RAMAN SPECTROSCOPY OF CYTOPLASMIC MUSCLE FIBER PROTEINS

Orientational Order

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ABSTRACT The polarized Raman spectra of glycerinated and intact single muscle fibers of the giant barnacle were obtained. These spectra show that the conformation-sensitive amide I, amide III, and C-C stretching vibrations give Raman bands that are stronger when the electric field of both the incident and scattered radiation is parallel to the fiber axis (I_{zz}). The detailed analysis of the amide I band by curve fitting shows that ~50% of the α-helical segments of the contractile proteins are oriented along the fiber axis, which is in good agreement with the conformation and composition of muscle fiber proteins. Difference Raman spectroscopy was also used to highlight the Raman bands attributed to the oriented segments of the α-helical proteins. The difference spectrum, which is very similar to the spectrum of tropomyosin, displays amide I and amide III bands at 1,645 and 1,310 cm^{-1}, respectively, the bandwidth of the amide I line being characteristic of a highly α-helical biopolymer with a small dispersion of dihedral angles. A small dichroic effect was also observed for the band due to the CH_2 bending mode at 1,450 cm^{-1} and on the 1,340 cm^{-1} band. In the C-C stretching mode region, two bands were detected at 902 and 938 cm^{-1} and are both assigned to the α-helical conformation.

INTRODUCTION

The conformation of the cytoplasmic proteins of large muscle cells has already been studied extensively by Raman spectroscopy (Pézolet et al., 1978, 1980; Caillé et al., 1983). The high concentration of protein needed for non-resonance Raman spectroscopy is not a limiting factor for studies on muscle fibers since the cellular macromolecules of these cells amount for 25% of their weight (Hinke et al., 1973). Moreover, barnacle muscle fibers are particularly suitable for Raman scattering since these striated muscle cells are large (diameter $\approx$1 mm) and, because of their optical quality, they give Raman spectra with a high signal-to-noise ratio. This experimental model is unique to study the conformation in vivo of oriented coiled-coil α-helical proteins composed of two linear α-helical chains wrapped around each other.

Oriented proteins and biopolymers have been studied by Raman scattering (Fanconi et al., 1969; Snyder, 1971; Wilser and Fitchen, 1975; Schachar and Solin, 1975; Hsu et al., 1976). It was shown that the CONH groups of the β-sheet conformation of proteins in bovine lenses (Schachar and Solin, 1975) and of feather keratin (Hsu et al., 1976) are preferentially oriented in directions orthogonal to the lens optic axis or the quill axis, respectively. A study of the polarization of the Raman scattering on oriented α-helical polypeptide films (Wilser and Fitchen, 1975) has given the orientation angle of the principal axis of the amide I Raman tensor.

The cytoplasm of barnacle muscle cells contains proteins like myosin, tropomyosin, and paramyosin (Levine et al., 1976), which display a superhelical coiled-coil structure and an orientational order such that the axes of the α-helical segments are almost parallel to the fiber axis.

Our objectives in this study were to characterize the Raman spectrum of the oriented helicoidal proteins, their conformation in vivo, and to detect orientation of their side chain residues. To accomplish this task, the polarized Raman spectra of glycerinated barnacle muscle fibers were measured with two orientations of the fibers with respect to the direction of propagation of the incident light. These spectra show that ~50% of the α-helical segments of the myofibrillar proteins are oriented along the fiber axis, which is in good agreement with the value expected from the composition and conformation of muscle fiber proteins. Difference Raman spectroscopy reveals that the spectrum

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This paper is dedicated to the memory of Dr. Jean-Pierre Caillé.

of these proteins is almost identical to that of α-helical tropomyosin and poly-L-lysine, except for the amide I band that is narrower in the difference spectrum of the oriented muscle proteins.

METHODS

Fiber-filled Capillaries

Single muscle fibers were isolated from the depressor muscle of the barnacle (*Balanus nubilus*) and kept in artificial seawater. These fibers were rinsed (2 h) in a choline-Ringer's solution which contained 450 mM choline chloride, 8 mM KCl, 30 mM $MgCl_2$, and 25 mM Tris(hydroxymethyl) aminomethane (THAM) so that the extracellular space, that represents 5% of the fiber volume (Hinke et al., 1973), is filled with this solution. Then a single fiber was introduced in a glass capillary chosen to fit the diameter of the fiber, and cut in 1.5-cm long segments whose open ends were sealed with thin glass plates. Raman spectra were immediately recorded on these fiber-filled capillaries.

