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# Effects of mutations in the calcium-binding sites of recoverin on its calcium affinity: evidence for successive filling of the calcium binding sites

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**A molecule of the photoreceptor $Ca^{2+}$-binding protein recoverin contains four potential EF-hand $Ca^{2+}$-binding sites, of which only two, the second and the third, are capable of binding calcium ions. We have studied the effects of substitutions in the second, third and fourth EF-hand sites of recoverin on its $Ca^{2+}$-binding properties and some other characteristics, using intrinsic fluorescence, circular dichroism spectroscopy and differential scanning microcalorimetry. The interaction of the two operating binding sites of wild-type recoverin with calcium increases the protein's thermal stability, but makes the environment around the tryptophan residues more flexible. The amino acid substitution in the EF-hand 3 (E121Q) totally abolishes the high calcium affinity of recoverin, while the mutation in the EF-hand 2 (E85Q) causes only a moderate decrease in calcium binding. Based on this evidence, we suggest that the binding of calcium ions to recoverin is a sequential process with the EF-hand 3 being filled first. Estimation of $Ca^{2+}$-binding constants according to the sequential binding scheme gave the values $3.7 \times 10^6$ and $3.1 \times 10^5$ $M^{-1}$ for third and second EF-hands, respectively. The substitutions in the EF-hand 2 or 3 (or in both the sites simultaneously) do not disturb significantly either tertiary or secondary structure of the apo-protein. Amino acid substitutions, which have been designed to restore the calcium affinity of the EF-hand 4 (G160D, K161E, K162N, D165G and K166Q), increase the calcium capacity and affinity of recoverin but also perturb the protein structure and decrease the thermostability of its apo-form.**

*Keywords*: metal-binding sites/recoverin/site directed mutagenesis/structure/thermal stability

## Introduction

Recoverin is an *N*-myristoylated calcium-binding protein with molecular mass of 23 kDa, which is involved in visual signal transduction. This protein modulates the $Ca^{2+}$-sensitive deactivation of rhodopsin (Gray-Keller *et al.*, 1993; Kawamura, 1993; Kawamura *et al.*, 1993; Klenchin *et al.*, 1995) via $Ca^{2+}$-dependent inhibition of rhodopsin kinase (Chen *et al.*, 1995) and thus participates in the regulation of the photoresponse duration. The amino terminus of recoverin is heterogeneously fatty acid acylated (mainly myristoylated) (Dizhoor *et al.*, 1992). This modification enhances the recoverin inhibitory efficiency with respect to rhodopsin kinase (Senin *et al.*, 1995). It is also essential for the binding of recoverin to membranes (Zozulya and Stryer, 1992) via the $Ca^{2+}$-dependent solvation of its myristoyl group (Hughes *et al.*, 1995).

The X-ray crystal structure of recoverin shows that the protein molecule is composed of two domains, each containing two EF-hand motifs (Flaherty *et al.*, 1993). Potential $Ca^{2+}$-binding sites are distributed relatively uniformly within the recoverin sequence and include amino acids between 36 and 48 (EF1), 73 and 85 (EF2), 109 and 121 (EF3) and 159 and 170 (EF4) (Flaherty *et al.*, 1993). Of the four potential $Ca^{2+}$-binding sites, only two (the second and the third) EF-hands are capable of binding $Ca^{2+}$, whereas the remaining two sites (the first and the fourth) do not possess this ability (Flaherty *et al.*, 1993; Ames *et al.*, 1995). The EF-hand structural motif consists of two perpendicularly placed α-helices and a connecting loop, that can be represented as a helix–loop–helix structure (Ikura, 1996). In the 'working' EF-hands of recoverin, six amino acid residues are involved in $Ca^{2+}$ binding. Five of them are located in the loop, while glutamate, the sixth residue, is in the second helix of the helix–loop–helix motif (Ames *et al.*, 1995).

Calcium binding by myristoylated recoverin has been shown to be a cooperative process with a Hill coefficient of 1.4–1.75 (Ames *et al.*, 1995; Baldwin and Ames, 1998). The intermediate value of the Hill coefficient suggests that calcium binding by myristoylated protein cannot be regarded as a totally uncooperative or fully cooperative process, thus raising the question of the exact mechanism of this process (Ames *et al.*, 1995).

Using site-directed mutagenesis, four mutants of recoverin with amino acid substitutions in the $Ca^{2+}$-binding sites have been designed (Alekseev *et al.*, 1998). Three of these mutants contained substitutions of glutamate to glutamine at the Z-position of the 'working' EF-hands in order to modify the $Ca^{2+}$-binding properties of these sites. As a result, the myristoylated recoverin mutants –EF2 (E85Q), –EF3(E121Q) and –EF2,3 (E85Q/E121Q) with the modified second, third and (second + third) EF-hands, respectively, were obtained. The fourth mutant, +EF4, had substitutions G160D, K161E, K162N, D165G and K166Q, which gave to the fourth potential $Ca^{2+}$-binding site of the recoverin the canonical EF-hand sequence. The appearance of enhanced $Ca^{2+}$ capacity has been expected for this mutant (Alekseev *et al.*, 1998). In our previous study, the ability of these mutants to bind to photoreceptor membranes and to inhibit the rhodopsin phosphorylation, catalyzed by rhodopsin kinase, was investigated (Alekseev *et al.*, 1998). It was established that

the –EF2, –EF3 and –EF2,3 mutants, with the modified ability to bind $Ca^{2+}$, were practically unable to interact with photoreceptor membranes and did not inhibit the rhodopsin phosphorylation in the micromolar range of $Ca^{2+}$ concentrations. The +EF4 mutantt showed a high affinity to membranes and inhibited rodopsin kinase even more effectively than the wild-type (wt) protein (Alekseev *et al.*, 1998).

Here we present results of detailed studies of the structural properties and $Ca^{2+}$-binding capabilities of these mutants. The recoverin species, the wt protein and its four mutants, were studied by intrinsic fluorescence spectroscopy, circular dichroism spectroscopy and differential scanning microcalorimetry in the absence and presence of $Ca^{2+}$. Several interesting features of the recoverin species were revealed. In particular, the data presented are consistent with the suggestion that the binding of calcium ions to recoverin is a sequential process, with EF3-hand being filled first. Also, recoverin exhibits spectral effects very uncommon for most calcium-binding proteins.

