The Secondary Structure of Calcium Pump Protein in Light Sarcoplasmic Reticulum and Reconstituted in a Single Lipid Component as Determined by Raman Spectroscopy*

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Raman spectra have been measured of the following samples: active calcium pump protein in light sarcoplasmic reticulum (SR) membranes, lipids extracted from light SR membranes, active calcium pump protein reconstituted in dielaidoylphosphatidylcholine (DEPC), and pure DEPC. The spectra of native SR lipids and of pure DEPC are different, and yet when these spectra are subtracted from the spectra of the respective protein-lipid complexes, the resulting amide I spectra of the calcium pump protein are the same, indicating that appropriate criteria have been chosen for subtraction of the spectrum of a lipid. This spectrum has been analyzed for secondary structure with the following results. The SR calcium pump protein contains 51 ± 5% helix, in agreement with a prediction of secondary structure obtained from an analysis of the sequence, and 21 ± 4% β-strand. In addition, the presence of protein broadens and lowers the main melting transition of DEPC.

Sarcoplasmic reticulum (SR) is a specialized membrane system that regulates muscle contraction and relaxation by controlling the intracellular calcium ion concentration (Tada et al., 1978). It consists of two distinct portions: the longitudinal and terminal cisternae that can be isolated as light and heavy SR respectively; light SR consists mainly of one protein component, the calcium pump protein (Meissner, 1975; Fleischer et al., 1979; Fleischer, 1985), have a subunit molecular weight of 115,000 (Møller et al., 1982).

The calcium pump of SR of fast skeletal muscle is one of the best studied biological pumping systems, and its pumping cycle has been described using both kinetic and thermodynamic approaches (Tanford, 1983; Pinkirt and Jencks, 1984; deMeis and Inesi, 1982). The calcium pump protein constitutes approximately 90% of the total protein, and in reconstituted membranes consisting of the calcium pump protein and DEPC.

MATERIALS AND METHODS

Preparation of Sarcoplasmic Reticulum and Phospholipid—Purified SR was prepared according to Meissner et al. (1975) from rabbit fast skeletal muscle and subfractionated as light and heavy SR according to Meissner et al. (1975). SR was solubilized with deoxycholate and exchanged with DEPC by the technique of Hymel et al. as described previously (Seelig et al., 1981). The single exchange replaced 90% of the SR phospholipids with the DEPC (obtained from Avanti Polar Lipids, Inc., Birmingham, AL) and the reconstitution procedure (Meissner and Fleischer, 1974) resulted in functional membrane vesicles with lipid/protein ratio of 20 μg of P/mg of protein. The light SR used in these studies had a lipid/protein ratio of 28 μg of P/mg of protein (Meissner et al., 1975). The SR samples were suspended in 0.1 M KCl, 5 mM HEPES, pH 7.0, prior to Raman spectroscopy. Phospholipids were extracted from SR with chloroform/methanol (2:1) and back-extracted to remove non-lipid materials as described previously (Meissner and Fleischer, 1974; Rouser and Fleischer, 1974). The lipids were microdispersed by sonication in aqueous medium (0.1 M KCl, 5 mM HEPES, pH 7.0). The samples were stored frozen at −70 °C in the same aqueous buffer.

Raman Spectroscopy—The Raman instrument consisted of a Coherent Innova 90 argon ion laser operating at 514.5 nm with typically 150 mW of light power focussed at the sample to a beam with a diameter of about 0.6 mm, thermostated sample holder, a 300-mm focal length f/0.6 aspheric lens for light collection optics, a polarization scrambler, a Spex 1403 double monochrometer with 1800 groove/mm holographic gratings, an RCA 31034 photo-multiplier tube, and a Spex Datamate coupled to a Cromemco Z80-based microcomputer.

1 The abbreviations used are: SR, sarcoplasmic reticulum; DEPC, dielaidoylphosphatidylcholine; Ca2⁺-ATPase, calcium pump protein, or Mg2⁺-Ca2⁺-activated ATP phosphohydrolase, EC 3.6.1.3; CD, circular dichroism; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.


