Multiple Equilibria Binding Treatment of Lipid and Detergent Interactions with Membrane Proteins. Application to Cytochrome *c* Oxidase Solubilized in Cholate†

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ABSTRACT: A modified multiple binding equilibria treatment is presented that allows determination of thermodynamic parameters of the interaction of phospholipids with integral membrane proteins solubilized in excess detergent. Lipid binding is modeled as a series of exchange reactions between lipid molecules and detergent molecules at the hydrophobic protein surface. A general equation is derived which expresses a relative association constant (K) and the total number of contact sites at the lipid–protein interface (N) in terms of experimentally measurable variables. A useful simplification of the general equation occurs when the amount of detergent is high relative to the total number of lipid binding sites in the sample. Computer simulations show that in cases we have examined there appears to be an experimentally accessible range of detergent to protein molar ratios where the approximation at high detergent is useful for analyzing experimental data. This model is used to examine the competition between cholate and spin-labeled phospholipids for the hydrophobic surfaces of bovine heart cytochrome *c* oxidase. We find, for example, that $K = 12 \pm 2$ for phosphatidylcholine relative to cholate (i.e., the cholate molecules are relatively easily displaced by membrane lipids). This helps to explain the experimental observation that cholate is an effective detergent both for solubilizing cytochrome *c* oxidase and for reconstituting this protein into a defined lipid bilayer environment. An excess of cholate readily displaces almost all of the native phospholipids, and the protein is dispersed in cholate micelles. However, when phospholipids are added back, the cholate molecules at the protein surface are replaced because of the higher relative binding of the phospholipids. Observed differences between the behavior of phosphatidylcholine and phosphatidylglycerol suggest that reconstitution in cholate is a selective process in which detergent molecules in localized areas on the protein surface are more readily displaced by certain phospholipids.

Membrane receptors, enzymes, and other membrane proteins require the use of detergents for isolation, reconstitution, and crystallization. For this reason, there is considerable interest in understanding how detergents interact with the hydrophobic surfaces of membrane proteins. A number of studies have examined the solubilization of membrane proteins and lipids by detergents [for a recent review, see Lichtenberg et al. (1983)]. However, one important aspect of this problem has not been treated in the literature, namely, the development of a quantitative method for comparing the relative binding of detergent and phospholipid molecules to membrane proteins. Our purpose here is to develop a form of the classical multiple equilibria binding model appropriate to this problem by extending the treatment described previously for membrane proteins in lipid bilayers by Brotherus et al. (1981). In contrast to the familiar treatment of water-soluble ligands binding to the active sites of water-soluble proteins, the solvent system in the present case is the detergent micelle phase, and the ligands are phospholipids or other amphipathic molecules competing for the hydrophobic surfaces of the membrane protein. We present here a general treatment appropriate for lipid–protein interactions in a detergent-solubilized system. As an example of an experimental application, the model is used to compare the binding of two phospholipids to the integral membrane protein cytochrome *c* oxidase solubilized in the detergent cholate.

Derivation of Binding Equations

General Equation. To derive a general equation for the binding of phospholipids to an integral membrane protein solubilized in excess detergent, our starting point is a competition between lipids and detergent for the hydrophobic surfaces of the membrane protein. For convenience, the points at which the lipids contact the protein surface are referred to as binding sites (B).[1] The detergent (D) is assumed to be present in sufficient excess so that each lipid binding site at the protein surface is occupied by detergent. Addition of phospholipid (L) will result in a series of exchange reactions. For a single lipid binding site, the exchange can be written as

$$BD_\beta + L \rightleftharpoons BL + \beta D \quad (1)$$

where the stoichiometry factor β represents the number of detergent molecules displaced by one lipid molecule. At equilibrium, the standard free energy change for this reaction is

$$\Delta G^\circ = -RT \ln K \quad (2)$$

with the association constant (K) defined as

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[1] List of symbols: A, water-soluble ligand; B, binding site at the protein surface; BD_β, moles of B occupied by detergent; β, number of detergent molecules displaced by one phospholipid molecule; BL, moles of B occupied by phospholipid; D, detergent; D_b, moles of bound detergent; D_f, moles of free detergent; D_t, total moles of detergent; F_b, fraction of the total ESR spectral intensity represented by the motion-restricted component; γ_j, activity coefficient of species j; K_i, association constant of the phospholipid relative to the detergent for class i; L, phospholipid; L_b, moles of bound phospholipid; L_f, moles of free phospholipid; L_t, total moles of phospholipid; N, total number of lipid binding sites per mole of protein; N_i, number of lipid binding sites per mole of protein in class i; P, total moles of protein; r, fractional occupancy of B with L; T, moles of all interacting species; $\bar{\nu}$, moles of phospholipid bound per mole of protein; X_j, mole fraction of species j.

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$$K = \frac{X_{BL}X_{D_f}^{\beta}}{X_{BD_\beta}X_{L_f}} \frac{\gamma_{BL}\gamma_{D_f}^{\beta}}{\gamma_{BD}\gamma_{L_f}} \quad (3)$$

where X and γ represent mole fractions and activity coefficients, respectively. The mole fractions are defined as

$$X_{BL} = \frac{\text{moles of BL}}{\text{moles of all interacting species } (T)} = \frac{BL}{BL + L_f + BD_\beta + D_f} \quad (4a)$$

and similarly

$$X_{D_f} = D_f/T \quad (4b)$$

$$X_{BD_\beta} = BD_\beta/T \quad (4c)$$

$$X_{L_f} = L_f/T \quad (4d)$$

where BL = moles of B occupied by L (i.e., moles of lipid bound, L_b), L_f = moles of free lipid, BD_β = moles of B occupied by detergent (which equals $\beta^{-1} \times$ moles detergent bound = $\beta^{-1}D_b$), and D_f = moles of free detergent.

The standard state for the detergent in the mixture is the micellar solution of pure detergent, while the standard state for the lipid is chosen such that the activity coefficient becomes unity when the mole fraction of the lipid approaches zero. Thus, for samples containing a large excess of detergent, the term containing the ratio of activity coefficients can be assumed to be unity, and K is defined in terms of the mole fractions of free and bound lipid and detergent only. Equation 3 can thus be rearranged to

$$X_{BL} = KX_{BD_\beta}X_{L_f}X_{D_f}^{-\beta} \quad (5)$$

Next we define a quantity r such that

$$r = \frac{\text{moles of B occupied by L}}{\text{moles of all forms of B}} = \frac{BL}{BL + BD_\beta} \quad (6)$$

Dividing the numerator and denominator of the right-hand side of eq 6 by $BL + L_f + BD_\beta + D_f$ and substituting eq 4a and 4c give

$$r = \frac{X_{BL}}{X_{BL} + X_{BD_\beta}} \quad (7)$$

Substitution of eq 5 into eq 7 gives

$$r = \frac{KX_{L_f}X_{D_f}^{-\beta}}{1 + KX_{L_f}X_{D_f}^{-\beta}} \quad (8)$$

Equation 8 is the general equation for a single binding site, relating the fractional occupancy (r) of that site with phospholipid (L) to the mole fractions of free phospholipid (X_{L_f}) and free detergent (X_{D_f}).

For a protein with a single class of binding sites, where N is the number of equivalent and independent sites, this becomes

$$\bar{\nu} = \frac{NKX_{L_f}X_{D_f}^{-\beta}}{1 + KX_{L_f}X_{D_f}^{-\beta}} \quad (9)$$

where $\bar{\nu}$ is the average number of moles of phospholipid bound per mole of protein. Note that N is defined as the number of exchangeable phospholipid binding sites; the corresponding number of detergent binding sites is therefore βN.