## Materials and methods

Recombinant myristoylated recoverins (wt protein and its mutants with substitutions in the second (E85Q), third (E121Q) and fourth (G160D, K161E, K162N, D165G and K166Q) calcium-binding sites) were produced in *Escherichia coli* as described previously (Alekseev *et al.*, 1998). Protein concentration was estimated spectrophotometrically using a molar extinction coefficient of $\varepsilon_{280\ nm} = 36\,400$ (Klenchin *et al.*, 1995). Apo-forms of recoverin species were prepared according to Blum *et al.* (1977). Residual $Ca^{2+}$ content was checked spectrofluorimetrically (Permyakov *et al.*, 1981) to be $<0.5$ mol per mole of the protein.

Absorption spectra were measured on a Specord UV–VIS spectrophotometer (Karl Zeiss, Jena, Germany) or a spectrophotometer designed and manufactured in the Institute for Biological Instrumentation (Pushchino, Russia).

Circular dichroism (CD) measurements were carried with a JASCO-600 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan), using cuvettes with pathlengths of 0.19 and 5.0 mm for far- and near-UV CD measurements, respectively. The protein concentration was kept at 0.64 mg/ml throughout all the experiments.

Fluorescence measurements were carried out on a laboratory-built spectrofluorimeter described earlier (Permyakov *et al.*, 1977). All spectra were corrected for the spectral sensitivity of the instrument and fitted to log-normal curves (Burstein and Emelyanenko, 1996) using non-linear regression analysis (Marquardt, 1963). The maximum positions of the spectra were obtained from the fits. The temperature inside the cell was monitored with a copper–constantan thermopile.

The apparent binding constants for $Ca^{2+}$ were evaluated from a fit of the fluorescence titration data to the specific binding scheme using non-linear regression analysis (Marquardt, 1963). The binding scheme was chosen on the 'simplest best fit' basis, also taking into consideration fluorescence phase plots (Burstein, 1977). The quality of the fit was judged by the randomness of the distribution of residuals. The accuracy of the determination of the calcium-binding constants was about half order of their values. The temperature dependence of intrinsic fluorescence was analyzed according to Permyakov and Burstein (1984).

Scanning microcalorimetric measurements were carried out on a DASM-4M differential scanning microcalorimeter (Institute for Biological Instrumentation of the Russian Academy of Sciences, Pushchino, Russia) in 0.48 ml cells at a 1 K/min heating rate. An extra pressure of 1.5 atm was maintained in order to prevent possible degassing of the solutions on heating. Protein concentrations were in the range 0.5–0.7 mg/ml. The heat sorption curves were baseline corrected by heating the measurement cells filled with the solvent only. Specific heat capacities of the proteins were calculated as described (Privalov, 1979; Privalov and Potekhin, 1986).

## Results

In order to choose the correct conditions to study the calcium-binding properties of wt recoverin and its mutants, it is important to investigate the effects of parameters such as pH and temperature on the structural properties of the proteins. It is crucial to ensure that there are no pH- or thermally induced transitions in the proteins under the conditions chosen for calcium-binding measurements. Such preliminary investigations can sometimes reveal substantial distortions of the protein structure caused by amino acid substitutions.

### *pH dependence of tryptophan fluorescence*

Our results on the pH dependence of the tryptophan fluorescence of recoverin and its mutants (Permyakov *et al.*, 1999) revealed the pH limits for the native recoverin structure, and also the regions of acidic and alkaline denaturation of the proteins. We found no spectral changes for any of the recoverin species studied in the pH range 7.5–8.5, so all measurements were carried out at pH $\approx$ 8.

### *Thermal denaturation*

It is also important to determine the regions of thermal denaturation for the recoverin species in both the presence and absence of calcium ions. This procedure excludes the possibility of $Ca^{2+}$-binding measurements at the middle of thermal transition and also enables us to compare the stability of the mutant proteins relative to the wild-type.

Figures 1 and 2 show the temperature dependences of the fluorescence maximum position and calorimetric scans for wt recoverin and the four mutants in the absence (1 mM EGTA) and presence (1 mM $CaCl_2$) of calcium. The heat sorption peaks and the temperature-induced red shifts of the fluorescence spectra demonstrate cooperative thermal unfolding of the proteins causing the tryptophans to be more accessible to water.

Interestingly, in contrast to other calcium-binding proteins [such as cod and whiting parvalbumin and human and bovine α-lactalbumin (Permyakov *et al.*, 1977, 1980)], the binding of calcium to the recoverins (wt protein and all the mutants) at temperatures below the thermal transition shifts their tryptophan fluorescence spectra toward longer wavelengths (compare the fluorescence data in Figures 1 and 2).

Figure 1 shows that all apo-forms of the proteins, except the apo-+EF4, have similar melting curves. The differences in the mid-temperatures of their transitions are within ~5°C. The apo-mutants are slightly more stable than the wt recoverin. The least thermostable mutant is +EF4, which is also characterized by the most red-shifted fluorescence spectrum at low temperatures. All this demonstrates that the mutations, except those in site 4, have only insignificant effects on the energetics of the apo-recoverin structure. This means that the mutations in the second and third $Ca^{2+}$-binding sites have not caused deleterious structural changes, which frequently take place if chelating residues are substituted for Ala.

