membrane for which the Ca^{2+} ATPase is predominately in the first phosphorylated intermediate state of the enzyme, minus the profile before ATP-initiated calcium transport, as adopted from reference (2).

In Fig. 2, we show the low-resolution, difference electron density profile for the isolated sarcoplasmic reticulum membrane for which the Ca^{2+} ATPase is transiently trapped in the first phosphorylated state of the enzyme minus the profile before ATP–initiated phosphorylation of the enzyme.

Simple inspection of the two difference profiles reveals a close similarity between them, and verifies the earlier detection of the profile structure of the first phosphorylated intermediate state of the enzyme under conditions of enzyme turnover (2). The smaller differences between the two difference profiles are currently being subjected to a model refinement analysis to provide the Ca^{2+} ATPase profile structure for the transiently trapped, first-phosphorylated intermediate state of the enzyme.

CONCLUSIONS

These time-resolved x-ray diffraction studies using intense synchrotron radiation sources appear to be fully capable of detecting and characterizing structurally distinct intermediate states within the ATP-driven calcium transport process in sarcoplasmic reticulum membranes. The ultimate time resolution achievable depends primarily upon the incident x-ray flux and the kinetics of the flash-photolysis reactions producing enzyme substrate, and possibly on radiation damage to the membrane multilayer.

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USE OF VIBRATIONAL SPECTROSCOPY IN DEFINING THE ROLE OF CLATHRIN IN COATED VESICLE FORMATION

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Clathrin, the major structural protein characteristic of the fibrous networks associated with the coat material of membrane pits and vesicles, has been implicated in the dynamics of a variety of endocytotic processes and intracellular transfer mechanisms (1). The basic unit of the membrane coat, the triskelion, occurs as a M_r = 650,000 protein trimer of clathrin subunits (M_r = 180,000) in combination with three associated proteins (M_r = 30,000–36,000). Under defined conditions, dissociated clathrin triskelions reassemble in the absence of the vesicle membrane into cagelike structures resembling the surface lattice of coated vesicle systems.

The sensitive, noninvasive molecular probes provided by infrared and Raman spectroscopy for examining lipid bilayer disturbances on the vibration time scale ($\sim 10^{-13}$ s) allow an assessment of the individual contributions of membrane bilayer components to the conformational, dynamical and functional properties of biological membrane assemblies. We have applied infrared spectroscopic techniques to clarify the pH-dependent conformational reorganizations paralleling the evolution of triskelions, isolated from bovine brain tissue, into the assembled high-molecular weight, latticelike species. Infrared spectral parameters were also used to determine the extent of the perturbation of the clathrin coat protein on the lipid matrix of the isolated coated vesicle membrane. In a complementary Raman spectroscopic study, we investigated the effects of clathrin on the lipid bilayer intrachain and intermolecular order/disorder characteristics of model systems comprised of unilamellar dipalmitoyl phosphatidylcholine (DPPC) dispersions.

MATERIALS AND METHODS

Coated vesicles were isolated from bovine brain by the method of Nandi et al. (2). The clathrin trimers were isolated and reassembled as described previously (3). The model membrane unilamellar systems were formed

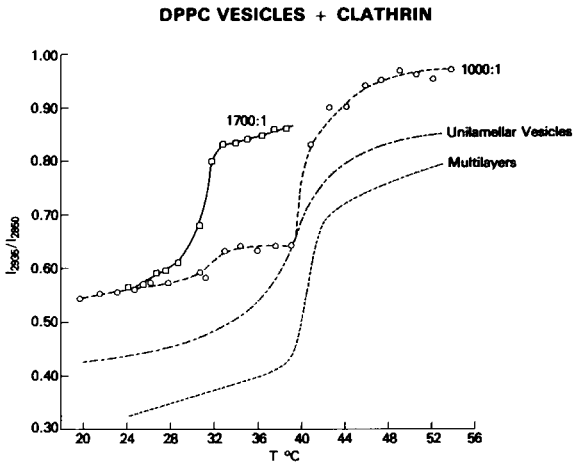

FIGURE 1 Temperature profiles comparing the 1,700:1 and 1,000:1 DPPC/clathrin mole ratio dispersions to unilamellar and multilamellar DPPC dispersions. The Raman spectral C–H stretching mode region I_{2935}/I_{2880} ($I_{disorder}/I_{order}$) peak height intensity ratios are used to construct the profiles. T_M for the four systems are 37.3, 39.9, 39.0, and 40.7°C, respectively. Note the relative gel and liquid crystalline disorder characteristics of the four systems.

