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THE PLANAR DISTRIBUTIONS OF SURFACE PROTEINS AND INTRAMEMBRANE PARTICLES IN *ACHOLEPLASMA LAIDLAWII* ARE DIFFERENTIALLY AFFECTED BY THE PHYSICAL STATE OF MEMBRANE LIPIDS *

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Summary

We have studied the influence of changes in lipid organization on the planar distribution of two classes of membrane proteins: integral proteins which have amino groups exposed to labelling at the membrane surface by the biotin-avidin-ferritin procedure, and those proteins which penetrate the lipid bilayer sufficiently to be seen as intramembranous particles by freeze-fracture electron-microscopy.

When the membranes are examined at temperatures below the lipid phase transition, the first class is dispersed and the second patched. At temperatures in the middle of the transition range, both classes are patched. At temperatures just above the phase transition the first class is dispersed and the second patched, and at temperatures well above the transition both classes are dispersed. Freeze-etch studies of avidin-ferritin-labeled membranes confirmed that the distribution seen by the labeling and the freeze-fracture techniques coexist in single membranes. Thus, there exist two distinct classes of membrane proteins with differential organizational responses to the lipid state.

Introduction

The existence in membranes of local regions of vastly different protein and lipid composition is suggested on both functional and structural grounds. It is therefore relevant to study molecular interactions which may give rise to different planar distributions in a membrane. In this study of *Acholeplasma*

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membranes we determine the influence of changes of lipid state on the organization of two classes of protein: those which have primary amino groups exposed at the membrane surface and those that give rise to intramembrane particles upon freeze-fracturing. Some proteins may be members of both classes, but our findings emphasize differences in behavior which show that the classes are not identical.

X-ray diffraction and differential scanning calorimetry have shown that lipids in some biological membranes undergo a thermal phase transition from an ordered paracrystalline state to a disordered fluid state at a temperature which depends on the fatty acid composition of the lipids [1,2]. We have demonstrated that, in *Acholeplasma laidlawii*, this change in lipid organization is accompanied by a change in the planar organization of membrane surface proteins [3]. In membranes from this organism, the biotin-avidin-ferritin procedure [4] specifically labels integral membrane proteins which are exposed, in part, on the cytoplasmic surface of the membrane [5], and permits visualization of these labeled sites in the electron microscope. Membranes were labeled with avidin-ferritin at temperatures above, below and in the middle of their transitions. In membranes labeled either above or below the transition, the labeled sites were relatively dispersed in the plane of the membrane, whereas in membranes labeled at temperatures in the mixed phase (mid-transition), patches of high and low density of label were found.

We now examine the influence of lipid state on those membrane proteins which are deeply embedded in, or which span the lipid bilayer. Freeze-etch electron microscopy permits observations of the behavior of intramembrane particles as well as avidin-ferritin labeled surface proteins in a single preparation. Previous reports [6—9] have suggested that the state of membrane lipids influenced the distribution of particles, but in a different manner than was observed for surface proteins: a relatively random distribution of particles existed far above the phase transition temperature; aggregation began at a temperature well above the transition and increased with decreasing temperature. The aggregation was complete at the lower end of the transition range, and no further changes were seen with decreasing temperature.

Thus, the reported distribution patterns of intramembraneous particles and surface proteins as a function of lipid state are significantly different. These differences could have resulted from several sources: variations between samples used in the two experiments, artefacts produced by one of the techniques which would result in false differences in distribution patterns, or the possibility that the two techniques detect the behavior of two classes of proteins (possibly overlapping subsets) which are influenced in different manners by the physical state of membrane lipids. To eliminate the possibility that the freeze-fracture or ferritin labelling techniques were producing artefacts which resulted in the different distribution patterns, or that variations in the experimental details of the sample preparation were responsible, it was important to establish the behavior of surface sites and intramembraneous particles in a single sample. This freeze-etch study of ferritin-labeled membranes demonstrates in a single sample that temperature-induced changes in the lipid state can cause different changes in the relative locations of different protein components parallel to the membrane surface.

Materials and Methods

(A). Organism and growth conditions

A. laidlawii B was grown at 37°C in lipid-poor medium which was supplemented with 120 μM/l erucic, palmitic, or eicosenoic acid, as previously described [3]. Cells were harvested in mid-log phase, as determined by following absorbance at 550 nm.

(B). Isolation and characterization of membranes

Membrane isolation and characterization procedures have been previously described [3,10].

Protein was determined according to the method of Lowry et al. [11]. The fatty acid composition of extracted lipids was analyzed by gas-liquid chromatography using a Perkin Elmer Model 990 chromatograph.

The phase transition temperature ranges of membranes were determined by X-ray diffraction using an Elliot toroid point focusing camera [12], with a thermally controlled sample holder. The first appearance of a broad 4.6 Å diffraction ring marked the onset of the disordered phase (lower boundary); the disappearance of the sharp 4.15 Å reflection associated with the paracrystalline phase indicated the upper boundary of the transition [1].

(C) Synthesis of reagents

Biotinyl-*N*-hydroxysuccinimide was synthesized according to the method of Becker et al. [13]. Avidin-ferritin was synthesized by the method of Heitzmann and Richards [4], as modified by Wallace et al. [3].

(D) Labeling of membranes

Membranes were labeled with biotinyl-*N*-hydroxysuccinimide as previously described [3]. Membranes were labeled in solution with avidin-ferritin as follows: A 0.5 ml suspension of biotinyl-*N*-hydroxysuccinimide-labeled membranes (approx. 1 mg protein/ml in 0.1 M $NaHCO_3$) was reacted with 0.3 ml avidin-ferritin (approx. 40 mg ferritin/ml) for 1 h, at a temperature in the middle of the phase transition range for each fatty acid supplement. This temperature is chosen since the local surface concentrations of labeled sites were highest under these conditions, which pose the largest steric constraints on the extent of labeling. Subsequent changes are, therefore, toward lower concentration so that steric effects of the label are minimized. Membranes were washed three times with 0.1 M $NaHCO_3$. Unlabeled membranes were removed by centrifugation on a 35—50% sucrose gradient in 0.1 M $NaHCO_3$ at 40 000 rev./min for 15 h in an SW 50.1 rotor. The denser, labeled membranes formed a pellet, while unlabeled membranes and small membrane fragments remained in the gradient. Labeled membranes were washed twice in 0.1 M $NaHCO_3$.

(E) Fixation

Unlabeled membranes (1 mg protein/ml) were fixed with a 2% glutaraldehyde solution in 0.1 M $NaHCO_3$ for 1 h at temperatures above, below, and in the middle of the phase transition in different experiments. They were then washed twice in 0.1 M $NaHCO_3$.

Freeze-etch methods

1. Sample preparation. Membranes were centrifuged at 40 000 rev./min for 30 min in an SW 50.1 rotor. A small amount of firm pellet was placed on a flat gold carrier (Balzers) and equilibrated at the experimental temperature for 20 min in a high humidity chamber. The samples were rapidly cooled in liquid nitrogen-cooled liquid Freon 22 (Matheson Gas) and then transferred to liquid nitrogen for storage.