Glycerinated Fibers

To eliminate the cellular membrane and the carotenoid pigments, and to introduce an internal intensity standard in the intracellular space, the isolated muscle cells rinsed in an isotonic sucrose solution were treated with glycerol. They were first incubated for 4 h at 10°C and then for 20–24 h at −10°C, in a mixture of a rigor solution and glycerol (1:1, vol/vol). The composition of the rigor solution was as the following: 50 mM K_2SO_4, 5 mM K_2HPO_4, 1.3 mM KH_2PO_4, 2 mM $MgCl_2$, 200 mM glycine, and 0.5 mM EDTA. A single glycerinated fiber was then gently pulled in the lumen of a glass capillary and cut into small sections of 6–8 mm. These small segments of glycerinated muscle cells were incubated for 24 h at 5°C in a relaxing solution containing 50 mM K_2SO_4, 5 mM K_2HPO_4, 1.3 mM KH_2PO_4, 2 mM Mg ATP, 150 mM glycine, 40 mM taurine, and 0.5 mM EGTA.

Electron Microscopy

Since the relaxing solution described above had not been used before, the morphology of the relaxed glycerinated fiber was studied. After incubation in the relaxing solution, the glycerinated muscle fibers were fixed for 60 min in the capillary with ice-cold 2.8% glutaraldehyde buffered with 0.1 M cacodylate. Then they were removed from the capillary and immersed in the fixative solution for 2 h. The samples were rinsed overnight in 0.1 M cacodylate buffer, postfixed in 2% osmium tetroxide buffered with 0.1 M cacodylate for 90 min, dehydrated, and embedded in epon. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM-300 electron microscope.

Raman Spectra

The Raman spectra were recorded with a model 1400 computerized spectrometer (Spex Industries, Inc., Edison, NJ) using the 514.5-nm line of a model 2020 argon ion laser (Spectra-Physics Inc., Mountain View, CA) as an excitation source. The direction of the monochromatic, linearly polarized incident radiation was either parallel (Fig. 1 *A*) or perpendicular (Fig. 1 *B*) to the long axis (z axis) of the muscle fiber. The scattered light collected at 90° to the incident laser beam was focused by an $f/2$ collection lens through an analyzer and a polarization scrambler on the entrance slit of the monochromator.

All spectra were obtained at ~10°C, with 150-mW laser power at the sample, a spectral resolution of 6 cm^{-1}, and an integration period of 2 s by 2 cm^{-1} step. They were not smoothed but some were corrected for a slight luminescent background by subtracting the appropriate polynomial function.

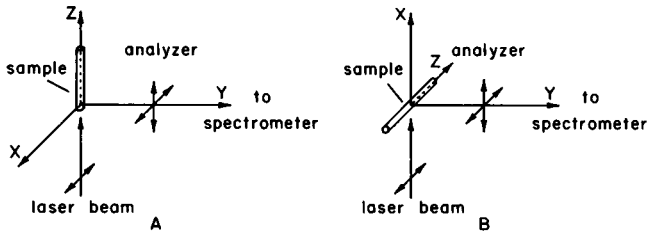

FIGURE 1 Orientations of the samples relative to the incident laser beam.