All calcium-loaded forms of recoverin can be divided

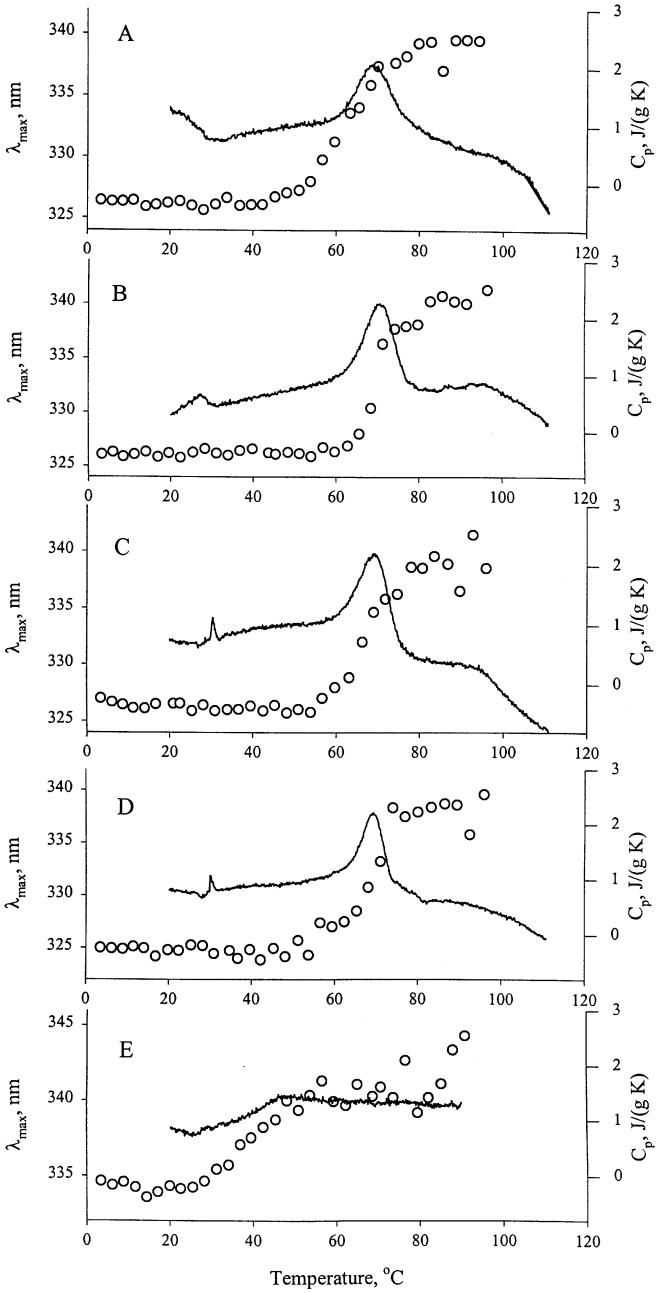


**Fig. 1.** Temperature dependence of fluorescence maximum position (○) and partial heat capacity (solid line) for wt recoverin (**A**) and mutant recoverins –EF2 (**B**), –EF3 (**C**), –EF2,3 (**D**) and +EF4 (**E**) in the presence of 1 mM EGTA. 10 mM HEPES–KOH or 50 mM $H_3BO_3$, pH 8.0. In the fluorescence experiments the excitation was at 280.4 nm.

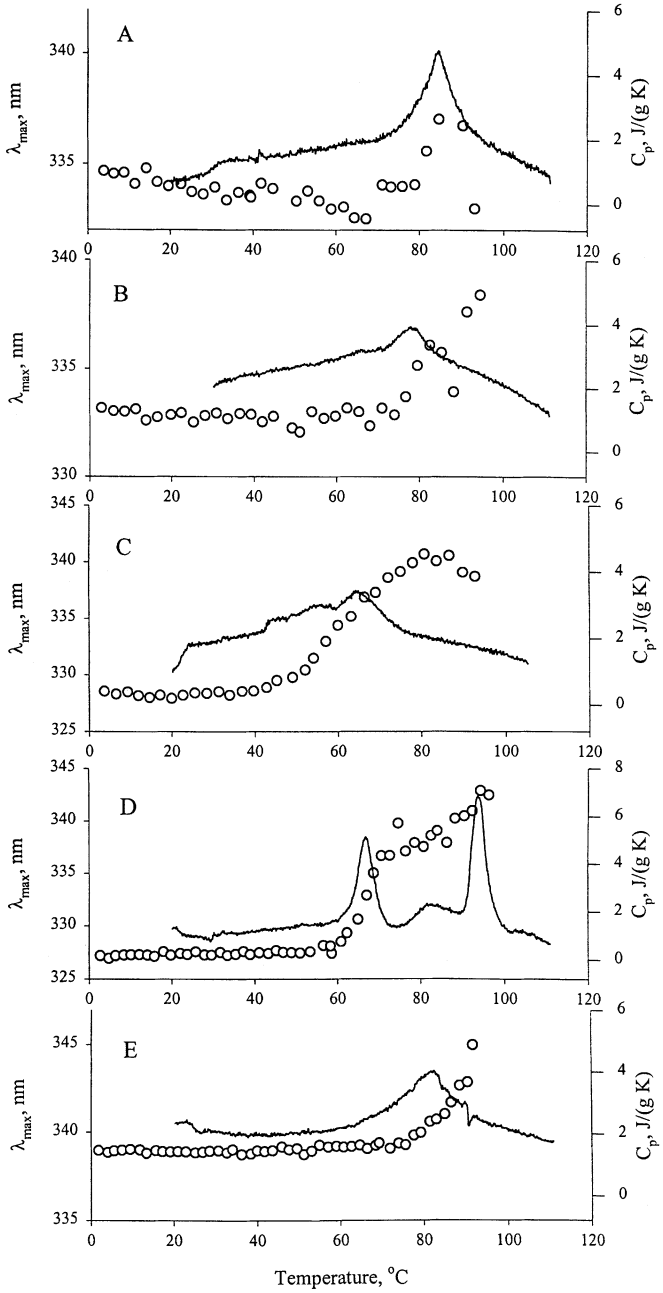


**Fig. 2.** Temperature dependence of fluorescence maximum position (○) and partial heat capacity (solid line) for wt recoverin (**A**) and mutant recoverins –EF2 (**B**), –EF3 (**C**), –EF2,3 (**D**) and +EF4 (**E**) in the presence of 1 mM $CaCl_2$. 10 mM HEPES–KOH or 50 mM $H_3BO_3$, pH 8.0. In the fluorescence experiments the excitation was at 280.4 nm.

into three groups according to their fluorescence maximum positions at low temperatures (Figure 2). +EF4 is characterized by the most red-shifted spectrum. The wt protein together with the –EF2 form the second group and the –EF3 and –EF2,3 have the most blue-shifted spectra. The proteins of the last group are the least thermostable. This thermostability is correct if additional high-temperature peaks for –EF2,3 are not taken into consideration, as these peaks are probably due to the melting of specific associates.

