Micelle–Vesicle Transition of Egg Phosphatidylcholine and Octyl Glucoside†

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ABSTRACT: The dissolution and formation of egg phosphatidylcholine (PC) vesicles by the detergent octyl glucoside were examined systematically by using resonance energy transfer between fluorescent lipid probes, turbidity, and gel filtration chromatography. Resonance energy transfer was exquisitely sensitive to the intermolecular distance when the lipids were in the lamellar phase and to the transitions leading to mixed micelles. Turbidity measurements provided information about the aggregation of lipid and detergent. Several reversible discrete transitions between states of the PC–octyl glucoside system were observed by both methods during dissolution and vesicle formation. These states could be described as a series of equilibrium structures that took the forms of vesicles, open lamellar sheets, and mixed micelles. As detergent was added to an aqueous suspension of vesicles, the octyl glucoside partitioned into the vesicles with a partition coefficient of 63. This was accompanied by leakage of small molecules and vesicle swelling until the mole fraction of detergent in the vesicles was just under 50% (detergent:lipid ratio of 1:1). Near this point, a transition was observed by an increase in turbidity and release of large molecules like inulin, consistent with the opening of vesicles. Both a turbidity maximum and a sharp increase in fluorescence were observed at a detergent to lipid mole ratio of 2.1:1. This was interpreted as the lower boundary of a region where both lamellar sheets and micelles are at equilibrium. At a detergent:lipid ratio of 3.0:1, another sharp change in resonance energy transfer and clarification of the suspension were observed, demarcating the upper boundary of this two-phase region. This latter transition is commonly referred to as solubilization. At [PC] > 2 mM, there were mixtures near the micellar phase boundary that separated into two bulk phases. On the basis of lipid and detergent composition, the upper phase was composed of lamellar structures. The structure of the lower phase, which was clear, viscous, and enriched in lipid and detergent, is yet to be determined.

Understanding the behavior of phospholipids and detergents in excess water is essentially a problem of physical chemistry with important implications for membrane protein reconstitution. To examine membrane proteins in an isolated but reasonably physiological state, they must be removed from their native membrane, purified, and resituated in a newly formed phospholipid membrane. Detergents are first added to solubilize the membrane components and then removed to permit membrane formation. How effectively the membrane protein becomes incorporated in the new membrane is determined by the micellar to lamellar transitions of the lipid as the detergent is removed.

The general scheme for lipid dissolution from a lamellar state to mixed lipid–detergent micelles is a succession of states as detergent partitions into the bilayer until, at saturation, the bilayer is converted into lipid-saturated detergent micelles. With further addition of detergent, the mole fraction of lipid in the micelles decreases (Dennis, 1974; Helenius & Simons, 1975; Tanford, 1980; Mazer et al., 1980; Lichtenberg et al., 1983; Jackson & Litman, 1985; Eidelman et al., 1988).

In this study, we examined solubilization and vesicle formation of egg phosphatidylcholine by the detergent octyl glucoside. The latter is frequently used for reconstitutions due to its relatively high critical micelle concentration. To define the structures and compositions of these particular lipid–detergent intermediates, several specific questions were addressed. How many discrete stages exist? What is the nature of the lipid–detergent aggregate at each stage? Are these affected by the rate or direction of octyl glucoside concentration change? What are the relative concentrations of lipid and detergent at each stage? How is the process affected by the phospholipid concentration? To answer these questions, we developed methods for continuously monitoring the process with optical techniques during addition or dilution of octyl glucoside. The aggregation state was followed by measuring optical density, and the interactions among the lipids were detected by the interactions between an energy-transfer pair of lipid probes, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE)[1] and Rho-PE. Since RET is related to probe surface density, it can be used to monitor the various transitions as the system goes from lamellar to micellar. Once the phase diagram could be drawn from these two measurements, several approaches were used to characterize the structures in each region.

Materials and Methods

Chemicals and Vesicle Preparation. Egg phosphatidylcholine (egg PC) and the fluorescent-labeled lipids *N*-(7-nitro-2,1,3-oxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidyl-

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[1] Abbreviations: $C_{12}E_9$, *n*-dodecyl nonaethylene glycol monoether; RET, resonance energy transfer; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine; Rho-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; SUV, small unilamellar vesicle(s); DUV, unilamellar vesicle prepared by dialysis; OD, optical density; DPPC, dipalmitoylphosphatidylcholine; ANS, 8-anilino-1-naphthalenesulfonate; cmc, critical micelle concentration.

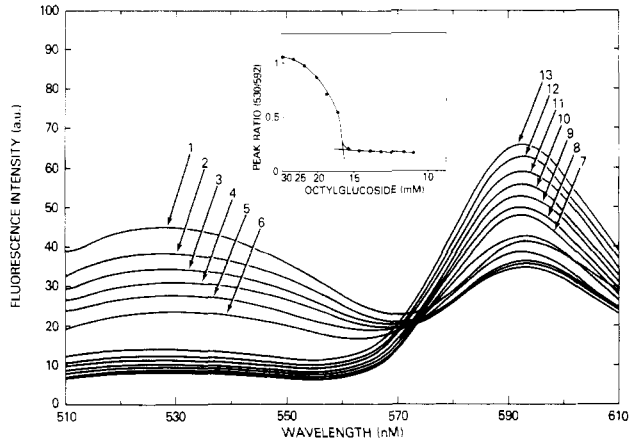

FIGURE 1: Emission spectra of NBD-PE and Rho-PE in PC–octyl glucoside. Spectra were taken 5 min after each addition of octyl glucoside free buffer in the order indicated by the numbers on the figure. Excitation was at 475 nm, and emission was scanned from 510 to 610 nm to observe the emission of NBD-PE (530 nm) and Rho-PE (592 nm). The ratio of the two peaks as a function of [octyl glucoside] is shown in the inset.

ethanolamine (*N*-Rho-PE) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Fluorescent probes were checked for purity by thin-layer chromatography. The detergents octyl glucoside and $C_{12}E_9$ were purchased from CalBiochem (Irvine, CA). Phospholipids and detergents were used without further purification. All preparation and measurements were done in 145 mM NaCl and 10 mM Na-HEPES, pH 7.4, with 0.02% sodium azide. In this buffer, the critical micellar concentration of pure octyl glucoside was measured by ANS (Sigma) fluorescence (Mast & Haynes, 1975) and found to be 20.5 mM at room temperature.

Small unilamellar vesicles (SUV) of egg PC were prepared by sonication. All vesicle preparations were filtered on 0.22-μm sterilization filters (Millex). The phospholipid and detergent concentrations were determined by weight or by total organic phosphate (Ames & Durbin, 1960).