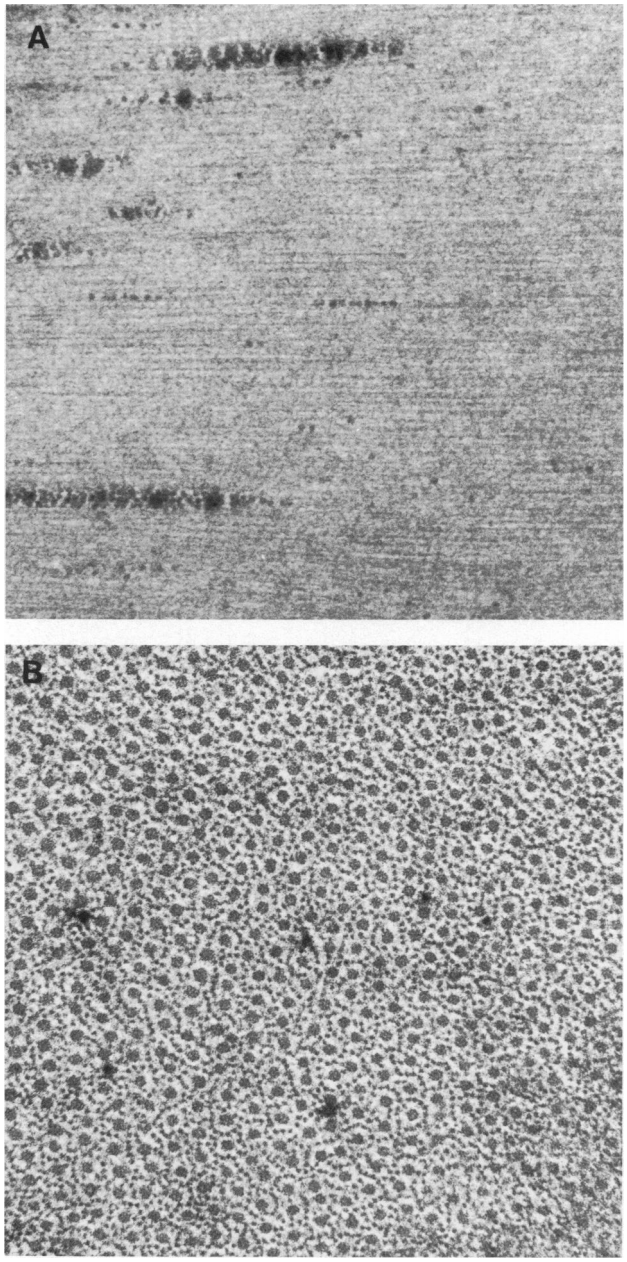

FIGURE 2. Electron micrographs of longitudinal (*A*) and transverse (*B*) sections of glycerinated barnacle muscle fibers. Magnification: (*A*) 84,900; (*B*) 79,800.

RESULTS AND DISCUSSION

Electron Microscopy of Glycerinated Fibers

The low carotenoid content of the glycerinated fibers is an important advantage to obtain spectra with a good signal-to-noise ratio. Considering that the myofilaments of the chemically skinned barnacle fibers may be damaged after incubation in relaxing salt solution for long periods of time (Clark et al., 1981), the morphology of the glycerinated fibers was verified. Two micrographs of a longitudinal and a transverse section of a glycerinated fiber incubated in the relaxing solution (24 h) are presented in Fig. 2. As can be seen, in the transverse section the thick filaments are packed hexagonally but not regularly as observed on amphibian striated muscle (Huxley, 1957), and the thin filaments clearly form "rings" around the thick filaments. Therefore, the removal of the sarcolemma by glycerol and the incubation in the relaxing solution do not affect the architecture of the contractile filaments observed on freshly isolated fibers (Gayton and Elliott, 1980; Clark et al., 1981), so that glycerinated fibers can be used to study the orientation of the myoplasm proteins.

