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The Thermal Stability of Recoverin Depends on Calcium Binding and Its Myristoyl Moiety As Revealed by Infrared Spectroscopy

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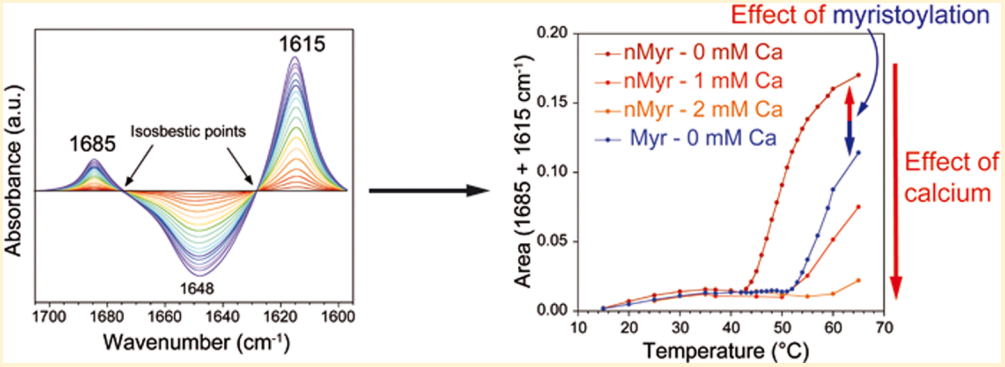
S *Supporting Information*



ABSTRACT: To evaluate the structural stability of recoverin, a member of the neuronal calcium sensor family, the effect of temperature, myristoylation, and calcium:protein molar ratio on its secondary structure has been studied by transmission infrared spectroscopy. On the basis of the data, the protein predominantly adopts α-helical structures (~50−55%) with turns, unordered structures, and β-sheets at 25 °C. The data show no significant impact of the presence of calcium and myristoylation on secondary structure. It is found that, in the absence of calcium, recoverin denatures and self-aggregates while being heated, with the formation of intermolecular antiparallel β-sheets. The nonmyristoylated protein (Rec-nMyr) exhibits a lower temperature threshold of aggregation and a higher intermolecular β-sheet content at 65 °C than the myristoylated protein (Rec-Myr). The former thus appears to be less thermally stable than the latter. In the presence of excess calcium ions (calcium:protein ratio of 10), the protein is thermally stable up to 65 °C with no significant conformational change, the presence of the myristoyl chain having no effect on the thermal stability of recoverin under these conditions. A decrease in the thermal stability of recoverin is observed as the calcium:protein molar ratio decreases, with Rec-nMyr being less stable than Rec-Myr. The data overall suggest that a minimal number of coordinated calcium ions is necessary to fully stabilize the structure of recoverin and that, when bound to the membrane, i.e., when the myristoyl chain protrudes from the interior pocket, recoverin should be more stable than in a Ca-free solution, i.e., when the myristoyl chain is sequestered in the interior.

Calcium is a ubiquitous intracellular messenger that allows the control of numerous cellular processes, such as fertilization, proliferation, development, learning and memory, contraction, and secretion (for a review, see ref 1). This is mainly achieved by proteins that specifically bind calcium.[2,3] Calcium-binding proteins (CaBP) make up a heterogeneous and wide group of proteins, which include proteins that contribute not only to the control of calcium concentration but also to the decoding of calcium signals, thus acting as calcium sensors.[4,5] Among these proteins, one can find intracellular CaBP containing[6] or lacking EF-hand motifs.[4] The EF-hand calcium-binding motif consists of an α-helix, a calcium-binding loop, and a second helix.[7] The proteins containing the EF-hand motif constitute a large and functionally diverse family of proteins.[8−10]

The neuronal calcium sensor (NCS) protein family makes up part of the EF-hand family. It contains 14 members, including recoverin.[11−13] Recoverin is a 23 kDa protein that acts as a calcium sensor in retinal rod cells.[14] In the dark, calcium-bound recoverin inactivates rhodopsin kinase.[15−17] However, light activation of rhodopsin results in a large decrease in the intracellular calcium content of rod photoreceptors and a large

Received: September 27, 2013
Revised: December 10, 2013
Published: December 12, 2013

   dx.doi.org/10.1021/bi401336g | *Biochemistry* 2014, 53, 48−56

conformational change in recoverin upon calcium dissociation. Consequently, recoverin dissociates from rhodopsin kinase, which then phosphorylates rhodopsin (see refs 18 and 19 for reviews on phototransduction).

Like other members of the NCS family, recoverin possesses four EF-hand motifs that can coordinate calcium ions. However, only two of these motifs can bind calcium (EF-hands 2 and 3). Calcium binding occurs in a successive manner, EF-hand 3 being coordinated first because of a higher association constant.[20−23] Moreover, recoverin contains an N-terminal myristoyl moiety that can be inserted within or extruded out of a hydrophobic cavity in a calcium concentration-dependent manner, a phenomenon called the calcium myristoyl switch.[24,25] It has been suggested that the myristoyl group can serve as a membrane anchor when it protrudes from the protein surface.[26]

The three-dimensional (3D) structure of myristoylated recoverin (Rec-Myr) has been determined in solution in the absence of calcium[27] and when coordinated with two calcium ions.[28] The 3D structure of nonmyristoylated recoverin (Rec-nMyr) has been determined in solution in the presence of calcium.[20] Recoverin mainly adopts an α-helical conformation in which two subdomains formed by EF-hand 1 and 2 (N-terminal domain) and EF-hands 3 and 4 (C-terminal domain) are joined by a U-shaped linker region.[29] Calcium binding of recoverin is associated with a rotation of 45° of the C-terminal domain relative to the N-terminal one and the exposure of many hydrophobic residues to the solvent.[28]

The structure of Rec-Myr and Rec-nMyr has been investigated in various environments, especially using circular dichroism (CD) and fluorescence spectroscopy. The data are contradictory as some of them suggest a change in the amount of α-helix content upon calcium binding,[30,31] whereas other studies detect virtually no structural modification.[23,32] N-Myristoylation does not seem to induce conformational changes in recoverin as indicated by CD.[31] However, it has been found by small-angle X-ray scattering (SAXS) that Rec-Myr and Rec-nMyr "aggregate" (oligomerize) at room temperature in the presence and absence of calcium, respectively.[31]

The study of the thermal stability is probably one of the simplest and most powerful ways of assessing the structural integrity of a protein, especially with regard to different protein environments. This type of information is also important because physiological processes occur at 37 °C and, from a more practical point of view, because it may serve as a reference for understanding the structural behavior of recoverin when it interacts with phospholipid model membranes. The study of the heat-induced stability of recoverin has only been performed for Rec-Myr using microcalorimetry,[23] fluorescence spectroscopy,[32] and CD.[33] The results have shown that recoverin is stabilized in the presence of calcium.

More generally, binding of calcium by proteins can have different effects on their stability. For example, it has been shown that the thermal stability of proteins containing EF-hand motifs is increased in the presence of calcium,[23,32,34−43] which is also true when no EF-hand motif is present in the structure of the protein.[44] However, a decrease in thermal stability has also been observed in the presence of calcium for some proteins bearing an unusual EF-hand motif[45,46] or in the absence of such a motif.[47] Calcium thus allows stabilization of the structure of most proteins assayed until now. However, none of these studies has carefully studied the effect of varying the molar ratio of calcium on protein thermal stability.