2. Freeze-etch conditions. Samples were fractured and etched, and replicas prepared in a Balzers freeze-fracture apparatus operating at a pressure of less than 10^{-6} torr. Pellets were fractured at −100°C with a microtome set at an advance speed of approx. 3 μm/s. Fracture faces were etched for 50—70 s. The etched samples were shadowed with platinum from a 45° angle, then coated with carbon perpendicular to the microtome stage. The thickness of the replica (20—25 Å of Pt, approx. 200 Å of carbon) was monitored automatically with a quartz crystal. Replicas were floated onto a hypochlorite solution (Clorox) and cleaned for 24 h to remove membrane debris. Avidin-ferritin-labeled membranes required additional cleaning with 40% chromic acid. Clean replicas were picked up on formvar-coated 200 mesh copper grids.

3. Electron microscopy. Grids were examined in a Philips EM300 electron microscope operating at 80 kV, and were photographed on Kodak medium contrast plates of Kodak S0-163 electron image film with an exposure time of 1 s. At least two different replicas of each sample frozen from the appropriate temperature were prepared and examined. When fractured, a relatively small proportion of the membranes on each replica showed fracture faces which are thought to arise from a splitting of the membrane between the halves of the bilayer [14]. A majority of the membranes present were cross-fractured. The relative proportion of the appearance of these two types of fractures did not seem to depend on the presence or absence of avidin-ferritin; however, more fracture faces were found in pellets that contained a lower concentration of membranes.

Results

(A) General characterization of membrane preparations

Supplementation of the growth medium with various fatty acids produces *A.*

TABLE I

PHASE TRANSITION TEMPERATURES AND FATTY ACID COMPOSITION

Supplementation	Phase transition temperature range (°C)	12 : 0	14 : 0	16 : 0	18 : 0	18 : 1	18 : 2	20 : 0	20 : 1	22 : 1
Erucate (22 : 1)	14–22	5.7	21.2	19.7	8.2	—	—	—	—	38.3
Eicosenoate (20 : 1, *cis*-11)	10—20	10.5	22.2	17.3	2.2	0.6	—	—	47.2	—
Palmitate (16 : 0)	27—37	2.0	3.0	54.0	14.4	10.7	14.2	1.6	—	—

laidlawii membranes which have different lipid phase transition temperatures. Membranes prepared under a given set of growth conditions are fairly homogeneous in fatty-acid composition and have relatively narrow phase transition temperature ranges, as determined by X-ray diffraction (Table I). Comparison of unlabeled and avidin-ferritin-labeled membranes indicates that the presence of the biotin and large avidin-ferritin labels have no effect on the lipid phase transition temperature ranges. For these studies, membranes were enriched with fatty acids that had been used for surface labeling studies: erucate (22 : 1), eicosenoate (20 : 1, *cis*-11) and palmitate (16 : 0). The membranes had widely differing, and overlapping, phase transition ranges: 14 to 22°C for erucate, 10 to 20°C for eicosenoate, and 27 to 37°C for palmitate. This permitted a determination of the intramembranous particle and surface protein distributions relative to the lipid phase transition temperature over different ranges of absolute temperature. For each supplement, unlabeled and

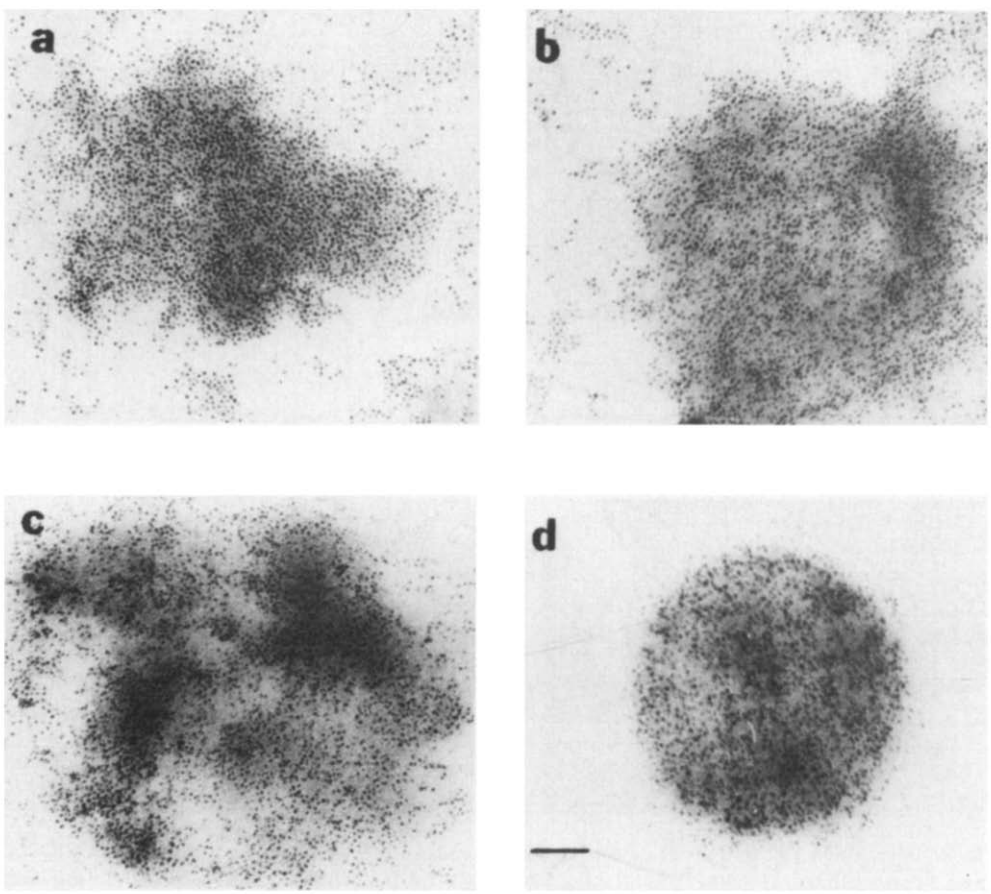

Fig. 1. Erucate-enriched membranes labeled with avidin-ferritin and dried at: (a) 37°C (well above), (b) 23°C (just above), (c) 17°C (middle), (d) 4°C (below) the transition range. The surface protein distribution is dispersed at temperatures below and above the lipid transition (just above and well above), whereas the distribution is patched when membrane lipids are partially through the transition. In all figures, the bars represent 0.1 μm.

avidin-ferritin-labeled membranes frozen from temperatures above, below, and in the middle of their phase transition range were examined by freeze-etch electronmicroscopy.