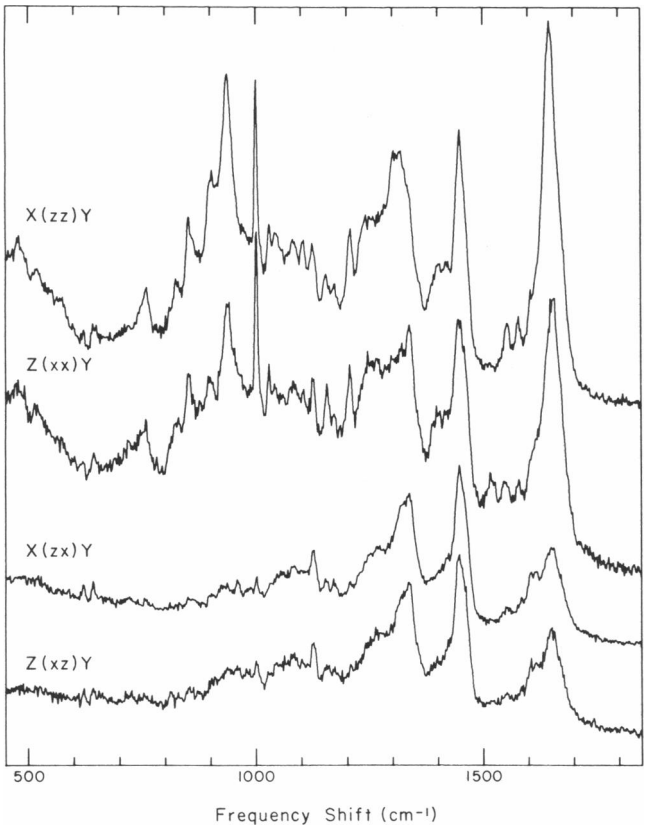

FIGURE 3 Polarized Raman spectra of an oriented glycerinated muscle fiber. The 980-cm^{-1} line of the sulfate ions present in the buffer was used as an internal intensity standard to subtract buffer contribution in the amide I region and to normalize the intensity of the I_{zz} and I_{xx} spectra. The labeling of the spectra is described in the text.

Glycerinated Muscle Fibers

Fig. 3 shows examples of the polarized spectra measured on a glycerinated fiber for the 90° geometry. The labeling of the spectra follows the convention of Damen et al. (1966). The first and last letters, written in upper case, designate the direction of propagation of the incident and scattered radiation, whereas the second and third letters, written in lower case, denote the polarization direction of the incident and scattered light. As seen on this figure, the scattering intensity due to the diagonal elements of the Raman tensor is much stronger than that due to the off-diagonal elements. In addition, since for several bands the I_{zz} component is significantly higher than the I_{xx} component, which should be equal to the I_{yy} component for proteins with axial symmetry, it is clear that the protein molecules are not randomly oriented in the muscle fiber.

According to the organization of the proteins which form the cytoplasm architecture of muscle fibers, the axis of the α-helical segments of these proteins are, in first approximation, parallel to the longitudinal axis of the fiber. Snyder (1970, 1971) has demonstrated that the induced polarizability changes along a bond may be quite different from those perpendicular to the bond. Since the transition moments of the amide I and III vibrations are oriented at ~39° to the α-helix axis (Tsuboi, 1962), it is expected that the bands associated with these vibrations are stronger when the polarization of both the incident and scattered light is parallel to the fiber axis. A similar dichroic effect has been reported for oriented α-helices of the biopolymer

TABLE I
INTENSITY RATIOS* OF SOME BANDS IN THE POLARIZED RAMAN SPECTRA OF THE GLYCERINATED MUSCLE FIBERS

ν	I_{zx}/I_{zz}	I_{xz}/I_{xx}	I_{zz}/I_{xx}	Assignments
cm^{-1}				
758	<0.1	<0.1	0.8–1.0	Trp
827	0	<0.1	0.8–1.0	Tyr
855	<0.1	<0.1	1.0–1.2	Tyr
902	<0.1	<0.1	2.0–2.3	C-C stretch
938	<0.1	<0.1	1.7–1.8	
1,002	≃0.1	≃0.1	0.9–1.0	Phe
1,210	≃0.1	≃0.1	0.9–1.1	Tyr, Phe
1,245	0.42	0.44	1.1–1.3	
1,270	0.48	0.53	1.1–1.3	Amide III
1,320	0.54	0.77	1.7–1.8	
1,336	0.77	0.76	0.9–1.0	Trp
1,450	0.67	0.83	1.2–1.3	CH_2 bending
1,555	0.36	0.45	1.2–1.5	Trp
1,580	0.47	0.58	1.2–1.5	Trp
1,612	0.65	0.59	0.9–1.0	Tyr, Phe
1,645	—	—	2.4	
1,660	—	—	0.9	Amide I
1,675	—	—	1.3	

*All intensity ratios were calculated from the peak height intensities except for the amide I bands for which the integrated intensities after band decomposition (Table II) were used.

poly-γ-benzyl-L-glutamate (Wilser and Fitchen, 1975) as well as for oriented β-sheets in intact bovine lenses (Schachar and Solin, 1975) and in feather keratin (Hsu et al., 1976). For these two latter cases, the intensity of the amide I band was stronger when the polarization of the incident and scattered light was perpendicular to the lens or quill axis since for the β-sheet structure the C=O bonds are perpendicular to the chain axis.

To obtain more quantitative results on the orientation of the myoplasm proteins, we have also calculated the relative intensity of the strongest polarized Raman bands of a muscle fiber (Table I). Even though the accuracy of these scattering ratios is not very high because some bands are poorly resolved and also because it is difficult to draw the baseline under the bands, they provide interesting information on the orientation of muscle fiber proteins.