For a protein with multiple classes of binding sites, the result is

$$\bar{\nu} = \sum_{i=1}^{m} \frac{N_iK_iX_{L_f}X_{D_f}^{-\beta_i}}{1 + K_iX_{L_f}X_{D_f}^{-\beta_i}} \quad (10)$$

The derivation of eq 8–10 is based on the following assumptions: (i) individual binding sites are independent and, within a given class, equivalent; (ii) the ratio of activity coefficients in eq 3 is unity; (iii) there are no unoccupied binding sites; (iv) there is equilibration of all components; and (v) lipid and detergent partition between no more than two thermodynamically different environments, i.e., in contact with the protein or free in the mixed micelle. The small amount of detergent present in the aqueous phase is neglected in this treatment because the experiments are performed at relatively high detergent concentrations, well above the critical micelle concentration, where almost all of the detergent is present in the micellar phase.

Comparison with the Water-Soluble Case. Equation 10 differs from the general binding equation commonly used in the case of water-soluble proteins and ligands [see, for example, Cantor & Schimmel (1980)] in that the amount of free solvent (detergent) in the system appears in the binding equation. Furthermore, all concentrations are expressed in mole fractions instead of molarities.

Equation 8, and similarly eq 9 and 10, can, however, easily be transformed into the form customarily used in solution biochemistry. In most cases, solvent (water) molecules will participate in interactions between water-soluble proteins and ligands in aqueous solution. Thus binding of a water-soluble ligand (A) to the binding site (B) of a water-soluble protein can be represented by an exchange reaction:

$$B(H_2O)_\beta + A \rightleftharpoons BA + \beta H_2O \quad (11)$$

and the binding equation equivalent with eq 8 is

$$r = \frac{KX_{A_f}X_{(H_2O)_f}^{-\beta}}{1 + KX_{A_f}X_{(H_2O)_f}^{-\beta}} \quad (12)$$

where X_{A_f} and $X_{(H_2O)_f}$ are the mole fractions of free ligand and free solvent, respectively. Experimentally, ligand binding studies are usually performed in fairly dilute aqueous solutions; that is, B and A are in the micromolar to millimolar concentration range while the solvent concentration is very close to that of pure water (55.5 M). It follows that the denominator $[BA + A_f + B(H_2O)_\beta + (H_2O)_f]$ of the mole fractions very nearly equals the total number of moles of water in the system so that

$$X_{(H_2O)_f} \cong 1 \quad \text{and} \quad X_{A_f} \cong \frac{A_f}{[H_2O]_{total}} \cong 55.5[A_f]$$

where $[A_f]$ is the concentration of free ligand in moles per liter. Substitution in eq 12 gives

$$r = \frac{55.5K[A_f]}{1 + 55.5K[A_f]} = \frac{K'[A_f]}{1 + K'[A_f]} \quad (13)$$

which is the familiar form of the binding equation for a single site in solution biochemistry with the factor 55.5 included in the binding constant K'.

Approximation at High Detergent Concentration. The comparison with the water-soluble case suggests that an important simplification of the general eq 10 occurs when the solubilizing detergent is present in high amounts relative to the total number of binding sites and lipid molecules in the system. In that case, we can make approximations similar to those described for dilute aqueous solutions in the previous section so that

$$X_{L_f} \cong L_f/D_t \quad \text{and} \quad X_{D_f} \cong 1$$

Substitution into eq 10 gives the simplified relationship

$$\bar{v} = \sum_{i=1}^{m} \frac{N_i K_i L_f D_t^{-1}}{1 + K_i L_f D_t^{-1}} \quad (14)$$

This approximation of the general binding equation in the limit of high detergent concentration results in the effective elimination of the stoichiometry factor β, analogous to the water-soluble case.

Exact Binding Equations. Evaluation of the approximation at high detergent concentration under specific experimental conditions requires a comparison of the approximate and exact binding equations. The latter are obtained as follows.

(A) Single Class of Sites with $\beta = 1$. Since the denominators in the mole fractions cancel, eq 9 reduces to

$$\bar{v} = \frac{NKL_f D_f^{-1}}{1 + KL_f D_f^{-1}} \quad (15)$$

where the quantities free lipid (L_f) and free detergent (D_f) are in moles. D_f is then eliminated by using the conservation equations

$$\text{total binding sites} = NP = BL + BD = L_b + D_b \quad (16)$$

$$\text{total detergent} = D_t = D_f + D_b \quad (17)$$

where P is total moles of protein. Since $\bar{v} = L_b/P$, we obtain from eq 16

$$D_b = NP - L_b = NP - P\bar{v}$$

Substitution into eq 17 yields

$$D_f = D_t - NP + P\bar{v}$$

which upon substitution in eq 15 gives

$$\bar{v} = \frac{NKL_f(D_t - NP + P\bar{v})^{-1}}{1 + KL_f(D_t - NP + P\bar{v})^{-1}} \quad (18)$$

Equation 18 can be rearranged to the quadratic equation

$$P\bar{v}^2 + \bar{v}(D_t - NP + KL_f) - NKL_f = 0 \quad (19)$$

(B) Single Class of Sites with $\beta = 2$. For this case, eq 9 reduces to

$$\bar{v} = \frac{NKX_{L_f}X_{D_f}^{-2}}{1 + KX_{L_f}X_{D_f}^{-2}} \quad (20)$$

where

$$X_{L_f} = \frac{L_f}{L_f + BL + BD_2 + D_f} \quad (21a)$$

and

$$X_{D_f} = \frac{D_f}{L_f + BL + BD_2 + D_f} \quad (21b)$$

From the conservation equations, we obtain

$$NP = BL + BD_2 = L_b + (1/2)D_b = P\bar{v} + (1/2)D_b$$

which gives

$$D_b = 2(NP - P\bar{v})$$

and

$$D_f = D_t - D_b = D_t - 2(NP - P\bar{v})$$

Substitution in eq 21a and 21b gives

$$X_{L_f} = \frac{L_f}{L_f + D_t - NP + 2P\bar{v}} \quad (22a)$$

and

$$X_{D_f} = \frac{D_t - 2NP + 2P\bar{v}}{L_f + D_t - NP + 2P\bar{v}} \quad (22b)$$

which upon substitution in eq 20 and rearranging result in the cubic equation

$$\bar{v}^3 + s\bar{v}^2 + q\bar{v} + r = 0 \quad (23)$$

with

$$s = D_t/P - 2N + (1/2)KL_f/P$$

$$q = (1/4)D_t^2/P^2 - ND_t/P + N^2 + (1/4)KL_fD_t/P^2 + (1/4)KL_f^2/P^2 - (3/4)NKL_f/P$$

$$r = (1/4)N^2KL_f/P - (1/4)NKL_f^2/P^2 - (1/4)NKL_fD_t/P^2$$

(C) Two Classes of Sites with $\beta_1 = \beta_2 = 1$. For this case, the general eq 10 reduces to

$$\bar{v} = \frac{N_1K_1L_fD_f^{-1}}{1 + K_1L_fD_f^{-1}} + \frac{N_2K_2L_fD_f^{-1}}{1 + K_2L_fD_f^{-1}} \quad (24)$$

D_f is eliminated as above under (A) by using the relationship

$$D_f = D_t - NP + P\bar{v}$$

Substitution followed by rearrangement results in the cubic equation

$$\bar{v}^3 + s\bar{v}^2 + q\bar{v} + r = 0 \quad (25)$$

with

$$s = 2D_t/P - 2N + K_1L_f/P + K_2L_f/P$$

$$q = D_t^2/P^2 + N^2 - 2D_tN/P + D_tK_1L_f/P^2 - NK_1L_f/P + D_tK_2L_f/P^2 - NK_2L_f/P - N_1K_1L_f/P - N_2K_2L_f/P + K_1K_2L_f^2/P^2$$

$$r = N_1K_1L_fN/P - N_1K_1L_fD_t/P^2 - N_2K_2L_fD_t/P^2 + N_2K_2L_fN/P - NK_1K_2L_f^2/P^2$$

and

$$N = N_1 + N_2$$

(D) Two Classes of Sites with $\beta_1 = \beta_2 = 2$. For this case, the general eq 10 becomes

$$\bar{v} = \frac{N_1K_1X_{L_f}X_{D_f}^{-2}}{1 + K_1X_{L_f}X_{D_f}^{-2}} + \frac{N_2K_2X_{L_f}X_{D_f}^{-2}}{1 + K_2X_{L_f}X_{D_f}^{-2}} \quad (26)$$

with the mole fractions X_{L_f} and X_{D_f} defined as in eq 21a and 21b, respectively. D_f is then eliminated from the expressions for the mole fractions as above under (B) by using the relation $D_f = D_t - 2(NP - P\bar{v})$ followed by substitution of the expressions for X_{L_f} and X_{D_f} into eq 26. Rearranging results in a fifth-order polynomial, which can be solved iteratively.