For the phase separation experiment, octyl glucoside and egg PC concentrations were determined by following added [^{14}C]octyl glucoside (CEN/SMM, Gif-sur-Yvette, France) and [^{3}H]DPPC (New England Nuclear). The mixture was left undisturbed for nearly 12 h, and then two aliquots of each phase were counted on a liquid scintillation counter (LS 3801, Beckman) and compared to the original mixture.

Spectroscopic Measurements. Resonance energy transfer (RET) between two fluorescent lipid probes, NBD-PE and Rho-PE, was measured with a spectrofluorometer (Perkin-Elmer, MPF 44B or SLM 8000) with excitation at 475 nm and emission either at 530 or at 592 nm (Struck et al., 1981). A 515-nm high-pass filter was used, when required, to reduce light scattering. As the lipid–detergent mixed micelles were diluted with detergent-free buffer, the relative fluorescence due to energy transfer between the two probes changed as seen from the spectra shown in Figure 1. The ratio F_{530}/F_{590} was calculated, as shown in the inset to Figure 1, to correct for changes in fluorescence intensity due to sample dilution (see below). Turbidity was measured on a Beckman DU-7 spectrophotometer at 350 nm. This wavelength was chosen because it is in a region where changes in the size of these particles will have a large effect on the optical density due to light scattering and where there is no specific lipid or detergent absorbance. Leakage from SUV containing self-quenching concentrations of carboxyfluorescein was followed by the increase in fluorescence as the dye is released (Weinstein et al., 1977).

Monitoring Vesicle Dissolution and Reconstitution in Octyl Glucoside. Measurements of RET and of turbidity were first performed at discrete detergent concentrations. Mixtures of lipid and detergent were prepared in fluorometer cuvettes, and fluorescence intensity or absorbance was measured as described above (Figure 1). It was observed that the fluorescence readings reached steady state in less than 1 min for a 5 mM change in octyl glucoside concentration at the transition region (not shown). Turbidity took somewhat longer, up to several hours to reach steady state in some domains (see below). When octyl glucoside concentrations were varied continuously at rates of 1 mM/min or slower, no differences could be detected between continuous monitoring and data collected at discrete points. Thus, most of the experiments were done by using the continuous procedure which yielded data at higher resolution.

For continuous measurements of solubilization, 1.2 mL of buffer containing the indicated concentration of lipid was placed in a cuvette with a paddle cuvette stirrer that did not interfere with the light path. A concentrated octyl glucoside solution (200, 400, or 500 mM) was slowly and continuously introduced through a thin tubing connected to a glass precision syringe (Hamilton, Reno, NV), which was pushed by a syringe pump (EDCO Scientific, Chapel Hill, NC).

For the reconstitution process, the lipid–detergent solutions were diluted 3-fold with detergent-free buffer using the same technique. Fluorescence was observed as the buffer was added at 530 or 592 nm and stored by a digital recorder. Ratios of the two values taken from paired runs were calculated.

Gel Exclusion Chromatography. Average size and size dispersity of vesicles were measured by gel exclusion chromatography over Sephacryl S1000 or TSK G6000, as previously described (Ollivon et al., 1986). In brief, 50-μL samples were chromatographed at 0.5 or 1.0 mL/min on TSK G6000 PW and/or G5000 PW columns and peaks detected at 254 nm. The columns were preequilibrated with the same medium as the sample.

Vesicle Closure. The concentration of octyl glucoside at which bilayers close to form vesicles impermeable to large molecules during detergent dilution was determined by inulin entrapment. Egg PC, octyl glucoside, and inulin were combined and vesicles formed by addition of detergent-free buffer. The inulin, PC, and detergent are diluted equally until the vesicles close whereas after closure, the inulin within the vesicles is not diluted further. The inulin dilution factor is equal to the concentration of entrapped inulin (I_v) relative to the original concentration (I_0), i.e., I_v/I_0. The octyl glucoside concentration at closure-to-inulin ($[OG]_c$) can be calculated from the inulin dilution factor and the original [octyl glucoside] ($[OG]_0$):

$$[OG]_c = (I_v/I_0)[OG]_0 \quad (1a)$$

To measure the entrapped inulin concentration, we needed a trapped volume marker or a reference solute [glucose (G)] that was present in both the original mixture and the dilution buffer so that its concentration remained constant. The relative concentrations were followed isotopically ([^{3}H]inulin; [^{14}C]-glucose (New England Nuclear)) so that the dilution factor was determined from the ^{3}H:^{14}C ratio or the relative concentrations $(I/G)_v$ and $(I/G)_0$ encapsulated and in the original mixture, specifically:

$$[OG]_c = \frac{(I/G)_v}{(I/G)_0}[OG]_0 \quad (1b)$$

The original mixture of egg PC (12.8 mM), octyl glucoside (40 mM), [^{3}H]inulin, and [^{14}C]glucose was diluted 11-fold

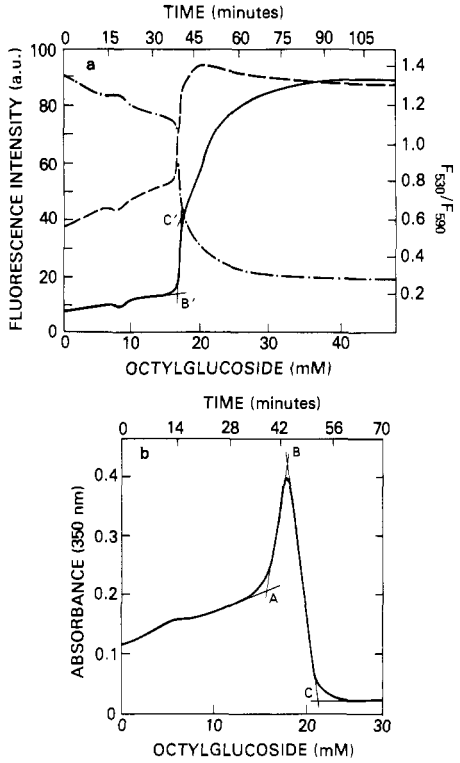

FIGURE 2: Dissolution of PC vesicles by octyl glucoside. (a) RET, indicated as F_{530}/F_{595}, during continuous addition of octyl glucoside to egg PC SUV. F_{530} (- - -) and F_{590} (— · —) were recorded directly, and the ratio F_{530}/F_{590} (—) was calculated to correct for changes in lipid concentration. Concentrated octyl glucoside (400 mM) was added at a constant rate of 0.42 mM/min to egg PC SUV (0.2 mM). Break points are indicated as B and C. (b) Optical density followed during continuous addition of octyl glucoside (0.42 mM/min) to egg PC SUV (2.3 mM). Break points are indicated by the letters A, B, and C.

