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Lens Cell-to-Cell Channel Protein: II. Conformational Change in the Presence of Calmodulin

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Summary. Lens fibers are coupled by communicating junctions, clusters of cell-to-cell channels composed of a 28-kD intrinsic membrane protein (MIP26). Evidence suggests that these and other cell-to-cell channels may close as a result of protein conformational change induced by activated calmodulin. To test the validity of this hypothesis, we have measured the intrinsic fluorescence emission and far-ultraviolet circular dichroism of the isolated components MIP26, calmodulin, and the MIP26-calmodulin complex, both in the absence and presence of Ca^{++}, an uncoupling agent. MIP26 shows no change in either fluorescence emission (primarily tryptophan and a measure of aromatic constitutivity) or in its circular dichroism spectrum. Calmodulin exhibits a 32% increase in fluorescence emission intensity with constant emission wavelength, entirely tyrosine, and a 44% increase in α-helicity, changes previously described. The MIP26-calmodulin complex, on the other hand, displays fluorescence emission and circular dichroism spectra which are slightly different from the sum of the two single components, but shows marked differences in both spectra upon Ca^{++} addition. This indicates a change in conformation in one or both of the two components. Spectral changes include a 5-nm blue-shift, a 50% increase in tyrosine fluorescence emission, a 25% decrease in tryptophan fluorescence emission, and a 5% increase in the α-helicity of the complex. These changes also occur about an isosbestic point and are fully reversible. These data provide additional evidence that activated calmodulin may modulate gating of cell-to-cell channels by affecting channel protein.

Key Words lens gap junctions · lens channel protein (MIP26) · communicating junctions · calmodulin and gap junctions · calmodulin/MIP26 interaction · conformational change—calmodulin/MIP26

Introduction

Communicating junctions are cell-to-cell gateable channels, which allow free transit of ions, metabolites, and messengers of less than 1 kDalton (Flagg-Newton, Simpson & Loewenstein, 1979). These channels exist within axially-paired intramembrane particles, believed to be composed of identical radially-arrayed, transmembrane protein subunits (reviewed in Peracchia, 1984*a*).

A number of treatments have been shown to close the cell-to-cell channel. The functional correlate of channel closure, cell-to-cell uncoupling, has long been associated with an increase in either $[Ca^{++}]_i$ or $[H^+]_i$ (Loewenstein, 1981; Turin & Warner, 1977, 1980; Spray, Harris & Bennett, 1981; Spray, Stern, Harris & Bennett, 1982). Channel closure is believed to occur via conformational change in the protein subunits. This is suggested by a number of observations showing structural changes in gap junctions (communicating junctions) with functional uncoupling or direct treatment with divalent cations or H^+ (reviewed in Peracchia, 1984*a*) and by recent evidence for a Ca^{++} effect on subunit orientation in isolated liver gap junctions (Unwin & Ennis, 1984).

In the companion paper (Girsch & Peracchia, 1985) both the rationale and evidence for Ca^{++}/H^+ mediation via calmodulin (CaM) in occluding reconstituted channels has been presented. To test the hypothesis that channel protein configuration is affected by activated CaM, we have studied the conformation of isolated lens channel protein (MIP26) by measuring the intrinsic protein fluorescence emission, which specifically reflects the constitutivity of aromatic residues, and the far-UV circular dichroism, not only an absolute measure of the current configuration of a given protein (α-helix, β-sheet, etc.) but a particularly sensitive monitor for conformational changes.