Recoverin has therefore been used in this study to clarify this issue. More specifically, we have used transmission infrared (IR) spectroscopy to study the effect of calcium and the N-myristoyl moiety on the secondary structure and thermal stability of recoverin in solution. IR spectroscopy is a powerful tool for investigating the secondary structure of proteins in various aqueous environments, because of the amide I band that originates from peptide bond vibrations. This technique has often been used to investigate the effect of calcium on the secondary structure of proteins.[48−51] In particular, the study of the antisymmetric COO^- stretching [$\nu_{as}(COO^-)$] vibration provides insight into the binding of amino acid side chain carboxylate groups.[52]

MATERIALS AND METHODS

Materials. Water used for buffer preparation was distilled and deionized (resistivity of 18.2 MΩ cm) using a Barnstead NANOpurII system (Boston, MA). Deuterium oxide (D_2O) was obtained from CDN isotopes (Pointe-Claire, QC). All solvents were of reagent grade or high-performance liquid chromatography grade [Commercial Alcohols, the industrial and beverage alcohol division of GreenField Ethanol (Brampton, ON) for ethanol and VWR International (Radnor, PA) for chloroform] and used without any further purification. Salts were of analytical grade and used as received. $CaCl_2$, NaOH, sodium myristate, and 2-mercaptoethanol were purchased from Sigma-Aldrich (Oakville, ON). Ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), NaCl, and NH_4HCO_3 were purchased from Laboratoire Mat (Québec, QC). HEPES was purchased from Fisher (Fair Lawn, NJ).

Expression and Purification of Recoverin and Removal of the Salts. Myristoylated recoverin and nonmyristoylated recoverin were expressed and purified as previously reported by Ray et al.[14] with the modifications of Desmeules et al.[53] Purified recoverin was then dialyzed four times to remove all salts. For samples in the absence of calcium, the protein sample was first dialyzed against 50 mM NH_4HCO_3 and 50 μM EGTA and then dialyzed three times against 50 mM NH_4HCO_3. For samples in the presence of calcium, the protein sample was dialyzed four times against 50 mM NH_4HCO_3 and 50 μM $CaCl_2$. All recoverin solutions were then concentrated, lyophilized, and stored at −20 °C. The concentration of recoverin was determined by UV−visible spectroscopy using a molar absorption coefficient (ε) of 23950 M^{-1} cm^{-1} at 280 nm.[54]

Transmission Infrared Spectroscopy. All samples were rehydrated with D_2O buffers (pH 7.5) containing 100 mM NaCl, 25 mM HEPES, and 1 mM 2-mercaptoethanol. Samples in the absence of calcium also contained 0.5 mM EGTA. The effect of calcium has been investigated at different calcium concentrations (1, 2, or 10 mM $CaCl_2$). The final protein concentration was 1 mM.

Measurements were taken using either a Magna 560 or 760 FTIR spectrometer from Nicolet (Thermo Scientific, Madison, WI) equipped with a nitrogen-cooled MCT A detector. The temperature was adjusted using a home-built temperature controller (±1 °C). The temperature was increased from 12−15 to 65 °C, with typical steps of 5 °C. For samples exhibiting aggregation, the experiments were performed with 1−2 °C increments in the temperature range where conformational

dx.doi.org/10.1021/bi401336g | Biochemistry 2014, 53, 48−56

changes occur. The stabilization time for each temperature was 3 min. A protein sample volume between 5 and 9 μL was deposited between two CaF_2 windows (Spectral Systems, Hopewell Junction, NY) using a Mylar film spacer of 13 μm (Goodfellow Cambridge Ltd., Huntingdon, U.K.). Spectra were recorded via the acquisition of 128 interferograms at a resolution of 4 cm^{-1} using a Happ-Genzel apodization.

All spectra were treated using GRAMS/7 AI (Galactic Industries Corp., Salem, NH). The appropriate buffer spectrum was first subtracted from each series of spectra. Water vapor was then subtracted, and the spectra were subsequently smoothed[55] when necessary. Then, a linear or cubic baseline was subtracted in the amide I′ and carboxylate asymmetric stretching regions, i.e., between 1720 and 1530 cm^{-1}. However, for samples showing aggregation, a linear baseline was further subtracted between ~1700 cm^{-1} (1701−1713 cm^{-1}) and ~1600 cm^{-1} (1595−1598 cm^{-1}). Then, the area of the amide I′ was normalized to unity between 1700 and 1600 cm^{-1} with a homemade program running with Grams. To evaluate the extent of aggregation, the spectrum of the series preceding the first appearance of the bands due to β-sheets (onset of aggregation) was subtracted from all the other spectra. The area of the bands due to aggregation was evaluated as a function of temperature using a homemade program, which calculates the positive area of the difference spectrum over the horizontal axis.

The curve fitting of the amide I′ band was performed using various initial sets of band parameters. The amide I′ band has been first decomposed using three Gaussian components, which were found to lead to improper fitting. In fact, the minimal number of components required to obtain a reasonable fit was six (located at 1676, 1663, 1648, 1633, 1617, and 1604 cm^{-1}). The two latter components most probably result from side chain vibrations. Because the curve-fitting software spontaneously tends to promote large bands, restrictions on the bandwidth have been imposed so that they could not exceed 20 cm^{-1}. The frequency was free to change during calculation but always remained within a ±1 cm^{-1} interval, suggesting that the spectral decomposition is robust. The area of the 1648 cm^{-1} component divided by the sum of the area of all amide I′ components was used for the determination of the α-helical content, assuming that the molar absorption coefficient is the same for all structures. The antisymmetric COO^- stretching [$\nu_{as}(COO^-)$] originating from the glutamic acid (Glu) and aspartic acid (Asp) band near 1570 cm^{-1} has also been fit to facilitate the spectral decomposition of the amide I′ band due to the overlap between these two spectral regions.

RESULTS

Effect of Calcium and Myristoylation on the Secondary Structure of Recoverin. Figure 1 shows the amide I′ and antisymmetric COO^- stretching [$\nu_{as}(COO^-)$] absorption bands of Rec-Myr and Rec-nMyr in the presence and absence of Ca^{2+}. The maximum of the amide I′ band for all spectra is located at 1647−1648 cm^{-1}, showing the predominance of α-helices, in agreement with the 3D structure of recoverin.[20,27,28] This value is 3−4 cm^{-1} lower than that obtained by Ozawa et al.[56] This difference is probably due to the fact that in this latter work, the protein was lyophilized after solubilization in D_2O buffer and then dissolved again into D_2O. This treatment may lead to a more complete H−D exchange of the peptide bonds, thus leading to a shift of the amide I′ band toward lower wavenumbers.