The binding of calcium to +EF4 causes the most pronounced stabilization of the protein (by ~35°C). For wt recoverin the calcium-induced shift of the thermal transition to higher temperatures is not very pronounced (~15°C) in comparison with the shifts for other calcium-binding proteins. The calcium-induced shift of the thermal transition for the mutant –EF2 is ~7°C, which seems to be due to a reduced calcium affinity of this species (see below). However, in this case the calcium binding leads to a decrease in the area under the main heat sorption peak and in the appearance of an additional transition at about 65°C. The addition of calcium to the –EF3 mutant shifts the main thermal transition to lower temperatures and induces an appearance of two small additional heat sorption peaks at 45 and 55°C. The addition of calcium to the mutant –EF2,3 results in a small shift of the main heat sorption peak to lower temperatures and the formation of two additional peaks at 87 and 96°C, which is most unusual. The appearance

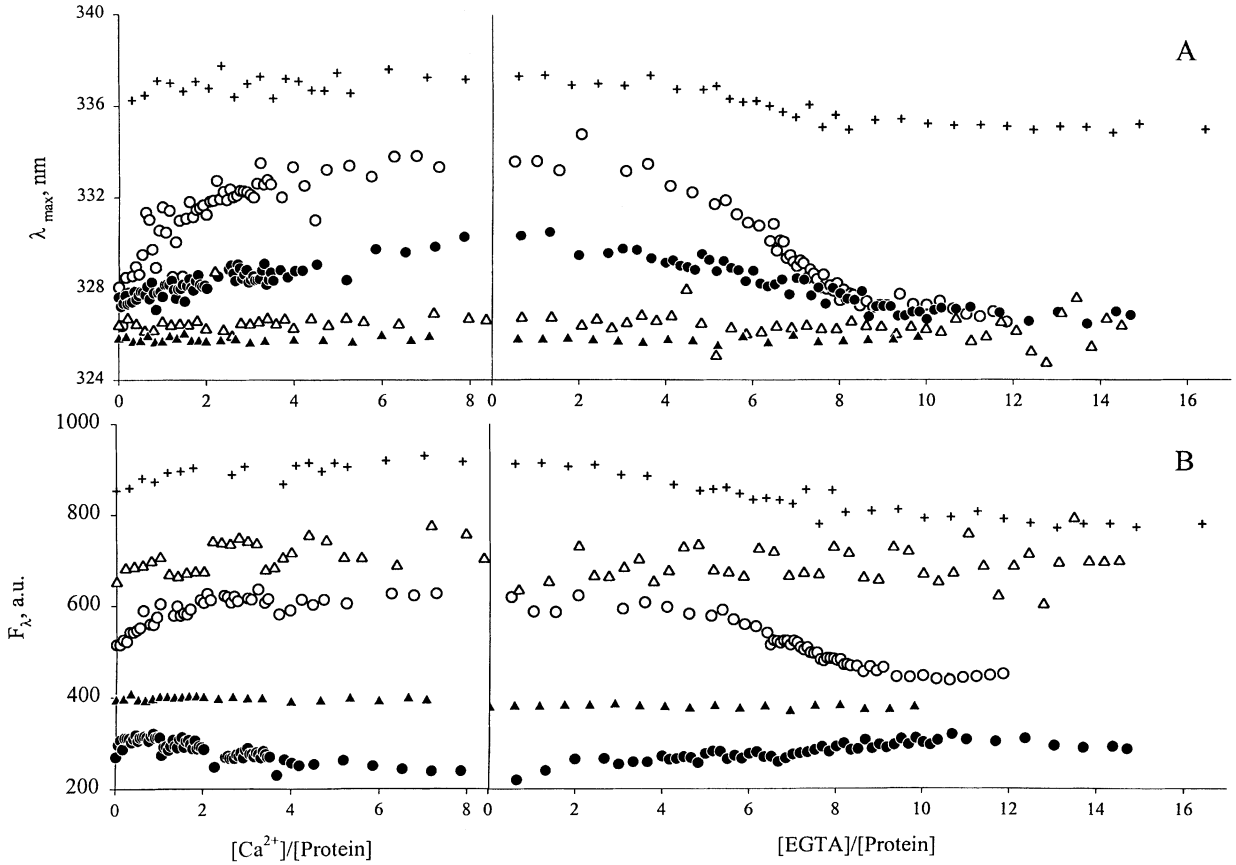


**Fig. 3.** Spectrofluorimetric calcium and EGTA titration of a wt recoverin (○) and the mutants –EF2 (●), –EF3 (△), –EF2,3 (▲) and +EF4 (+). (**A**) Fluorescence maximum position; (**B**) fluorescence intensity at 350–366 nm. 10 mM HEPES–KOH, pH 8.1, 14°C.

of the additional heat sorption peaks in the case of the –EF3 and –EF2,3 mutants seems to be due to a low-affinity calcium binding (see below) or aggregation effects (Kataoka *et al.*, 1993).

The results of these studies demonstrate that at pH 8 all the proteins are stable up to at least 30°C regardless of calcium content, thus allowing us to use room temperature for all titration experiments.

*Calcium binding to recoverin species*

Figure 3 presents the results of the spectrofluorimetric titration of calcium-free wt recoverin and its mutants by calcium and then by the calcium chelator EGTA. The dependence of the fluorescence maximum position of the proteins on the relative calcium and EGTA concentrations is shown in Figure 3A. Figure 3B shows the corresponding dependences of the fluorescence intensities at fixed wavelengths. The measurements were carried out at pH 8.0 and 14°C.

The titration of wt apo-recoverin by calcium induces a 7 nm red shift of the tryptophan fluorescence spectrum and a slight increase in fluorescence intensity (and also in relative fluorescence quantum yield). The curves reach a plateau at a calcium to protein molar ratio of ~2, which corresponds to the binding of two calcium ions per protein molecule. The titration of the calcium-loaded protein by EGTA (or EDTA) reverses the spectral effects. The mutation in the third EF-hand or in both the second and third EF-hands results in the disappearance of the calcium- and EGTA-induced spectral effects in these concentration ranges owing to the absence of the strong calcium binding by these mutants. The mutation in the second EF-hand leads only to a decrease in the magnitude of the spectral effects observed for the wt protein; the calcium-induced red spectral shift in this case is about 3.5 nm.