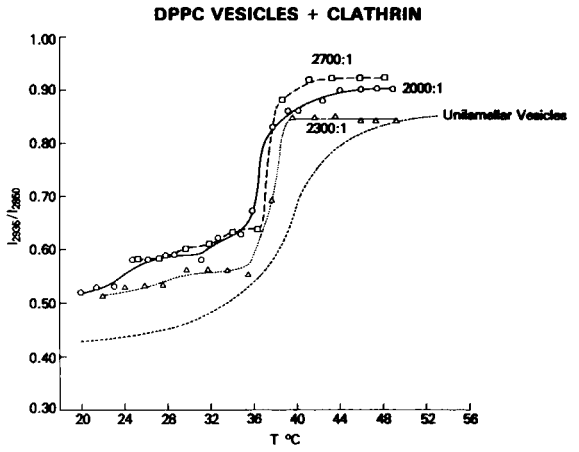

FIGURE 2 Temperature profiles for 2,700:1, 2,300:1, and 2,000:1 DPPC/clathrin mole ratio dispersions. T_M for the three systems are 37.3, 37.5, and 36.5°C, respectively.

from sonicated and sized DPPC disperions. Vibrational infrared spectra were obtained with a Perkin-Elmer 580B spectrophotometer (Perkin-Elmer Corp. Instrument Div., Norwalk, CT) controlled by a local LSI-11 based computer with a Tektronix 4006 graphics terminal (Tektronix, Inc., Beaverton, OR). Procedures for infrared cell manipulations and computer subtraction techniques have been described previously (3). The laser Raman spectroscopic procedures, based upon a Spex Ramalog 6 spectrometer (Spex Industries Inc., Metuchen, NJ) equipped with holographic gratings and interfaced to a Nicolet NIC-1180 data system (Nicolet Instrument Corp., Madison, WI) have also been described in detail (4).

RESULTS AND DISCUSSION

The secondary structures of the clathrin triskelions, their reassembled, water-soluble, cagelike networks, and the clathrin coat of intact vesicles are characterized by the infrared spectroscopic amide I (~1,650 cm^{-1} spectral region) and amide II (~1,545 cm^{-1} region) bands. Since the amide I vibrational bands lie beneath a strong water-solvent feature, special techniques are required for obtaining reliable spectra. Spectral frequency and intensity changes indicate a decrease in α-helical content of the triskelion polypeptide chains as they assemble into cages. These cages, reassembled in the absence of membrane components, reflect spectra identical to that of the clathrin coat of intact vesicles. The close correspondence between the spectra of the integral and peripheral protein components present in uncoated vesicles and the spectra of reassembled clathrin cages suggest the existence of similar secondary structures within the different aqueous and hydrophobic environments.

Temperature profiles, derived from Raman C–H stretching region spectral parameters and displayed in Figs. 1 and 2, were obtained for the unilamellar DPPC vesicles interacting with clathrin at lipid:protein ratios ranging from 2,700:1 to 1,000:1. The forms of the temperature curves and the 1–8°C depressions in T_m, the gel-to-liquid crystalline phase transition, reflect substantial acyl chain disorder within the hydrophobic region of the model membranes. The characteristics of the gel-to-liquid crystalline phase transition region, in terms of cooperativity and entropy changes of the transition, suggest headgroup interactions whose effects are propagated to the hydrocarbon chain regions through structural alterations within the lipid matrix.

In examining the membrane lipid response to the coat protein, we also focused on the infrared spectral frequency characteristics and temperature dependence of the lipid acyl chain symmetric methylene carbon-hydrogen stretching modes (at ~2,850 cm^{-1}) of isolated clathrin-coated vesicles, uncoated vesicles, and synaptic membranes. The analysis of the spectral behavior in terms of lipid intrachain disorder clearly indicates increased numbers of *gauche* chain conformers (i.e., a greater lipid chain disorder) for the clathrin-coated vesicles compared with the other two membrane assemblies at each temperature studied (5). Because the lipid environments of the coated pit and coated vesicle are not significantly different from uncoated membrane domains, the bilayer disorder induced by the clathrin coat may be one of the major factors responsible for membrane invagination and coated vesicle formation.