It is clear from Table I that the bands that display the highest diagonal scattering ratios, I_{zz}/I_{xx}, are due to the backbone vibrations of the contractile proteins. Since the amide I band is the strongest conformation-sensitive spectral feature, and also because this band has been studied for several proteins (Hsu et al., 1976; Schachar and Solin, 1975; Williams, 1983) and polypeptides (Wilser and Fitchen, 1975), we have decomposed the amide I band profile to determine the relative spectral contribution of the α-helical conformation using the method of Pitha and Jones (1967) and the Cauchy-Gauss sum function (60% Cauchy and 40% Gauss). For these calculations, only the α-helical, β-sheet, and unordered structures were considered to have the minimum number of component bands. The starting frequencies for the amide I bands were those of native and denatured tropomyosin (1,645 and 1,660 cm^{-1}, respectively) for the α-helical and unordered conformations (Pézolet, M., M. Pigeon, J. Nadeau, and J.-P. Caillé, unpublished results), and of intact bovine lenses (1,673 cm^{-1}) for the β-sheet structure (Schachar and Solin, 1975). In addition, a band due to the presence of carotenoid pigments was included at 1,520 cm^{-1} (Pézolet et al., 1978) as well as contributions due to aromatic side-chains at ~1,550, 1,580, 1,605, and 1,617 cm^{-1} (Hsu et al., 1976). The results of the curve fitting analysis are shown in Fig. 4 for the I_{zz} and I_{xx} spectra, and the spectral characteristics of the components are given in Table II. As can be seen, the frequency and halfwidth determined for each component band are in good agreement with those of the amide I Raman bands of proteins and polypeptides of known structure (Carey, 1982; Tu, 1982) and do not change with the orientation of the sample. The I_{zz}/I_{xx} ratios calculated from the integrated intensity of the individual bands from the decomposed spectra (Fig. 4) are given in Table I. As expected, within the experimental uncertainty, this ratio is very close to unity for the unordered conformation, which adds confidence to the curve fitting analysis. The results show that the β-sheet content is relatively low in muscle fiber proteins and that this structure is not oriented. On the other hand, the I_{zz}/I_{xx} ratio for the 1,645-cm^{-1} band due to

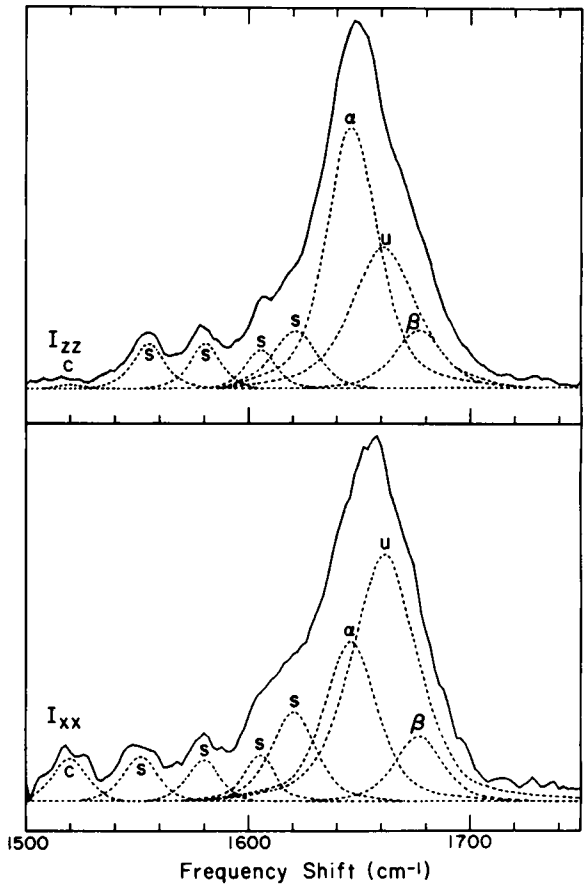

FIGURE 4 Original amide I band contours (*solid curves*) with individual component bands (*broken curves*) for the I_{zz} and I_{xx} spectra of Fig. 3. The symbols α, β, u, s, and c stand for α-helices, β-structures, unordered structure, aromatic side-chains, and carotenoid pigments, respectively.