(B) Microscopy of avidin-ferritin-labelled, dried membranes reveals lipid state-dependent distributions of surface proteins

The results obtained for these membrane samples were consistent with those previously reported. Erucate-enriched membranes are used here as an illustration; however, similar results were also obtained for other fatty acid supplements. Membranes were labeled with biotinyl-*N*-hydroxysuccinimide and avidin-ferritin and dried on grids as previously described [3] at temperatures well above (37°C), just above (23°C), in the middle (17°C), and below (4°C)

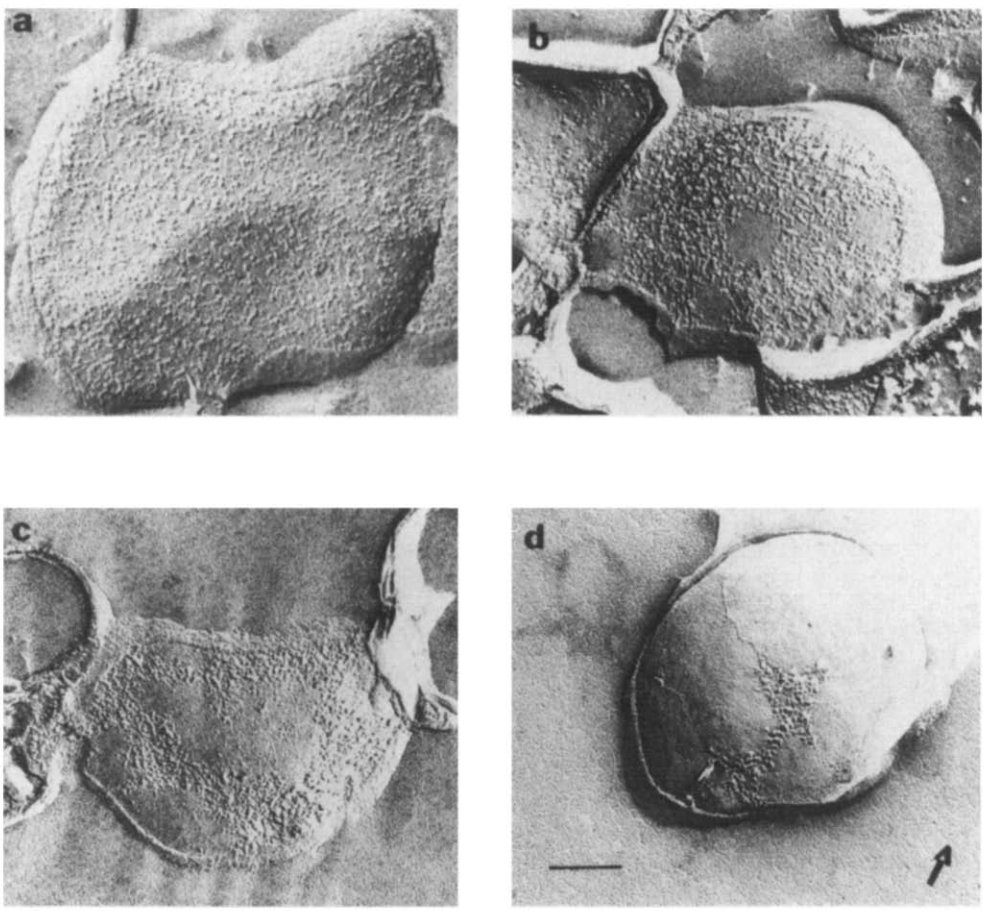

Fig. 2. Freeze-dried preparations of erucate-enriched membranes. Membranes were frozen from (a) 37°C (well above), (b) 26°C (just above), (c) 17°C (middle), (d) 4°C (below) the transition range. The intramembranous particle distribution is dispersed in the samples frozen from a temperature far above the transition, whereas the distribution is patched in samples frozen from just above, below, and in the middle of the transition. The degree of patching appears to increase somewhat with decreasing temperature. Similar particle distributions at corresponding temperatures relative to the phase transition were found for eicosenoate-enriched membranes. In this case, the temperatures were 4°C (below), 16°C (middle), 25°C (just above), and 37°C (well above). The arrow indicates the direction of platinum shadowing.

the lipid phase transition (Fig. 1). At temperatures well above, just above, and below the transition, the surface protein distribution (as indicated by the ferritin-labeled sites) is dispersed. In the middle of the transition, the surface proteins are patched.

(C) Freeze-fracturing shows different lipid state-dependent distributions for intramembranous particles

Our freeze-fracture results correlate well with the findings of James and Branton [9]. We found that at a temperature well above the transition (i.e., 37°C for erucate-supplemented membranes), the particles are dispersed; particle aggregation began at a temperature just above the transition (23°C) and increased with decreasing temperature (Fig. 2). The behavior of membranes supplemented with palmitate and eicosenoate is similar. It is therefore clear that the apparent conflict of previous observations on surface proteins and intramembranous particles was not a consequence of different experimental preparations. Furthermore, the distributions of particles on fracture faces of avidin-ferritin-labeled and unlabeled membranes are identical at all temperatures, thereby demonstrating that the large avidin-ferritin label does not significantly disrupt the membrane protein organization.

(D) Freeze-etching demonstrates that the drying and fracturing observations characterize two sets of proteins with different responses to lipid state

To determine if the results obtained were the result of an artefact produced either by the freeze-etching technique or by the avidin-ferritin labeling and drying technique, freeze-etch experiments were done on avidin-ferritin-labeled membranes frozen from various temperatures relative to the phase transition. This technique permits us to discount the possibility that differences seen are the result of freeze-fracturing or avidin-ferritin drying artefacts, since intramembranous particles and surface proteins can be detected simultaneously in a single sample. To summarize the results (Figs. 3–6): at temperatures below the transition surface proteins are dispersed and particles are patched; in the middle of the transition, both particles and surface proteins are patched; just above the transition surface proteins are dispersed, particles are patched; and well above the transition both surface proteins and particles are dispersed (Table II).

Since the distributions of avidin-ferritin particles on the surfaces of freeze-etched preparations and dried preparations were essentially the same and the distribution of particles in labeled and unlabeled membrane samples were also identical, this argues that the patterns seen were also not induced by the

TABLE II

MEMBRANE PROTEIN DISTRIBUTIONS AS A FUNCTION OF LIPID STATE

Temperature relative to to lipid phase transition	Surface protein distribution	Intramembrane protein distribution
Well above	Dispersed	Dispersed
Above	Dispersed	Patched
Middle	Patched	Patched
Below	Dispersed	Patched

