^i \sum_{\substack{i_1+...+i_n=i \\ 0\le i_1\le 1 \\ 0\le i_n\le 1}} \delta_{i_1,...,i_n}$$

Let

$$d_i = \sum_{\substack{i_1+...i_n=i \\ 0\le i_1\le 1 \\ 0\le i_n\le 1}} \delta_{i_1,...,i_n}$$

and

$$\beta_i = K_1...K_i$$

with these notations

$$\beta_i = K_1 \ldots K_i = k^i d_i$$

$$K_i = \frac{\beta_i}{\beta_{i-1}} = \frac{d_i}{d_{i-1}} k \tag{16}$$

d_i/d_{i-1} appears equivalent to the parameters n of Saroff used by Crouch & Klee (1980).

In the absence of cooperativity, $\delta_{i_1,\ldots,i_n}$ are all equal to 1 and since C_n^i is the number of combinations made with i objects taken among n

$$\frac{d_i}{d_{i-1}} = \frac{C_n^i}{C_{n-1}^i} \tag{17}$$

The deviation of the numbers of Saroff from these equalities is indicative of the existence of cooperativity. In the general case where no assumptions exist on the intrinsic constants and the cooperative factors, eq 14 composes a system of n equations with $2^n - 1$ unknowns. Except in the case where $n = 1$, the system needs several assumptions in order to be solved. In the case of a multimeric protein, the system can be solved for a protein with two subunits with one site on each subunit.

Analysis of Effect of an Ion on Binding of Another Ion. Let us consider one macromolecule able to bind two ligands L^1 and L^2, with n_1 sites saturated by the first ligand and n_2 sites saturated by the second. The average number of moles of ligand L^1 bound per mole of protein is measured.

From (5), we have

$$\nu_1 = \frac{x \dfrac{\partial P(x,y)}{\partial x}}{P(x,y)} \tag{18}$$

where $P(x,y)$ is the binding polynomial associated to this system

$$P(x,y) = \sum_{i_1=0}^{n_1} x^{i_1} \sum_{i_2=0}^{n_2} \beta_{i_1,i_2} y^{i_2} \tag{19}$$

On the other hand, following the same reasoning as in the previous section, we say that we are able to determine to which sites the ligand L^1 or the ligand L^2 is bound. We consider that the macromolecule M is able to bind n_1 ligands $L_1^1 \ldots L_1^{n_1}$ each on one site and n_2 ligands $L_2^1 \ldots L_2^{n_2}$ each on one site. The free concentration of n_1 first ligands $L_1^1 \ldots L_1^{n_1}$ is depicted by $x_1,\ldots,x_{n_1}$ and of the n_2 other ligands by $y_1,\ldots,y_{n_2}$. We associate to this system a binding polynomial Q

$$Q(x_1,\ldots,x_{n_1},y_1,\ldots,y_{n_2}) = \sum_{\substack{0\le i_1\le 1 \\ 0\le i_{n_1}\le 1}} x_1^{i_1},\ldots,x_{n_1}^{i_{n_1}} \sum_{\substack{0\le j_1\le 1 \\ 0\le j_{n_2}\le 1}} \beta_{1_1,\ldots,i_{n1}j_1,\ldots,j_{n2}} y_1^{j_1},\ldots,y_{n_2}^{j_{n_2}} \tag{20}$$

The amount of the ligand L^1 bound to the protein is

$$\nu = \nu_{x_1} + \ldots + \nu_{x_{n1}} = \frac{x_1 \dfrac{\partial Q(x_1,\ldots,x_{n_1},y_1,\ldots,y_{n_2})}{\partial x_1} + \ldots + x_{n_1} \dfrac{\partial Q(x_1,\ldots,x_{n_1},y_1,\ldots,y_{n_2})}{\partial x_{n_1}}}{Q(x_1,\ldots,x_{n_1},y_1,\ldots,y_{n_2})} \tag{21}$$