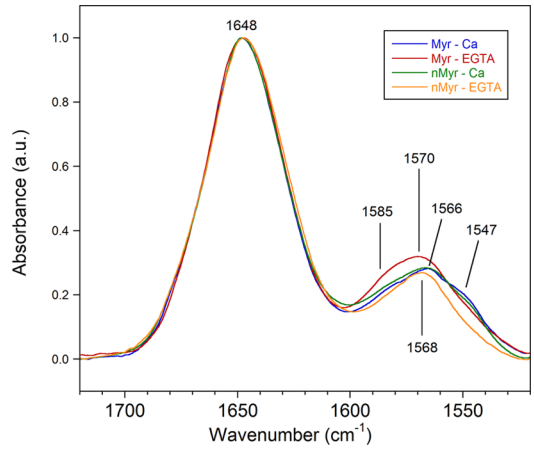


Figure 1. Spectra of Rec-Myr and Rec-nMyr (1 mM in D_2O buffer) in the presence of Ca^{2+} (10 mM Ca^{2+}) and in its absence (0.5 mM EGTA) at 25 °C, in the 1720−1520 cm^{-1} frequency range. Spectra are normalized with respect to the peak maximum of the amide I′ band.

The width at half-height of the amide I′ band is 42 cm^{-1}, which indicates the presence of other band components on each side of the main α-helix band, thus showing that other secondary structure elements such as turns and random structures are also present. The amide I′ band shows no significant difference in shape for the different samples, suggesting no major modification of the secondary structure because of myristoylation or the presence or absence of Ca^{2+}. The lack of a change in secondary structure upon calcium binding is consistent with the absence of or the very small[23,32,57] structural changes reported by circular dichroism but is in contrast with other conclusions.[30,31,33,58] Besides these apparent discrepancies, because infrared spectroscopy does not reveal changes in secondary structure upon calcium binding, it may be hypothesized that the structural changes of recoverin are mainly related to tertiary alterations. Indeed, the tertiary structure changes occurring in recoverin upon calcium binding as described in ref 29 may be detected by CD or fluorescence but cannot be detected by infrared spectroscopy.

To obtain more information about the secondary structure of recoverin, the amide I′ band at 25 °C has been decomposed using different components. It appeared that four amide I′ components located at 1676, 1663, 1648, and 1633 cm^{-1} were necessary to obtain a reasonable curve fitting. The band at 1648 cm^{-1} is assigned to α-helices, whereas those at 1633, 1663, and 1676 cm^{-1} correspond to contributions from β-sheets, turns, and disordered structures, respectively.[59,60] These structural components can be found in the high-resolution structure of recoverin.[20,27,28]

A typical spectral decomposition is shown in Figure 2 for nonmyristoylated recoverin in 10 mM Ca^{2+} buffer. Using different initial band parameters, the curve-fitting calculations show that the α-helical component represents approximately 45−50% of the secondary structure content of recoverin. These values are smaller than those reported by circular dichroism showing that the amount of α-helical structure corresponds to 52 and 65% of the secondary structures in the absence and presence of calcium, respectively.[30,31] However, our data are rather consistent with the 43−60% α-helical content determined by NMR or X-ray diffraction for both the calcium-free recoverin or the one coordinated with two calcium ions.[20,27,28]

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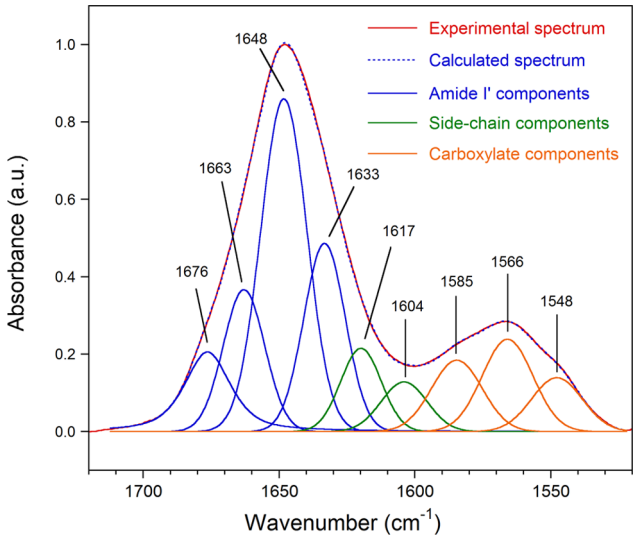

Figure 2. Decomposition of the spectrum of Rec-nMyr (1 mM in D_2O buffer) in the presence of 10 mM Ca^{2+} at 25 °C, in the 1720−1520 cm^{-1} frequency range.

As opposed to the amide I′ band, the $\nu_{as}(COO^-)$ vibration near 1560−1570 cm^{-1} exhibits spectral alterations depending on the presence or absence of calcium and a myristoyl group (Figure 1). This band originates from the vibrations of glutamic acid and aspartic acid side chains that arise near 1558 and 1585 cm^{-1}, respectively.[61,62] Histidine residues may also contribute in this region, although they seem to be in too small number (three residues) with respect to glutamic acid (25 residues) and aspartic acid (11 residues). This vibrational mode is well-known to be sensitive to the binding of divalent cations and to the coordinated structure formed.[63] More generally, this vibration may also be affected by its environment, especially by the formation of hydrogen bonds with water.

In the absence of calcium, the maximum of this band is similar for Rec-Myr and Rec-nMyr (1570 and 1568 cm^{-1}, respectively). A shoulder can also be seen in this band for both proteins near 1585 cm^{-1}. Despite these similarities, the spectra also display differences in this region, especially near 1550 cm^{-1}. This band is indeed broader on the low-wavenumber side for Rec-Myr, suggesting that its carboxylate groups experience more diversified environments. The 3D representations of recoverin suggest that all aspartic and glutamic acid side chains