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Spectral bandpass was set to 6 cm⁻¹. Data points were taken at every 1 cm⁻¹ at 2 cm⁻¹ with multiple scans totaling about 1 min integration time/cm⁻¹. Samples were held in melting point capillary tubes in 0.1 M KCl, 5 mM HEPES, pH 7.0. Samples were pelleted in a microhematocrit centrifuge for 1-2 min to obtain a translucent pellet with a volume of from 3 to 5 aliters. These samples were colorless and exhibited relatively low background fluorescence when exposed to the laser beam. Data collection was started immediately upon exposure of the sample to the beam so that photobleaching of trace fluorescent impurities could be monitored. Photobleaching was essentially complete after 20 min or two scans. The first two scans were discarded, and up to 80 scans were collected. An average of the first 10 scans was identical to an average of the last 10 scans, indicating no detectable change in the spectral properties of the samples during the course of the measurements.

After measurement of the Raman spectrum, the SR samples were resuspended in 0.3 M sucrose, 0.1 M KCl, 5 mM HEPES, pH 7.0, for analysis. The samples retained Ca²⁺-stimulated ATPase activities comparable to the original samples prior to spectroscopy, i.e. (2.2 ± 0.2 μmol of ATP hydrolyzed)/(min × mg of protein) for both light SR and DEPC-exchanged SR.

The amide I region Raman spectra (1500-1800 cm⁻¹) of the light SR and the DEPC-reconstituted calcium pump were collected at 12°C. Data in this same spectral region for light SR lipids and DEPC were collected at 16°C. Under these conditions the lipids appear to be fully melted in both the protein complex and in the pure lipid samples and do not exhibit spectral changes indicative of phase changes at higher temperatures. By differential scanning calorimetry, we determined that the onset of the main melting transition of the sample of DEPC used in these studies to be 12.1°C. The Raman spectra referable to the C=H stretching mode (2800-3000 cm⁻¹) were recorded for DEPC, and DEPC-exchanged SR, at four temperatures ranging from 4 to 16°C.

Analysis of Raman Spectra—Spectra of lipids were subtracted from spectra of lipid-protein complexes to satisfy the following criteria. The intensity of the lipid ester C=O stretch at 1735 cm⁻¹ was reduced to zero, and the resulting difference spectrum was determined to have a minimum number of inflections near the peak frequency of the sharp lipid C=C stretching band. To clarify this second criterion: the lipid C=C band of DEPC's narrow relative to the amide I band of proteins, and the over- or under-subtraction of this band from the spectrum of a lipid-protein complex gives a sharply inflected curve in the difference spectrum.

Another example of lipid subtraction can be seen in Yager et al. (1984).

Amide I spectra of the calcium pump were analyzed for secondary structure information according to Williams (1983, 1986). Briefly, fractions of helix, β-strand, and reverse turn are estimated from the Raman spectrum by a procedure similar to ones recently developed for the analysis of CD spectra (Provencher and Glöckner, 1981; Hennessey and Johnson, 1981). Solvent spectrum and side-chain bands are subtracted from the amide I region. The spectrum is then normalized and fitted with a linear combination of amide I spectra of proteins whose structures are known by x-ray diffraction. The secondary structure is then calculated as a sum of fractions of the reference proteins. C=H stretching spectra were analyzed for the number of linearly independent components using a singular value analysis by the procedure described elsewhere (Lawson and Hanson, 1974; Williams, 1983, 1986).

Assays—Protein was determined by the procedure of Lowry et al. (1951). Total phosphorus was measured by a modification (Rouser and Fleischer, 1967) of the method of Chen et al. (1966) and was used to estimate lipid phosphorus. Ca²⁺-stimulated ATPase was measured at 55°C using a coupled enzyme system (Warren et al., 1974) in 50 mM potassium phosphate buffer, pH 7.0, 5 mM MgSO₄, 50 mM KCl, 50 mM CaCl₂, 2 mM phosphoenolpyruvate, 200 mM NADH, and 1 mM ATP (sodium salt). Pyruvate kinase and lactate dehydrogenase were added at approximately 9 and 13 units/ml, respectively. The ATPase activity was calculated from the rate of oxidation of NADH in the coupled assay.

### RESULTS AND DISCUSSION

Raman spectra of light SR and of DEPC-reconstituted calcium pump protein are dominated by the C=C stretching band of the lipid components (Fig. 1). Since the C=C stretching frequency of SR lipids (Fig. 1A) are at a lower frequency than that observed for DEPC (Fig. 1B), the amide I region

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**Fig. 1.** A, a comparison of Raman spectra of light SR (solid line) and lipids extracted from light SR (line with dot). B, DEPC-reconstituted calcium pump protein (solid line) and DEPC (line with dot), showing that the amide I region is dominated by the C=C stretching band from the lipids. The 1730 cm⁻¹ band is from the lipid ester C=O stretch. The samples were suspended in 0.1 M KCl, 5 mM HEPES, pH 7.0.