Definition of the Coupling Factor. Following the same reasoning that we have used to define the "cooperative factor" in the previous section, we write

$$\beta_{i_1,\ldots,i_{n1}j_1,\ldots,j_{n2}} = \epsilon_{i_1,\ldots,i_{n1}j_1,\ldots,j_{n2}} \beta_{i_1,\ldots,i_{n1},0,\ldots,0} \beta_{0,0,\ldots,0,j_1,\ldots,j_{n2}} \tag{22}$$

By definition, $\epsilon_{i_1,\ldots,i_{n1}j_1,\ldots,j_{n2}}$ is called the *coupling factor* associated with the complex $(ML_{i_1}^1 \ldots L_{i_{n1}}^1 \cdot L_{j_1}^2 \ldots L_{j_{n2}}^2)$.

If we use the notations of the preceding sections for the intrinsic constants and the cooperative factors associated to the first ligand and the same notations with a prime for the second ligand, we can write

$$\beta_{i_1,\ldots,i_{n1}j_1,\ldots,j_{n2}} = \epsilon_{i_1,\ldots,i_{n1}j_1,\ldots,j_{n2}} \delta_{i_1,\ldots,i_{n1}} \delta'_{j_1,\ldots,j_{n2}} k_1^{i_1} \ldots k_{n_1}^{i_{k1}} k_1^{j_1} \ldots k_{n_2}^{j_{n2}} \tag{23}$$

The binding polynomial becomes

$$Q(x_1,\ldots,x_{n_1},y_1,\ldots,y_{n_2}) = \sum_{\substack{0\le i_1\le 1 \\ 0\le i_{n_1}\le 1}} \delta_{i_1,\ldots,i_{n1}} k_1^{1_1} \ldots k_{n_1}^{i_{n1}} x_1^{i_1} \ldots x_{n_1}^{i_{n1}} \times \sum_{\substack{0\le j_1\le 1 \\ 0\le j_{n_2}\le 1}} \epsilon_{i_1,\ldots,i_{n1}j_1,\ldots,j_{n2}} \delta'_{j_1,\ldots,j_{n2}} k_1'^{j_1} \ldots k_{n_2}'^{j_{n2}} y_1^{j_1} \ldots y_{n_2}^{j_{n2}} \tag{24}$$

If we identify the expressions 18 and 21 by using expression 24, we get the following relationships between the apparent macroscopic constants, experimentally determined, and the intrinsic binding constants:

$1 \le i \le n_1$

$$\beta_i^{app} = \frac{\displaystyle\sum_{i_1+\ldots+i_{n_1}=i} \delta_{i_1,\ldots,i_{n1}} k_1^{i_1} \ldots k_{n_1}^{i_{n1}} \sum_{j=0}^{n_2} \gamma_0^j}{\displaystyle\sum_{j=0}^{n_2} y_0^j \sum_{j_1+\ldots+j_{n_2}=j} \delta_{j_1,\ldots,j_{n_2}}' k_1'^{j_1} \ldots k_{n_2}'^{j_{n2}} \sum_{j_1+\ldots+j_{n_2}=j} \epsilon_{i_1,\ldots,i_{n1}j_1,\ldots,j_{n2}} \delta'_{j_1,\ldots,j_{n2}} k_1'^{j_1} \ldots k_{n_2}'^{j_{n2}}} \times \tag{25}$$

If we consider a protein able to bind two ligands L^1 and L^2 on n sites (as in the case of competitive binding), the coupling factors present the obvious property: if e exists such that $i_e + j_e = 2$, then

$$\epsilon_{i_1,\ldots,i_{n1}j_1,\ldots,j_{n2}} = 0$$

Equation 25 is the general expression giving the relationships between the intrinsic constants, the cooperative factors, and the coupling factors, on one hand, and the apparent macroscopic constants accessible experimentally, on the other hand. We are now going to examine some specific cases which are applicable to the binding of ions to calmodulin.