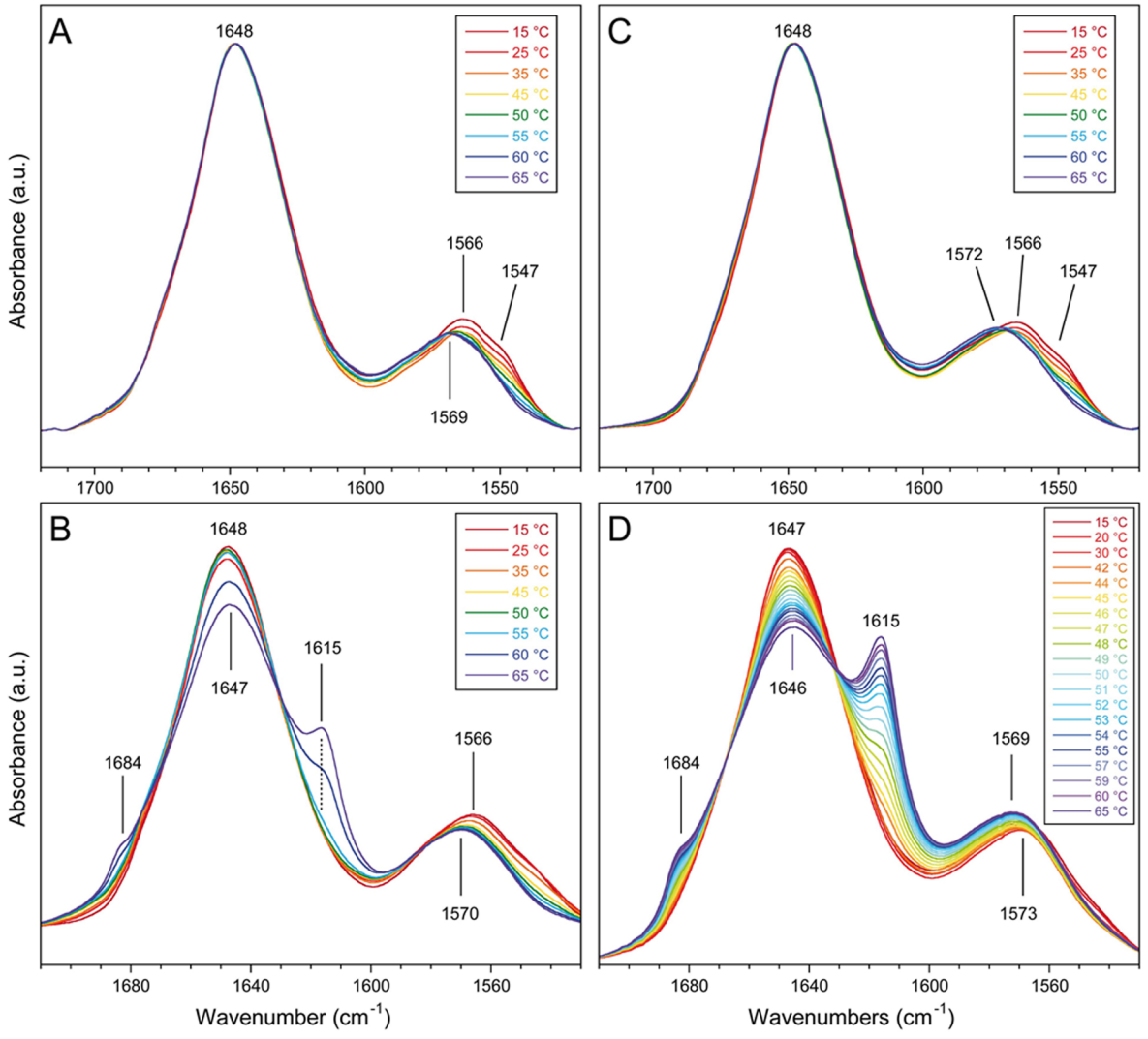

Figure 3. Spectra of (A and B) Rec-Myr and (C and D) Rec-nMyr (1 mM in D_2O buffer) as a function of temperature in the (A and C) presence (10 mM Ca^{2+}) and (B and D) absence of Ca^{2+} (0.5 mM EGTA), in the 1720−1520 cm^{-1} frequency range.

dx.doi.org/10.1021/bi401336g | Biochemistry 2014, 53, 48−56

are water-exposed except those located close to the myristoyl chain, notably those that belong to EF-hand 1.[29] It is probable that the presence of the myristoyl chain located inside the protein prevents these COO^- groups from being exposed to the aqueous environment, leading to a more heterogeneous environment of the carboxylate groups. The structure of Rec-nMyr suggests that its carboxylate groups experience a more homogeneous environment that is consistent with the expectation that all side chains are equally solvent-exposed in the absence of the myristoyl chain.[29]

In the presence of calcium, the $\nu_{as}(COO^-)$ band is virtually identical for both Rec-Myr and Rec-nMyr, indicating that the absence of the myristoyl chain has no effect on the binding of calcium. Calcium binding results in a small shift of the maximum of the band from 1568−1570 to 1566 cm^{-1} and the appearance of a shoulder near 1547 cm^{-1}. These differences are in agreement with the literature[56] and are due to the binding of calcium to the carboxylate groups of glutamic acid and/or aspartic acid. The 1547 cm^{-1} component has been assigned to the coordination of calcium with the COO^- group of glutamic acid in the bidentate state.[56]

Effect of Calcium and Myristoylation on the Thermal Stability of Recoverin. In the presence of calcium, no significant modification of the amide I′ band is observed with temperature, showing a total thermal stability for Rec-Myr (Figure 3A) up to 65 °C. The small shift of the $\nu_{as}(COO^-)$ band from 1569 to 1566 cm^{-1} suggests a weakening of the interactions of the carboxylate groups with both water and calcium with an increase in temperature (Figure 3A).

In the absence of calcium, no strong modification of the amide I′ band occurs until 52−54 °C (Figure 3B). However, from this temperature, one can observe the appearance of two well-defined bands at 1684 and 1615 cm^{-1} with intensities that increase with temperature. Simultaneously, the intensity at ∼1648 cm^{-1} decreases. These spectral changes are due to the formation of ordered intermolecular antiparallel β-sheets[59] at the expense of the native α-helical structural components. This phenomenon is irreversible (data not shown). It is often observed for proteins and is characteristic of the denaturation of proteins accompanied by aggregation.[64−67] These data clearly demonstrate that recoverin is stabilized by calcium, although the binding and removal of this cation do not seem to affect the protein secondary structure at 25 °C (Figure 1). Moreover, it can again be observed that the $\nu_{as}(COO^-)$ band shifts toward higher wavenumbers with temperature, thereby indicating a weakening of the interactions of the carboxylate groups with its environment.

In the case of Rec-nMyr, the spectral evolution observed in the 1720−1520 cm^{-1} frequency range with temperature is very similar to that of Rec-Myr (Figure 3). No significant change in the amide I′ band can be observed in the presence of calcium, showing again that the protein, either myristoylated or not, is highly stabilized when it is coordinated by calcium. Thus, the myristoylation of recoverin appears to have no impact on the thermal stability of recoverin when calcium is present. However, this conclusion is only valid in the temperature range presented here and at high calcium concentrations. Indeed, as will be shown below, the thermal stability of Rec-Myr and Rec-nMyr is different at lower calcium concentrations.

In the absence of calcium, Rec-nMyr behaves the same as Rec-Myr (Figure 3). Indeed, the amide I′ band of Rec-nMyr remains stable until two components appear because of β-aggregation. The position of these bands is the same as those of Rec-Myr, thus suggesting that Rec-nMyr forms the same type of β-sheets as Rec-Myr. However, the β-sheet components arise at lower temperatures for Rec-nMyr, and its extent of aggregation is higher as judged from the intensity of these components at 65 °C (see below). Thus, the myristoyl moiety reduces the extent of denaturation of recoverin in the absence of calcium.

To characterize more quantitatively the thermal stability of myristoylated and nonmyristoylated recoverin, the area of the β-sheet component at 1685 and 1615 cm^{-1}, A(1685 + 1615 cm^{-1}), has been measured as a function of temperature. To this end, a linear baseline has been subtracted under the amide I′ band (1710−1590 cm^{-1} range) and the spectrum of the series preceding aggregation (onset of aggregation) has been subtracted from all subsequent spectra. A typical series of difference spectra is shown in Figure 4 for Rec-nMyr in the presence of 0.5 mM EGTA (the conditions under which recoverin aggregates most). This analytical procedure magnifies the progressive appearance of the bands at 1685 and 1615 cm^{-1} with heating and the concomitant disappearance of the native amide I′ band. Two isosbestic points can be seen near 1675 and 1628 cm^{-1} and are also observed under other conditions (see Figures S1−S4 of the Supporting Information). Such isosbestic points suggest that the aggregation of recoverin is a two-state process, the native structures being progressively converted into intermolecular β-sheets without any intermediate.