Experimental Procedures

Cytochrome c Oxidase Preparation. Phospholipid-depleted bovine heart cytochrome *c* oxidase was prepared from Keilin–Hartree particles as described previously (Yu et al., 1975). Cytochrome *c* oxidase preparations were stored at −196 °C in 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA)[2] and 50 mM phosphate, pH 7.4 (SPE buffer), containing 1.0% (w/v) sodium cholate.

Materials. Cholic acid (Sigma) was purified by recrystallization from hot 95% ethanol as described by Silvius et al. (1984). Purity was verified by thin-layer chromatography

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; PC, phosphatidylcholine; PG, phosphatidylglycerol; PC*, 1-palmitoyl-2-(14-proxylstearoyl)-*sn*-glycero-3-phosphocholine; PG*, 1-palmitoyl-2-(14-proxylstearoyl)-*sn*-glycero-3-phosphoglycerol; DPG, diphosphatidylglycerol (cardiolipin).

(HETLC, Analtech) with chloroform/methanol/glacial acetic acid (40:10:1, by volume) as the solvent. The phospholipid spin-labels PC* and PG* were prepared essentially as described by Griffith et al. (1986). PC was prepared from fresh chicken egg yolks (Singleton et al., 1965). Part of the PC preparation was converted into PG via transphosphatidylation with phospholipase D and purified on CM-cellulose (Whatman, CM-52) as described by Comfurius and Zwaal (1977). Purity of phospholipids was checked by using HETLC (Analtech) with chloroform/methanol/water (65:25:4, by volume) as the solvent. Phospholipids were stored in chloroform/methanol (99:1, by volume) under Ar at −20 °C. Special enzyme-grade ammonium sulfate and sucrose were procured from Schwartz/Mann. EDTA was from Matheson Coleman and Bell. All other reagents were of the highest grade commercially available.

Analytical Methods. Protein was determined essentially by the method of Lowry et al. (1951). Bovine serum albumin (Miles Pentex fraction V, fatty acid poor) was used as the standard, and corrections were made for color augmentation due to the presence of lipid. Heme *a* content of the cytochrome *c* oxidase was estimated by the method of Kuboyama et al. (1972). Phospholipid was extracted by the method of Bligh and Dyer (1959) and quantitated by the method of Lowry and Tinsley (1974), except that samples were digested for 6 h in the presence of perchloric and nitric acid at 130 °C (Mrsny et al., 1985). $NaH_2PO_4 \cdot H_2O$ (J. T. Baker) was used as the standard for phosphate analysis. Characterization of phospholipids was performed by two-dimensional thin-layer chromatography on silica gel G (Merck) analytical plates using the solvent system of Parsons and Patton (1967). Cytochrome *c* oxidase activity was measured polarographically in the presence of 0.5% Tween 80 as described by Vik and Capaldi (1980).

Preparation of Samples for ESR. Phospholipids (including the phospholipid spin-label) were dried from organic solvent under a stream of argon gas at the bottom of a small Potter-type homogenizer. A small volume of SPE buffer containing 20% cholate was added, and the mixture was carefully homogenized by using a hand-held glass pestle followed by bath sonication for 10 min under argon. A volume of cytochrome *c* oxidase in SPE buffer containing 1.0% cholate was then added, giving a final sample containing approximately 25 mg of cytochrome *c* oxidase/mL, 4.0% cholate, and varying amounts of added lipid in a total volume of 75 μL. After mixing and equilibration at room temperature for 1 h, samples were drawn into capillary tubes, which were sealed and placed in the cavity of the ESR spectrometer. ESR spectra were collected on a Varian E-Line Century Series 9.5-GHz spectrometer interfaced with a 32K Varian 620/L100 computer. The sample temperature for each experiment was maintained between 30.0 and 30.2 °C and monitored by a thermocouple inserted into the cavity at the level of the sample. Spectra were collected at a power setting of 5 mW with a scan time of 16 min, a filter time constant of 0.128 s, a modulation amplitude of 1.25 G, and a scan range of 100 G. Following collection of the spectra, samples were removed from the capillaries and analyzed for protein and phospholipid content. Control experiments showed that protein and phospholipid contents were the same when measured before introduction of the samples into the capillaries and after retrieval of the samples from the capillaries.

ESR Spectral Analysis. ESR data analysis, integration, and spectral subtraction were performed as previously described (Jost & Griffith, 1978; Silvius et al., 1984). Reference line shapes used to determine the end points of spectral subtractions were obtained at different temperatures from samples containing 4.0% cholate in SPE with a cholate to phospholipid molar ratio of either 15:1 or 25:1 and a phospholipid:spin-label ratio of about 50:1.

Data Treatment. From the ESR spectral analysis in combination with lipid phosphorus and protein analysis of the samples, sets of data points were computed by using the relationships:

$$\bar{\nu} = L_b/P = F_b L_t/P \tag{27}$$

and

$$L_f/D_t = (1 - F_b)L_t/D_t \tag{28}$$

where F_b is the fraction of the total spectral intensity represented by the motion-restricted component, $\bar{\nu}$ is the moles of phospholipid bound per mole of protein, and L_f, L_t, P, and D_t are the moles of free phospholipid, total phospholipid, protein, and total detergent present in the sample, respectively. Experimental or simulated data were fit to a function representing the appropriate binding equation using a nonlinear least-squares method to provide values for the thermodynamic binding parameters. A curve-fitting program based on the Marquardt algorithm [Marquardt, 1963; see also Levenberg (1944)] was translated into BASIC for use with an IBM Personal Computer and adapted to the specific requirements of this study (a listing of the modified or original program is available upon request). For fits to experimental data, convergence was reached usually within 5–10 iterations. The goodness of fit was evaluated by requiring that (i) experimental data showed random distribution about the calculated fit and (ii) the standard deviation of the experimental points from the calculated curve was at a minimum.

In calculating the data points used in the curve fits, it was assumed that the cardiolipin (DPG) endogenous to the cytochrome *c* oxidase preparation (roughly four cardiolipin molecules or 8 PC or PG equiv per enzyme monomer) was nonexchangeable. This was based on the fact that this negatively charged phospholipid is difficult to remove from cytochrome *c* oxidase by solubilization in cholate or other detergents (Yu et al., 1975; Watts et al., 1978; Robinson et al., 1980). No attempt was made to exchange the endogenous DPG for added dimyristoyl-PC as described by Powell et al. (1985). The question of exchangeability of the endogenous DPG in cytochrome *c* oxidase is still an open one. If all the lipid is exchangeable, the 8 equiv of endogenous lipid phosphorus should not be subtracted from the experimental total phosphorus. This case is, however, complicated by the fact that the binding behavior of the endogenous DPG will most likely be different from that of the added lipids (PC or PG).