Calmodulin Models. The effects of K^+ or Mg^{2+} on the Ca^{2+} binding to calmodulin were studied. Let us consider calmodulin as a protein with four sites which bind Ca^{2+}, K^+, and Mg^{2+}. First, the coupling factors are supposed to be equal to either 0 or 1. This means that the binding of Ca^{2+} to a specific site is not dramatically altered by the binding of K^+ or Mg^{2+} to the other sites. Finally, we suppose that the cooperative factors associated with K^+ or Mg^{2+} binding to one of the four sites do not modify the binding of K^+ or Mg^{2+} on the other sites.

In this case, if we keep our previous notations, eq 25 becomes

$$\beta_1^{app} = k_1\left(\frac{1}{1 + k_1'y}\right) + k_2\left(\frac{1}{1 + k_2'y}\right) + k_3\left(\frac{1}{1 + k_3'y}\right) + k_4\left(\frac{1}{1 + k_4'y}\right)$$

$$\beta_2^{app} = \delta_{1,1,0,0} k_1 k_2 \left(\frac{1}{1 + k_1'y}\frac{1}{1 + k_2'y}\right) + \delta_{1,0,1,0} k_1 k_3 \left(\frac{1}{1 + k_1'y}\frac{1}{1 + k_3y}\right) + \ldots$$

$$\beta_3^{app} = \delta_{1,1,1,0} k_1 k_2 k_3 \left(\frac{1}{1 + k_1'y}\frac{1}{1 + k_2'y}\frac{1}{1 + k_3'y}\right) + \ldots$$

$$\beta_4^{app} = \delta_{1,1,1,1} k_1 k_2 k_3 k_4 \left(\frac{1}{1 + k_1'y}\frac{1}{1 + k_2'y}\frac{1}{1 + k_3'y}\frac{1}{1 + k_4'y}\right)$$

With this model, $1/\beta_1^{app}$ (as a function of y) gives a straight line if $k_1' = k_2' = k_3' = k_4' = k'$ or if $k_2 = k_3 = k_4 = 0$. In the first case, $\beta_1^{app}/\beta_2^{app}$, $\beta_2^{app}/\beta_3^{app}$, and $\beta_3^{app}/\beta_4^{app}$ as a function of y also give straight lines, and the intercepts on the x axis of all the curves are equal to $1/k'$. In the other case, when $k_2 = k_3 = k_4 = 0$, $1/\beta_1^{app}$ as a function of y gives us the intrinsic constants k_1 and k_1'. Then we consider the function $\beta_1^{app}/\beta_2^{app}$. A straight line, is indicative of two possibilities: (1) $k_1' = k_2' = k_3' = k_4' = k'$ (this is the case examined before) or (2) $\delta_{1,1,0,0} = +\infty$, $\delta_{1,1,0,0}k_1k_2 = \beta_2$, and $\delta_{1,0,1,0} = \delta_{1,0,0,1} = \delta_{0,1,1,0} = \delta_{0,0,1,1} = \delta_{0,1,0,1} = 0$.

This last case can be interpreted as a sequential binding of ligand L^1 to the two first sites. The same reasoning can be applied to the functions $\beta_2^{app}/\beta_3^{app}$ and $\beta_3^{app}/\beta_4^{app}$. To summarize, if we consider a sequential binding of Ca^{2+} to calmodulin detected by the scheme

$$\mathrm{CaM} \underset{}{\overset{\beta_1}{\rightleftharpoons}} \mathrm{CaMCa} \overset{\beta_2/\beta_1}{\rightleftharpoons} \mathrm{CaMCa_2} \overset{\beta_3/\beta_2}{\rightleftharpoons} \mathrm{CaMCa_3} \overset{\beta_4/\beta_3}{\rightleftharpoons} \mathrm{CaMCa_4}$$