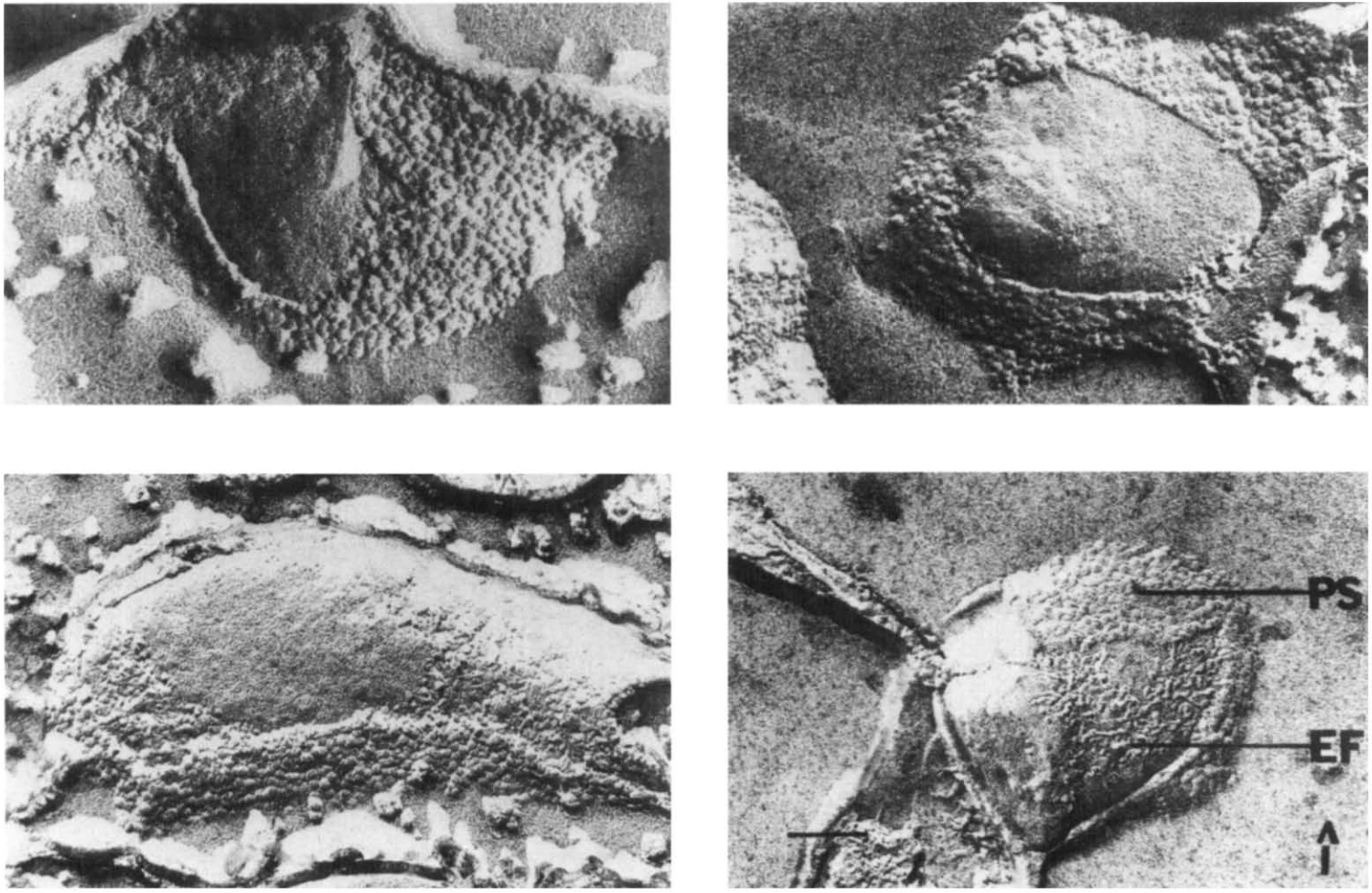

Fig. 3. Freeze-etched preparations of avidin-ferritin membranes frozen from a temperature below the lipid transition range. Erucate-enriched membranes were labeled in solution with avidin-ferritin and frozen from 4°C. The distribution of intramembranous particles is detected in the fracture face. The particles are patched. The distribution of avidin-ferritin-labeled sites is seen in the etched surface adjacent to the fracture face. The avidin-ferritin label appears to be dispersed. Thus, the distribution patterns of labeled surface proteins and intramembranous particles are not identical at this temperature.

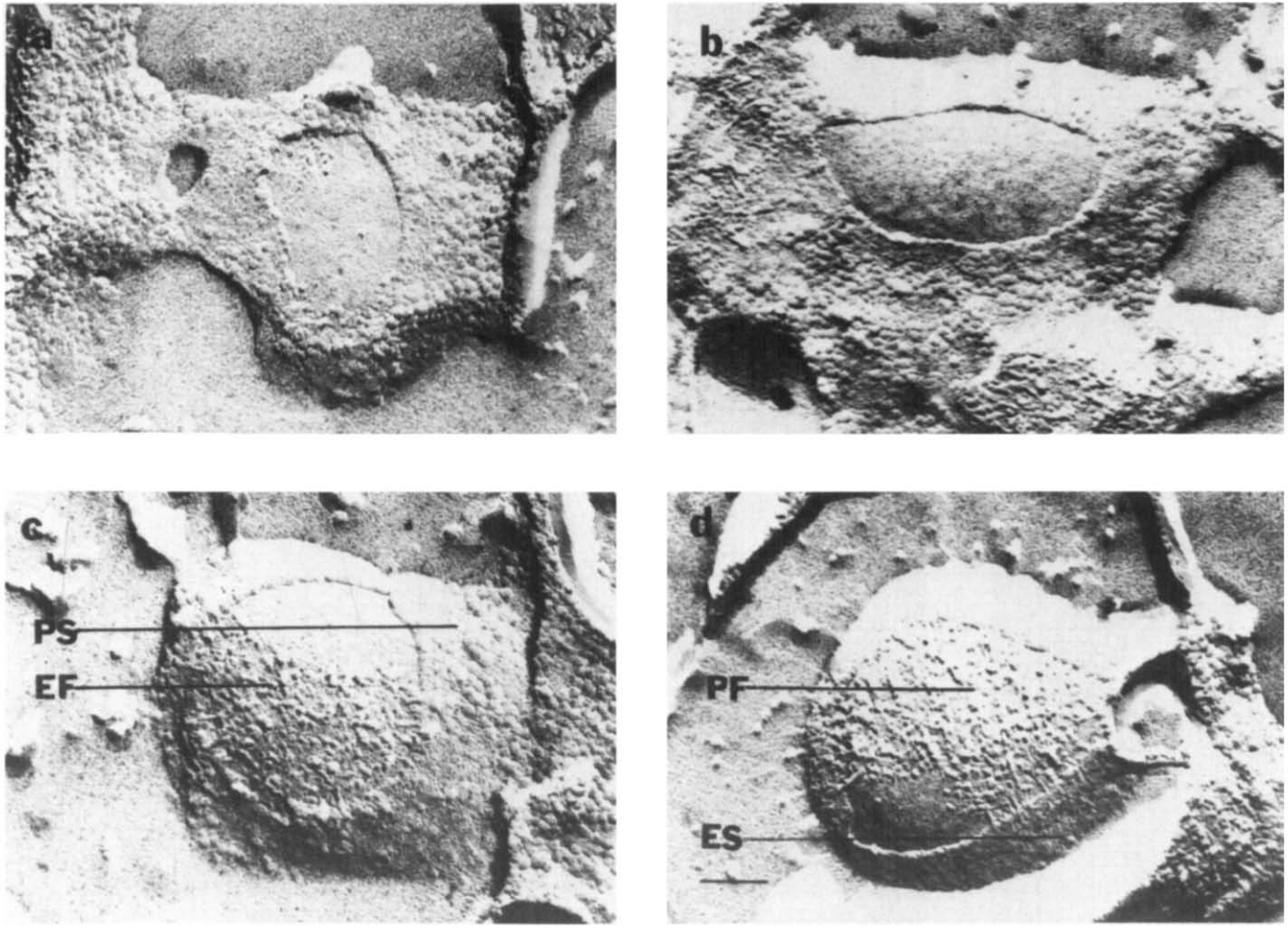

Fig. 4. Freeze-etched preparations of avidin-ferritin-labeled membranes frozen from a temperature within the lipid transition. Erucate-enriched membranes were labeled in solution with avidin-ferritin and frozen from 17°C. The intramembranous particles exposed in the fracture face are patched, as are the avidin-ferritin-labeled sites exposed on the etched surface. In panels a—c it is possible to see fracture faces representing the outer (exoplasmic) leaflet of the bilayer (EF) adjacent to cytoplasmically exposed etched faces (PS) containing ferritins. In d the inner (protoplasmic) leaflet to the bilayer (PF) is seen adjacent to the ecoplasmic etch face (ES). At this temperature, surface proteins and intramembranous particles are both patched, although the patches void of particles and free of label are not necessarily contiguous.