The mutation in the third EF-hand abolishes the high calcium affinity of recoverin and the mutation in the second EF-hand does not cause any essential drop in calcium affinity. This is consistent with assumption that the binding of calcium ions to recoverin is sequential, with the third EF-hand being filled first:

$$\text{protein} + Ca^{2+} \overset{K_1}{\Longleftrightarrow} \text{protein}\cdot Ca$$

$$\text{protein}\cdot Ca + Ca^{2+} \overset{K_2}{\Longleftrightarrow} Ca\cdot\text{protein}\cdot Ca$$

where $K_1$ and $K_2$ are calcium binding constants for the third and second EF-hands, respectively.

The data on the calcium and EGTA titrations were fitted simultaneously and the fit was achieved by variation of the binding constants $K_1$ and $K_2$ (Permyakov *et al.*, 1999). In the case of the EGTA titration an equilibrium equation for the binding of calcium to EGTA with the well-known binding constant $K_{EGTA}$ (Permyakov *et al.*, 1977) was added:

$$\text{EGTA} + Ca^{2+} \overset{K_{EGTA}}{\Longleftrightarrow} \text{EGTA}\cdot Ca$$

The values of $K_1$ and $K_2$ which give the best fit are presented in Table I, which contains similar data for the mutant proteins. It should be noted that the first binding constant for the –EF2 is determined with poor accuracy because the fluorescence response to the filling of the third EF-hand is very small.

**Table I.** Values of calcium-binding constants for wt and mutant recoverins, obtained from the fit of experimental data by theoretical curves computed according to the two-site binding scheme (10 mM HEPES–KOH, pH 8.1, 14°C)

| Recoverin species | $K_1$ ($M^{-1}$) | $K_2$ ($M^{-1}$) |
|---|---|---|
| Wt | $3.7 \times 10^6$ | $3.1 \times 10^5$ |
| –EF2 | $2.4 \times 10^5$ | $9.9 \times 10^4$ |
| –EF3 | ~$10^3$[a] | – |
| –EF2,3 | ~$10^3$[a] | – |

[a]These are apparent binding constants evaluated from spectrofluorometric titrations of the recoverin species by millimolar calcium concentrations.

The mutations in the fourth EF-hand, constructed to 'repair' its calcium-binding affinity, shift the fluorescence spectrum toward longer wavelengths. Small values of the calcium-induced effects do not permit the study of the binding quantitatively; nevertheless, the data presented in Figure 3 are consistent with the conclusion that this mutant binds calcium with fairly high affinity. Analysis of the calcium-titration curves for +EF4 shows that they reach a plateau at a calcium to protein ratio of ~3. This means that the overall calcium capacity of this mutant is higher than that for the wt protein.

It should be noted that –EF3 and –EF2,3 have no high-affinity calcium-binding sites, yet they can bind calcium with low affinity. Calcium titration of –EF3 and –EF2,3 in the region of millimolar concentrations (not shown) causes small spectral effects allowing the evaluation of the binding constants for the low-affinity binding sites (~$10^3$ $M^{-1}$). The existence of such sites is reflected in the different patterns of the thermal transitions for these mutants in the presence and absence of calcium (see above).

*Calcium-induced changes of CD spectra of wt recoverin and its mutants*

Figure 4 shows the near- (A) and far-UV (B) CD spectra for recoverin and its mutated forms in the absence of calcium (1 mM EGTA). It is known that a native protein with a rigid tertiary structure is characterized by a pronounced near-UV CD spectrum owing to the asymmetric environment of its aromatic amino acid residues. The far-UV CD spectra allow the characterization of the secondary structure of the protein. Figure 4 shows that the CD spectra of wt recoverin, –EF2, –EF3 and –EF2,3 in the absence of calcium are practically coincidental with each other, in both the near- and far-UV regions. This suggests that neither the tertiary nor secondary structure of the recoverin molecule is affected by the amino acid substitutions.

Another situation is observed for the +EF4 mutant. Considerable reduction of the near-UV CD signal and characteristic changes in the far-UV CD spectrum shape and intensity are consistent with the conclusion that the structure of this protein is altered. The same conclusion was drawn from the analysis of the intrinsic fluorescence data.

An unusual feature of recoverin is that the calcium binding induces a pronounced red shift in the tryptophan fluorescence spectrum. Such behavior can be explained by an increase in the exposure of tryptophan residues to the solvent. It is logical to assume that such intramolecular transformations should be accompanied by changes in the protein near-UV CD spectrum. Figure 5A shows the CD spectra for all the species of recoverin in the presence of 1 mM $CaCl_2$. Comparison of these data with the results presented in Figure 4A shows that the

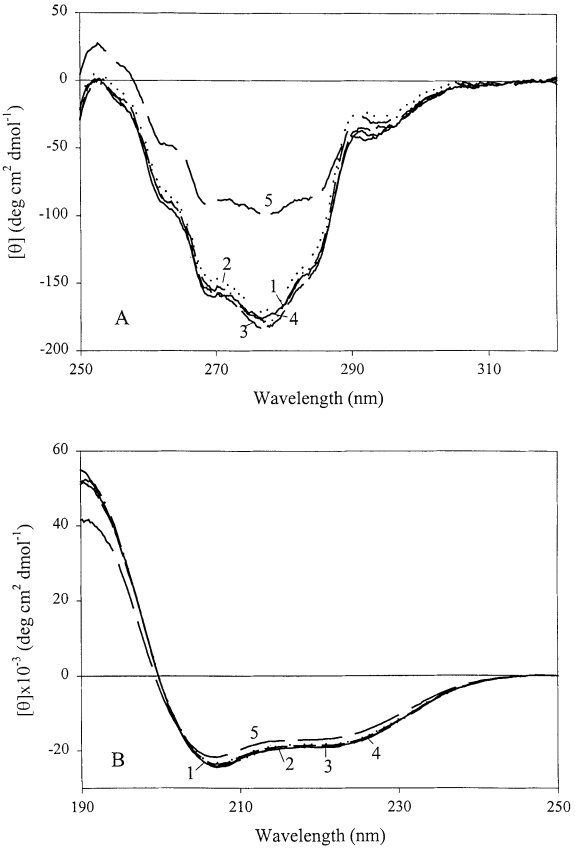


**Fig. 4.** Near- (**A**) and far-UV (**B**) CD spectra measured for various recoverin species in the absence of calcium (1 mM EGTA): wt protein (1), –EF2 (2), –EF3 (3), –EF2,3 (4) and +EF4 (5). 50 mM $H_3BO_3$, pH 8. Protein concentration, 0.64 mg/ml.