Materials and Methods

MIP26

Young beef eyes were obtained from a local abattoir; lenses were removed and decapsulated within 2 hr of death. MIP26 was prepared as previously described (Girsch & Peracchia, 1985) except that 2% SDS was used as MIP26 extractant since yields and

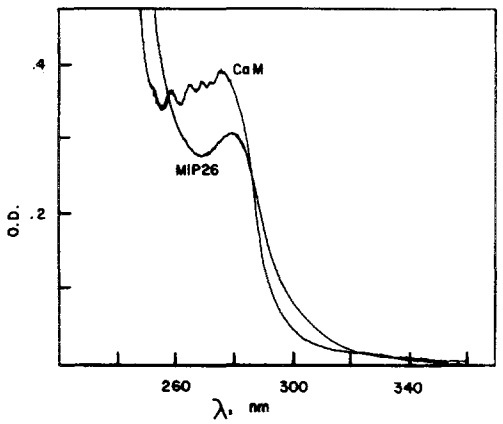

Fig. 1. Near UV absorbance spectra of MIP26 and CaM (×20). CaM is characterized by aromatic fine structure due to phenylalanine and tyrosine, usually masked by tryptophan in other proteins. MIP exhibits a spectrum primarily from tryptophan. Concentrations: MIP26 = 50 μM, CaM = 35 μM

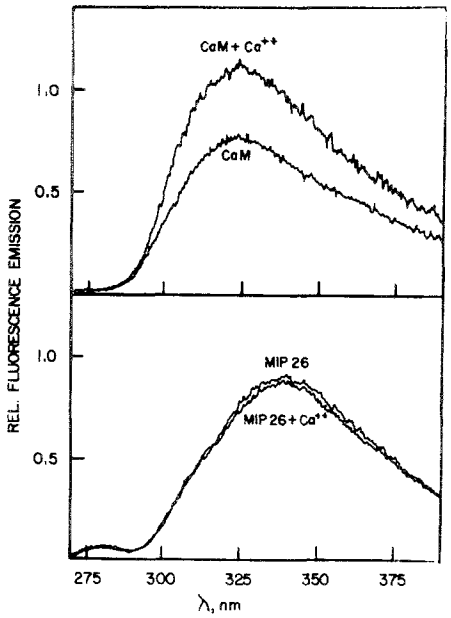

Fig. 2. (*Upper panel*): Relative fluorescence emission spectra of CaM both in absence and presence of Ca^{++} (1 μM). Emission is primarily due to tyrosine and increases 25% on Ca^{++}-addition. (*Lower panel*): Relative fluorescence emission spectra of MIP26 both in absence and presence of Ca^{++} (1 μM). Little change in relative fluorescence emission occurs. Excitation is at 278 nm

optical clarity were superior to octyl-POE. For most studies the clarified preparation was judged adequate, being better than 95% pure. Stock solution was between 25–40 mg/ml. This was separated into 20 μl aliquots (stored at 4°) and was stable for several months.

Calmodulin

CaM was either purchased from CAABCO, Houston, TX, or obtained from Dr. M.J. Welsh. Purity was assessed using near-UV absorbance and SDS-PAGE profile. Stock solution was 500 μg/ml. HEPES, EGTA, Tris, and SDS were purchased from Sigma, St. Louis, MO. Inorganic salts were obtained from Fisher, Fair Lawn, NJ. All solutions were made with deionized distilled water.

Assay System

The reconstitution buffer used was 20 mM HEPES, 2 mM EGTA, 10 mM KCl for fluorescence studies, and 2.5 mM Tris, 0.2 mM EGTA, 2.5 mM KCl for circular dichroism studies (where buffer transparency was needed in the far UV (200–240 nm)). The two components, MIP26 and CaM, are markedly different in their solubilities. For this reason conditions for mixing were critical. Usually not more than 1 μl of stock MIP26 could be added *slowly*, and with rapid microbar stirring to a cuvette containing 1 ml of reconstitution buffer. Other components were added afterward. Changing any of the parameters, for example adding CaM first, resulted in precipitation. The stability of the test solutions was monitored by Rayleigh scatter at 278 nm. The micellar dispersion of MIP26 was only semistable, since after 30 min precipitation began to occur.

Free Calcium Determination

[Ca^{++}] was determined using Ca^{++}-EGTA equilibrium nomograms generated by Rose and Loewenstein (1976) using the constants of Chaberek and Martell (1959). Measurement of pH was made before and after addition of each component, and free Ca^{++} calculated for each pH and Ca^{++} addition.