**Fig. 2.** A comparison of the Raman amide I spectra of calcium pump protein obtained by subtracting the spectrum of light SR lipids from the spectrum of light SR (solid line), and the spectrum of DEPC from the spectrum of DEPC reconstituted calcium pump protein (line with dot). Each of these spectra are obtained by subtraction (see text) of the spectra shown in Fig. 1. Spectra of these two protein-lipid complexes differ by 15 cm⁻¹. However, when the lipid spectra are subtracted from the spectrum of the complexes, the resulting amide I spectra of calcium pump protein are virtually identical (Fig. 2).

A technique has been developed to represent the difference between two amide I Raman spectra as a single normalized number, the ratio of variances for points from 1630 to 1700 cm⁻¹ and from 1750 to 1800 cm⁻¹ in the difference spectrum. When this comparison is applied to 60 difference spectra representing four distinct groups, experiments repeated over a period of several years on different instruments, the same protein under different conditions such as in crystals and

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1 R. W. Williams and W. Hendrickson, unpublished experiments.
solutions, related proteins such as ribonucleases A and S, and unrelated proteins, a statistically significant discrimination is observed between groups. Proteins that differ in secondary structure content by only 5% fail outside of the category for the same protein. When this comparison is applied to the spectra shown in Fig. 2, the null hypothesis that they are of the same protein under different conditions cannot be rejected at the 0.01 level.

The structure of the calcium pump protein in reconstituted DEPC vesicles is similar to that in native SR membranes, since the reconstituted pump protein is active (Meissner and Fleischer, 1974; Seelig et al., 1981) and the calcium pump protein both in normal and reconstituted SR is dimeric (Hymel et al., 1984). That the spectra of native and reconstituted calcium pump protein are the same indicates both that the criteria for subtraction of the lipid spectrum is reliable and that the secondary structures of these forms are similar if not identical, i.e. they differ by less than 5%.

Secondary structure estimates for the calcium pump protein in native SR and reconstituted in DEPC are listed in Table 1. Two quite different samples, giving quite different spectra dominated by lipid bands, yield the same results. The secondary structure is about 50 ± 5% helix, 21 ± 4% β-strand, and 17 ± 2% reverse turn, with the remainder undefined. The sample of SR is well-defined in that it contains approximately 90% calcium pump protein in the membrane. Even if the other remaining proteins are all quite different in secondary structure content from the main constituent, the calcium pump protein, these estimates accurately represent the pump protein structure.

Hardwicke and Green (1974) have estimated from CD spectra that the secondary structure of the calcium pump protein (calcium ATPase) consists of about 35% helix. Similar results have been obtained for the detergent-solubilized preparation by Fromicelli et al. (1984) who also observe that at mM levels of Ca++ minimize changes in CD spectra referable to partial denaturation. These reported values for the helix content of the calcium pump protein may be inaccurate since they have been determined using one of the earliest methods to be developed for quantitation of secondary structure from CD spectra (Greenfield and Fasman, 1969). Refinements in the x-ray diffraction structures of some of the proteins used to validate the Greenfield and Fasman (1969) method indicate that the original parameters yield an underestimate of the helix content of proteins. For lysozyme, ribonuclease A, and carboxypeptidase accepted x-ray values for helix are now 41, 23, and 37% respectively (Chang et al., 1978). These values are approximately 15% higher than the values originally estimated from CD spectra (29, 12, and 15% respectively) (Greenfield and Fasman, 1969). The previous CD estimates of about 35% helix for the calcium pump protein also appear to be about 15% low based on the results reported here. Raman amide I measurements of SR in the presence of different amounts of calcium show no detectable change (no change greater than 4%) as a function of Ca++ concentration, which differs from the results obtained by CD (Fronticelli et al., 1984). Lippert et al. (1981) measured the Raman spectrum of SR under a variety of conditions and tentatively reported small intensity changes (6%) in bands in the amide III region. They interpreted these changes as indicating a decrease in protein helix content induced by temperature, Ca++ and Mg++. They also observed that these changes were not much greater than the experimental noise and for this reason they declined to quantify the changes in terms of structure. Lippert et al. (1981) could not estimate the overall secondary structure content of SR proteins because of interference from the intense lipid bands at 1302 cm⁻¹ in the amide III region and at 1655 cm⁻¹ in the amide I region. Subtraction of overlapping lipid bands to allow quantitation of protein structure had not been attempted at that time.