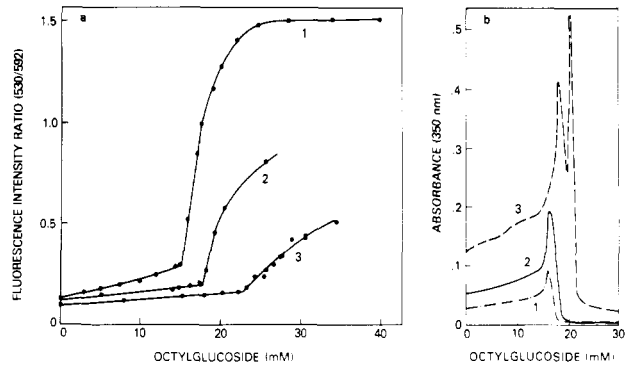

FIGURE 3: Effect of PC concentration on RET and OD during octyl glucoside addition. (a) Energy transfer (F_{530}/F_{590}) as a function of PC concentration during dissolution with octyl glucoside. The initial concentrations of lipid are (1) 0.01, (2) 1.1, and (3) 4.0 mM. These curves were generated by discrete fluorescence measurements made 5 min after each addition of detergent. (b) Turbidity at three different PC concentrations, (1) 0.7 mM (-·-), (2) 1.3 mM (—), and (3) 2.4 mM (- - -), as a function of increasing [octyl glucoside]. The second turbidity peak observed at 2.4 mM PC appeared only at the higher concentrations of lipid.

with detergent-free buffer containing equivalent concentrations of [^{14}C]glucose and unlabeled inulin (to prevent colloid osmotic problems), at a maximal rate of 0.5 mM octyl glucoside per minute (and about 0.1 mM/min near the point of closure). Thus, the specific activity of [^{14}C]glucose was kept constant, while that of [^{3}H]inulin diluted in parallel to octyl glucoside. To rapidly remove residual detergent without losing [^{14}C]-glucose from the vesicles, 1 mL of the mixture was applied on a Sephadex G25 column (PD 10, Pharmacia, Sweden) which was prewashed with buffer containing [^{14}C]glucose and egg PC vesicles. The diluted mixture was then eluted over a G-150 Sephadex column (1.5 × 15 cm) to remove the remaining octyl glucoside, and the external inulin and glucose. Aliquots of the initial mixture and washed vesicle fractions were taken for liquid scintillation counting.

Results

Solubilization by Detergent. Solubilization of egg PC SUV was followed at the molecular level by RET between lipid probes (Figure 2a) and at the supramolecular level by the turbidity of the solution (Figure 2b). The state of the lipid–detergent–water mixture appeared by either technique to fall into four domains separated by apparent break points as noted in the figures. The last phase of the fluorescence change to a steady plateau (i.e., after the break marked "C") was very dependent on the fluorescent lipid probe concentrations, whereas the others, B and C, were independent of the mole percent of probe and could thus be considered true break points. Two of these breaks, designated B and C, occurred at identical octyl glucoside concentrations for both measurements when the PC concentration was the same (see Figure 4 below).

Initially, as octyl glucoside was added to the SUV, both F_{530}/F_{590} and OD increased gradually. The change in RET indicates increased separation of the probes, and the OD change indicates an increase either in the average diameter or in the number of particles. There was a perturbation in the linear increase in fluorescence seen in Figure 2 at about 8 mM octyl glucoside which appeared to be dependent on the total lipid concentration and on the mole fractions of the fluorescent probes (not shown). We have no good explanation for this perturbation (see Discussion). The first break point, A, was observed only by OD and was marked by a sharp increase in the turbidity of the solution over a small increase in [octyl glucoside]. Thus, a reorganization of the assembly occurred that did not disturb the associations among lipid molecules on a molecular scale.

The OD reached a maximum value at B, a point that corresponded to the first sharp change in RET (Figure 2a,b). (The height but not the position of B increased over time.) From point B, F_{530}/F_{590} increased linearly to the next break point denoted C. OD decreased sharply in the same region to a break point at C, after which turbidity continued to decrease very gradually with additional octyl glucoside. However, F_{530}/F_{590} continued to increase as a function of [octyl glucoside] after break point C, reaching a plateau level of fluorescence. No further transitions were observed in the range of [detergent] (0–30 mM) and [lipid] (0–5 mM) beyond break point C. This region is the subject of a subsequent paper on micelle composition (Eidelman et al., 1988).

Dependence on the Phospholipid Concentration. The shape of the OD and fluorescence curves remained the same as a function of increasing PC concentration, but the break points shifted to higher [octyl glucoside] (Figure 3a,b). The plots in Figure 3a were constructed from discrete points after stepwise additions of octyl glucoside. The additions were not in small enough steps to discern point C easily in these experiments. The amount of octyl glucoside required between each break point increased with [PC] as seen, for example, by the broadening of the turbidity peaks in Figure 3b. An additional turbidity peak was observed at [PC] > 2 mM (see below).

The phase diagram for mixtures of egg PC and octyl glucoside in excess water is shown in Figure 4. There appear to be several discrete phases between the two extremes: detergent micelles in the absence of lipid and vesicles in the

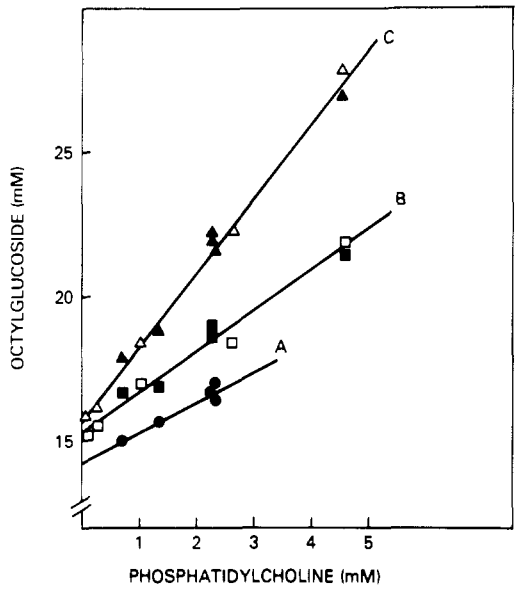

FIGURE 4: Octyl glucoside–PC phase diagram. The total octyl glucoside concentrations at break points A (circles), B (squares), and C (triangles) are shown as a function of PC concentration. The closed points are from turbidity and the open ones from RET measurements taken during either vesicle dissolution (see Figure 3) or formation (see Figure 5).