Absorbance Measurements

Optical densities were made with a Beckman model 25 spectrophotometer using 0.5 ml microcuvettes and 2 mm slits.

Spectrophotofluorometry

Spectra were obtained using an Aminco-Bowman SPF spectrophotofluorometer equipped with elliptical optics. Slit arrangement was 1-2-1 mm at both the entrance and exit ports. Excitation was usually at 278 nm, with relative emission spectra recorded from 300–450 nm on strip chart.

Circular Dichroism (CD)

Spectra were obtained using a microprocessor-controlled Jasco 41A dual-beam recording instrument. Ellipticity was measured at 2-nm intervals, with 1,000 determinations per interval. Additionally, three successive spectra were averaged before the resultant spectrum was stored. A PDP 11/34 was used for computed averaged transients, storage, and manipulation of spectra. Measurements were made using circular cells with 2 mm pathlength to keep light scattering to a minimum in the far UV. Molar ellipticities were calculated by using the equations of Fasman (1976).

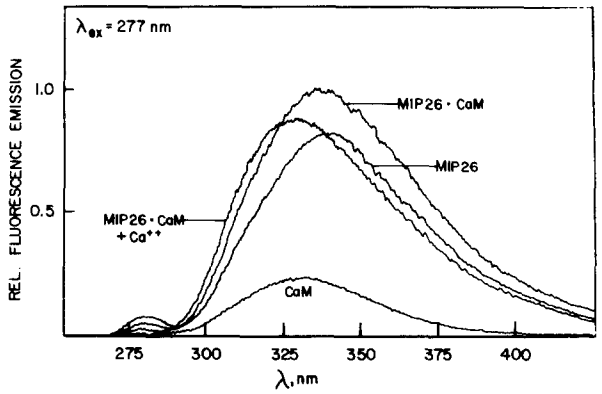

Fig. 3. Relative fluorescence emission spectra of MIP26 · CaM (equimolar) both in absence and presence of Ca^{++} (1 μM). The two spectra are markedly different, characterized by a decrease in tryptophan emission (solely MIP26), an increase in tyrosine emission (primarily CaM) and a blue shift in intensity maximum of 20 nm. Individual fluorescence emission spectra of MIP26 and CaM are shown for reference. Excitation is at 278 nm

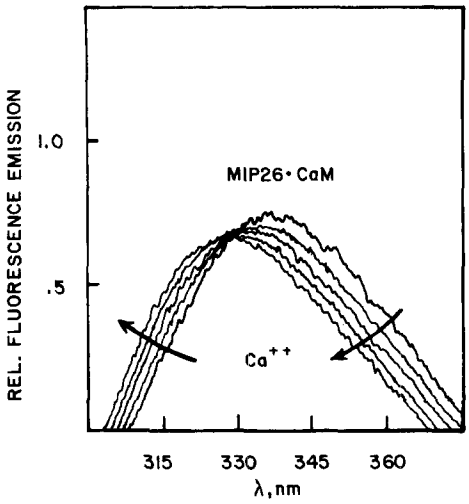

Fig. 4. The conversion of MIP26 · CaM to the Ca^{++}-activated form by incremental addition of Ca^{++}, final conc. = 1.5 μM. The fluorescence changes observed are reversible and pass through an isosbestic point at 330 ± 7 nm. Excitation is at 278 nm

pH Measurements

pH was measured using a Fisher Accumet 620 pH meter equipped with flat-tip Ingold combination microelectrode (6025-04). Measurements were made in the cuvette, both before and after addition of aliquots.

Results

Fluorescence

In Fig. 1 the near-UV absorption spectra for MIP26 and CaM (×20) are shown. CaM contains eight phenylalanines (λ_{max} = 252, 259, 265, and 269 nm), two tyrosines (*Y*) (λ_{max} = 275 nm), and no tryptophan (*W*). MIP26, on the other hand, contains at least 10 *W*'s (λ_{max} = 290 nm) and very little *Y* (B. Nicholson, *personal communication*). The absorption envelopes, and hence fluorescence excitation (ϕ_{ex}) spectra, of MIP26 and CaM are therefore markedly different, allowing an independent measure of the intrinsic fluorescence emission (ϕ_{em}) spectra of the two proteins simultaneously.