Estimates of protein secondary structure from Raman spectra appear at this time to be less susceptible to artifacts than those from CD spectra. The CD spectrum of a protein in a membrane is affected by disulfide bonds, light scattering, and absorption (Mao and Wallace, 1984). Interpretation of CD spectra is also influenced by uncertainties in protein concentration and assumptions regarding dielectric constant. Some of these artifacts can be avoided or corrected, but there are notable exceptions. Raman has not been widely used to analyze protein structure, and so cases of large error have not appeared. Of the proteins studied thus far, Raman-based estimates for proteins with a high helix content tend to be low on the average (Williams, 1983, 1986), with the largest error being about 8% low for the helix content of one protein out of 15 with known x-ray structures. It would seem likely that the estimate of approximately 35% helix from the analysis of CD spectra (Hardwicke and Green, 1974) is a low value, since we obtain 50 ± 5% helix from the analysis of the Raman amide I spectra that, in our experience, tends to underestimate the helix content. The 52% helix content anticipated from an analysis of amino acid sequence (MacLennan et al., 1985; Brandl et al. 1986) is in agreement with the Raman estimate. This may be fortuitous since predictions of percent secondary structure content obtained from an analysis of sequence are unreliable; however, a prediction of structure from sequence analysis is likely to be more reliable when assignments of secondary structure are constrained to agree with measurements of percent content (Chang and Williams, 1986).

The approach we have used to analyze structure, involving the subtraction of the lipid spectrum, is in some ways new and may be subject to unpredictable sources of error. The accuracy of estimates made for pure proteins has been predicted based on comparisons with known x-ray structures (Williams, 1983, 1986), but comparisons may not be valid for membrane proteins. Subtraction of lipid bands from the Raman spectrum of a lipid-protein complex in order to estimate the protein secondary structure has been reported only once before in a study of the acetylcholine receptor (Yager et al., 1984), and x-ray structures for colorless intrinsic membrane proteins are still not available. Under these circumstances it is appropriate to discuss here some of the reasons why the
Raman spectrum of a membrane protein may be different from that of soluble proteins.

The dielectric constant and protic nature of the environment will influence the frequency of some vibrational modes (Shih and Williams, 1986), but not much is known about the amide I band in different solvents. It has been shown that the amide I spectra of the small proteins crambin and purothionin do not change over a wide range of alcohol concentrations (Williams and Teeter, 1984). Also, the amide I spectrum of bovine pancreatic trypsin inhibitor is the same in solution and lyophilized forms. This evidence implies that the amide I spectrum of a protein is relatively independent of the dielectric constant of the medium.

Subtraction of the lipid band from the amide I region is a possible source of error. The frequency of the C=C band near 1670 cm⁻¹ is changed as the lipid goes through its main melting transition, and the presence of protein contaminants is known to shift the melting transition of lipids. For subtraction to be successful, the lipid must be in the same phase or state of order for both the pure lipid spectrum and the lipid-protein complex. We have been careful to see that the lipid for both samples was completely melted (above the gel to liquid-crystalline phase transition). While the temperature of the lipid-protein sample here is close to the melting transition of the pure lipid, it is clearly above the melting transition of the lipid with protein present. Spectra of the complex collected at higher temperatures were identical. Also, when the spectrum of the pure lipid does not match the spectrum of the lipid in the complex, subtraction produces a derivative-like curve in the difference spectrum (not shown).

The main melting transition of DEPC, normally at 12 °C, has apparently broadened and shifted to a lower temperature in the lipid-protein complex. The spectrum of the DEPC-reconstituted membrane at 11 °C is virtually identical to the spectrum taken at 16 °C (Fig. 3). These results are consistent with those obtained in a study of the calcium pump protein reconstituted with dipalmitoylphosphatidylcholine (Mendelsohn et al., 1984). When these spectra of the C–H stretching region from 2800 to 3000 cm⁻¹, taken at different temperatures through the melting transition, were analyzed using a singular value analysis to determine the number of linearly independent components present in the spectra, the following results were obtained. The four spectra of DEPC-reconstituted calcium pump protein contain two components. The four spectra of DEPC alone contain two components. The eight spectra, including DEPC-reconstituted calcium pump protein and DEPC alone, contain three components. The components in the spectra do not have a one to one correspondence with the components in the DEPC-protein sample. The number of independent components obtained by singular value analysis of the spectra are an indication of the number of different phases that may exist in the sample. However, the singular value analysis gives no information about what phases these components represent. The independent components are related to the eigenvectors of the data from which they are derived and may bear little resemblance to the original spectra. While we may assign these phases to the gel and liquid crystalline states of the lipid matrix, doing so involves the assumption that the protein spectrum does not change. These results indicate that new or intermediate phases possibly introduced by the protein (Lentz et al., 1985), could not be detected.