Table I: Free and Lipid-Associated Octyl Glucoside at the Break Points (T = 25 °C)[a]

break point	$[OG]_{free}$ (mM)	OG:PC mol:mol ratio
A	14.3	1.1
B	15.2 ± 0.2	2.1 ± 0.6
C	15.7 ± 0.1	3.0 ± 0.5

[a] The intercept and slope values for the B and C transitions are the weighted averages from three sets of experiments.

absence of detergent. The physical meaning for each region will be considered under Discussion. The concentrations of octyl glucoside at which all of the break points, A, B, and C, occurred depend on lipid concentration in a linear fashion (Figure 4). The same is true for the "bump" before A (not shown). The concentration of free octyl glucoside at each of those points was estimated from the intercept of the respective line (see Figure 4 and Table I). These concentrations were all far below the cmc of 20.5 mM determined for octyl glucoside at this temperature. The molar ratio of octyl glucoside to PC in the lipid–detergent complexes at the respective transitions can be determined from the slopes of the lines in Figure 4 (see Table I).

At egg PC concentrations larger than about 2 mM and [octyl glucoside] close to C, another turbidity increase was observed, followed by a sharp decrease (Figure 3b). The width of this peak increased as a function of lipid concentration (data not shown). When lipid–detergent mixtures at higher PC concentrations (i.e., >10 mM PC) in this region of the phase diagram were left undisturbed for about 30 min at room temperature (23 °C), there was a macroscopic phase separation resulting in a clear and viscous lower phase and a turbid upper phase. The relative volumes of the upper and lower phases were about 1:3, respectively. In the lower phase, the lipid (35.6 mM) and octyl glucoside (130 mM) were more concentrated than in the initial solution (21.1 and 61.4 mM, respectively), and the relative concentration of octyl glucoside compared to PC was somewhat greater than in the original mixture. Therefore, in the upper phase, the lipid (16.6 mM), and especially octyl glucoside (38.2 mM), was depleted relative to the original concentration.

Vesicle Formation. Vesicles (DUV) were formed by slowly diluting a mixture of octyl glucoside and PC with detergent-free buffer. The transitions noted during dissolution are observed for the formation process as well, but since the concentration of PC was lowered by this procedure, the direct recordings show an apparent shift in the break points (Figure 5a,b). After correction for the appropriate [PC], the octyl glucoside concentrations at the break points (B and C) for vesicle formation coincided with those seen during vesicle dissolution (Figure 4).

However, after vesicle formation, the OD amplitudes at and below B were greater than the corresponding values when SUV were dissolved. Moreover, when SUV were dissolved, there was a continuous, slow increase in the amplitude of the turbidity at B. However, the positions of the break points, A, B, and C, remained the same regardless of the rate of octyl glucoside addition. After 6 h, the OD stabilized at the value observed when B is approached by octyl glucoside dilution, at the same PC concentration. Vesicles formed by rapid detergent removal have a diameter of 610 Å (see below). Thus, this change in turbidity is consistent with an increase in size of the scattering particle from that just sufficient to form an SUV (250-Å diameter). When vesicles formed by removal of octyl glucoside were redissolved, the turbidity pattern was different from that in Figure 2b. For any given [PC], the initial turbidity and the height at B were greater, corresponding to the values observed during vesicle formation (Figure 5b).

Vesicle Size at Low [Octyl Glucoside]. Vesicles in the presence or absence of 10 mM octyl glucoside were chromatographed on a gel exclusion column (TSK G6000) equilibrated with buffer with or without 10 mM octyl glucoside, respectively. The average diameter increased in the presence of detergent. For example, egg PC SUV with an original diameter of 229 Å eluted at a position equivalent to 256 Å in octyl glucoside. Thus, the surface area increased by 25%. This was consistent with the increase in size of 23% estimated from the RET changes by using the relationship (Walter et al., 1986)

$$\Delta A/A_0 = [\ln (F_0/F_{max})/\ln (F_x/F_{max})] - 1 \qquad (2)$$

where the fractional change in area ($\Delta A/A_0$) can be calculated directly from the original NBD-PE fluorescence (F_0) and that at the condition of interest (F_x), i.e., 10 mM octyl glucoside. F_{max} is the fluorescence at infinite probe dilution. The elution times of the PC–octyl glucoside structures in 20 mM octyl glucoside from the TSK G6000 column corresponded to significantly smaller structures (<200 Å).

Lipid Exchange. In order to determine what interactions occurred among the particles at low detergent concentration, lipid exchange was measured. This was done by comparing F_{530} when all the vesicles were labeled (equivalent to Figure 2a) to F_{530} when 90% of the vesicles were replaced with unlabeled ones. Any differences in the fluorescence changes between these two protocols would be due to the NBD dequenching as the probes were diluted among unlabeled lipids by lipid exchange between the labeled and unlabeled vesicles. Addition of octyl glucoside to a mixture of labeled and unlabeled (1:9) vesicles resulted in a rapid increase in F_{530} reaching, within 1 min, the stable fluorescence dequenching level shown in Figure 6. This lipid mixing occurred at detergent concentrations as low as 3 mM and was 80% in the presence of 10 mM octyl glucoside (Figure 6).

Lipid mixing could be mediated by monomer exchange among lipid particles or by fusion between vesicles or micelles. To distinguish between these two possibilities, vesicle size after

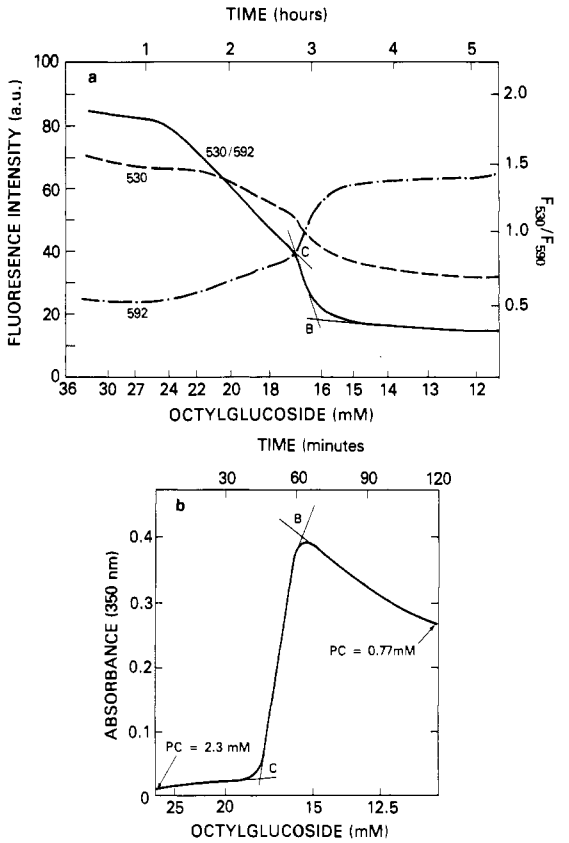

FIGURE 5: Vesicle formation by continuous dilution. Initial mixtures of lipid dissolved in 35 mM octyl glucoside were diluted 2.3-fold with detergent-free buffer. (a) RET monitoring of vesicle formation ($[PC]_{initial}$ = 25 μM); F_{530} (---) and F_{590} (–·–) were recorded directly, and the ratio F_{530}/F_{590} (—) was calculated to correct for changes in labeled lipid concentration. (b) Absorbance measurements during dilution of the sample ($[PC]_{initial}$ = 2.3 mM, $[PC]_{final}$ = 0.77 mM).