In Fig. 2 (upper panel) is shown the fluorescence emission spectrum of CaM. The $\phi_{em}\lambda_{max}$ is approximately 322 nm, 20 nm blue-shifted from that of MIP26 (lower panel). When 1 μM calcium ion is added to CaM, ϕ_{em} intensity increases, while the position of λ_{max} remains constant. No further changes are seen with additional calcium.

In Fig. 2 (lower panel) is shown the ϕ_{em} spectrum of MIP26. The spectrum is characterized by a broad mono-Gaussian with emission maximum at 342 nm. When Ca^{++} (1 μM) is added, no change occurs in the emission envelope. Raising $[Ca^{++}]$ to 10–100 μM does not alter fluorescence emission, but it does hasten precipitation.

In Fig. 3 are shown the single components MIP26 and CaM, under equimolar conditions. The highest trace shows the resulting spectrum when MIP26 and CaM are mixed together. The resultant trace is slightly different from the arithmetic addition of the two separate emission envelopes. There is a slight decrease in the *W* side of the spectrum, coupled with an enhancement on the *Y* side of the envelope. When calcium ion is added to the mixture, a dramatic change can be seen in the ϕ_{em} profile. There is an approximate 25% decrease in *W* emission (solely MIP26) and a 50% increase in *Y* ϕ_{em} (primarily contributed by CaM).

Figure 4 provides evidence that the Ca^{++}-induced fluorescence changes occur in a complex of MIP26-CaM and not in one or both components singly. As the $[Ca^{++}]$ is raised by 0.25-μM increments, changes occur in an orderly and progressive manner, passing through an isosbestic point at approximately 330 nm.

To assess whether the changes observed with Ca^{++} are nonspecific divalent ion effects, Mg^{++} was substituted for Ca^{++} and added to MIP26, CaM, and the MIP26-CaM complex. Figure 5 shows the fluorescence emission spectrum of MIP26-CaM both before and after Mg^{++} addition. Even with $[Mg^{++}]$ as high as 100 μM little change in the emission envelope occurs. No effects were seen in the ϕ_{em} of either MIP26 or CaM when Mg^{++} was added (*not shown*).

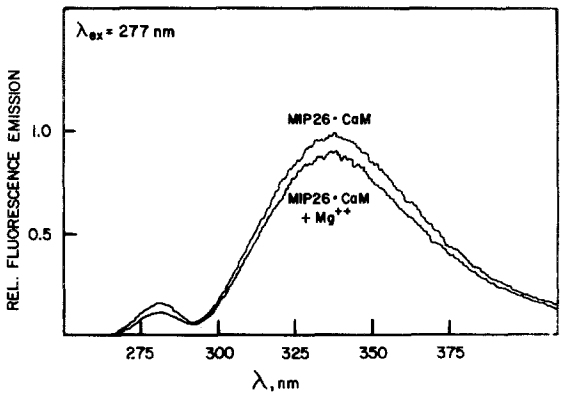

Fig. 5. The relative fluorescence emission spectra of MIP26 · CaM both in absence and presence of Mg^{++} (10 μM). Excitation is at 278 nm

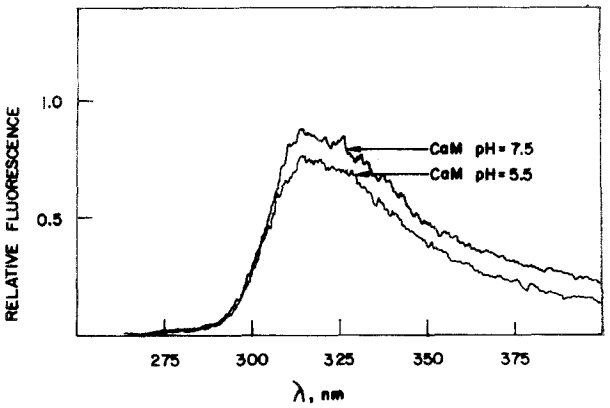

Fig. 7. The relative fluorescence emission spectra of CaM both at pH 7.5 and 5.5. Only a small change in fluorescence intensity is seen, with no change in the position of intensity maximum. Excitation is at 278 nm