The binding treatment presented here strictly applies only to those situations where trapped lipid is not a problem; i.e., either there is no endogenous lipid or its behavior is known and properly taken into account. It is, however, useful to estimate the effect of an improper subtraction. For this purpose, let $L'_t = L_t - nP$, where n is the number of phosphorus equivalents of endogenous lipids per mole of protein. Equations 27 and 28 become $\bar{\nu}' = F_b(L_t - nP)/P$ (eq 27′) and $L'_f/D_t = (1 - F_b)(L_t - nP)/D_t$ (eq 28′), where primes indicate the new (incorrect) values. By combining these equations, it follows that $\bar{\nu}'(D_t/L'_f) = \bar{\nu}(D_t/L_f)$. That is, in the Scatchard-type plots, the y-axis values do not change because the subtraction occurs both in the numerator (from the $\bar{\nu}'$ term) and in the denominator (from the D_t/L'_f term) and cancels out. However, the x-axis values are shifted to the left as is clear from eq 27′. By combining eq 27 and 27′, the relationship between $\bar{\nu}'$ and $\bar{\nu}$

is $\bar{\nu}' = \bar{\nu} - nP\bar{\nu}/(L_f + P\bar{\nu})$. Thus, for a single class of binding sites, the Scatchard-type plot is no longer strictly linear. However, over a broad region ($0 < \bar{\nu}' < 0.8N$), the curve is nearly linear and parallel to the original curve so there is very little change in K. The value of N extrapolated from this region is lowered at the most to $N - n$. Similarly, if the endogenous lipid is nonexchangeable and no subtraction is performed, there will be an error in the opposite direction; that is, $\bar{\nu}'' = \bar{\nu} + nP\bar{\nu}/(L_f + P\bar{\nu})$, and the extrapolated value becomes at the most $N + n$, with little or no change in K. If the extrapolations are performed by using data nearer the x-axis intercept, the differences in the estimated number of binding sites are even smaller. For two classes of sites, we find that the total N ($N_1 + N_2$) is affected very little by the choice of assumption of exchangeability or nonexchangeability of the endogenous lipid, but that the resulting K values change more than in the single class of sites case. If one models the behavior as two classes of sites and two competing phospholipids with different binding constants (an extension of the work presented here) with the cardiolipin free to exchange but having a much higher binding constant than the PC or PG, this lipid will preferentially occupy the higher affinity binding sites. In the limit of very high relative affinity, the cardiolipin will behave in these experiments as trapped or silent lipid since it will not be displaced by the PC or PG, and the data treatment presented here would again be appropriate (i.e., subtracting the endogenous lipid from the total experimental phosphorus). In any case, the corrections are small, and the modeling does not preclude that all or part of the residual cardiolipin exchanges with detergent and added phospholipid.

Calculated Binding Isotherms. A program written in BASIC was used to generate binding curves according to the approximate or exact binding equations derived in this work. The program includes separate subroutines for solving the quadratic and cubic binding equations, while the fifth-order polynomial obtained in the case of two classes of binding sites with $\beta = 2$ is solved iteratively by using the solutions of the corresponding approximate equation as the starting values. Values for $\bar{\nu}$ were calculated over a range of values for L_f at selected values for β, D_t, P, K_i, and N_i.

RESULTS

Comparison of Approximate and Exact Binding Equations for One Class of Sites. The validity of the approximate eq 14 was tested over an experimentally accessible range of detergent, lipid, and protein concentrations[3] by computer simulation. For a protein with a single class of binding sites, two cases were examined, corresponding to the ones most likely encountered in the analysis of experimental data: (a) lipid and detergent exchange in a 1:1 stoichiometry ($\beta = 1$), and (b) lipid and detergent exchange in a 2:1 stoichiometry ($\beta = 2$). For each case, binding isotherms were calculated from the approximate eq 14 and compared with those obtained from the exact equation pertaining to each specific case (eq 19 and 23 for $\beta = 1$ and $\beta = 2$, respectively). Our purpose here is to provide a method of measuring K, not the factor β. Nevertheless, it is necessary to include β in the derivation in order to test the accuracy of the useful approximate solution to the binding equations. These calculations were performed for a

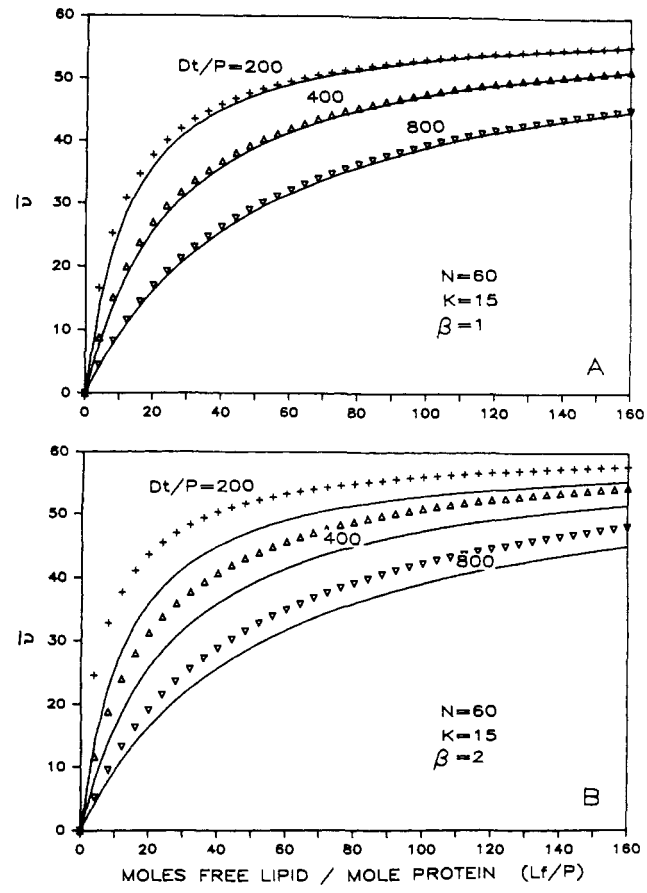

FIGURE 1: Comparison of binding isotherms for one class of binding sites calculated by using the approximate eq 14 (solid lines) with those calculated from the exact binding eq 19 (A, symbols) or eq 23 (B, symbols). The quantity $\bar{\nu}$, moles of lipid bound per mole of protein, is plotted vs. the moles of free lipid per mole of protein for three values of the ratio of total detergent to protein (D_t/P). The top set of curves (A) assumes a detergent:lipid exchange stoichiometry of 1:1 ($\beta = 1$), and the bottom set (B) is for $\beta = 2$. The total number of binding sites (N) for the protein was set at 60, with a relative binding constant (K) of 15.

hypothetical protein of molecular weight 200 000 with a total number of binding sites of $N = 60$ at a constant protein concentration of 25 mg/mL (125 μM), which corresponds to a concentration of total binding sites of 7.5 mM. This rather high protein concentration was chosen because experimentally it allows for more accurate determination of the fraction of phospholipids in contact with the protein by increasing the signal to noise ratio.

Figure 1A shows binding curves calculated for three different detergent to protein molar ratios (D_t/P) with $\beta = 1$ and $K = 15$, while the corresponding binding curves for $\beta = 2$ are depicted in Figure 1B. The solid lines are calculated according to the approximate eq 14 with $m = 1$, while the symbols represent binding curves generated with eq 19 (Figure 1A) or eq 23 (Figure 1B). The difference between pairs of binding curves (i.e., solid lines and symbols) for both sets of data decreases with increasing detergent concentration, indicating that, as expected, the approximation becomes better at higher detergent:protein ratios. Furthermore, the difference between pairs of binding curves is greater when $\beta = 2$ than when $\beta = 1$.

That the differences between the approximate and exact binding curves are rather small when $\beta = 1$ is illustrated in a more quantitative way by the data summarized in Table I (top three rows). These data are the results of simulation experiments, where sets of data points generated with the exact

[3] The term concentration (moles of substance per aqueous volume of sample) is used here for convenience. This quantity does not appear in the binding equations; the internal molar ratios of protein, lipid, and detergent are the relevant quantities. The aqueous concentration of detergent was well above the critical micelle concentration (cmc) in all experiments.