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NUCLEAR MAGNETIC RESONANCE, BIOCHEMICAL, AND MOLECULAR GENETIC STUDIES OF THE MEMBRANE-BOUND D-LACTATE DEHYDROGENASE OF *ESCHERICHIA COLI*

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D-Lactate dehydrogenase (D-LDH) of *Escherichia coli* is an integral membrane protein which is activated by lipids and detergents. This flavin adenine dinucleotide (FAD)-containing enzyme catalyzes the oxidation of D-lactate in electron transfer reactions that are coupled to the active transport of various amino acids and sugars into the cell. We are carrying out a systematic investigation of D-LDH using techniques of ^{19}F nuclear magnetic resonance (NMR), biochemistry, and molecular genetics. To obtain sufficient quantities of D-LDH, we have constructed a recombinant plasmid that overproduces D-LDH by 300-fold over wild-type levels. To construct mutants and to aid in the interpretation of our NMR data, we have determined the primary structure of D-LDH by nucleotide sequencing of the cloned gene (1). The protein has 571 amino acid residues and a molecular weight of 65,000.

RESULTS AND DISCUSSION

By adding 4-, 5-, or 6-fluorotryptophan at the time of heat induction of D-LDH activity, we can incorporate high levels of these ^{19}F-labeled analogs into the enzyme. ^{19}F NMR spectra of these labeled proteins are shown in Fig. 1. The spectrum of 5F-Trp-labeled D-LDH shows five distinct Trp resonances, in agreement with the five Trp residues expected from the nucleotide sequence, at positions 59, 384, 407, 469, and 567. The intensities of the peaks in the spectra of 4- and 6F-Trp-labeled protein also indicate the presence of five resonances. Thus, each Trp in D-LDH must have a different environment. Also, the linewidth of each of the five resonances is different, suggesting that there are differences in the motional properties of each Trp residue.

In 100 mM lysolecithin, dipalmitoyl phosphatidylglycerol (DPPG), or dipalmitoyl phosphatidylcholine (DPPC), the spectra of 5F-Trp-labeled D-LDH are as shown in Fig. 1. In 1% Triton X-100, we find that peak 3 shifts 0.5 ppm downfield. In 1% Triton X-100 plus 0.1% SDS, we find that peak 2 shifts 1 ppm downfield.

We have performed a number of experiments on the 5F-Trp-labeled enzyme, in the presence of lysolecithin, to determine the structural and dynamic properties of the Trp residues in D-LDH. By changing the ratios between D_2O and H_2O in the sample, we can induce a change in the ^{19}F chemical shift of the fluorotryptophan. When we perform this experiment on D-LDH, we find that peaks 1, 2, and 3 show no chemical shift changes. Peaks 4 and 5 show a change in chemical shift which indicates that they are 60% (peak 4) and 100% (peak 5) exposed to the solvent. We have also observed the ^{19}F resonances under conditions of broad-band proton irradiation, and we find that the five resonances are reduced in intensity by different amounts. In particular, resonances 4 and 5 show the least amount of reduction, which implies that they are the most mobile of the five Trp residues. This is consistent with the fact that resonances 4 and 5 are also exposed to the solvent.

When the substrate, D-lactate, is added to 5F-labeled enzyme, we find that peak 2 shows a large increase in linewidth and is shifted 0.5 ppm downfield. When oxalate, a competitive inhibitor, is added, there is no effect on the spectrum. This indicates that peak 2 is sensitive to reduction of the flavin but not to the binding of the substrate.

To summarize, we find that resonances 2 and 3 are sensitive to the lipid environment. Resonance 2 is also sensitive to the oxidation state of the FAD cofactor. In addition, we find that resonances 4 and 5 are on the surface of the enzyme.

The relationship between this information and an understanding of the structure and function of D-LDH is not simple. To obtain a "working model" for the structure