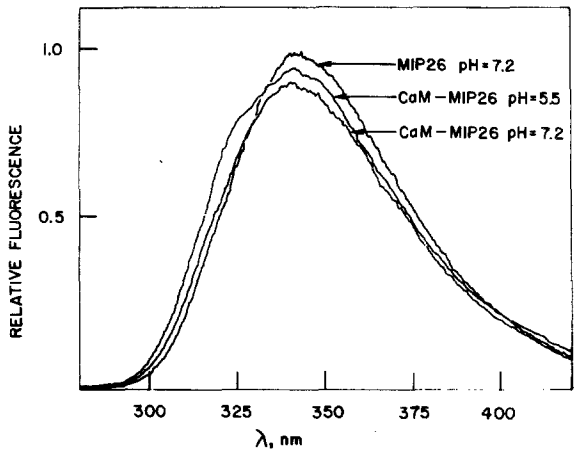

Fig. 6. The relative fluorescence emission spectra of MIP26 · CaM at both pH 7.2 and pH 5.5. MIP26 fluorescence is shown for reference. Some enhancement of tyrosine fluorescence emission intensity is observed in the complex at the lower pH

To test the possibility that fluorescence changes occur by way of a nonspecific CaM-protein interaction, BSA, lens soluble protein, and opsin were substituted for MIP26 in the cuvette. In all cases only the expected CaM $Y\phi_{em}$ intensity increase was seen, i.e., no shifts in $Y\lambda_{max}$, or changes in $W\phi_{em}$ occurred (*not shown*).

The effect of pH on the MIP26-CaM complex was evaluated by adjusting pH in the cuvette, with HCl, to achieve 0.2 pH unit decrements. As with Ca^{++} there is a shift in the $\phi_{em}\lambda_{max}$, although the $Y\phi_{em}$ appears more as a distinct shoulder, but the $W\phi_{em}$ shows a slight increase (~3%) (Fig. 6). Lowering the pH to 5.5 has some effect on CaM alone, causing a decrease in ϕ_{em} intensity, but not a change in λ_{max} (Fig. 7), and no effect on MIP26 emission (*not shown*).

In Fig. 8 is shown the effect of Ca^{++} on the near-UV CD spectrum of MIP26. Molar ellipticity of the native protein is approximately −10,900 deg-cm^2 dmol^{-1}, corresponding to an alpha-helical content of 37%. Addition of Ca^{++} has no effect on the helicity of the protein. The only other value cited for lens channel helicity is 58%, measured recently by Horwitz and Bok (1983).

In Fig. 9 is shown the far-UV CD spectrum of CaM. The negative, slightly bimodal peak indicates that CaM contains ~33% alpha-helix (molar ellipticity $(\theta)_{222\,nm}$ = −9,800 deg-cm^2 dmol^{-1}). After Ca^{++} addition, helicity increases to 44% ($(\theta)_{222\,nm}$ = −13,200 deg-cm^2 dmol^{-1}). The CD spectrum in the near-UV region, where aromatic residues absorb, shows little change on Ca^{++} addition, even though $Y\phi_{em}$ exhibits a dramatic increase. Both Wolff et al. (1977) and Walsh et al. (1979) have described similar results.

In Fig. 10 is shown the near-UV CD spectrum of MIP26-CaM complex, both with and without Ca^{++} ion addition. The expected and observed molar ellipticities are approximately the same before Ca^{++} (≃−21,000 deg-cm^2 dmol^{-1}). After Ca^{++} addition, however, the measured molar ellipticity is approximately −28,000 deg-cm^2 dmol^{-1}, while that calculated for the complex is −24,000 deg-cm^2 dmol^{-1}, representing an increase in α-helicity of approximately 5% above that for the proteins when noninteraction is assumed.