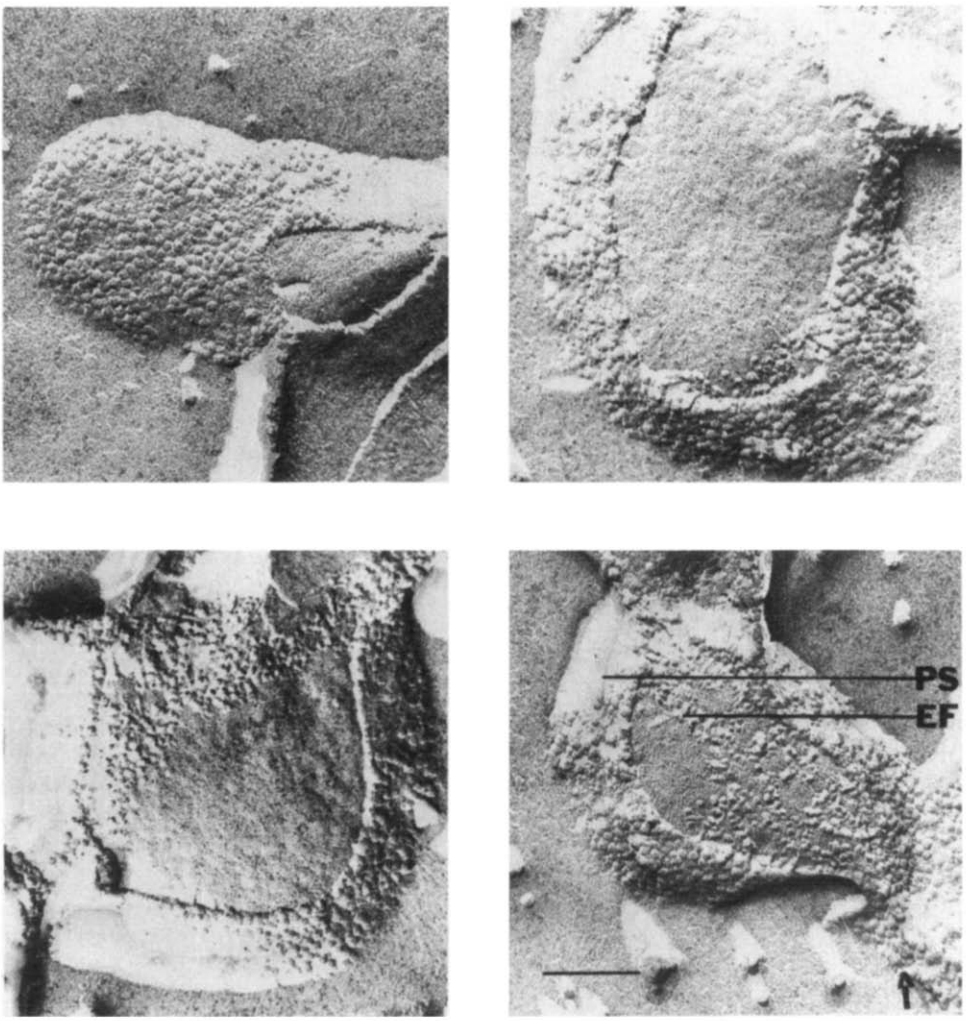

Fig. 5. Freeze-etched preparations of avidin-ferritin-labeled membranes frozen from a temperature just above the lipid transition. Erucate-enriched membranes were labeled in solution with avidin-ferritin and frozen from 23°C. Intramembranous particles are patched, while the avidin-ferritin-labeled surface sites are dispersed.

drying, labeling or freeze-etching techniques. These techniques are, therefore, revealing the behavior of two classes of membrane protein sites which behave in different manners as a function of the physical state of the membrane lipids. That these two classes are not identical can best be illustrated at temperatures below (Fig. 3) or just above (Fig. 5) the phase transition, where surface proteins labeled by avidin-ferritin are dispersed, while particles are extensively patched. For comparison, it can be seen at temperatures in the middle of the transition (Fig. 4) that surface proteins are patched (there exist regions of membrane surface essentially devoid of ferritins), while particles are also patched. However, even in this case, the patches of ferritins on etched faces

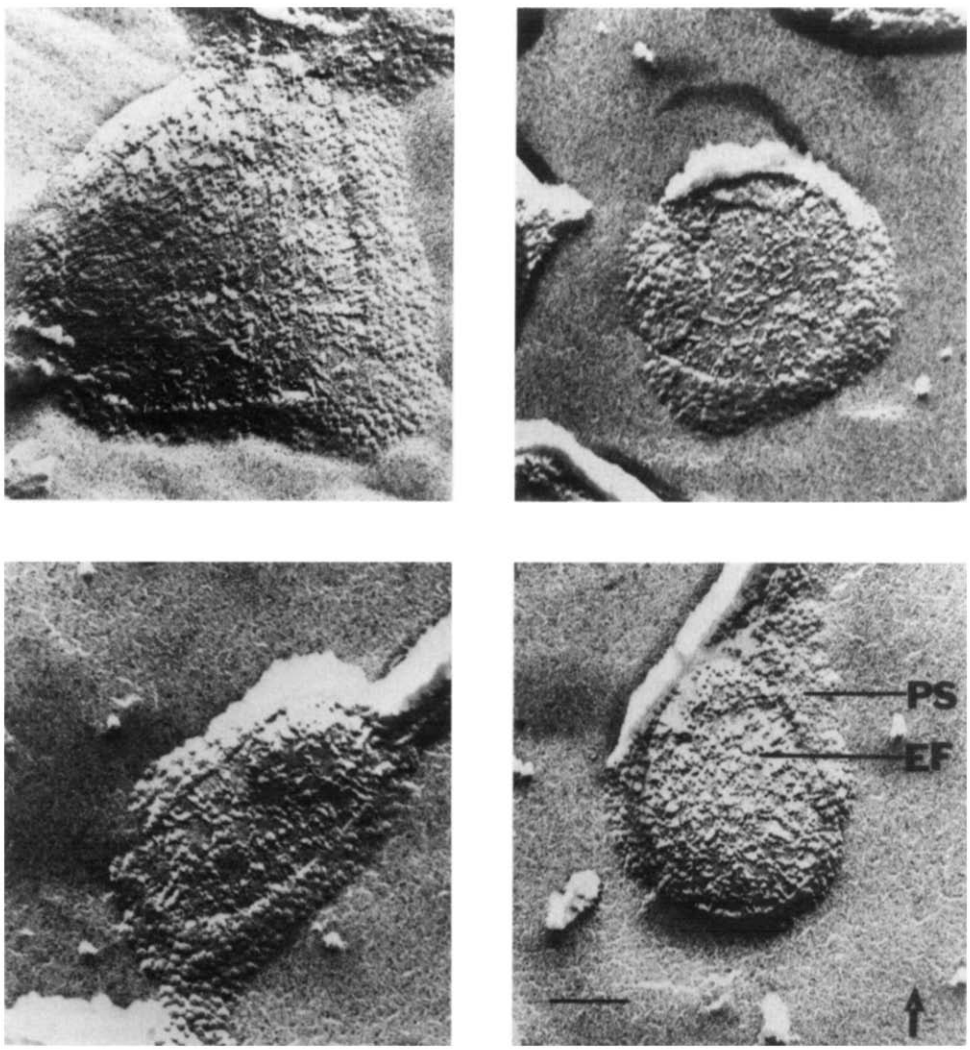

Fig. 6. Freeze-etched preparations of avidin-ferritin-labeled membranes frozen from a temperature well above the lipid transition. Erucate-enriched membranes were labeled in solution with avidin-ferritin and frozen from 37°C. Both the intramembranous particles and avidin-ferritin-labeled sites are dispersed at this temperature.