interaction with calcium induces significant changes in both shape and intensity of the recoverin near-UV CD spectrum. Interestingly, the largest calcium-induced effect is observed above 270 nm, which is the region where the largest tryptophan contribution is expected. At the same time, Figure 5A indicates that the interaction with calcium does not significantly affect the environment of other aromatic amino acid residues (phenylalanines and tyrosines).

We can also conclude that the larger calcium affinity of the protein induces more pronounced spectral changes. Figure 5A shows that the near-UV CD spectra of –EF3 and –EF2,3 are practically independent of the calcium addition, whereas the interaction of the wt protein with $Ca^{2+}$ is characterized by the largest spectral changes.

The same situation is observed for the far-UV CD spectra of the proteins (Figure 5B). However, in this case the largest spectral changes are observed for +EF4. Analysis of the data presented in Figures 4B and 5B allows one to conclude that for all the proteins the interaction with calcium is accompanied by minor changes in the far-UV CD spectrum intensity. This observation is consistent with a very small increase in α-helical structure in the protein molecule.

## Discussion

Our experimental data on calcium binding to wt recoverin and its mutants suggest that the filling of the two binding sites of recoverin by calcium ions is a sequential process with the third EF-hand being occupied first. Ames *et al.* (1995)

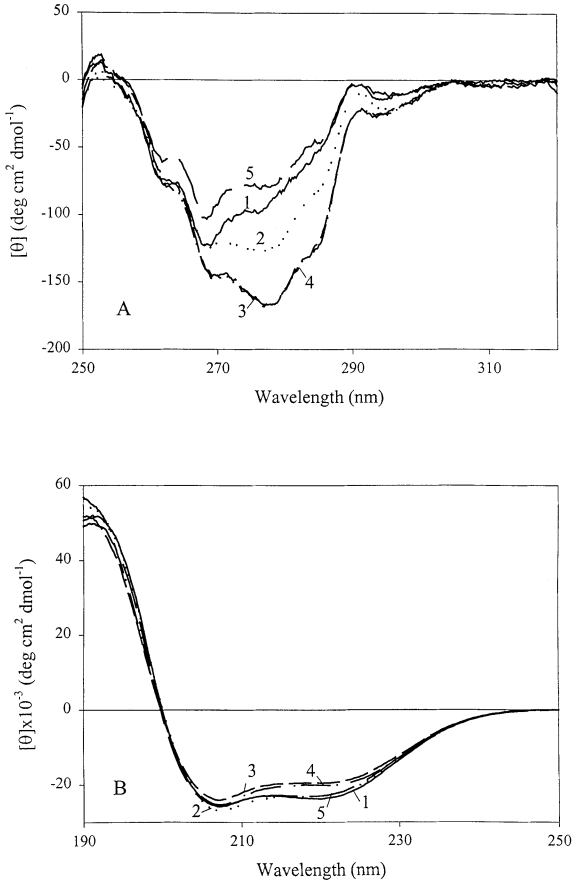


**Fig. 5.** Near- (**A**) and far-UV (**B**) CD spectra measured for various recoverin species in the presence of calcium (1 mM $CaCl_2$): wt protein (1), –EF2 (2), –EF3 (3), –EF2,3 (4) and +EF4 (5). 50 mM $H_3BO_3$, pH 8. Protein concentration, 0.64 mg/ml.

proposed a sequential binding scheme for myristoylated recoverin, where the myristoyl group is or is not exposed to the solvent, respectively. The scheme also takes into consideration the equilibrium between R and T conformational states. According to their data, the binding constants of the two sites of myristoylated recoverin in the R-state are $9.1\times10^6$ and $1.4\times10^5$ $M^{-1}$, which is very close to our results ($3.7\times10^6$ and $3.1\times10^5$ $M^{-1}$ for the wt protein). A similar sequential binding scheme was also proposed for whiting parvalbumin (Permyakov *et al.*, 1980), which belongs to the same family of calcium-binding proteins as recoverin and contains three EF-hand domains, only two of which bind calcium.

It should be noted that Kataoka *et al.* (1993) have shown that in the absence of calcium, myristoylated recoverin is monomeric and globular, whereas addition of calcium ions brings about aggregation. The aggregation effect should perturb experimental estimations of calcium binding constants.

Our data are in line with the results of Matsuda *et al.* (1998) on E85M and E121M mutants of a recoverin subfamily member, S-modulin. According to their data, E121M, which has a mutation in the EF3-hand, neither binds calcium nor inhibits phosphorylation, whereas E85M binds one calcium and has the same membrane affinity as wt S-modulin. However, this mutant has lost the ability to inhibit rhodopsin phosphorylation.