Table I: Quantitative Evaluation of Differences between Approximate and Exact Binding Equations Using Curve Fitting to Calculated Data Points

One Class of Binding Sites						
input[a]		output[b]			no. of data points[c]	D_t/P[d]
K	N	K	N	β		
15	60	18.5	58.4	1	13	200
15	60	16.2	59.0	1	15	800
15	60	15.5	59.6	1	10	1600
15	60	18.1	60.9	2	15	800
150	60	174	59.9	2	16	800
1500	60	1689	59.9	2	13	800

Two Classes of Binding Sites										
input[a]				output[b]					no. of data points[c]	D_t/P[d]
K_1	N_1	K_2	N_2	K_1	N_1	K_2	N_2	β		
15	45	1500	15	15.7	44	1519	15.2	1	13	800
15	45	1500	15	16.7	46	1665	15.4	2	13	800

[a] Values of the input parameters K and N used to calculate the data points. [b] Best-fit parameter values obtained when a set of data points, calculated with the exact equation, are fit to the approximate equation. The difference between the input and output parameter values provides an estimate of the error in the fit parameter values obtained when, under the given conditions, the approximate equation is used for analysis of experimental data. [c] Number of data points generated and used in the curve fit. Data points were selected in a range including the region of maximal curvature of the binding isotherm. [d] The molar ratio of total detergent to protein used to calculate the data points and used in the curve fit.

equation were used in a curve fit to the approximate equation. (This procedure is technically simpler than fitting the approximate to the exact equation because the exact equation is a higher order polynomial.) The difference between the best-fit values for the parameters K and N obtained from the fit and the input values for these same parameters provides a measure of how well the true binding curve is described by the approximation. The data of rows 1–3 of Table I indicate that a fit to the approximate equation of data points generated with the exact equation results in output parameter values that are close to the chosen input values. The difference between the input and output parameter values is dependent on the detergent:protein ratio and is less at higher values of the latter.

Similar results are obtained for the case of a protein with a single class of binding sites and where lipid and detergent exchange in a 1:2 stoichiometry (Table I, rows 4–6). In this case, the differences between the input and output parameter values are also fairly small, with the most significant deviation in the binding constant K. The relative difference between the input and output values of K decreases as K increases.

The results of Figure 1 and the top section of Table I together indicate that, under the conditions described here and for a membrane protein with the stated characteristics, analysis of experimental binding data with the approximate eq 14 will result in reasonable estimates of the binding parameters. The differences between the input and output K values at detergent to protein molar ratios of about 800, which can be experimentally achieved, are probably of minor significance when the experimental errors in real data points are taken into account.

At constant high D_t, the shape of the binding curve for a membrane protein with a single class of sites is determined by the number of binding sites (N value) and the relative binding affinity (K value) for a given lipid, allowing determination of these parameters by curve fitting. In Figure 2A,B, curves calculated by using the approximate eq 14 are plotted for the binding of lipids to a hypothetical protein with a single class of binding sites, keeping N constant while varying K.

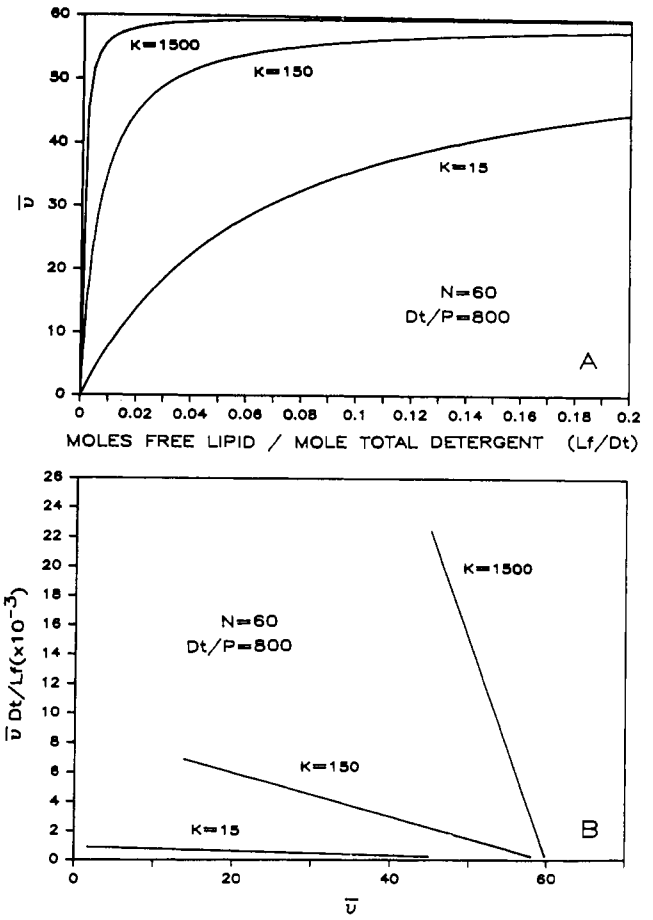

FIGURE 2: Calculated binding isotherms for three values of the binding constant (K) for a membrane protein with a single class of binding sites interacting with phospholipids. Values for $\bar{\nu}$ were computed by using the approximate eq 14 at a constant total detergent:protein molar ratio (D_t/P = 800) for N = 60. (A) $\bar{\nu}$ plotted vs. the moles of free lipid per moles of total detergent and (B) the same data shown in the form of linear Scatchard-type plots.

Because D_t is a constant, it is convenient to plot the quantity L_f/D_t on the x axis. Data can be readily presented in a linearized form by means of a Scatchard-type graph as in Figure 2B. These plots are similar to the ones obtained for water-soluble proteins and ligands except that L_f/D_t, the molar ratio of free ligand and total solvent, is used as the concentration variable instead of moles of free ligand per liter of aqueous solution.

Comparison of Approximate and Exact Binding Equations for Two Classes of Sites. Equation 14 was similarly tested for a hypothetical membrane protein with two classes of binding sites. As before, two cases were examined. Figure 3A shows the results for the case where detergent and lipid exchange in a 1:1 ratio (β = 1), and the data for β = 2 are shown in Figure 3B. The solid lines were calculated according to the approximate eq 14 with m = 2, while the symbols represent binding curves generated with the exact eq 25 (Figure 3A) or 26 (Figure 3B). The bottom section of Table I summarizes the results of curve fits obtained when sets of data points generated with the exact equations are fit to the approximate equation.

These simulation results show that for a membrane protein with two classes of binding sites, analysis of experimental binding data with the high detergent approximation will also result in reasonable estimates of the binding parameters N_1, N_2, K_1, and K_2.

Examples of the types of binding isotherms that can be observed for the binding of phospholipids to a membrane