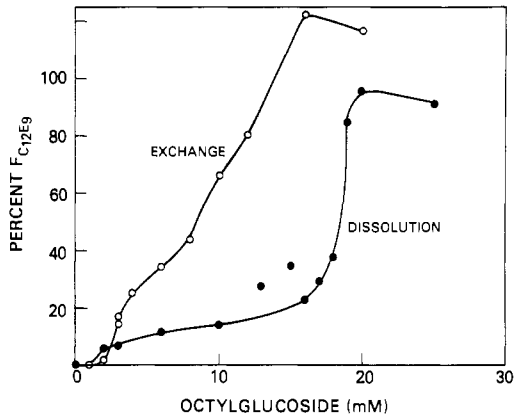

FIGURE 6: Energy-transfer changes measured as F_{535} of PC vesicles (1 mM total lipid) at discrete concentrations of octyl glucoside when all vesicles are labeled with NBD-PE and Rho-PE (closed circles) and when only 1 in 10 vesicles are labeled (open circles). All values are the percentage change 8 min after octyl glucoside addition and are present as the percentage change from the initial value of fluorescence to the value when the system was fully dispersed by $C_{12}E_9$.

exposure to different octyl glucoside concentrations was compared to that of the initial SUV by gel filtration chromatography (Sephacryl S1000). Vesicles exposed to ≤10 mM octyl glucoside and rapidly returned to low detergent were eluted at the same position as the original SUV (230-Å diameter, chromatograms not shown). This indicated that the swelling observed in the presence of detergent (see Vesicle Size at Low [Octyl Glucoside]) was reversible and not due to net vesicle growth. Since there was no evidence for fusion among vesicles exposed to 10 mM or less octyl glucoside, the relief of NBD quenching observed at less than 10 mM octyl glucoside in the unlabeled vesicle experiment must be due to monomer exchange among the vesicles. However, exposure of SUV to 20 mM or more octyl glucoside followed by rapid dilution to low detergent concentration resulted in significantly larger vesicles (610-Å diameter) than SUV.

Vesicle Integrity and Closure. Carboxyfluorescein leakage was measured as a function of octyl glucoside concentration. The fraction of trapped carboxyfluorescein released was a continuous function of [octyl glucoside]. At 1 mM, the lowest octyl glucoside concentration tested, 17% was released in 8 min, while at 10 mM, 90% was lost in 0.5 min (not shown). Assuming that carboxyfluorescein leakage observed at low [octyl glucoside] (i.e., <10 mM) was due to minor disturbances in the membrane structure, and that larger molecules are not released under the same conditions, it is possible to determine the [octyl glucoside] at which sheets of bilayer lipids close to form vesicles. The concentration of octyl glucoside at which inulin became entrapped was determined by slowly adding 10 volumes of detergent-free buffer containing [^{14}C]glucose to a detergent–lipid mixture with both [^{3}H]inulin and [^{14}C]-glucose in the aqueous buffer. The concentration of octyl glucoside at closure-to-inulin, determined from the dilution of inulin relative to the entrapped volume marker glucose as described under Materials and Methods, depended on lipid concentration. At low concentrations of PC (1.05 and 1.15 mM), closure occurred at 11.5 and 12.6 mM octyl glucoside, respectively, while at higher [PC] (3.3 and 5.65 mM) more octyl glucoside was required for closure. This method for determining closure is rather prone to slight errors in sampling and counting. Nevertheless, it is clear that the concentrations of octyl glucoside at which closure occurred for a given [PC] were all somewhat below the corresponding A break point (Figure 4).

Discussion

In this study, we continuously monitored the dissolution and formation of phospholipid vesicles in aqueous solutions of octyl glucoside by the turbidity of the solution and energy transfer between fluorescent lipid probe molecules. Discrete break points, that were taken to indicate changes in the state of the lipid–detergent complexes, could be identified by both types of measurement at particular lipid–detergent compositions. Both dissolution and vesicle formation follow essentially the same pathway since no hysteresis in the break point positions was observed. Thus, the process we studied involves sequential progress through successive states during the lamellar-to-micellar transition.

On the basis of our data, we propose the scheme shown in Figure 7 for the phase transitions of an aqueous solution of egg PC and octyl glucoside at low amphiphile concentrations. In summary, as detergent is added to an aqueous dispersion of lipid, it partitions into the lamellar phase (i) until, at a critical detergent mole fraction, the vesicles open (ii), breaking into sheets composed of lipid (L_α phase) and detergent in the lamellar plane probably with detergent stabilizing the edges (iii). At a higher critical detergent mole fraction, these sheets, now lipid saturated with detergent, transform into micelles that are detergent saturated with lipid (iv) and, most likely, with a maximum thickness determined by the detergent. At higher total [OG], the micelles (v) are composed of progressively less lipid and a higher mole fraction of detergent (Eidelman et al., 1988). The particulars are unique to egg PC and octyl glucoside, the system examined, but both the sequence of events and methods used may be generalized to other lipid and detergent systems. For example, the concept of lamellar disks

and disk-shaped micelles has been proposed for cholate–lecithin mixtures [e.g., see Small et al. (1969)], and similar overall schemes for dissolution and vesicle formation have been demonstrated for bile salts and lecithin [e.g., see Mazer et al. (1980)] and Triton and lecithin [e.g., see Dennis (1974)]. The evidence for the scheme that we propose is discussed below.

Supramolecular Intermediates of Solubilization and Vesicle Formation. (A) Vesicles Swell during Equilibration with Detergent. When pure egg PC is dispersed in excess water by sonication, it forms an L_α bilayer phase that bends into closed spherical vesicles of limit size. When small amounts of octyl glucoside are added, the detergent monomers partition between the aqueous and PC phases. The partition coefficient estimated from the octyl glucoside concentration in the lipid (assuming an average lipid density of 1 g/cm^3) in the structures up to point A is about 63 (concentration ratio) which compares favorably with the value of 59 determined previously by equilibration and centrifugation of vesicles in the presence of detergent at 25 °C (Jackson et al., 1982).