Comparison of NMR structures obtained both for $Ca^{2+}$-loaded and apo-forms of recoverin (Tanaka *et al.*, 1995; Ames *et al.*, 1997) reveals that loop regions of both functional EF-hands of the protein are equally accessible to solvent, thus making them equally attractive for calcium ions. If this is the case, then why is the third EF-hand filled first? Binding of two $Ca^{2+}$ by recoverin results in a substantial change in the relative position of helices for all EF-hands of the protein, except the last (Yap *et al.*, 1999). Hence such structural rearrangements are necessary for both working EF-hands to form geometries of residues capable of $Ca^{2+}$ binding. At the same time, all the helices of the EF-hands 1 and 2 in $Ca^{2+}$-free recoverin are involved in forming a hydrophobic cavity with the myristoyl group (Tanaka *et al.*, 1995). This can potentially prevent them from making any displacements facilitating $Ca^{2+}$ binding. On the other hand, the third EF-hand is located in the interdomain region of the polypeptide chain: the N-terminal helix is involved in the formation of the same hydrophobic pocket (Tanaka *et al.*, 1995), while the C-terminal helix is located in the C-terminal domain. The position of the EF3 hand is likely to promote relative displacement of its helices, assisting $Ca^{2+}$ binding. The binding of the first calcium ion induces some structural changes, which alter the position of the EF2 helices so that the binding of the second $Ca^{2+}$ becomes geometrically favorable. The myristoyl group might be exposed to water upon the binding of the first ion, but our previous study of the ability of –EF2 recoverin mutant to bind to photoreceptor membranes (Alekseev *et al.*, 1998) showed a significant decrease in comparison with wt protein. Nevertheless, this effect may be the case for some members of the recoverin subfamily. For example, an S-modulin mutant with modified EF2-hand binds one $Ca^{2+}$ and has the same membrane affinity as wt S-modulin (Matsuda *et al.*, 1998), indicating the extrusion of the myristoyl group upon loading of the EF3 hand.

The idea that the hydrophobic pocket, comprising the myristoyl group of recoverin, could prevent the EF2-hand helices from relative displacement, facilitating $Ca^{2+}$ loading of the EF2-hand, is supported by data of Baldwin and Ames (1998). Hydrophobic core mutations W31K and I52A/Y53A aimed at destabilization of the N-terminal domain resulted in an increase in the apparent calcium-binding constant and a decreased cooperativity of binding (Hill coefficient in the range 1.0–1.2).

Analysis of the near-UV CD spectra of recoverins shows that the interaction of these proteins with $Ca^{2+}$ is accompanied by a decrease in the asymmetry of the tryptophan residue environment. It is important to note that such a structural transformation is very unusual. Since the rigidity of the environment for other aromatic residues is minimally affected by the calcium binding (Figures 4A and 5A), we can assume that such spectral changes reflect only the increased solvent accessibility of Trp residues. This suggestion is confirmed by the fact that the binding of calcium induces a red shift in the tryptophan fluorescence spectrum. This is also unusual for calcium-binding proteins.

Examination of the tertiary structure of recoverin (Tanaka *et al.*, 1995; Ames *et al.*, 1997) shows that the spectral changes observed can be due to a calcium-induced increase in the degree of solvation of all the tryptophan residues (Trp31, Trp104 and Trp156). This is also in accordance with the finding that the quenching of the tryptophan fluorescence of myristoylated recoverin by acrylamide is more efficient for the calcium-loaded state than the apo-state (Hughes *et al.*, 1995). The more complete exposure to solvent of Trp31 and Trp104 is likely to be a consequence of $Ca^{2+}$-induced disruption

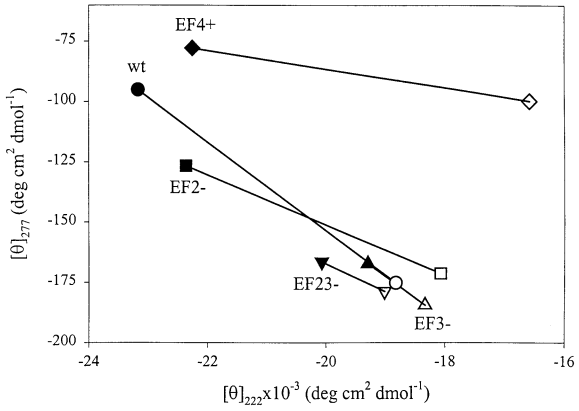


**Fig. 6.** Comparison of the effects of the calcium binding on near- and far-UV CD spectra measured for various recoverin species in the absence (open symbols) and presence (filled symbols) of calcium.

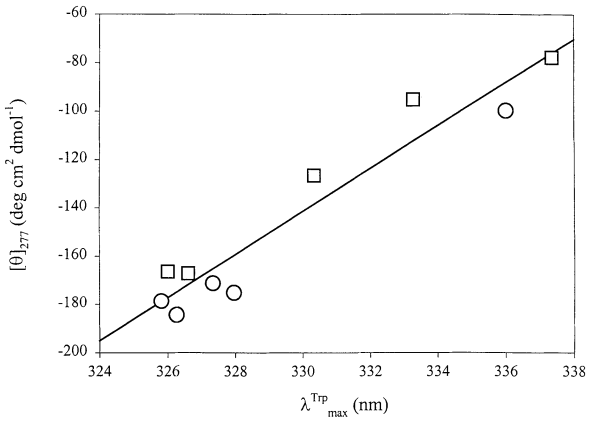


**Fig. 7.** Correlation of near-UV CD spectral changes with the tryptophan fluorescence spectral shifts for various recoverin species. Circles and squares correspond to apo- and calcium-loaded forms of the proteins, respectively.

of the hydrophobic cavity, formed by the myristoyl group along with helices of the first three EF-hands in calcium-free recoverin (Tanaka *et al.*, 1995).

Figure 6 compares the effects of calcium binding on the near-UV CD spectra of different recoverin species with those on the far-UV CD spectra ($[\theta]_{277}$ versus $[\theta]_{222}$ dependences). It is seen that the binding of calcium to any recoverin species is accompanied by an increase in the far-UV CD spectrum intensity and by a decrease in the aromatic CD signal. The observed increase of the far-UV CD signal reflects an increase in the α-helical structure of recoverin upon $Ca^{2+}$ binding. This indicates that the $Ca^{2+}$-loaded protein is more ordered than the apo-form. A $Ca^{2+}$-induced decrease in the total size of the protein (Kataoka *et al.*, 1993) also supports this idea. Thus, in spite of the manifestation of extraordinary spectral effects of $Ca^{2+}$, the overall structural behavior of recoverin is very similar to that of other $Ca^{2+}$-binding proteins. The only peculiarity of the behavior of recoverin is the exposure of hydrophobic residues on calcium binding. This is manifested in the appearance of hydrophobic ANS binding surfaces in both myristoylated and unmyristoylated recoverin (Hughes *et al.*, 1995). The myristoyl group becomes solvent exposed upon $Ca^{2+}$ binding, thus making the N-terminal domain residues, which interact with myristoyl in the apo-form, available for binding to membrane targets.