Some information regarding the nature of the lipid in the presence of the protein can be obtained from a consideration of the Raman lateral packing parameter Sₜₕ (Gaber and Peticolas, 1977). This parameter provides a relative measure of the lateral interchain packing and is derived from the ratio of the heights of the symmetric and asymmetric CH₂ stretching bands at 2880 and 2850 cm⁻¹, respectively. From Table II it can be seen that at temperatures below Tₚ for DEPC, a smaller Sₜₕ is obtained in the presence of the calcium pump protein indicating that the lipid in the presence of the protein is somewhat more disordered (in the sense of chain packing) than is the pure lipid. This is consistent with d-NMR studies of similar preparations (Seeig et al., 1981). In addition, for pure DEPC there is a small but real increase in Sₜₕ (0.42–0.45) with temperature decreasing from 7 to 4 °C; in the presence of calcium pump protein, a comparable change in Sₜₕ is not observed. This suggests that while the pure lipid can continue to become more ordered below Tₚ, the interaction with protein restricts the ability of the chains to become more ordered.

A question sometimes arises concerning the effect of intense laser light on the integrity of biological structures. Damage could occur to the colorless samples normally exposed to 514.5 nm (green) light (proteins, lipids, and nucleic acids) from energy absorbed by chromophoric impurities and transferred to the sample as heat. The temperature of some samples, such as pelleted SR vesicles, cannot be measured directly with confidence, since thermal gradients around the laser beam can involve spaces smaller than the dimensions of measuring devices. However, it has been determined that colorless samples illuminated with a low flux beam do not absorb enough energy to produce changes in the temperature of the sample. The melting transition temperatures of pure lipids as measured by Raman have been less than 1 °C lower than calorimetric values.

The melting transition of DEPC-reconstituted calcium pump protein and DEPC alone, contain three components. The components in the spectra do not have a one to one correspondence with the components in the DEPC-protein sample. The number of independent components obtained by singular value analysis of the spectra are an indication of the number of different phases that may exist in the sample. However, the singular value analysis gives no information about what phases these components represent. The independent components are related to the eigenvectors of the data from which they are derived and may bear little resemblance to the original spectra. While we may assign these phases to the gel and liquid crystalline states of the lipid matrix, doing so involves the assumption that the protein spectrum does not change. These results indicate that new or intermediate phases possibly introduced by the protein (Lentz et al., 1985), could not be detected.

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**Table II**

Lateral packing parameter, Sₜₕ, for DEPC with and without the calcium pump protein

<table>
<thead>
<tr>
<th>Temperature</th>
<th>DEPC</th>
<th>DEPC + Ca²⁺-pump</th>
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<tr>
<td>°C</td>
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<tr>
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<tr>
<td>16</td>
<td>0.30</td>
<td>0.30</td>
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*DEPC was prepared by sonication in aqueous buffer (0.1 M KCl, 5 mM HEPES, pH 7.0).
metric determinations (Gaber and Peticolas, 1977), and protein crystals, even when highly fluorescent, remain undamaged when exposed to more than 500 mW of green laser light for more than 24 h (Williams, 1983), indicating that the structure of these proteins is not changed. The samples of SR studied here are colorless and show the low fluorescence characteristic of the very pure lipids and proteins that are not influenced by laser light. While we have not made Raman measurements of the lipid melting transitions here, we observe from a comparison of Table II with the 12 °C melting transition for DEPC that the temperature of the sample in the laser beam was less than 4 °C higher than the thermostated sample holder (The S_ref parameter shows that the DEPC is melted at 16 °C and markedly more ordered at 11 °C). With these observations in mind, we believe that the structure of the calcium pump protein has not been altered by the laser Raman measurements made here. Further, the spectra were comparable at the beginning and end of the measurements, and the samples retained enzymic function after the Raman spectra were recorded.

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REFERENCES