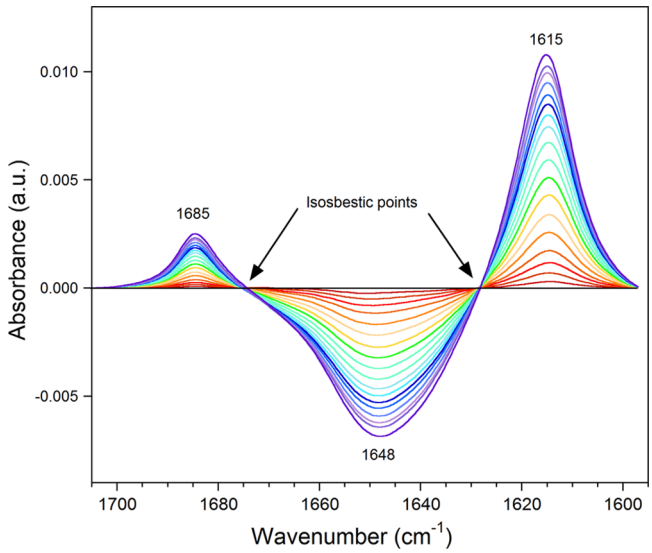

Figure 4. Difference spectra of Rec-nMyr (1 mM in D_2O buffer) as a function of temperature from 42 to 65 °C in the absence of calcium (0.5 mM EGTA), in the 1700−1600 cm^{-1} frequency range. The difference spectra are obtained by subtracting the spectrum of the series preceding aggregation from all subsequent spectra.

The measurement of the area of these bands as a function of temperature allows a semiquantitative evaluation of the extent and kinetics of aggregation under different conditions. Moreover, to better understand the effect of calcium on the structure of recoverin, its thermal stability has been investigated as a function of calcium concentration (1−10 mM Ca^{2+}). The data are shown in Figure 5 (spectra are shown in Figures S1−S4 of the Supporting Information).

The data are not shown for Rec-Myr and Rec-nMyr in the presence of 10 mM calcium and for Rec-Myr in the presence of 2 mM calcium because no aggregation occurs in these cases, so

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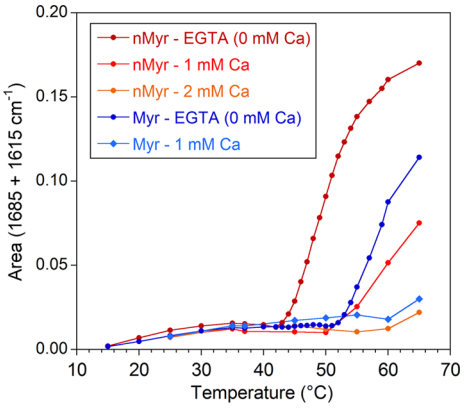

Figure 5. Area at 1685 and 1615 cm^{-1} [$A(1685 + 1615\ cm^{-1})$], corresponding to the formation of β-sheets, of Rec-Myr and Rec-nMyr (1 mM in D_2O buffer) as a function of temperature and calcium concentration. The uncertainty in $A(1685 + 1615\ cm^{-1})$ is ±0.02.

that $A(1685 + 1615\ cm^{-1})$ is zero at all temperatures, thus showing that above a certain calcium concentration threshold, the protein is thermally stable (up to 65 °C). The data shown in Figure 5 confirm that, in the absence of calcium (0.5 mM EGTA), Rec-Myr and Rec-nMyr self-aggregate and that Rec-nMyr is more prone to aggregation than Rec-Myr. In fact, Rec-nMyr aggregates at a temperature (42−44 °C) lower than that of Rec-Myr (52−54 °C), and the level of aggregation at 65 °C is higher for Rec-nMyr. It can also be seen that the extent of aggregation decreases with an increase in calcium concentration for both Rec-Myr and Rec-nMyr. In any case, the amount of intermolecular β-sheets is larger for Rec-nMyr than for Rec-Myr for a given calcium concentration.

Rec-Myr is relatively stable at 1 mM calcium (1:1 recoverin:Ca^{2+} molar ratio), as a slight formation of intermolecular β-sheets appears only at 65 °C. As mentioned above, no aggregation occurs at 2 mM calcium (1:2 recoverin:Ca^{2+} molar ratio that corresponds to the ratio allowing binding of both functional EF-hands of recoverin by calcium). In contrast, Rec-nMyr still exhibits bands at 1685 and 1615 cm^{-1} at this calcium concentration, although with a weak intensity. The results are detailed in Figure 6, where the evolution of the β-sheets bands at 65 °C is plotted as a function of calcium concentration. This figure highlights the fact that

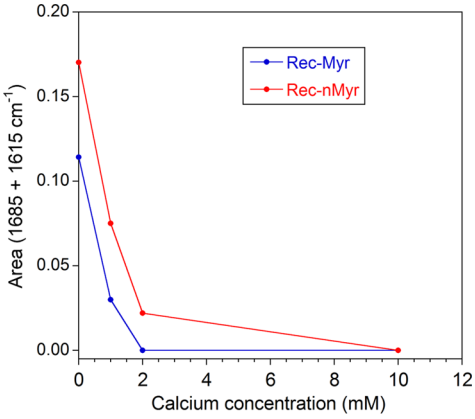

Figure 6. $A(1685 + 1615\ cm^{-1})$ of Rec-Myr and Rec-nMyr (1 mM in D_2O buffer) at 65 °C as a function of calcium concentration. The uncertainty in $A(1685 + 1615\ cm^{-1})$ is ±0.02.

Rec-Myr is more stable than Rec-nMyr and the fact that the β-sheet content of these proteins at 65 °C decreases as the calcium concentration increases.

DISCUSSION

Infrared spectroscopy was used in this study to investigate the effect of calcium concentration and myristoylation on the thermal stability of recoverin. Both the nonmyristoylated and myristoylated forms of recoverin were studied at calcium concentrations of 1, 2, and 10 mM, while the recoverin concentration was kept at 1 mM.

These data show that in excess calcium, Rec-Myr and Rec-nMyr are totally stable up to 65 °C, suggesting that there is no or only a weak influence on the thermal stability of the protein regardless of whether the myristoyl moiety protrudes from the protein hydrophobic pocket or is absent. Indeed, a small shift in the midtransition temperature of 0.6 °C has recently been observed by CD spectroscopy upon myristoylation.[33] Below a certain calcium concentration threshold, the protein denatures and undergoes β-aggregation. This type of aggregation is different from that proposed by Kataoka et al.,[31] which has to be associated with oligomerization. This calcium concentration threshold is 2 mM for Rec-Myr. Because the protein concentration is 1 mM in these experiments, this means that when two calcium cations or more are available for the myristoylated protein, it is thermally stable (under these heating conditions).