the α-helical conformation has a value of 2.4 compared with 3.7 ± 0.5 for the amide I band of highly oriented α-helical poly-γ-benzyl-L-glutamate (Wilser and Fitchen, 1975). If one assumes that the α-helical segments of the cytoplasmic proteins are either oriented along the fiber axis or completely unoriented, which should give amide I bands with an I_{zz}/I_{xx} ratio of 3.7 and 1.0, respectively, the amount of oriented α-helical segments calculated for a I_{zz}/I_{xx} ratio of 2.4 is 52% of the total α-helical content. In muscle fibers like those of the giant barnacle, the α-helical proteins that can be oriented are paramyosin, tropomyosin, and the myosin rod, which includes the S-2 moiety of myosin (Yu et al., 1985). Unfortunately, the relative amount of proteins has not yet been determined for the barnacle muscle fibers. For mammalian muscles, tropomyosin and the myosin rod account for only 45% of the total α-helical proteins (Yates and Greaser, 1983). For the barnacle muscle fibers, this percentage should be higher since there should be one paramyosin molecule for two myosin heavy chains (Levine et al., 1976). Therefore, the amount of oriented α-helical segments determined from the polarized Raman spectra of barnacle muscle fibers is in

TABLE II
FREQUENCY (ν), BANDWIDTH ($\Delta\nu_{1/2}$), AND RELATIVE INTENSITY (I) OF THE AMIDE I REGION BAND COMPONENTS FOR TWO ORIENTATIONS OF A BARNACLE MUSCLE FIBER*

I_{zz}			I_{xx}		
ν	$\Delta\nu_{1/2}$	I	ν	$\Delta\nu_{1/2}$	I
cm^{-1}	cm^{-1}	%	cm^{-1}	cm^{-1}	%
1,517	18	1	1,519	18	8
1,554	17	11	1,551	19	8
1,579	16	10	1,579	16	7
1,604	16	9	1,604	17	8
1,620	26	19	1,619	26	20
1,645	31	100	1,645	32	41
1,660	45	68	1,660	45	79
1,675	29	22	1,675	29	16

*The frequency, bandwidth, and integrated intensity relative to the 1,645-cm^{-1} band of each component were determined from the band-fitted spectra (Fig. 4).

very close agreement with that predicted from the protein compositions and conformations of the myofibrils, even though there should be a 5° deviation introduced by the coiled-coil structure of the oriented α-helical proteins of muscle cells (Schulz and Schirmer, 1979).

A surprising result from Fig. 3 and Table I is that the I_{zz}/I_{xx} ratio is greater than unity for the 1,450-cm^{-1} band assigned to the CH_2 bending mode of the methylene groups. Since the glycine content of the α-helical proteins is low (Chou and Fasman, 1974; Squire, 1981), our results suggest that some of the methylene groups of the amino acid side-chains are oriented. This orientation may be due to the fact that the coiled-coil structure is stabilized by electrostatic interactions between positively and negatively charged residues on adjacent chains. This result indicates that the intensity of the 1,450-cm^{-1} band cannot be used as an internal intensity standard for the Raman spectra of proteins with orientational order as it is used for proteins in solution (Lippert et al., 1976; Pézolet et al., 1976).

Difference Raman Spectroscopy

Difference Raman spectroscopy was used to highlight the Raman bands attributed to the oriented segments of the α-helical proteins and the orientational order of the side-chains. The I_{zz}/I_{xx} difference Raman spectra were obtained from intact and glycerinated fibers. An example of such spectrum is presented in Fig. 5. The intensity at 760, 1,414, and 1,450 cm^{-1} in the I_{zz} spectrum in this figure is smaller than that observed on intact fibers (Caillé et al., 1987). This is due to a lower concentration of free amino acids in glycerinated fibers and to the absence of trimethylamine in the relaxing solution. The difference spectrum (I_{zz}-I_{xx}) was obtained using the intensity of the 980-cm^{-1} band due to sulfate ions (relaxing solution) as an internal standard. After the subtraction, there is no residual intensity due to phenylalanine residues (1,003 and 1,210 cm^{-1}) or to the presence of soluble molecules, like taurine (1,040 cm^{-1}) and glycine (1,414 cm^{-1}). The subtraction (Fig. 5) also cancelled out the 1,637-cm^{-1} band due to the bending vibration of H_2O since the spectra were measured on the same fiber. The difference spectrum obtained on freshly isolated fibers using the 715-cm^{-1} choline band as an internal standard is very similar to that