Discussion

The data presented here show that lens junction protein (MIP26) changes in conformation in the presence of Ca^{++}-activated CaM. MIP26 exhibits

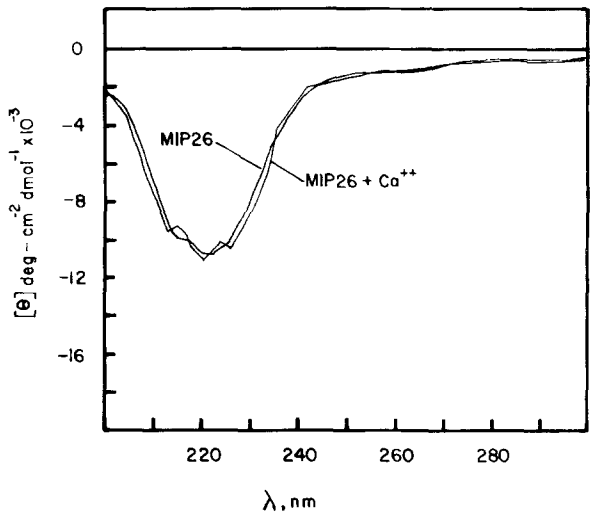

Fig. 8. Far-UV CD spectra of MIP26 both in absence and presence of Ca^{++} (1 μM). Molar ellipticity remains virtually unchanged

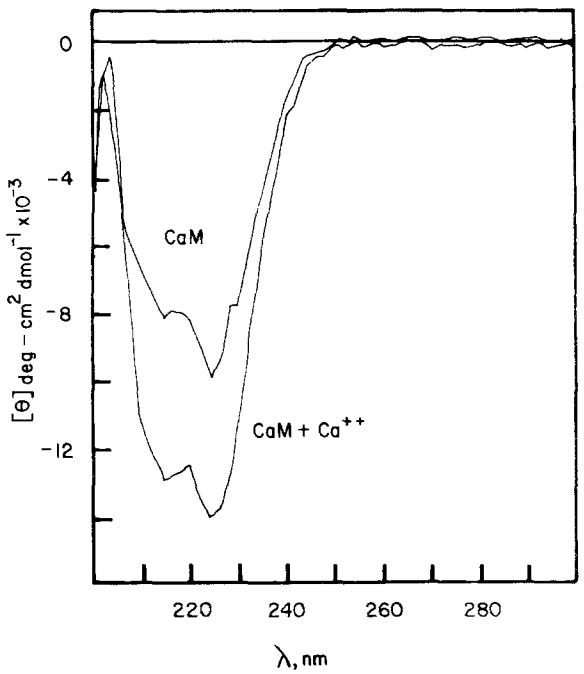

Fig. 9. Far-UV CD spectra of CaM both in absence and presence of Ca^{++} (1 μM). CaM enters a tighter conformation on Ca^{++} binding, increasing helicity ~40%

no change in either aromatic constitutivity or CD spectrum when just Ca^{++} is added, suggesting that Ca^{++} alone does not directly affect its conformation. Although CaM alone exhibits changes in both fluorescence and ellipticity, these spectral alterations cannot account for changes observed in the MIP26-CaM-Ca^{++} complex.

Fluorescence

Purified MIP26 showed no change in ϕ_{em} with [Ca^{++}] as high as 100 μM. The SDS micellar dispersion of this very hydrophobic channel protein may have caused alteration or denaturation, which in turn could have prevented Ca^{++} binding and/or induced conformational change. However, sodium dodecylsulfate (SDS) solubilized protein can be reconstituted after extensive dialysis, forming channels in vesicles (Girsch & Peracchia, 1983), suggesting that the SDS-extracted channel protein retains a native-like configuration. A second possibility is that Ca^{++} binding and/or activation of MIP26 does occur, but this change is not reflected in the tryptophan emission profile. However, it is difficult to imagine Ca^{++} binding and conformational change *without* modifications in the microenvironment of any of the aromatic residues.