β^{app} must obey

$$\frac{1}{\beta_1^{app}} = \frac{(1 + k_1'y)}{\beta_1} \qquad \frac{\beta_2^{app}}{\beta_3^{app}} = \frac{\beta_2(1 + k_3'y)}{\beta_3}$$

$$\frac{\beta_1^{app}}{\beta_2^{app}} = \frac{\beta_1(1 + k_2'y)}{\beta_2} \qquad \frac{\beta_3^{app}}{\beta_4^{app}} = \frac{\beta_3(1 + k_4'y)}{\beta_4} \tag{26}$$

To satisfy the last model, binding of Ca^{2+} to calmodulin must exhibit the following properties. Calmodulin must have four specific Ca^{2+} binding sites. An increase in the concentration of Mg^{2+} of K^+ must decrease the affinity of Ca^{2+} for calmodulin in a nonsaturable manner. The representation of $1/\beta_1^{app}$, $\beta_1^{app}/\beta_2^{app}$, $\beta_2^{app}/\beta_3^{app}$, and $\beta_3^{app}/\beta_4^{app}$ as a function of y must give straight lines with different intercepts on the x axis.

General Conclusions. Finally, we can draw the following conclusions. The experimental data of ion binding to a macromolecule can be analyzed by using a stepwise equilibrium model. More generally, if we have a system described by a stepwise equilibrium model, the same analysis can be done, if we can associate to each complex a signal (for instance spectrophotometric signal, average number of ligand bound per molecule, reaction rate for an enzymatic reaction, etc.). The information given by the experimental data allows us to propose a kinetic scheme, the first step in the kinetic analysis of a given system. The use of effectors (other ions, pH, temperature, etc.) acting in a different way on each step of the kinetic scheme allows the determination of the molecular mechanism of ion binding. In the case where the signal is a spectrophotometric one, some information on the molecular mechanism of conformational changes can also be obtained.

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Interaction of Apolipoprotein B from Human Serum Low-Density Lipoprotein with Egg Yolk Phosphatidylcholine†

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ABSTRACT: A binary complex of apolipoprotein B and egg yolk lecithin has been formed which contains 250–350 mol of lipid/500 000 g of protein. This particle retains many of the structural properties of native human low-density serum lipoprotein (LDL) as evidenced by the state of association of the protein, the circular dichroic spectrum, and immunological characteristics. Apolipoprotein B does not interact with lipid vesicles but rather binds a small number of phospholipid molecules in water-soluble form. This study represents the first partial reconstitution of native LDL from the delipidated apoprotein and is the initial step in a systematic investigation of the lipid binding properties of apolipoprotein B.

Apolipoprotein B (apo B)[1] from human serum low-density lipoprotein (LDL) has been delipidated by substituting a number of different detergents for the naturally occurring amphiphilic ligands (phospholipid, cholesterol, cholesteryl esters, and triglycerides) (Helenius & Simons, 1971; Ikai & Hasegawa, 1978; Steele & Reynolds, 1979a; Watt & Reynolds, 1980). In all detergents thus far investigated the protein maintains its native dimeric state (500 000 g/mol of complex). In nonionic detergents the secondary structure is also similar to that of the native protein as evidenced by the circular dichroic spectra and by immunological studies. The large alteration in apparent helical and β pleated sheet content which has been reported when sodium dodecyl sulfate ($NaDodSO_4$) is bound is readily reversed by exchanging the ionic detergent for $C_{12}E_8$ (Watt & Reynolds, 1980).

In the absence of bound amphiphiles apo B aggregates irreversibly, and a large increase in β pleated sheet structure is observed. Consequently, any study of the interaction of naturally occurring lipids with apo B must be carried out as a competition binding experiment, i.e., lipid must be substituted for the bound detergent. Exchange of bound amphiphiles

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[1] Abbreviations used: LDL, human low-density serum lipoproteins; HDL, human high-density serum lipoproteins; VLDL, human very low density serum lipoproteins; apo B, apolipoprotein B; PL, phospholipid; Apo B–PL, soluble complex of apolipoprotein B and phospholipid; $C_{12}E_8$, octaethylene glycol *n*-dodecyl monoether; $NaDodSO_4$, sodium dodecyl sulfate; cmc, critical micelle concentration.

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