It is well-established that two calcium cations can bind recoverin at EF-hands 2 and 3[8,29,68] in a successive manner.[23] This coordinated configuration seems then to correspond to the most stable structure. The stabilization effect of calcium was also demonstrated by the study of the effect of amino acid substitutions in EF-hand sites of recoverin on its calcium binding properties.[23] On the other hand, the increase in the thermal stability of recoverin in the presence of calcium was reported in microcalorimetric,[23] fluorescence,[32] and CD[33] studies. In these studies, the calcium-induced increase in stability ranges between 5−6 and 15 °C. These IR data show an ∼10 °C stability increase in an excess of calcium (1:10 recoverin:Ca^{2+} molar ratio). The temperature difference may be due to the fact that these techniques do not probe the same parameters and physical phenomena. Calorimetry provides thermodynamic parameters, while spectroscopy is sensitive to changes at the molecular level. In addition, CD and even more fluorescence spectroscopy are sensitive to tertiary structural unfolding, while the IR amide I′ band is affected by changes in secondary structure resulting from aggregation. It is interesting to note that the effect of calcium on protein stability has also been observed for other proteins of the NSC family, namely with guanylate cyclase activating protein-2 (GCAP-2)[39] and guanylyl cyclase activating protein 1 (GCAP-1).[40,43] Other EF-hand-containing proteins such as S100A2[41] and α-amylase[42] have also demonstrated an increased thermal stability with an increasing calcium concentration.

The situation is slightly different for Rec-nMyr compared to that for Rec-Myr at low calcium concentrations. For Rec-nMyr, a slight aggregation is observed at 2 mM calcium, showing that Rec-nMyr is not as stable as Rec-Myr. Thus, it seems that the structure of Rec-nMyr is less stable than Rec-Myr when coordinated. In the latter case, this would suggest that the myristoyl chain plays a significant role in recoverin stability. In both cases, Rec-nMyr appears to be less stable than Rec-Myr at low calcium concentrations and in the absence of calcium.

dx.doi.org/10.1021/bi401336g | Biochemistry 2014, 53, 48−56

These observations thus suggest that the N-terminal myristoyl chain stabilizes recoverin, which is in line with crystallographic data obtained with Rec-nMyr[69] showing that the myristoyl modification significantly stabilizes the conformation of the calcium-free protein. From IR data, this stabilization cannot be explained by differences in the "native" (i.e., before heating) secondary structure, but major tertiary structure changes may result from the absence of the myristoyl chain.[20,27,28] It is also possible that the myristoyl moiety perturbs some structural elements of its surrounding hydrophobic cavity that are important for protein thermal stability.

Our IR data therefore show that the thermal stability of recoverin decreases at lower calcium contents, i.e., upon myristoyl sequestration. The results further suggest that the release of the myristoyl chain and consequent binding to lipid bilayer membranes (i.e., at high calcium concentrations) may lead recoverin to exhibit a structural stability enhanced compared to that of its myristoyl-sequestered form.

CONCLUSION

IR spectroscopy appears to be a useful technique for investigating the stability of recoverin. Overall, the results show that although calcium concentration and myristoylation have a weak effect on the secondary structure of recoverin, they have a significant impact on its thermal stability, thus suggesting changes in the intramolecular interactions and/or tertiary structure. Such structural alterations may be important for the biological function of recoverin. The fact that the structural integrity of recoverin is particularly high when the myristoyl chain is extruded from its hydrophobic pocket may be necessary for the protein to be not too strongly affected by membrane binding, thus to keep a functional structure. This conclusion has to be confirmed by a study of the secondary structure of recoverin upon interaction with model membranes.

ASSOCIATED CONTENT

Supporting Information

Difference spectra at various calcium:protein ratios (Figures S1−S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by the Natural Science and Engineering Research Council (NSERC) of Canada. The Regroupement québécois de recherche sur la fonction, la structure et l'ingénierie des protéines (PROTEO), the Centre de recherche sur les matériaux avancés (CERMA), the Centre québécois sur les matériaux fonctionnels (CQMF), and the Centre de recherche du CHU de Québec are thanked for the infrastructure they provide for the performance of the research. PROTEO and CQMF are supported by the Fonds de recherche du Québec-Nature et Technologies (FRQ-NT). K.P.-F. is the recipient of a scholarship from the FRQ-NT and NSERC, whereas G.V.-P. held a Frederick Banting and Charles Best Graduate Scholarship from the Canadian Institutes of Health Research (CIHR) as well as from the Réseau de recherche en santé de la vision.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Jean-François Rioux-Dubé for technical assistance and Philippe Calvez for helpful discussions.

ABBREVIATIONS

CaBP, calcium-binding proteins; CD, circular dichroism; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetra-acetic acid; FTIR, Fourier transform infrared; GCAP-1, guanylyl cyclase activating protein 1; GCAP-2, guanylate cyclase activating protein-2; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; NCS, neuronal calcium sensor; NMR, nuclear magnetic resonance; Rec-nMyr, recoverin nonmyristoylated; Rec-Myr, recoverin myristoylated; SAXS, small-angle X-ray scattering.

REFERENCES

(1) Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol. 1*, 11−21.

(2) Permyakov, E. A., and Kretsinger, R. H. (2011) *Calcium Binding Proteins*, John Wiley & Sons, Inc., Hoboken, NJ.

(3) Kretsinger, R. H. (1976) Calcium-binding proteins. *Annu. Rev. Biochem. 45*, 239−266.

(4) Yanez, M., Gil-Longo, J., and Campos-Toimil, M. (2012) Calcium Binding Proteins. In *Calcium Signaling* (Islam, M. S., Ed.) Springer, Dordrecht, The Netherlands.

(5) Haynes, L. P., McCue, H. V., and Burgoyne, R. D. (2012) Evolution and functional diversity of the calcium binding Proteins (CaBPs). *Front. Mol. Neurosci. 5*, 9.

(6) Lewit-Bentley, A., and Rety, S. (2000) EF-hand calcium-binding proteins. *Curr. Opin. Struct. Biol. 10*, 637−643.

(7) Braunewell, K. H., and Gundelfinger, E. D. (1999) Intracellular neuronal calcium sensor proteins: A family of EF-hand calcium-binding proteins in search of a function. *Cell Tissue Res. 295*, 1−12.

(8) Gifford, J. L., Walsh, M. P., and Vogel, H. J. (2007) Structures and metal-ion-binding properties of the Ca^{2+}-binding helix-loop-helix EF-hand motifs. *Biochem. J. 405*, 199−221.

(9) Grabarek, Z. (2006) Structural basis for diversity of the EF-hand calcium-binding proteins. *J. Mol. Biol. 359*, 509−525.

(10) Bhattacharya, S., Bunick, C. G., and Chazin, W. J. (2004) Target selectivity in EF-hand calcium binding proteins. *Biochim. Biophys. Acta 1742*, 69−79.

(11) Burgoyne, R. D. (2004) The neuronal calcium-sensor proteins. *Biochim. Biophys. Acta 1742*, 59−68.

(12) Burgoyne, R. D., O'Callaghan, D. W., Hasdemir, B., Haynes, L. P., and Tepikin, A. V. (2004) Neuronal Ca^{2+}-sensor proteins: Multitalented regulators of neuronal function. *Trends Neurosci. 27*, 203−209.

(13) Burgoyne, R. D., and Weiss, J. L. (2001) The neuronal calcium sensor family of Ca^{2+}-binding proteins. *Biochem. J. 353*, 1−12.

(14) Ray, S., Zozulya, S., Niemi, G. A., Flaherty, K. M., Brolley, D., Dizhoor, A. M., McKay, D. B., Hurley, J., and Stryer, L. (1992) Cloning, expression, and crystallization of recoverin, a calcium sensor in vision. *Proc. Natl. Acad. Sci. U.S.A. 89*, 5705−5709.