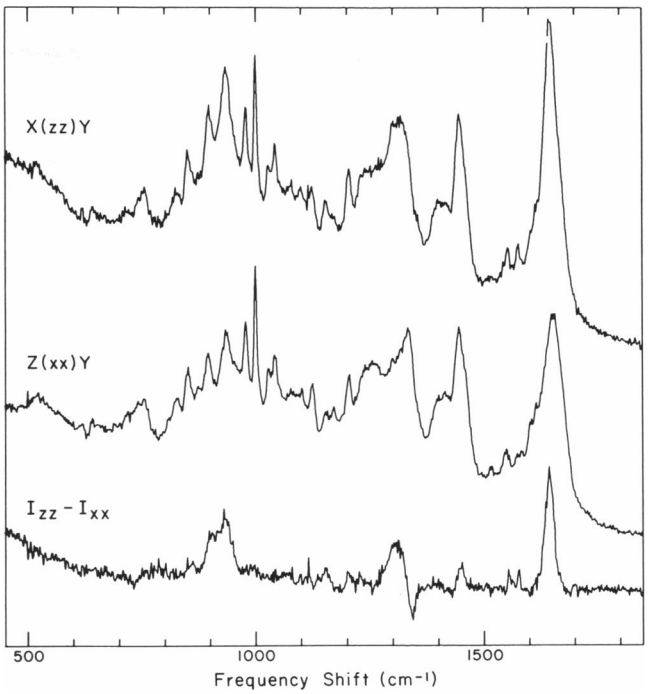

FIGURE 5 Difference between the I_{zz} and I_{xx} spectra of a glycerinated barnacle muscle fiber. The difference spectrum was calculated using the 980-cm^{-1} sulfate band as an internal standard.

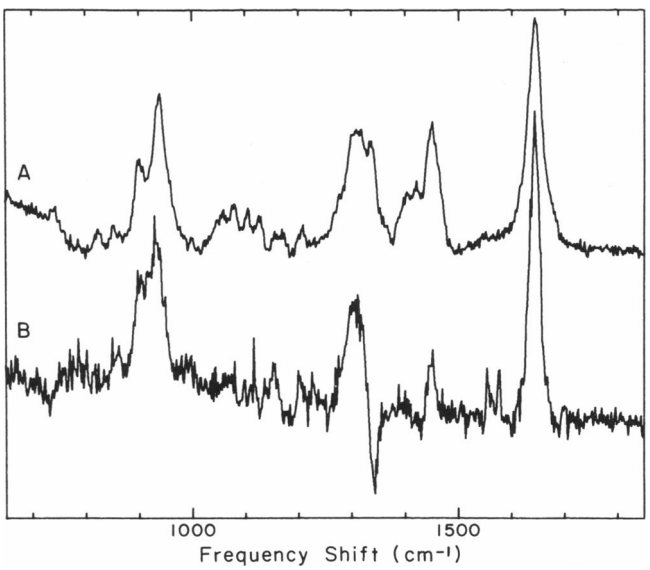

FIGURE 6 Comparison between (A) the Raman spectrum of tropomyosin (Pézolet, M., M. Pigeon, J. Nadeau, and J.-P. Caillé, unpublished results) and (B) the difference spectrum of Fig. 5.

observed on glycerinated fibers, except for residual scattering intensity at 1,340 cm^{-1} (data not shown). As seen in Fig. 6, the difference spectrum of Fig. 5 is almost identical to that of the contractile protein tropomyosin, which is almost 100% α-helical. The main difference occurs at 1,417 cm^{-1}, where a band assigned to the symmetric COOH-stretching vibration of the acidic residues is present in the spectrum of tropomyosin but not in the difference spectrum, which indicates that these residues are not oriented in muscle fibers.

We will now consider the main features of the difference spectrum that are the bands due to the amide I, amide III, and C-C stretching vibrations.