When CaM and MIP26 are mixed together, the fluorescence emission envelope is different from that of the sum of individual components. Addition of Ca^{++} to the CaM-MIP26 complex results in a significantly different emission envelope. Moreover, these changes are fully reversible, occur in an isosbestic manner, and cannot be accounted for by the Ca^{++}-induced changes in CaM. For example, the 25% decrease in the $W\phi_{em}$ region of the envelope (350 nm) cannot be due to CaM, since it contains no *W*. In like manner, the $Y\phi_{em}$ is blue-shifted, which cannot be due to a CaM-Ca^{++} interaction but must result from a change in the aromatic constitutivity of the complex.

Since the activation of the MIP26-CaM complex by Ca^{++}, as monitored by successive fluorescence emission scans, passes through a single crossover point, the MIP26-CaM complex behaves as one emitting species:

$$(XZ) \xrightarrow{Ca^{++}} (XZ)^*.$$

If Ca^{++} altered only the ϕ_{em} of one component:

$$X + Z \xrightarrow{Ca^{++}} X^* + Z$$

the ϕ_{em} profile would not pass through a single crossover point during the titration with Ca^{++} since there are three emitting species. Finally, these fluorescence changes occur only when all three components are present: Ca^{++}, CaM, and MIP26. Substituting other proteins for channel protein or adding other divalent ions yields ϕ_{em} profiles, which are only the sum of individual components ϕ_{em} profiles, indicating no interaction among the components.

Lowering the pH of the complex gave similar,

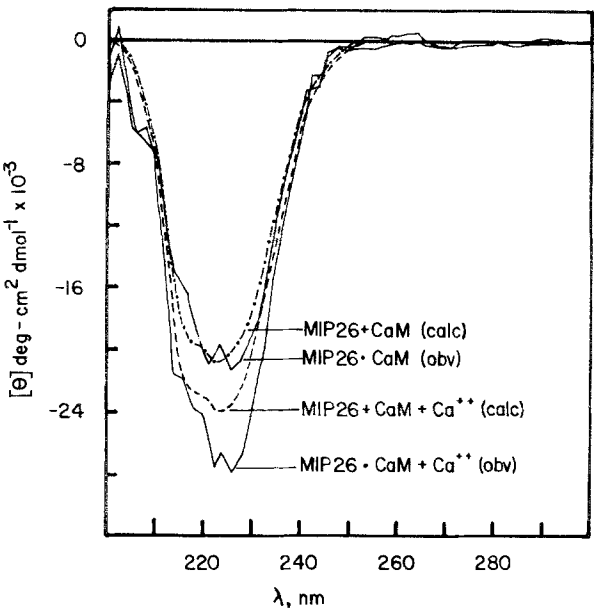

Fig. 10. Far-UV CD spectra of the MIP26 · CaM complex both in the absence and presence of Ca^{++} (1 μM). Before Ca^{++} addition the computed spectrum (–·–·) and measured spectrum (—) are virtually identical (≃−21,000 deg-cm² dmol⁻¹). After Ca^{++} addition the complex exhibits a molar ellipticity (≃−28,000 deg-cm² dmol⁻¹) 15% greater than that computed for the Ca^{++}-activated complex (– – –) (≃−24,000 deg-cm² dmol⁻¹)

but not identical, results to those discussed for Ca^{++}. The spectral changes indicate that, as with Ca^{++}, the $Y\phi_{em}$ of CaM undergoes a blue shift as well as an increase in intensity. Unlike with Ca^{++}, however, the *W* side of the emission envelope changes very little, suggesting that the aromatic constitutivity of MIP26 is altered only slightly.