When the amount of detergent in the vesicles is 5 mol %, the vesicles become leaky to moderately sized solutes such as carboxyfluorescein (376 g/mol), suggesting some changes in the membrane structure occur. Inulin, however, is retained at these detergent concentrations. A graded but transient increased permeation rate for small solutes also was reported during cholate addition to PC at cholate:PC ratios greater than 0.15 (Schubert et al., 1986).

There is also lipid exchange among vesicles at low [octyl glucoside] as indicated by lipid–probe movement among vesicles with no concomitant irreversible vesicle growth as would be expected for a process such as fusion. This is in contrast to the situation with cholate, which induces a net lipid transfer from smaller to larger vesicles until equilibrium size is reached (Almog et al., 1986). However, in the *presence* of detergent, the vesicles swell as measured by RET (Figures 2a and 5a), turbidity (Figures 2b and 5b), and gel filtration chromatography. The increase in surface area reported by the lipid probes is linear with [octyl glucoside] until the mole fraction of detergent in the membrane is about 0.45 and the surface area has increased by 50%.

The first feature to be observed is a small bump in the fluorescence data that is seen by both probes. We do not know what this means. However, it is probably not a change in state, since the position of the bump varies with the mole percent of the fluorescent probe used (data not shown) and is therefore a method-dependent feature. It *might* be explained as a change in the disposition of probes in the membrane, resulting in more quenching, countervailing the effect of increased probe separation on RET.

(B) Vesicle-to-Sheet Transition. When the total octyl glucoside concentration is high enough for the fraction associated with the lipids to be about 50 mol %, vesicles begin to break and open into sheets or shells as indicated by the initial sharp increase in turbidity (A). Our estimates of the position of vesicle closure from inulin entrapment are also close to, but below, break point A, corroborating our interpretation that this break marks vesicle opening and the commencement of flattening into lamellar sheets. Inulin leakage would mark the appearance of large, detergent-stabilized pores, a necessary precedent to vesicle flattening. This opening is not accompanied by a change in RET, indicating that there is no significant change in lamellar organization. Others have envisioned sheets or disks as part of the phase diagram for aqueous dispersions of lipid and detergent. For example, cholate–phospholipid mixtures are proposed to form disks (Small et al., 1969; Mazer et al., 1980). Fromherz and Ruppel (1985) observed cholate-stabilized disks by electron microscopy. Proteins such as Apo A1 and melittin also are known to stabilize disks of lamellar phase lipids (Klausner et al., 1985; Dufourcq et al., 1986).

The dramatic OD increase seen in conjunction with lamellar sheet formation may represent the appearance of larger structures that must contain more lipid than a single SUV. The opened vesicles probably "fuse" to decrease their curvature while maintaining relatively low proportions of "edge", where presumably octyl glucoside would be at high local concentrations. Obviously, the thermodynamically favored size of these sheets is larger than that of a SUV. Since the maximum OD observed at point B occurs during vesicle formation from lipid–detergent micelles *and* is equal to the OD observed during DUV dissolution (or after a long time during SUV dissolution), one might infer that the most stable egg PC and octyl glucoside sheet has the same number of lipids as a DUV. From estimates of lipid and detergent numbers and surface areas, these sheets should be about 190 nm in diameter. Growth of the sheets from SUV to a stable size is relatively slow, occurring over about 6 h at 25 °C, whereas during vesicle formation (DUV) large sheets form from mixed micelles in minutes.

A turbidity peak that occurs during SUV dissolution has been reported for Triton X-100–PC, octyl glucoside–PC, and other detergent–lipid mixtures [e.g., see Alonso et al. (1982), Goni et al. (1986), and Paternostre et al. (private communication)]. Jackson et al. (1982) observed a turbidity increase when DUV or SUV are dissolved. However, their [PC] was greater than 6.1 mM in all cases, and we suspect the turbidity peak they observe to include the second peak we find in our samples (Figure 3b) that occurs in the two-phase domain at high [PC]. In general, the turbidity has been taken to mean aggregation or an increase of vesicle size (Jackson et al., 1982) based on increased average diameters observed after detergent removal [e.g., see Alonso et al. (1982)]. Since inulin is not trapped at these concentrations, our interpretation is that these structures have large holes and are not proper vesicles so that the growth process should be described as fusion among sheets or shells rather than among vesicles.

(C) Sheet-to-Micelle Transition. At the turbidity maximum (designated B), a break is seen also by RET (see Figure 2a,b). When the molar ratio of octyl glucoside to PC becomes 2.1:1 (Table I), there is an increase of the rate of fluorescence change with rising [octyl glucoside], while the OD starts to decrease. The turbidity decrease indicates the appearance of smaller particles while the dramatic change in RET shows that these particles have a very different lipid packing from the original L_α bilayer phase. We interpret this transition as the beginning of the supramolecular rearrangement from detergent-saturated sheets of lamellar phase lipids to lipid-saturated micelles. The shape is probably still flattened [see Eidelman et al. (1988)], but instead of an L_α arrangement of the lipids, we envision the thickness to be determined by the octyl glucoside and the lipids interdigitated with a lot of detergent separating each molecule. The lower boundary for conversion from bilayers to mixed micelles with octyl glucoside was determined to be at a detergent to lipid ratio of 1.5:1 by Jackson et al. (1982), somewhat lower than the value we observe.

The last phase boundary is identified at higher octyl glucoside concentrations by the appearance of a clarified solution and a somewhat shallower increase in F_{530}/F_{590} as a function of [octyl glucoside]. The break point (both in OD and in RET) plotted as a function of PC concentration (Figure 4) again shows a linear dependence on phospholipid concentration.

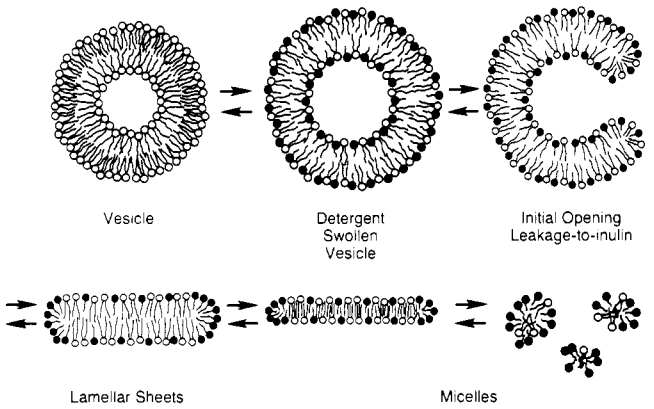

FIGURE 7: These cartoons represent the structures proposed for the sequential states of the lipid–detergent complexes during vesicle dissolution and formation. See text for further explanation.