Amide I Vibration. The amide I band appears in the difference as a strong and sharp feature centered at 1,645 cm^{-1}, which is in very good agreement with the frequency of the amide I vibrations in the spectra of predominantly α-helical poly-L-lysine (Carrier and Pézolet, 1984), tropomyosin (Fig. 6), and coat proteins of filamentous viruses (Thomas et al., 1983). However, the bandwidth of the amide I lines for these proteins is ~10 cm^{-1} higher than that observed in the difference spectrum, 33 cm^{-1} compared with 23 cm^{-1}. We believe that this difference is due to the fact that, because of the three-dimensional organization of the proteins in a muscle fiber, the dispersion of the dihedral angles (ϕ and ψ angles) for the oriented α-helical coiled-coil segments is narrower than that of a coiled-coil protein like tropomyosin in solution.

Amide III Vibration. The amide III vibration is also very sensitive to the conformation of proteins. However, this spectral region is complicated by the presence of bands due to aromatic and aliphatic side chains. The advantage of using difference Raman spectroscopy to study the amide III region of the oriented α-helices is that all the bands due to the unoriented side-chains should be cancelled in the difference spectrum. As seen in Fig. 6, the amide III band of the α-helix appears ~1,310 cm^{-1} with no significant contribution below 1,275 cm^{-1}. Therefore, it is clear from these results that the amide III vibration contributes significantly to the scattering intensity above 1,300 cm^{-1} in the Raman spectra of highly helical proteins such as coat proteins of filamentous viruses (Thomas et al., 1983) and tropomyosin (Fig. 6). From deuteration experiments, Carew et al. (1983) also came to the conclusion that the amide III band of the α-helix is located at ~1,304 cm^{-1} in tropomyosin and light meromyosin. A negative peak is also detected at 1,340 cm^{-1} in the difference spectrum. The precise assignment of this band is difficult to make because it may be due to different vibrations such as the C-H bending mode of oriented methine groups and CH_2 twisting modes (Chen and Lord, 1976) or from vibration of the tryptophan side-chains (Yu, 1974; Kitagawa et al., 1979). Bands observed at 1,556 and 1,580 cm^{-1} can also be associated with the tryptophan residues, but the intensity of the 1,580 cm^{-1} band is too high to be attributed only to these side-chains.

C-C Stretching Vibrations. As for the amide III region, the 900–1,000-cm^{-1} region, where the conformational-sensitive C-C stretching vibrations give Raman bands, is complicated by contributions from the aliphatic side-chains. It is now well accepted that a band at ~940 cm^{-1} is associated with the presence of α-helical conformation (Yu et al., 1973; Pézolet et al., 1978). A band at 902 cm^{-1} has also been assigned to the presence of β-sheet structure (Barrett et al., 1978), and it was found that this band increases in intensity when calcium ions are added to myosin (Barrett et al., 1978) or when this protein is aggregated (Carew et al., 1983). It is clear from the difference spectrum of Fig. 6 that both the 938 and 902 cm^{-1} bands show a strong dichroism. Since we have no evidence from neither the amide I nor the amide III regions that some β-sheets are oriented in muscle fibers, we assigned both the 902 and 940 cm^{-1} to C-C stretching vibrations of the proteins segments with α-helical conformation. The splitting of this spectral feature may be due to either a factor group coupling of the C-C vibration of the backbone of the α-helical conformation or to C-C stretching vibrations involving both the protein backbone or some side-chains. Since the 902-cm^{-1} band seems to be more sensitive to the state of aggregation of myosin than does the 938-cm^{-1} band (Carew et al., 1983), we favor the second interpretation.

CONCLUSION

This study demonstrates that Raman spectroscopy can be a valuable tool to investigate the degree of orientation of contractile proteins in vivo and can, therefore, be used to detect changes in orientation of these proteins during muscle contraction. Approximately 50% of the α-helical proteins found in muscle tissues appear to have their backbone oriented along the fiber axis. Most side-chains seem unoriented, but a small dichroism was detected for the 1,450-cm^{-1} band due to the CH_2 bending vibration as well as for some tryptophan lines. The spectrum of the oriented α-helical segments detected by difference spectroscopy shows that the amide I band is centered at 1,645 cm^{-1} and is narrower than that of coiled-coil α-helical proteins in solution. The amide III band is at 1,310 cm^{-1} with essentially no contribution below 1,300 cm^{-1}. Finally, the C-C stretching vibrations give bands at 938 and 902 cm^{-1} that are both characteristic of the α-helix. All these features are found in the Raman spectrum of the highly α-helical protein tropomyosin.

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