(15) Chen, C. K., Inglese, J., Lefkowitz, R. J., and Hurley, J. B. (1995) Ca^{2+}-dependent interaction of recoverin with rhodopsin kinase. *J. Biol. Chem. 270*, 18060−18066.

(16) Kawamura, S., Cox, J. A., and Nef, P. (1994) Inhibition of rhodopsin phosphorylation by non-myristoylated recombinant recoverin. *Biochem. Biophys. Res. Commun. 203*, 121−127.

(17) Kawamura, S., Hisatomi, O., Kayada, S., Tokunaga, F., and Kuo, C. H. (1993) Recoverin has S-modulin activity in frog rods. *J. Biol. Chem. 268*, 14579−14582.
(18) Korenbrot, J. I. (2012) Speed, sensitivity, and stability of the light response in rod and cone photoreceptors: Facts and models. *Prog. Retinal Eye Res. 31*, 442−466.
(19) Burns, M. E., and Pugh, E. N. (2010) Lessons from Photoreceptors: Turning Off G-Protein Signaling in Living Cells. *Physiology 25*, 72−84.
(20) Flaherty, K. M., Zozulya, S., Stryer, L., and McKay, D. B. (1993) 3-Dimensional structure of recoverin, a calcium sensor in vision. *Cell 75*, 709−716.
(21) Ames, J. B., Porumb, T., Tanaka, T., Ikura, M., and Stryer, L. (1995) Amino-terminal myristoylation induces cooperative calcium-binding to recoverin. *J. Biol. Chem. 270*, 4526−4533.
(22) Senin, I. I., Fischer, T., Komolov, K. E., Zinchenko, D. V., Philippov, P. P., and Koch, K. W. (2002) Ca^{2+}-myristoyl switch in the neuronal calcium sensor recoverin requires different functions of Ca^{2+}-binding sites. *J. Biol. Chem. 277*, 50365−50372.
(23) Permyakov, S. E., Cherskaya, A., Senin, I. I., Zargarov, A. A., Shulga-Morskoy, S. V., Alekseev, A. M., Zinchenko, D. V., Lipkin, V. M., Philippov, P. P., Uversky, V. N., and Permyakov, E. A. (2000) Effects of mutations in the calcium-binding sites of recoverin on its calcium affinity: Evidence for successive filling of the calcium binding sites. *Protein Eng. 13*, 783−790.
(24) Ames, J. B., Tanaka, T., Ikura, M., and Stryer, L. (1995) Nuclear magnetic resonance evidence for Ca^{2+}-induced extrusion of the myristoyl group of recoverin. *J. Biol. Chem. 270*, 30909−30913.
(25) Hughes, R. E., Brzovic, P. S., Klevit, R. E., and Hurley, J. B. (1995) Calcium-dependent solvation of the myristoyl group of recoverin. *Biochemistry 34*, 11410−11416.
(26) Valentine, K. G., Mesleh, M. F., Opella, S. J., Ikura, M., and Ames, J. B. (2003) Structure, topology, and dynamics of myristoylated recoverin bound to phospholipid bilayers. *Biochemistry 42*, 6333−6340.
(27) Tanaka, T., Ames, J. B., Harvey, T. S., Stryer, L., and Ikura, M. (1995) Sequestration of the membrane-targeting myristoyl group of recoverin in the calcium-free state. *Nature 376*, 444−447.
(28) Ames, J. B., Ishima, R., Tanaka, T., Gordon, J. I., Stryer, L., and Ikura, M. (1997) Molecular mechanics of calcium-myristoyl switches. *Nature 389*, 198−202.
(29) Ames, J. B., and Lim, S. (2012) Molecular structure and target recognition of neuronal calcium sensor proteins. *Biochim. Biophys. Acta 1820*, 1205−1213.
(30) Johnson, W. C., Palczewski, K., Gorczyca, W. A., RiazanceLawrence, J. H., Witkowska, D., and Polans, A. S. (1997) Calcium binding to recoverin: Implications for secondary structure and membrane association. *Biochim. Biophys. Acta 1342*, 164−174.
(31) Kataoka, M., Mihara, K., and Tokunaga, F. (1993) Recoverin alters its surface-properties depending on both calcium-binding and N-terminal myristoylation. *J. Biochem. 114*, 535−540.
(32) Uversky, V. N., Permyakov, S. E., Senin, I. I., Cherskaya, A. M., Shulga-Morskoy, S. V., Zinchenko, D. V., Alekseev, A. M., Zargarov, A. A., Lipkin, V. M., Philippov, P. P., and Permyakov, E. A. (2000) Point amino acid substitutions in the Ca^{2+}-binding sites of recoverin. II. The unusual behavior of the protein upon the binding of calcium ions. *Bioorg. Khim. 26*, 173−178.
(33) Marino, V., Astegno, A., Pedroni, M., Piccinelli, F., and Dell'Orco, D. (2014) Nanodevice-induced conformational and functional changes in a prototypical calcium sensor protein. *Nanoscale 6*, 412−423.
(34) Spurway, T. D., Morland, C., Cooper, A., Sumner, I., Hazlewood, G. P., Odonnell, A. G., Pickersgill, R. W., and Gilbert, H. J. (1997) Calcium protects a mesophilic xylanase from proteinase inactivation and thermal unfolding. *J. Biol. Chem. 272*, 17523−17530.
(35) Yamniuk, A. P., Nguyen, L. T., Hoang, T. T., and Vogel, H. J. (2004) Metal ion binding properties and conformational states of calcium- and integrin-binding protein. *Biochemistry 43*, 2558−2568.
(36) Zhou, Y. B., Tzeng, W. P., Yang, W., Zhou, Y. M., Ye, Y. M., Lee, H. W., Frey, T. K., and Yang, J. (2007) Identification of a Ca^{2+}-binding domain in the rubella virus nonstructural protease. *J. Virol. 81*, 7517−7528.
(37) Ye, Y. M., Lee, H. W., Yang, W., Shealy, S. J., Wilkins, A. L., Liu, Z. R., and Yang, J. J. (2001) Metal binding affinity and structural properties of an isolated EF-loop in a scaffold protein. *Protein Eng. 14*, 1001−1013.
(38) Wendt, B., Hofmann, T., Martin, S. R., Bayley, P., Brodin, P., Grunstrom, T., Thulin, E., Linse, S., and Forsen, S. (1988) Effect of amino-acid substitutions and deletions on the thermal-stability, the pH stability and unfolding by urea of bovine calbindin-D_{9K}. *Eur. J. Biochem. 175*, 439−445.
(39) Schroder, T., Lilie, H., and Lange, C. (2011) The myristoylation of guanylate cyclase-activating protein-2 causes an increase in thermodynamic stability in the presence but not in the absence of Ca^{2+}. *Protein Sci. 20*, 1155−1165.
(40) Lim, S., Peshenko, I., Dizhoor, A., and Ames, J. B. (2009) Effects of Ca^{2+}, Mg^{2+}, and myristoylation on guanylyl cyclase activating protein 1 structure and stability. *Biochemistry 48*, 850−862.
(41) Botelho, H. M., Koch, M., Fritz, G., and Gomes, C. M. (2009) Metal ions modulate the folding and stability of the tumor suppressor protein S100A2. *FEBS J. 276*, 1776−1786.
(42) Sadeghi, L., Khajeh, K., Mollania, N., Dabirmanesh, B., and Ranjbar, B. (2013) Extra EF Hand unit (DX) mediated stabilization and calcium independency of α-amylase. *Mol. Biotechnol. 53*, 270−277.
(43) Dell'Orco, D., Behnen, P., Linse, S., and Koch, K. W. (2010) Calcium binding, structural stability and guanylate cyclase activation in GCAP1 variants associated with human cone dystrophy. *Cell. Mol. Life Sci. 67*, 973−984.
(44) Turnay, J., Olmo, N., Gasset, M., Iloro, I., Arrondo, J. L. R., and Lizarbe, M. A. (2002) Calcium-dependent conformational rearrangements and protein stability in chicken annexin A5. *Biophys. J. 83*, 2280−2291.
(45) Heredia, P., and De Las Rivas, J. (2003) Calcium-dependent conformational change and thermal stability of the isolated PsbO protein detected by FTIR spectroscopy. *Biochemistry 42*, 11831−11838.
(46) Chen, C. P., Lee, L., and Chang, L. S. (2006) Effects of metal-binding properties of human Kv channel-interacting proteins on their molecular structure and binding with K_v4.2 channel. *Protein J. 25*, 345−351.
(47) del Valle, L. J., Escribano, C., Perez, J. J., and Garriga, P. (2002) Calcium-induced decrease of the thermal stability and chaperone activity of α-crystallin. *Biochim. Biophys. Acta 1601*, 100−109.
(48) Jackson, M., Haris, P. I., and Chapman, D. (1991) Fourier-transform infrared spectroscopic studies of Ca^{2+}-binding proteins. *Biochemistry 30*, 9681−9686.
(49) Dong, A., Caughey, B., Caughey, W. S., Bhat, K. S., and Coe, J. E. (1992) Secondary structure of the pentraxin female protein in water determined by infrared-spectroscopy: Effects of calcium and phosphorycholine. *Biochemistry 31*, 9364−9370.
(50) Wu, G., Gao, Z., Dong, A., and Yu, S. (2012) Calcium-induced changes in calmodulin structural dynamics and thermodynamics. *Int. J. Biol. Macromol. 50*, 1011−1017.
(51) Saba, R. I., Goormaghtigh, E., Ruysschaert, J. M., and Herchuelz, A. (2001) Conformational changes of the 120-kDa Na^+/Ca^{2+} exchanger protein upon ligand binding: A Fourier transform infrared spectroscopy study. *Biochemistry 40*, 3324−3332.
(52) Nara, M., Morii, H., and Tanokura, M. (2013) Coordination to divalent cations by calcium-binding proteins studied by FTIR spectroscopy. *Biochim. Biophys. Acta 1828*, 2319−2327.
(53) Desmeules, P., Penney, S. E., and Salesse, C. (2006) Single-step purification of myristoylated and nonmyristoylated recoverin and substrate dependence of myristoylation level. *Anal. Biochem. 349*, 25−32.
(54) Xu, X. Z., Ishima, R., and Ames, J. B. (2011) Conformational dynamics of recoverin's Ca^{2+}-myristoyl switch probed by ^{15}N NMR relaxation dispersion and chemical shift analysis. *Proteins 79*, 1910−1922.