From the slope, we calculate an average of three octyl glucoside molecules per PC molecule at this boundary. Jackson et al. (1982), using NMR techniques, have identified the same boundary at an octyl glucoside:lipid ratio of 3:1 and observed an isotropic dispersion of lipid in that state. Lamellar regions are no longer stable structures, and further changes will be in the size and composition of mixed micelles. How these change as octyl glucoside is added will be addressed in a subsequent publication (Eidelman et al., 1988).

From break points B to C, the linearity of both the turbidity decrease and RET increase is consistent with the existence of two populations in equilibrium as also shown by NMR (Jackson et al., 1982). This happens when the two structures are different enough so that a continuous transition from one to another cannot occur. Thus, the observed linearity of both parameters (OD and RET), which have different behaviors as a function of particle size, makes the alternative hypothesis of a sheet to micelle transition via a continuous reduction in size less likely. Instead, the data show the transition occurring through a two-phase equilibrium with the detergent-saturated sheets of lamellar phase lipids transforming into lipid-saturated mixed micelles. Mixed populations of lamellar and micellar species were also observed for Triton X-100 at intermediate stages of solubilization of sphingomyelin [by electron microscopy and sedimentation velocity (Yedgar et al., 1974)] and Triton–phosphatidylcholines [column chromatography (Dennis, 1974) and calorimetry (Goni et al., 1986)]. Bayerl et al. (1986) identified a coexistence region by ^{31}P NMR when solubilizing hepatic microsomes with octyl glucoside.

At high lipid concentrations (above 2 mM), the mixture of lamellar sheets and mixed micelles can also form what we believe are extended structures composed of mixed micelles or a combination of mixed micelles and lamellar structures. These are indicated by a second sharp absorbance peak adjacent to the boundary at C in the absence of a break in the RET measurements (Figure 3) and a region where two macroscopic phases coexist. The upper phase is turbid with PC and octyl glucoside concentrations consistent with the values predicted at break point B, indicating a region where the predominant structure is lamellar. The lower phase is more of a quandary. It is somewhat viscous, although water rich, and its conductivity is about 80% of the upper phase. It presents an X-ray scattering pattern corresponding to an unoriented ordered structure (M. Ollivon, unpublished results). The detergent to lipid ratio observed for this phase corresponds to the micellar domain (see below). The various observations taken together (detergent to lipid ratio, X-ray, viscosity, and conductivity) indicate the existence of arrays of mixed micelles, perhaps including a small fraction of lamellar lipid. This arrangement might be analogous to the C-mesophase recently observed in amphiphile mixtures (Tabony et al., 1987) or to the network of stacked structures observed by negative-stain electron microscopy (Fromherz & Ruppel, 1985) that were interpreted to be bilayer lamellae of cholate–egg PC mixtures. We have also considered the possibility that this is a hexagonal phase, perhaps as seen by Small et al. (1969) for cholate–PC mixtures; however, the aqueous content and the very weak X-ray pattern make this unlikely. Regardless, the evidence for any structural interpretation is still rather weak, and, therefore, this curious phase is currently under investigation.

(*D*) *Large to Small Mixed Micelles.* At phase boundary C, the solution is clarified, and all the lipid is in lipid-rich detergent micelles. Turbidity decreases only slightly, meaning either that the micelle size does not decrease much further or that the sizes are too small to be discriminated at the scattering wavelength used. The RET continues to change as detergent is added until the two lipid probes are completely dispersed among micelles that contain an increasing proportion of detergent. The lipid–detergent compositions of these micelles will be reported in a subsequent paper (Eidelman et al., 1988).

Thermodynamic and Kinetic Considerations of the Transitions. The use of RET between lipid probes as a means of studying the state of lipids in micelles proved very efficacious. This technique discriminates among states and provides specific information regarding the interactions among the lipid molecules. For example, the RET information corroborates the turbidity changes, which are indicative of vesicle swelling at low [octyl glucoside]. Moreover, RET shows that the increase in area is linear with [octyl glucoside]. RET clearly demonstrates that the lipid structure remains lamellar immediately after vesicles break into sheets. The NMR techniques used previously to explore egg PC–octyl glucoside lamellar–micellar transitions could not provide an unambiguous interpretation of the nature of the change occurring at this point (Jackson et al., 1982). Of note is the observation made after this work was completed (Eidelman et al., 1988) that the fluorescence of NBD-PE alone is sensitive to solubilization, and clearly reports transitions B and C. This is further corroboration that these transitions are true changes in the molecular arrangements of the lipids and detergents. Thus, fluorescent techniques, and especially RET, are useful tools for studying a wide variety of lipid–lipid interactions.

The energy-transfer techniques, combined with turbidity measurements, indicate that there are two types of intermediate structures between the L_α and micellar stages and that these are equally accessible by detergent dilution or addition, although not at equal rates. Thus, the structures described are equilibrium structures, and the process is thermodynamically and not kinetically controlled, at least for the rates of change in [detergent] of 1 mM/min or slower. However, the turbidity measurements indicate that the structure at B (but not the position of B or the other break points), when reached during SUV dissolution, is not an equilibrium structure since the size increases with time toward the value seen for vesicle formation. At high rates of detergent removal or dilution, when equilibration is not achieved, the final product is indeed kinetically determined as reported by Mimms et al. (1981). We also found that rapid dilution of PC–octyl glucoside mixtures results in heterogenous vesicle preparations [e.g., see Walter, Zimmerberg, and Harris (unpublished results)].

The general scheme for the vesicle–micelle transition is similar to that previously proposed for various bile salt–PC mixtures as well as solubilization by Triton X-100 and octyl

glucoside. However, the bile salts seem to require long equilibration times when mixed with PC and behave differently at vesicle closure. Thus, cholate takes hours (Lichtenberg et al., 1983) to days (Schurtenberger et al., 1984) to equilibrate, whereas octyl glucoside–PC mixtures generally equilibrate in minutes as observed by Jackson et al. (1982) and by us. Vesicles formed from cholate also grow after closing (Almog et al., 1986) in contrast to PC–octyl glucoside mixtures which grow to equilibrium size during the disk stage and do not fuse (at an appreciable rate) once vesicles are formed. It is likely that the smaller cholate–PC disk size and instability of the cholate-saturated vesicles are related to the shape and low aggregation number of the detergent micelles. Triton X-100, a nonionic detergent with a much lower cmc than octyl glucoside, appears to follow the same pattern during solubilization at similar detergent:PC ratios. Bilayers remain intact in the presence of Triton X-100 at levels up to 50 mol % (near our break point A), exist in a mixed lamellar and micellar phase at mole ratios from 1:1 to 2:1, and are fully solubilized when the structures contain somewhere between 2:1 and 3:1 Triton:PC ratios (Dennis, 1974). Similarly, we see solubilization with octyl glucoside–PC at ratios of 3.0:1 (break point C).