adjacent to the fracture faces rarely seem to be contiguous with the patches of particles. Therefore, the class of surface proteins represented by ferritin-labeled sites is not identical to the class of proteins represented by particles, although there may be proteins which are members of both classes.

(E) The protein distribution changes are a consequence of changes in lipid organization

The possibility that the distribution changes result from thermal changes in membrane organization other than those associated with lipid state was tested

by studies of membranes with different phase transition ranges. Eicosenoate-enriched membranes were examined at four temperatures relative to their phase transition. In this case the appropriate temperatures were: 4°C (below), 16° (middle), 25°C (just above), and 37°C (well above). At a temperature well above the transition, intramembranous particles are dispersed in the plane of the membrane. Just above the transition, they become slightly patched. At lower temperatures the particles are patched and the degree of patching seems to increase with decreasing temperature. Palmitate-enriched membranes were frozen from three temperatures relative to their phase transition: 19°C (below), 32°C (middle) and 40°C (just above). Membranes from this supplement were not frozen from a temperature well above their transition, as this would have required subjecting them to temperatures substantially above the temperature at which their proteins denature. In palmitate-enriched membranes frozen from these three temperatures, intramembrane particles are extensively patched. The degree of particle patching at the three temperatures is virtually indistinguishable by inspection. Thus, at a temperature far above the upper end of the phase transition range (37°C), the particles in erucate and eicosenoate membranes are dispersed. This temperature is lower than the highest temperature (40°C) at which particles are patched in palmitate membranes. These results demontrate that the behavior of particles is, in fact, a function of the lipid state (as had been previously found for surface proteins [3]), rather than of absolute temperature.

(F) Fixation shows that freezing preserves the protein distribution present at a given temperature

To show that the particle distribution patterns observed were not a result of diffusion of protein or rearrangements in the membrane during the freezing process, unlabeled membranes were fixed with glutaraldehyde at temperatures well above and below the phase transition prior to freezing from these temperatures. Fixed and unfixed membranes frozen from well above the transition have a dispersed particle distribution (Fig. 7, a and b); fixed and unfixed membranes frozen from below the transition have a patched distribution.

Glutaraldehyde fixation may not be an altogether satisfactory method of restricting diffusion because of its specificity for primary amino groups, none of which are found in the lipids or carbohydrates of *Acholeplasma*, so it must react with the proteins. It seems possible that the glutaraldehyde could cross-link proteins, possibly causing more extensive patching than seen in unfixed membranes. This was not the case. To demonstrate that the glutaraldehyde fixation was effective in preventing diffusion, however, membranes were fixed at a temperature below the phase transition (patched condition), then subjected to dispersing conditions (well above) prior to freezing. A patched distribution is seen (Fig. 7c), indicating that fixation had prevented the redistribution. In similar experiments with unfixed membranes, in which membranes were incubated at one temperature, then shifted to and incubated at another temperature prior to freezing, the particle distribution is characteristic of patterns typically seen at the latter temperature, which shows that redistribution was possible in these unfixed membranes. Therefore, since no difference in particle distribution is seen between fixed and unfixed prepara-

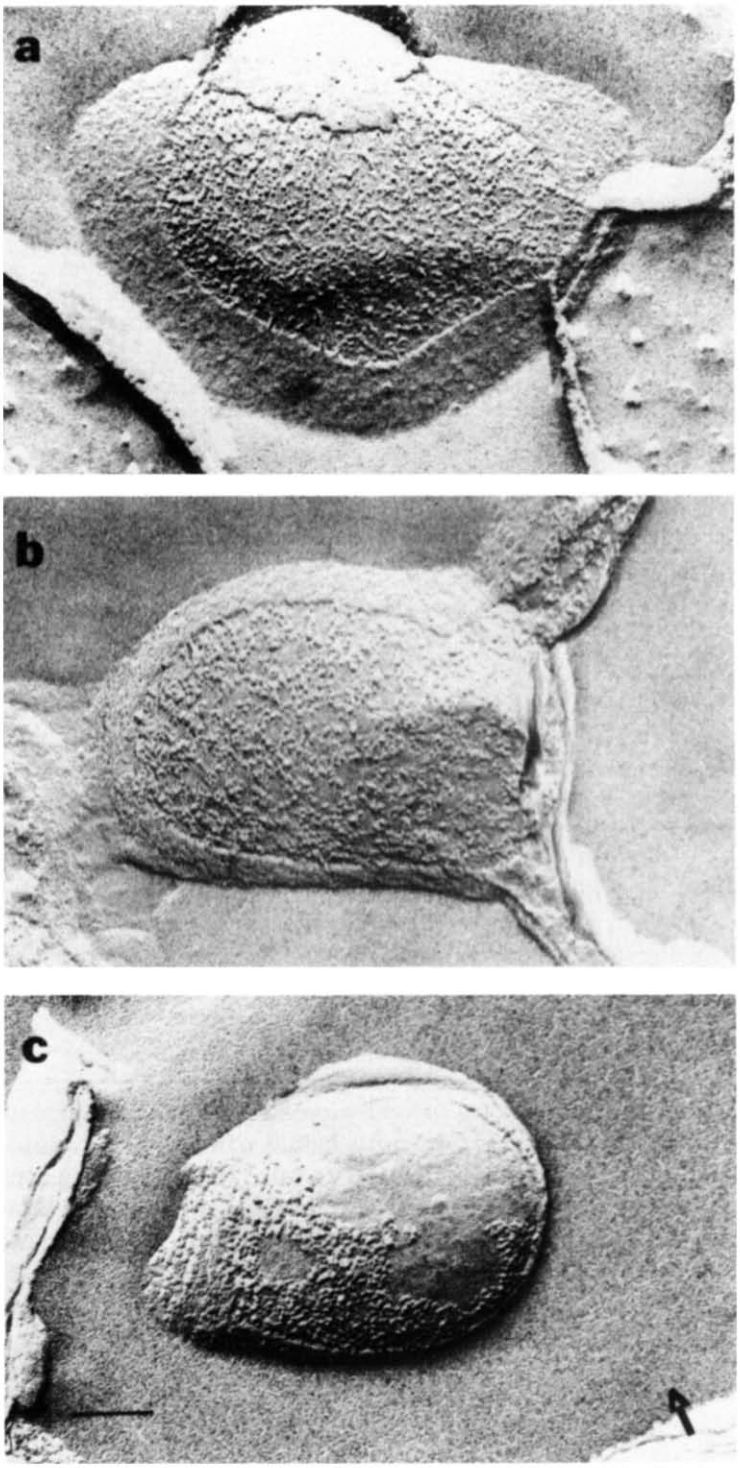

Fig. 7. Freeze-etched preparations of fixed and unfixed membranes. (a) Unfixed erucate-enriched membranes were frozen from 37°C (well above the lipid transition). Membranes were fixed with glutaraldehyde at either 37°C (b) (well above) or 4°C (below) and then incubated at and frozen from 37°C. The intramembranous particles are dispersed, in both unfixed membranes and membranes fixed at 37°C. Intramembranous particles in membranes fixed at 4°C are patched, even though the membranes have been incubated at 37°C prior to freezing. Thus, fixation must immobilize the proteins; moreover, since unfixed samples and samples fixed and frozen from the same temperature show identical particle distributions, it is likely that substantial protein diffusion does not occur over the time scale of the freezing process