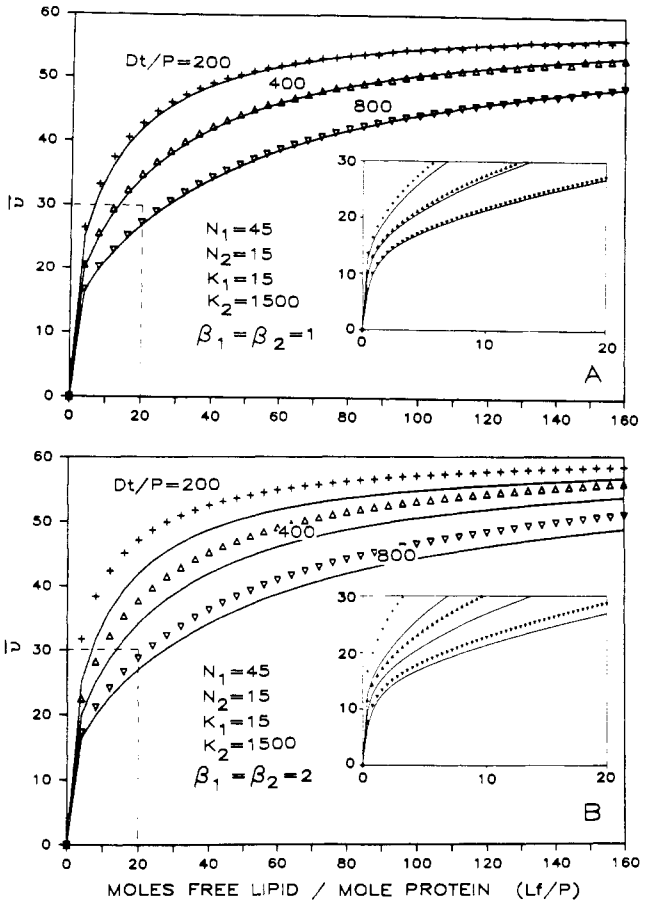

FIGURE 3: Comparison of binding isotherms for two classes of sites calculated by using the appropriate eq 14 (solid lines) with those calculated from the exact binding eq 25 (A, symbols) or eq 26 (B, symbols). Of the 60 binding sites, 45 (N_1) have a binding constant of 15 (K_1), and 15 (N_2) have a binding constant of 1500 (K_2). The top set of curves (A) was calculated for the case where one detergent molecule can occupy a lipid binding site (β = 1), and the bottom set (B) is for the case where two detergents can replace one lipid (β = 2). The inserts show the initial parts of the binding curves, indicated by the dashed lines in (A) and (B), in more detail.

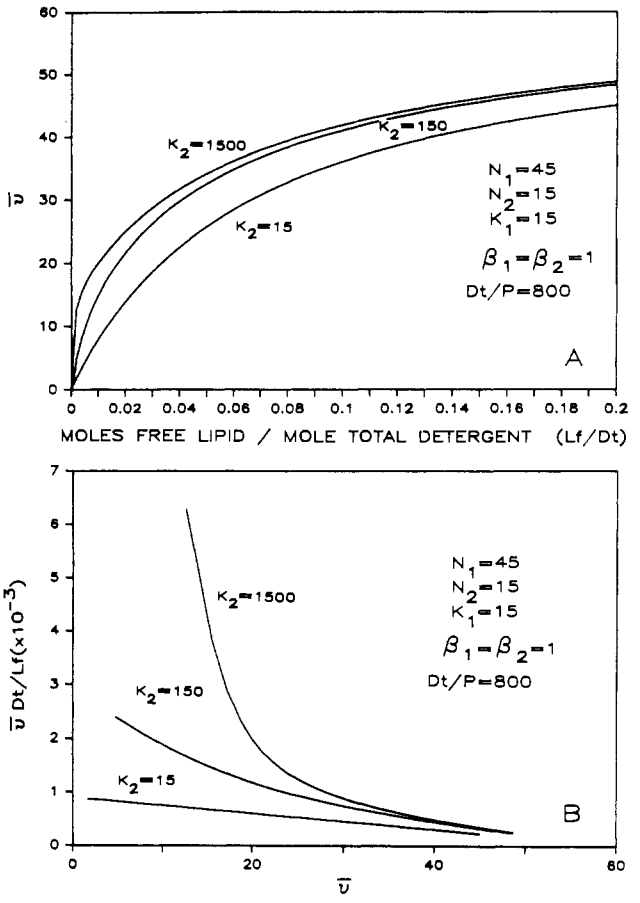

FIGURE 4: Calculated binding isotherms for a membrane protein containing two classes of binding sites, for three values of the binding constant of the high-affinity class (K_2). Values for $\bar{\nu}$ as a function of moles of free lipid were computed by using the approximate eq 14 (m = 2) at a constant total detergent:protein molar ratio (D_t/P = 800). Values of N_1, N_2, and K_1 were held constant as indicated. (A) $\bar{\nu}$ plotted vs. L_f/D_t and (B) the same data shown in the form of Scatchard-type plots.

protein with two classes of binding sites in excess detergent are shown in Figure 4A with the corresponding Scatchard-type plots in Figure 4B. Curves were calculated from eq 14 at a constant detergent to protein molar ratio of 800 and with values for the binding parameters as indicated. Evidently, a ratio of relative affinity constants (K_2/K_1) of 10 or more is required in order to distinguish between one or two classes of binding sites.

The graphs of Figure 4B illustrate the need for nonlinear curve fitting to obtain correct values for the parameters in the case of two classes of sites. Extrapolation of the linear portion of the curve will only give a rough idea of the number of binding sites. For example, extrapolation of the steep linear portion of the top curve in Figure 4B gives an intercept on the abscissa of N_2 = 20, whereas this curve was calculated with N_2 = 15. This problem is well-known in the conventional Scatchard plots for ligand binding in aqueous solution (Munson & Rodbard, 1983) and results from a competitive occupancy of both classes of sites as the concentration of ligand is increased, rather than filling one class of sites completely before occupying the second class. Simple extrapolations can be used when there is only one class of sites. For example, in Figure 2B, extrapolation of any curve yields the correct value of N (in this case, N = 60, the input value).

Experimental Binding Data. The equilibrium binding treatment was used to examine the competition of two phospholipids relative to the detergent cholate for the hydrophobic lipid contact sites (the boundary region) of bovine heart cytochrome *c* oxidase. The two phospholipids chosen were the ubiquitous and neutral (zwitterionic) phosphatidylcholine (PC) and the negatively charged phosphatidylglycerol (PG). The enzyme preparation used in these studies contained approximately 10 nmol of heme *a*/mg of protein (corresponding to a monomer molecular weight of 200 000) and had an electron-transfer activity of 90 mol of oxygen per second per mole of cytochrome *c* oxidase following dilution into the assay mixture. Analysis of endogenous phospholipids demonstrated approximately four cardiolipin (diphosphatidylglycerol), two phosphatidylethanolamine, and two phosphatidylcholine molecules per cytochrome *c* oxidase monomer.

Information on the binding of PC and PG to cholate-solubilized cytochrome *c* oxidase was obtained from ESR spectral analysis of samples containing cytochrome *c* oxidase, small amounts of spin-labeled phospholipid (PC* or PG*, 1–2 mol/mol of cytochrome *c* oxidase), and varying amounts of the corresponding unlabeled lipids in 4% cholate. Interaction of the phospholipids with the protein surface was assumed not to be affected by the presence of the spin-label moiety, as has been shown for the combination PC and PC* in lipid vesicles (Silvius et al., 1984). Exposure of cytochrome *c* oxidase to the 4% cholate concentrations used in these binding experiments did not affect the final enzyme activity measured after dilution into the assay mixture, suggesting this high detergent

concentration did not denature the enzyme complex. All spectra were composed both of a component characteristic of lipid spin-labels in a fluid environment and of a component with a much broader splitting, characteristic of lipid spin-labels in a more motion-restricted environment. Addition of the corresponding non-spin-labeled phospholipid to the samples, with protein and detergent concentrations kept constant, resulted in a decrease of the motion-restricted component (F_b) and an increase of the fluid component. These changes in line shape as a function of the lipid:protein ratio in the sample are consistent with previous ESR studies of lipid–protein interactions in membrane vesicles (Jost & Griffith, 1978; Marsh & Watts, 1982) and suggest that the broad component represents spin-labeled phospholipids in contact with the hydrophobic surface of the protein.

By combining quantitative spectral analysis with lipid phosphorus and protein determinations of the samples, values for $\bar{\nu}$ as as function of L_f/D_t were obtained from which values for the binding parameters were derived by curve fitting. The results are shown as a direct plot of $\bar{\nu}$ vs. L_f/D_t in Figure 5A and in the form of a Scatchard-type graph in Figure 5B. The symbols represent the individual data points with their associated experimental errors while the solid lines are the binding curves calculated with the best-fit values for the parameters K and N. These fits are done by using sets of data points consisting of values for $\bar{\nu}$ and L_f/D_t. The differences between the PC and PG data show up most clearly in the Scatchard-type graph drawn in Figure 5B. For the PC data, a good fit is obtained by assuming a single class of binding sites with $N = 57 \pm 5$ and $K = 12 \pm 2$. For the PG data, on the other hand, a curve fit assuming one class of binding sites was unsatisfactory. A reasonably good fit is obtained, however, by assuming a two classes of sites model with the following parameters: $N_1 = 70 \pm 20$, $K_1 = 6 \pm 4$, $N_2 = 8 \pm 5$, and $K_2 \cong 2 \times 10^2$.