Critical Mixed Micelle Concentration. The pure octyl glucoside critical micelle concentration (cmc) depends on the medium's salt concentration and temperature. In our buffer (145 mM NaCl), at 25 °C, we found a cmc of 20.5 mM, using a fluorometric method (Mast & Haynes, 1975), which is in agreement with the original value of 25 mM (Shinoda et al., 1961). The B and C phase boundaries, which correspond to the first appearance of mixed micelles and to complete solubilization of the bilayers, respectively, extrapolate at zero [PC] and to 15.2 and 15.7 mM octyl glucoside (Figure 4, Table I). The critical mixed micelle concentration observed in the presence of PC is clearly well below the pure detergent cmc. Since both PC and octyl glucoside are amphiphiles and the phospholipid maximum solubility is several orders of magnitude lower than that of octyl glucoside (Tanford, 1980), the intermediate value observed is not surprising. Indeed, this behavior is predicted for mixtures of two amphiphiles or a solubilizing agent in the presence of a solute. Tanford (1980) proposed that if one amphiphile is much less soluble than the other, it can be considered a solute, and if one assumes ideal mixing within the micelle, then one can apply Raoult's law to estimate the aqueous concentration (i.e., effective cmc) for the detergent. Thus, the aqueous concentration is calculated by

$$C_{aq} = \chi_{mic}\text{cmc}^0 \quad (3)$$

where C_{aq} is the aqueous detergent concentration, cmc^0 is the detergent cmc in the absence of solute, and χ_{mic} is the mole fraction of detergent in the micelle. From our value of χ_{mic}, we estimate the free octyl glucoside concentration to be 15.5 mM at vesicle solubilization which is between the detergent concentration at the initial appearance of mixed micelles (15.2 mM, Table I) and the upper boundary of the lamellar–micellar mixed phase (15.7 mM, Table I). This is quite remarkable in light of the assumptions, especially that of ideal mixing within the micelle which is not likely considering the differences between the two molecules.

The reduction in octyl glucoside cmc due to the presence of PC that we observe is also in agreement with the observations reported by Stubbs and Litman (1978), Alonso et al. (1982), and Rivnay and Metzger (1982). In the latter case, the critical mixed micelle concentration of 22 mM octyl glucoside at 4 °C is actually 10–11 mM below the pure detergent cmc at this temperature (A. Walter et al., unpublished results).

Conclusions. With this methodology to determine the lipid and detergent behavior during solubilization and vesicle formation, it is feasible to add a third component and study the process of protein incorporation at a molecular level. We are presently studying the incorporation of the spike glycoprotein from vesicular stomatitis virus into a defined phospholipid environment.

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Two Components of an Extracellular Protein Aggregate of *Clostridium thermocellum* Together Degrade Crystalline Cellulose†

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ABSTRACT: The extracellular cellulase system of *Clostridium thermocellum* ATCC 27405 was fractionated on a Sepharose 2B gel filtration column by using assays based on the hydrolysis of Avicel and (carboxymethyl)cellulose (CMC). Four A_{280} peaks were eluted, with most of the Avicelase (cellulase active on crystalline cellulose such as Avicel) and (carboxymethyl)cellulase (CMCase) activities coinciding with the second peak (approximate M_r 6.5×10^6). A lower molecular weight CMCase was also detected. Avicelase-containing fractions displayed six major protein bands (M_r 60000–250000) and many other minor bands on a sodium dodecyl sulfate (SDS) gel, indicating that Avicelase exists as a multisubunit protein aggregate. The Avicelase-containing protein aggregate was dissociated by mild SDS treatment, and the resulting subunits or subcomplexes were resolved on an Ultrogel AcA 34 gel filtration column in the presence of 0.1% SDS. Although none of the individual fractions obtained displayed significant Avicelase activity, Avicel was degraded by a combination of two fractions after the removal of SDS. One of the fractions showed only a single major protein species (M_r 82000, designated S_S) on an SDS gel. The other fraction was a mixture of at least six protein species of higher molecular weights. The major protein species (M_r 250000, designated S_L) of the latter fraction was purified by elution from an SDS gel and displayed no Avicelase activity. However, S_L degraded Avicel when combined with S_S. These results indicate that the degradation of crystalline cellulose by *C. thermocellum* can be accomplished by combining two protein components (S_S and S_L) existing in the extracellular protein aggregate. The roles, if any, of the other peptides present in the protein aggregate are not known, but we can say that they are not required for activity on the substrate Avicel under the conditions of our assay.

Clostridium thermocellum produces an extracellular cellulase system capable of degrading crystalline cellulose such as cotton and Avicel (Johnson et al., 1982b). Studies of the *C. thermocellum* cellulase system are important due to the potential use of this bacterium in the direct conversion of cellulosics to liquid fuel or chemicals (Avgerinos & Wang, 1980). Two endo-β-glucanases (1,4-β-D-glucan glucanohydrolase; EC 3.2.1.4) with molecular weights of 56000 (endoglucanase A; Petre et al., 1981) and 83000–94000 (Ng & Zeikus, 1981) have been purified and characterized from this organism. A gene (*celA*) coding for endoglucanase A has been cloned and expressed in *Escherichia coli* (Cornet et al. 1983) and in *Saccharomyces cerevisiae* (Sacco et al. 1984); its nucleotide sequence has been determined (Beguin et al., 1985). Another gene (*celB*) coding for another endoglucanase (endoglucanase B) has also been cloned in *E. coli* (Cornet et al., 1983), and its gene product with a molecular weight of 66000 has been purified from *E. coli* (Beguin et al., 1983). Five additional genes coding for endoglucanases have also been cloned into *E. coli* (Millet et al., 1985). These five genes differ from one another and from *celA* and CelB in their restriction maps. Recently, endoglucanase C (M_r 39000; Petre et al., 1986) and endoglucanase D (M_r 65000; Joliff et al., 1986) were purified from the *E. coli* clones that harbor *cel* genes. The endoglucanases that have been studied degrade (carboxymethyl)cellulose (CMC),[1] a soluble cellulose derivative.

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, (carboxymethyl)cellulose; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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