Thus, lowered pH induces structural changes in CaM which mimic the Ca^{++} form of the protein. Unlike the changes with Ca^{++}, the changes in CaM with pH, as monitored by $Y\phi_{em}$, occur only when MIP26 is present. While changes in MIP26 fluorescence occur with Ca^{++}-activated CaM as the conformer, in the studies with pH the two protein components act in a mutually cooperative manner to achieve a configuration similar to the Ca^{++}-activated form of the MIP26-CaM complex. Earlier work by Lehrer and Leavis (1974) showed such changes in troponin C with lowered pH as evidenced by $Y\phi_{em}$ and light scattering. More recently, Steiner, Lamboy and Sternberg (1983), using such criteria as $Y\phi_{em}$ relaxation time, CD, and viscosity measure, showed that CaM in lowered pH also mimics conformationally its Ca^{++}-activated form. In agreement with our observations, they found that CaM ϕ_{em} intensity changes only slightly with pH from 3–9 (except for a narrow spiked increase centered at pH 4.5, and beyond the region of biological interest).

Circular Dichroism

The technique of far-UV CD spectrophotometry provides a powerful tool for studying protein-protein interactions. While the measure of ϕ_{em} gives information on the microenvironment of the aromatic residues, the information to be gleaned from CD tells more about the structural backbone of the proteins in question. In the case of MIP26, the direct involvement of Ca^{++} would be expected to initiate tertiary changes in the channel protein, but in fact none were observed. The theory of direct Ca^{++} involvement may not be dismissed, however, since SDS is known to induce α-helix formation in some hydrophobic proteins. The possibility that SDS micellar MIP26 is screened from Ca^{++}-induced conformational changes cannot be dismissed.

The near-UV CD results with Ca^{++}-activated MIP26-CaM complex, like the fluorescence data, indicate that an interaction occurs between the two proteins. MIP26 does not change in α-helicity with Ca^{++}, but CaM undergoes a dramatic (33%) increase in α-helicity after Ca^{++} addition. Assuming noninteraction, the calculated $[\theta]_{220\,nm}$ for the Ca^{++}-activated complex would be −24,000 deg-cm² dmol⁻¹. However, a value of ≃−28,000 deg-cm² dmol⁻¹ is measured. This would indicate that either CaM or MIP26 forms additional α-helix or that portions of both proteins have entered a new conformation containing 5% additional α-helix. Experiments are planned which will examine the interaction of MIP26 and CaM by far-UV CD at several pH and Ca^{++}-EGTA buffer values.

All the data presented in this paper are consistent with the concept that Ca^{++}-activated calmodulin induces conformational change in the channel protein. In the companion paper we have shown that Ca^{++} – CaM closes the channels of MIP26 reconstituted vesicles (Girsch & Peracchia, 1984). Evidence for a structural change in channel protein with uncoupling agents has been previously obtained. Changes in gap junction structure (crystallization) have been described with functional uncoupling in many systems. In the isolated lens junction, particle crystallization is apparently linked to both divalent ions and hydrogen ion concentrations (Peracchia, 1978; Alcala et al., 1979; Peracchia & Peracchia, 1980*a,b*; Kistler & Bullivant, 1980; Benedetti, Dunia, Ramaekers & Kíbbélaar, 1981). In the liver Unwin and Ennis (1984) have shown that Ca^{++} is associated with subunit tilting. CaM is a likely candidate for involvement in the control of

communicating junction permeability. Indeed, lens junction crystallization could be prevented by a CaM inhibitor trifluoperazine (TFP) (Peracchia, Bernardini & Peracchia, 1981), and more recently the electrical uncoupling of amphibian embryonic cells could be inhibited by TFP (Peracchia et al., 1981, 1983) as well as calmidazolium (Peracchia, 1984*b*), a more specific inhibitor of CaM. The CaM involvement in gating communicating channels is also supported by its capacity to bind to the lens (Welsh et al., 1981, 1982; Hertzberg & Gilula, 1981) and liver (Hertzberg & Gilula, 1981) gap junction protein.

In conclusion, the complex MIP26-CaM exhibits new and unique spectroscopic properties on addition of Ca^{++} or H^+, which are not the sum of the expected changes in the individual components. This indicates conformational changes in MIP26 induced by activated CaM, which are likely to be part of the mechanism which closes cell-to-cell channels. This *in vitro* system will be a useful model for exploring molecular events, occurring during channel closure, that cannot be studied *in vivo*.

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