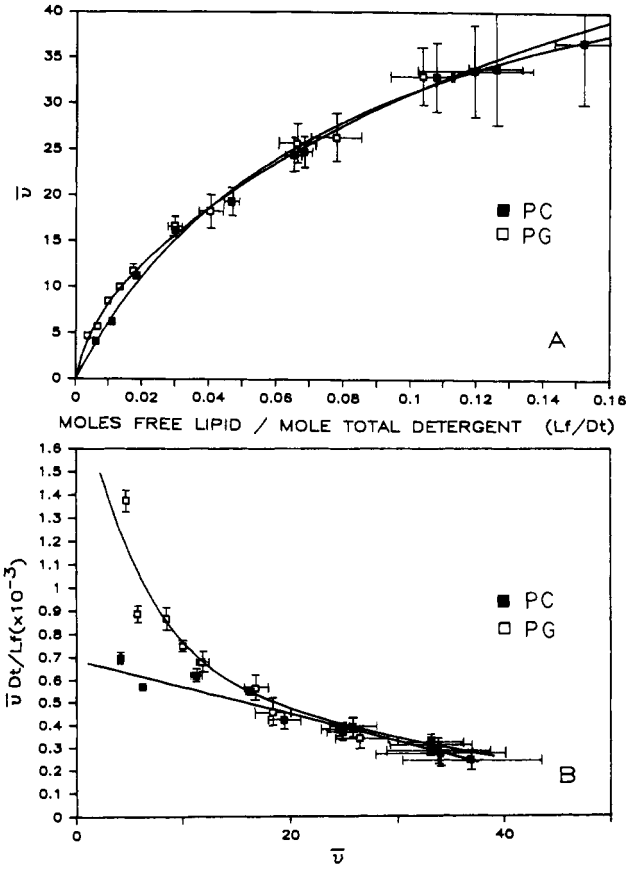

FIGURE 5: Experimental results for the phospholipids PC (closed squares) and PG (open squares) interacting with bovine heart cytochrome *c* oxidase in the presence of the detergent cholate. The error bars were calculated for each data point separately by combining (square root of the sum of squares) the estimated error in F_b with the standard deviation in the means ($n = 4$) of both total phospholipid and protein determinations. (A) The solid lines are best fits to the experimental data calculated from the equilibria binding equations. For PC, a good fit was obtained with the single class of sites case (eq 14, $m = 1$) whereas for PG a much better fit was obtained with the two classes of sites model (eq 14, $m = 2$). (B) The same data and calculated best-fit binding isotherms shown in the form of a Scatchard-type graph. Samples were at 30 °C in SPE buffer (pH 7.4). The aqueous detergent concentration was held constant at 4% (by weight; 93 mM), while the aqueous protein concentrations (determined for each data point separately) were in the range between 22 and 27 mg/mL, giving a total detergent:protein molar ratio in each sample of about 740. On the basis of phospholipid analysis, the cytochrome *c* oxidase preparation was assumed to contain 8 lipid–phosphorus equiv of nonexchangeable lipid, and this number was subtracted from the total moles of phospholipid before performing the curve fits and plotting the experimental data.

Discussion

The goal of this study was to develop a method of characterizing the relative binding of detergents and lipids to membrane proteins, as a step toward attaining a better understanding of the solubilization and lipid reconstitution procedures used in membrane biochemistry today. Solubilization and lipid depletion involve the replacement of the phospholipid bilayer normally surrounding the hydrophobic surfaces of membrane proteins by nondenaturing detergents such as sodium cholate. The intact membranes are dispersed to form mixed micelles of protein and lipids in detergent which can be manipulated in purification, reconstitution, and crystallization steps. Our present knowledge of the size and shapes of the mixed micelles is far from complete, and the structures undoubtedly depend on temperature, ionic strength, and lipid to protein ratios as well as the specific structure of the detergent and proteins involved. However, it is useful to consider a simple model to describe one important aspect of these complex mixed micelle systems: the dynamic equilibria of detergent–lipid exchange at the hydrophobic protein surface. The approach presented here is based on established thermodynamic principles and is formally equivalent to the equilibrium binding treatment commonly used to analyze multiple equilibria involving water-soluble proteins and ligands [see, for example, Cantor & Schimmel (1980)]. There are necessarily some differences due to the particular nature of the detergent-solubilized system. First, even though the components participating in the exchange equilibria are dispersed in an aqueous medium, water does not directly participate in the equilibria under study. The transfer of detergent molecules from the aqueous phase to the micelles or protein is not the relevant factor here. The number of sites and equilibrium constants reported here should not be confused with the familiar detergent–protein binding studies and protein unfolding experiments (e.g., with sodium dodecyl sulfate) where water is the solvent and the hydrophobic effect is a primary driving force (Tanford, 1980). In the present model, the exchange occurs between two fairly hydrophobic environments, the detergent micelle and the protein surface. The detergent is considered to be the solvent in the equilibrium binding treatment. Second, the molar ratios of the protein and detergent, and not the aqueous concentrations, are the relevant quantities in determining the equilibrium distribution of the interacting species. In contrast to equilibria involving water-soluble molecules where, in general, addition of more solvent produces a shift in the equilibrium, in this model changes in the aqueous volume of the detergent-solubilized

system have no effect on the equilibrium distribution of lipid and detergent at the protein surface. This should be a good approximation when the detergent concentration is sufficiently far above the critical micellar concentration so that no gross structural changes occur in the mixed micelle as the result of changing the aqueous detergent concentration over a limited range of values.

The central assumption in this study is that membrane proteins have a defined hydrophobic surface exposed to lipids (the boundary region). Studies in many laboratories have shown that lipid exchange at the hydrophobic surface of membrane proteins in phospholipid vesicles can be analyzed according to a multiple equilibria binding model (Brotherus et al., 1981; Knowles et al., 1981; London & Feigenson, 1981a,b; Caffrey & Feigenson, 1981; Griffith et al., 1982; Marsh & Watts, 1982; Marsh et al., 1982; Thomas et al., 1982; Ellena et al., 1983; Pember et al., 1983; Silvius et al., 1984; Brophy et al., 1984). Here we extend the model to include nondenaturing detergents displacing lipids at these hydrophobic surfaces of integral membrane proteins. By analogy with the water-soluble equilibrium model, the sites are called "binding sites", but a more appropriate description would be contact sites, since strong interactions would be expected to be the exception rather than the rule in detergent solubilization and lipid–protein binding.

The equations derived here are more general than those required to describe the situation in pure lipid bilayers where only phospholipid molecules compete for the binding sites. Because of the structural similarity between most phospholipids, it is reasonable to assume that in the latter case exchange of phospholipids at the hydrophobic surface of the protein will occur in a one to one stoichiometry with, for example, one molecule of PC* replacing one molecule of PC or one molecule of PG replacing one molecule of PC. With detergents that can have a geometry very different from that of a phospholipid, a strict one to one stoichiometry for detergent–phospholipid and phospholipid–detergent exchange is not necessarily maintained, and this leads to more complicated binding equations. The general eq 10, however, readily reduces to an equation derived previously (Brotherus et al., 1981; eq 6) to describe the binding of spin-labeled phospholipids to membrane proteins in phospholipid bilayers. By setting $\beta = 1$, the denominators in the mole fractions in eq 10 cancel, and by replacing phospholipid with solute lipid and detergent with solvent lipid, the resulting equation is written in terms of the molar ratio of solute and solvent lipids.