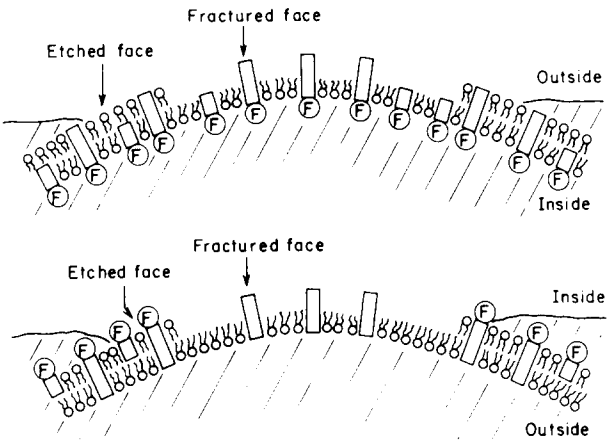

Fig. 8. Schematic representation of freeze-fractured and etched faces revealing intramembranous particle distributions on the inside and outside halves of the bilayer. The boxes represent integral membrane proteins. F indicates the presence of an avidin-ferritin-labeled sites on the cytoplasmic surface of the membrane. Fracture faces with adjacent etched faces that do not contain avidin-ferritin sites reveal the distribution of intramembranous particles on the cytoplasmic leaflet of the bilayer. Fracture faces with adjacent etched faces containing avidin-ferritin sites reveal the particle distribution on the exoplasmic leaflet of the bilayer. Thus, the behavior of intramembranous particles in both halves of the bilayer may be established by examination of the adjacent etched surfaces.

tions, and since surface ferritin site distributions are identical in frozen and dried preparations, it appears that diffusion of intramembrane proteins over the time period of freezing is not significant.

(G) Intramembrane particles occur with equal frequency and have similar distributions on exoplasmic and protoplasmic fracture faces

We have demonstrated that the avidin-ferritin-labeled sites are integral membrane proteins exposed, in part, on the cytoplasmic surface of the *Acholeplasma* membrane [5].

This characterization of the labeled sites provides a means of determining the distribution of intramembranous particles on both halves of the bilayer. Since avidin-ferritin labels only the cytoplasmic surface, fracture faces with adjacent etched faces containing ferritins must represent the outer (exoplasmic) leaflet of the bilayer (EF); correspondingly, fracture faces with adjacent etched faces void of ferritins must represent the inner (protoplasmic) leaflet of the bilayer (PF). This is shown schematically in Fig. 8. In our samples, etched faces containing ferritins and void of ferritins may be seen within a single replica. The distribution of proteins on both leaflets of the bilayer is very similar at all temperatures observed (Fig. 4, c and d). Virtually all the particles are detected as bumps; few pits are seen.

(H) Problems exist in the interpretation of the freeze-fracture and etching experiments

The interpretation of the freeze-fracture and etching experiments may be somewhat weakened by sampling problems.

A majority of the membranes viewed in the replicas had been cross-fractured (cleaved at right angles to the bilayer, exposing their profile, but not fracture faces). Depending on the specific preparation, only 5—20% of the membranes present at a fracture plane had fracture faces. The relative proportion of the appearance of these two types of fractures did not seem to depend on the presence or absence of avidin-ferritin. However, more fracture faces compared to cross-fractured membranes were seen in fixed samples than had been seen in unfixed samples.

Additionally, etching was not extensive at the periphery of fracture faces, and thus the regions of membrane surface viewed were small. It was, however, possible to determine the distribution patterns from observations of a number of these regions and from etched surfaces which had not been fractured in the same replica.

Lastly, it was difficult to quantitate absolutely the degree of patching at any one temperature because there was substantial heterogeneity in the extent of patching within a single replica. However, at all three temperatures which had patched particle distributions, no membranes were found in the replicas which had dispersed particle distributions, nor were any patched distributions seen in replicas from well above the transition (dispersed condition).

Thus, these results must be evaluated in the light of the sampling problem inherent in the freeze-fracture technique, since only a small fraction of the membranes present in any replica surface have fracture faces. The membranes observed may be a special subset of the total population, or regions of a membrane may vary with respect to their ability to cleave, and this ability to cleave may be influenced by the nature of their protein and/or lipid organization. Thus, this method could potentially be selecting particular membrane structures. Also, the area exposed upon fracturing and etching is small in comparison to the usual avidin-ferritin views of entire membranes, so distribution patterns on a scale that is comparable to the whole membrane are not easily observed in freeze-fracture replicas. Our general level of confidence in the avidin-ferritin labeling technique is greater than in the freeze-etch technique on statistical grounds. However, since our observations from the two techniques are in essence indistinguishable, we expect the freeze-etch results may be representative of the membrane population.

Conclusions

When the state of the lipid in a membrane is varied, the distribution patterns of surface proteins, detected by avidin-ferritin labeling, and of intramembranous particles, detected by freeze-fracturing, are not identical. To summarize our results on freeze-etched preparations of avidin-ferritin-labeled *Acholeplasma* membranes (see Table II), at temperatures below and just above the phase transition, surface proteins are dispersed, while intramembranous particles are patched; at temperatures in the middle of the transition, both particles and surface proteins are patched, and at temperatures well above the transition, both are dispersed.