(55) Savitzky, A., and Golay, M. J. E. (1964) Smoothing and differentiation of data by simplified least squares procedures. *Anal. Chem. 36*, 1627−1639.
(56) Ozawa, T., Fukuda, M., Nara, M., Nakamura, A., Komine, Y., Kohama, K., and Umezawa, Y. (2000) How can Ca^{2+} selectively activate recoverin in the presence of Mg^{2+}? Surface plasmon resonance and FT-IR spectroscopic studies. *Biochemistry 39*, 14495−14503.
(57) Permyakov, S. E., Cherskaya, A. M., Wasserman, L. A., Khokhlova, T. I., Senin, I. I., Zargarov, A. A., Zinchenko, D. V., Zernii, E. Y., Lipkin, V. M., Philippov, P. P., Uversky, V. N., and Permyakov, E. A. (2003) Recoverin is a zinc-binding protein. *J. Proteome Res. 2*, 51−57.
(58) Hughes, R. E., Brzovic, P. S., Dizhoor, A. M., Klevit, R. E., and Hurley, J. B. (1998) Ca^{2+}-dependent conformational changes in bovine GCAP-2. *Protein Sci. 7*, 2675−2680.
(59) Krimm, S., and Bandekar, J. (1986) Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. *Adv. Protein Chem. 38*, 181−364.
(60) Goormaghtigh, E., Cabiaux, V., and Ruysschaert, J.-M. (1994) Determination of soluble and membrane protein structure by Fourier transform spectroscopy. II. Experimental aspects, side-chain structure and H/D exchange. In *Physico-Chemical Methods in the Study of Biomembranes* (Hilderson, H. J., and Ralston, G. B., Eds.) pp 363−403, Plenum Press, New York.
(61) Venyaminov, S. Y., and Kalnin, N. N. (1990) Quantitative IR spectrophotometry of peptide compounds in water (H_2O) solutions. 1. Spectral parameters of amino-acid residue absorption-bands. *Biopolymers 30*, 1243−1257.
(62) Barth, A. (2000) The infrared absorption of amino acid side chains. *Prog. Biophys. Mol. Biol. 74*, 141−173.
(63) Deacon, G. B., and Phillips, R. J. (1980) Relationships between the carbon-oxygen stretching frequencies of carboxylato complexes and the type of carboxylate coordination. *Coord. Chem. Rev. 33*, 227−250.
(64) Susi, H., Timasheff, S. N., and Stevens, L. (1967) Infrared spectra and protein conformations in aqueous solutions. I. Amide I band in H_2O and D_2O solutions. *J. Biol. Chem. 242*, 5460−5466.
(65) Casal, H. L., Kohler, U., and Mantsch, H. H. (1988) Structural and conformational-changes of β-lactoglobulin-B: An infrared spectroscopic study of the effect of pH and temperature. *Biochim. Biophys. Acta 957*, 11−20.
(66) Fabian, H., Falber, K., Gast, K., Reinstadler, D., Rogov, V. V., Naumann, D., Zamyatkin, D. F., and Filimonov, V. V. (1999) Secondary structure and oligomerization behavior of equilibrium unfolding intermediates of the lambda Cro repressor. *Biochemistry 38*, 5633−5642.
(67) Clark, A. H., Saunderson, D. H. P., and Suggett, A. (1981) Infrared and laser-Raman spectroscopic studies of thermally-induced globular protein gels. *Int. J. Pept. Protein Res. 17*, 353−364.
(68) Ames, J. B., Tanaka, T., Stryer, L., and Ikura, M. (1996) Portrait of a myristoyl switch protein. *Curr. Opin. Struct. Biol. 6*, 432−438.
(69) Weiergraber, O. H., Senin, I. I., Philippov, P. P., Granzin, J., and Koch, K. W. (2003) Impact of N-terminal myristoylation on the Ca^{2+}-dependent conformational transition in recoverin. *J. Biol. Chem. 278*, 22972−22979.