This increase in the hydrophobic surface of calcium-bound recoverin should result in an increase in enthalpy, associated with hydration of the apolar surface. Nevertheless, thermal denaturation scans exhibit a stabilization of the protein molecule on binding of calcium to recoverin, indicating that such an increase in enthalpy is compensated by the entropy increase associated with the release of the myristoyl group. In fact, the rest of the molecule is even more ordered in the $Ca^{2+}$-bound state and contributes to an entropy change with the opposite sign. Such uncommon energetics of calcium binding arise from the presence of the myristoyl group. This may perhaps explain why calcium binding to wt recoverin and to –EF2 shifts their thermal denaturation transitions by only 15 and 7°C, respectively. These are unexpectedly small shifts in comparison with the calcium-induced shifts for other calcium-binding proteins [>50–60°C for parvalbumin, >30°C for α-lactalbumin (Permyakov, 1993)]. Hence the contribution of calcium binding to the conformational stability of recoverin is less than this contribution in other calcium-binding proteins.

Another important point is the existence of a very good correlation between calcium-induced changes in fluorescence and the near-UV CD spectra of recoverin. Figure 7 shows the $[\theta]_{277}$ versus $\lambda^{Trp}_{max}$ dependence obtained from the analysis of near-UV CD and fluorescence spectra of different species of recoverin in the absence and presence of calcium. One can see that the more red shifted the tryptophan fluorescence spectrum of the protein, the less intense is the CD spectrum in the near-UV region.

It is intriguing that the amino acid substitutions in the EF4-hand of the recoverin resulted in destabilization of the protein molecule (the thermal transition for the apo-protein shifts by almost 20°C); nevertheless, this significant structure perturbation does not preclude high-affinity calcium binding.

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## References

Alekseev,A.M. *et al.* (1998) *FEBS Lett.*, **440**, 116–118.
Ames,J.B., Porumb,T., Tanaka,T., Ikura,M. and Stryer,L. (1995) *J. Biol. Chem.*, **270**, 4526–4533.
Ames,J.B., Ishima,R., Tanaka,T., Gordon,J.I., Stryer,L. and Ikura,M. (1997) *Nature*, **389**, 198–202.
Baldwin,A.N. and Ames,J.B. (1998) *Biochemistry*, **37**, 17408–17419.
Blum,H.E., Lehky,P., Kohler,L., Stein,E.A. and Fisher,E.H. (1977) *J. Biol. Chem.*, **252**, 2834–2838.
Burstein,E.A. (1977) *Intrinsic Protein Fluorescence: Origin and Applications. Biophysics*, Vol. 7. VINITI, Moscow.
Burstein,E.A. and Emelyanenko,V.I. (1996) *Photochem. Photobiol.*, **64**, 316–320.
Chen,C.K., Inglese,J., Lefkowitz,R.J. and Hurley,J.B. (1995) *J. Biol. Chem.*, **270**, 18060–18066.
Dizhoor,A.M., Ericsson,L.H., Johnson,R.S., Kumar,S., Olshevskaya,E., Zozulya,S., Neubert,T.A., Stryer,L., Hurley,J.B. and Walsh,K.A. (1992) *J. Biol. Chem.*, **267**, 16033–16036.
Flaherty,K.M., Zozulya,S., Stryer,L. and McKay,D.B. (1993) *Cell*, **75**, 709–716.
Gray-Keller,M.P., Polans,A.S., Palczewski,K. and Detwiler,P.B. (1993) *Neuron*, **10**, 523–531.
Hughes,R.E., Brzovic,P.S., Klevit,R.E. and Hurley,J.B. (1995) *Biochemistry*, **34**, 11410–11416.
Ikura,M. (1996) *Trends Biochem. Sci.*, **21**, 14–17.
Kataoka,M., Mihara,K. and Tokunaga,F. (1993) *J. Biochem.*, **114**, 535–540.

Kawamura,S. (1993) *Nature*, **362**, 855–857.
Kawamura,S., Hisatomi,O., Kayada,S., Tokunaga,F. and Kuo,C.H. (1993) *J. Biol. Chem*., **268**, 14579–14582.
Klenchin,V.A., Calvert,P.D. and Bownds,M.D. (1995) *J. Biol. Chem*., **270**, 16147–16152.
Marquardt,D.W. (1963) *J. Soc. Ind. Appl. Math*., **11**, 431–441.
Matsuda,S., Hisatomi,O., Ishino,T., Kobayashi,Y. and Tokunaga,F. (1998) *J. Biol. Chem*., **273**, 20223–20227.
Permyakov,E.A. (1993) *Calcium Binding Proteins*. Nauka, Moscow.
Permyakov,E.A. and Burstein,E.A. (1984) *Biophys. Chem*., **19**, 265–271.
Permyakov,E.A., Burstein,E.A., Sawada,Y. and Yamazaki,I. (1977) *Biochim. Biophys. Acta*, **491**, 149–154.
Permyakov,E.A., Yarmolenko,V.V., Emelyanenko,V.I., Burstein,E.A., Gerday,C. and Closset,J. (1980) *Eur. J. Biochem*., **109**, 307–315.
Permyakov,E.A., Yarmolenko,V.V., Kalinichenko,L.P., Morozova,L.A. and Burstein,E.A. (1981) *Biochem. Biophys. Res. Commun*., **100**, 191–197.
Permyakov,S.E. *et al.* (1999) *Bioorg. Chem. (Moscow)*, **25**, 742–746.
Privalov,P.L. (1979) *Adv. Protein Chem*., **33**, 167–241.
Privalov,P.L. and Potekhin,S.A (1986) *Methods Enzymol*., **131**, 4–51.
Senin,I.I., Zargarov,A.A., Aiekseev,A.M., Gorodovikova,E.N., Lipkin,V.M. and Philippov,P.P. (1995) *FEBS Lett*., **376**, 87–90.
Tanaka,T., Ames,J.B., Harvey,T.S., Stryer,L. and Ikura,M. (1995) *Nature*, **376**, 444–447.
Yap,K.L., Ames,J.B., Swindells,M.B., Ikura,M. (1999) *Proteins: Struct. Funct. Genet.*, **37**, 499–507.
Zozulya,S. and Stryer,L. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11569–11573.

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