These results correspond well to those reported for surface protein studies using dried avidin-ferritin-labeled membranes [3] and to the previously

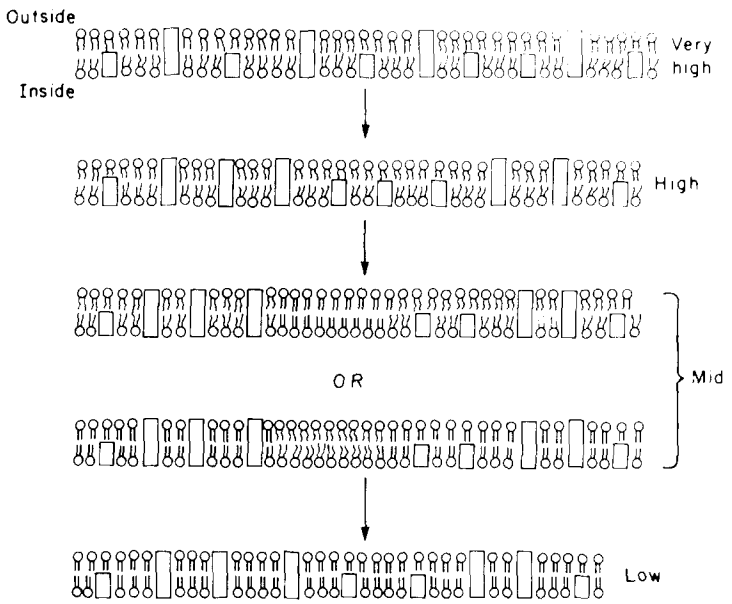

Fig. 9. Model for the effect of the lipid transition on the distribution of surface proteins and intramembranous proteins within the plane of the lipid bilayer. The large boxes represent intramembranous proteins which are deeply embedded in or which span the bilayer. The small boxes represent surface proteins which do not span the membrane. The physical state of fatty acid chains adjacent to the protein molecules may differ from that of the bulk lipids of the membrane. However, for the sake of clarity, the presence of these lipids have not been indicated in the model. Far above the transition, both surface proteins and intramembranous proteins are dispersed in the bilayer. Just above the transition, intramembranous proteins are patched, while surface proteins are dispersed. In mid-transition both intramembranous proteins and surface proteins are patched, and below the transition, surface proteins are dispersed, while intramembranous proteins are patched. The two classes of proteins are affected in different manners by the physical states of the membrane lipid (see text).

reported behavior of intramembranous particles detected in freeze-fracture preparations [7–9]. The freeze-etch results support the notion that the particles and the avidin-ferritin label reveal the relative locations of different subsets of membrane proteins, and that the different distribution patterns observed using the two techniques are not due to differences in samples, or an artefact produced by one of the techniques.

In addition, the location of the avidin-ferritin sites on the protoplasmic surface of the membrane [5], permits identification of the behavior of intramembranous particles exposed in each leaflet of the bilayer. Despite the asymmetric distribution of labeled protein sites between the inside and outside surfaces of the membrane, those proteins represented by particles in both halves of the membrane have virtually identical distributions parallel to the plane of the membrane.

To summarize the different distribution patterns of surface proteins and intramembrane particles with lipid state the following model is proposed (Fig. 9). Two classes of proteins exist which are differently influenced in their distribution by the state of the membrane lipid. The first class of proteins consists of all integral membrane proteins having primary amino groups exposed at the cytoplasmic membrane surface. This is the set of proteins

observed by avidin-ferritin labeling and may include all proteins in the membrane. The second class consists of proteins which penetrate deeply into the bilayer and are seen as particles in freeze-fracture. This is a subset of the total set of proteins and possibly a subset of the first class.

It is clear that while proteins which are deeply embedded in (or which span) the membrane, and integral proteins which are exposed at the surface may comprise intersecting subsets of membrane proteins, these two classes may well be influenced in different manners by lipids as they undergo changes in physical state. In addition to the change in the order of the hydrocarbon chains of the lipid, the lipid bilayer thickness and surface area per lipid molecule change dramatically as a consequence of the change of state [1,15]. These changes may well exert a differential influence on proteins which interact with the bilayer in different ways.

Intramembranous particles are influenced by the lipids on both the cytoplasmic and exoplasmic halves of the bilayer, whereas the remainder of the surface proteins detected by avidin-ferritin are influenced primarily by the cytoplasmic lipids. Since the lipids may have an asymmetrical distribution with respect to the bilayer profile, the behavior of lipids in both leaflets of the bilayer may not be identical. It has been suggested that the intramembrane particles are "squeezed out" by the hydrocarbon portion of the lipids as they form a solid phase, thus creating regions of high protein density and regions of high lipid density in the hydrophobic portion of the bilayer. At very high temperatures, the proteins are free to float in the fluid bilayer and thus can redistribute to a more uniform protein and lipid density. It was previously postulated [3] that the distributions of surface proteins at different temperatures are dependent on a phase separation of lipids and the preferential association of protein with lipids in either the smectic or paracrystalline states. For temperatures in which all lipids are in a single phase, surface proteins have no preferential associations and are distributed randomly in the bilayer.

Thus, the following protein distribution patterns are found: at temperatures below the transition when all lipids are in a single state, surface proteins are randomly distributed in the cytoplasmic half of the membrane. However, a subset of those proteins, the intramembranous particles, are "squeezed out" by the paracrystalline hydrocarbon chains of lipids in both halves of the bilayer, forming patches.

At temperatures in the middle of the transition, the surface proteins preferentially associate with one phase (either smectic or paracrystalline) and are patched. The particles remain "squeezed out" by the hydrocarbon chains, and are themselves patched. These results permit us to elaborate on our previous proposal that surface proteins preferentially associate with one lipid phase [3]. In that work, we were unable to determine with which phase the proteins had a higher energy of association. This study, however, suggests that the proteins may be associated with the fluid phase. If it is assumed that the intramembranous particles are a small subset of the total set of surface proteins, and thus are also labeled by avidin-ferritin, and that their distribution behavior in a mixed phase system is representative of the surface proteins, then the exclusion of the particles from the paracrystalline phase at temperatures below and in the middle of the transition can be extrapolated to describe the

organization of surface proteins. Since the particles are associated with the fluid phase (or excluded from the paracrystalline phase), then the patches of high protein concentration on the membrane surface (represented by high density of ferritin label) should also be associated with the fluid phase. While this is clearly not proof of which lipid phase the proteins have preference for (and thus both possibilities are included in our model, see Fig. 8), it does suggest that the less ordered lipid state contains a higher concentration of protein than the paracrystalline state lipids.

At temperatures just above the transition, as in the case just below the transition, surface proteins are randomly distributed and do not preferentially associate with any lipids. At this temperature, the intramembranous particles may remain patched. The possibility that protein-protein interactions are responsible for this association is argued against by the different distributions observed in palmitate and erucate membranes incubated at approximately the same temperature.

Finally, at temperatures well above the transition, neither the particles nor the surface proteins are constrained by or preferentially associated with lipids, and their equilibrium distribution is dispersed.

Such behavior would result in the distribution patterns observed for intramembranous particles and surface proteins at the four temperatures utilized in these experiments.

Variation in architectural arrangements of different proteins in the plane of the membrane with lipid state may present the cell with one mechanism for regulation of membrane function. Selective incorporation of exogeneously supplied fatty acids or biosynthesis of a range of different fatty acids may permit the organism to regulate the physical state of its membrane lipids. We have demonstrated that such changes in lipid state affect the lateral organization of membrane proteins. Local rearrangements of proteins in the plane of the membrane could offer a selective advantage to the organism. Two opposing factors are relevant: activity, especially of multimeric enzymes, may increase with membrane fluidity, while fragility of the membrane also increases dramatically above the transition temperature (Wallace, B., unpublished observations). Cells could compensate for the latter disadvantage by producing membranes which are less fluid but which have patches of higher protein concentration. This could be accomplished by changes in the lipid state which influence the protein organization.

Our results show that temperature-induced changes in the physical state of membrane lipids can cause changes in the relative location of surface protein and intramembrane protein components parallel to the membrane surface. The behavior of avidin-ferritin-labeled proteins and intramembrane particles represent the different influence of the lipid organization on two subsets of integral membrane proteins.

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