Comparison with the water-soluble case shows that the more complicated binding equations can be avoided by adopting conditions where the amount of solvent (detergent) in the system far exceeds that of the other species. In that case, the stoichiometry factor β is effectively eliminated, and the resulting expression (eq 14) contains the molar ratio of free lipid and total detergent as the concentration variable. The results presented here do not yield direct information on the number of detergent contact sites at the protein surface (i.e., βN). This information requires determination of β, which is outside the scope of the present study. By analogy, in the case of water-soluble proteins and ligands, the number of water molecules participating in the equilibria is usually not determined.

Because it is experimentally not possible to attain detergent concentrations that even approach the concentration of water in dilute aqueous solution (about 55.5 M), it was crucial to examine under what experimental conditions use of the approximate eq 14 was justified. The results of the computer simulations (Figures 1 and 3 and Table I) show that, even at high concentrations of a typical membrane protein, sufficiently high detergent concentrations can be reached experimentally (e.g., 100 mM) to warrant use of the approximate equation in analyzing the binding data.

For an experimental test of this equilibrium binding treatment, the integral membrane protein cytochrome *c* oxidase, a multisubunit enzyme complex responsible for the terminal electron-transfer step in the mitochondrial respiratory chain, was selected. Cholate is frequently used in the solubilization, purification, and reconstitution of this enzyme into defined phospholipid vesicles (Casey, 1984). During the solubilization and subsequent lipid depletion steps, the membranes are dispersed, and the cholate displaces most of the native phospholipids, which are then removed. At this point, the protein molecules are solubilized in cholate micelles. Reconstitution involves the addition of the appropriate amount of defined phospholipids and removal of the cholate by, for instance, dialysis, leaving the protein in vesicles of defined composition (Marsh & Watts, 1982; Silvius et al., 1984).

The equilibrium binding results provide some additional insight into why the detergent cholate is effective in solubilization and reconstitution of membrane proteins. Cholate is an amphipathic molecule with a hydrophilic side lined with hydroxyl groups and a nonpolar side that can bind to the hydrophobic protein surface. Cholate also has a fairly high cmc ($\sim$14 mM; Lichtenberg et al., 1983) which facilitates its removal during reconstitution. Our data show that the cholate does not bind as effectively as do natural phospholipids to the hydrophobic surfaces of cytochrome *c* oxidase. For example, for phosphatidylcholine $K \sim 12$, that is, PC has some 12 times greater affinity for the protein surface than cholate. This allows the cholate to be effectively replaced by phospholipid molecules during reconstitution. On the other hand, the K value is low enough to facilitate efficient removal of PC from the protein surface during solubilization with excess detergent.

It is interesting that the relative binding constants are not the same for all lipids. For example, the negatively charged PG requires a model with two classes of sites. One class has a large N and a K similar to that of PC. The other class consists of only a few sites on the protein surface but with a much higher binding constant ($K \cong 2 \times 10^2$). Thus, both phospholipids bind with a somewhat higher affinity than cholate, but a few sites on the protein show selectivity for the negatively charged PG. Some selectivity for negatively charged lipids has been observed previously for cytochrome *c* oxidase in phospholipid vesicles, but the differences in binding constants were much smaller (Marsh, 1985; Powell et al., 1985; Devaux & Seigneuret, 1985). Any selectivity will affect the order of displacement of lipids by cholate in the equilibrium mixture. Therefore, in addition to providing a method of measuring the relative binding of lipids and detergents to membrane proteins, these results point out reason for caution in using only one radioactive lipid (e.g., PC) to monitor the level of displacement of lipids in solubilization procedures.

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Registry No. Cytochrome *c* oxidase, 9001-16-5.

REFERENCES

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Brophy, P. J., Horvath, L. I., & Marsh, D. (1984) *Biochemistry 23*, 860–865.

Brotherus, J. R., Griffith, O. H., Brotherus, M. O., Jost, P. C., Silvius, J. R., & Hokin, L. E. (1981) *Biochemistry 20*, 5261–5267.

Caffrey, M., & Feigenson, G. W. (1981) *Biochemistry 20*, 1949–1961.

Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, Chapter 15, W. H. Freeman, San Francisco, CA.

Casey, R. P. (1984) *Biochim. Biophys. Acta 768*, 319–347.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

Devaux, P. F., & Seigneuret, M. (1985) *Biochim. Biophys. Acta 822*, 63–125.

Ellena, J. F., Blazing, M. A., & McNamee, M. G. (1983) *Biochemistry 22*, 5523–5535.

Griffith, O. H., Brotherus, J. R., & Jost, P. C. (1982) in *Lipid–Protein Interactions* (Jost, P. C., & Griffith, O. H., Eds.) Vol. 2, pp 225–238, Wiley-Interscience, New York.

Griffith, O. H., McMillen, D. A., Keana, J. F. W., & Jost, P. C. (1986) *Biochemistry 25*, 574–584.

Jost, P. C., & Griffith, O. H. (1978) *Methods Enzymol. 49*, 369–418.

Knowles, P. F., Watts, A., & Marsh, D. (1981) *Biochemistry 20*, 5888–5894.

Kuboyama, M., Yong, F. C., & King, T. E. (1972) *J. Biol. Chem. 247*, 6375–6383.

Levenberg, K. (1944) *Q. Appl. Math. 2*, 164–168.

Lichtenberg, D., Robson, R. J., & Dennis, E. J. (1983) *Biochim. Biophys. Acta 737*, 285–304.

London, E., & Feigenson, G. W. (1981a) *Biochemistry 20*, 1932–1938.

London, E., & Feigenson, G. W. (1981b) *Biochemistry 20*, 1939–1948.

Lowry, D. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lowry, R. J., & Tinsley, I. J. (1974) *Lipids 9*, 491–492.

Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431–444.

Marsh, D. (1985) in *Progress in Protein–Lipid Interactions* (Watts, A., & De Pont, E. H. H. J. M., Eds.) Vol. 1, pp 143–172, Elsevier, Amsterdam, The Netherlands.

Marsh, D., & Watts, A. (1982) in *Lipid–Protein Interactions* (Jost, P. C., & Griffith, O. H., Eds.) Vol. 2, pp 53–126, Wiley-Interscience, New York.

Marsh, D., Watts, A., Pates, R. D., Uhl, R., Knowles, P. F., & Esmann, M. (1982) *Biophys. J. 37*, 265–274.

Mrsny, R. J., Volwerk, J. J., & Griffith, O. H. (1985) *Chem. Phys. Lipids 39*, 185–191.

Munson, P. J., & Rodbard, D. (1983) *Science (Washington, D.C.) 220*, 979–981.

Parsons, J. G., & Patton, S. (1967) *J. Lipid Res. 8*, 696.

Pember, S. O., Powell, G. L., & Lambeth, J. D. (1983) *J. Biol. Chem. 258*, 3198–3206.

Powell, G. L., Knowles, P. F., & Marsh, D. (1985) *Biochim. Biophys. Acta 816*, 191–194.

Robinson, N. C., Strey, F., & Talbert, L. (1980) *Biochemistry 19*, 3656–3661.

Silvius, J. R., McMillen, D. A., Saley, N. D., Jost, P. C., & Griffith, O. H. (1984) *Biochemistry 23*, 538–547.

Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53–56.

Tanford, C. (1980) *The Hydrophobic Effect*, 2nd ed., Wiley-Interscience, New York.

Thomas, D. D., Bigelow, D. J., Squier, T. C., & Hidalgo, C. (1982) *Biophys. J. 37*, 217–225.

Vik, S. B., & Capaldi, R. A. (1980) *Biochem. Biophys. Res. Commun. 94*, 348–354.

Watts, A., Marsh, D., & Knowles, P. F. (1978) *Biochem. Biophys. Res. Commun. 81*, 403–409.

Yu, C., Yu, L., & King, T. E. (1975) *J. Biol. Chem. 250*, 1383–1392.