Structural stabilization of protein 4.1R FERM domain upon binding to apo-calmodulin: novel insights into the biological significance of the calcium-independent binding of calmodulin to protein 4.1R

Wataru NUNOMURA*1, Daisuke SASAKURA†2, Kohei SHIBAÇ, Shigeyoshi NAKAMURAÇ, Shun-ichi KIDOKORO§ and Yuichi TAKAKUWA*

*Department of Biochemistry, Tokyo Women’s Medical University, Kawada 8-1, Shinjuku, Tokyo 162-8666, Japan, †Bruker Optics K.K., Taitou 1-6-4-6F, Taitou, Tokyo 110-0016, Japan, §Symex Corporation, Takatsukadai 4-4-4, Nishiki, Kobe 651-2271, Japan, and §Department of Bioengineering, Nagasaki University of Technology, Kamitomioka 1603-1, Nagasaki, Niigata 940-2188, Japan

In erythrocytes, 4.1R80 (80 kDa isoform of protein 4.1R) binds to the cytoplasmic tail of the transmembrane proteins band 3 and GPC (glycophorin C), and to the membrane-associated protein p55 through the N- (N-terminal), α- (α-helix-rich) and C- (C-terminal) lobes of R30 [N-terminal 30 kDa FERM (4.1/ezrin/radixin/moesin) domain of protein 4.1R] respectively. We have shown previously that R30 binds to CaM (calmodulin) in a Ca2+-independent manner, the equilibrium dissociation constant (Kd) for R30–CaM binding being very similar (in the submicromolar range) in the presence or absence of Ca2+. In the present study, we investigated the consequences of CaM binding on R30’s structural stability using resonant mirror detection and FTIR (Fourier-transform IR) spectroscopy. After a 30 min incubation above 40°C, R30 could no longer bind to band 3 or to GPC. In contrast, R30 binding to p55, which could be detected at a temperature as low as 34°C, was maintained up to 44°C in the presence of apo-CaM. Dynamic light scattering measurements indicated that R30, either alone or complexed with apo-CaM, did not aggregate up to 40°C. FTIR spectroscopy revealed that the dramatic variations in the structure of the β-sheet structure of R30 observed at various temperatures were minimized in the presence of apo-CaM. On the basis of Kd values calculated at various temperatures, ΔC and ΔG = −10 kJ·K−1·mol−1 and −38 kJ·mol−1 at 37°C (310.15 K) respectively. These data support the notion that apo-CaM stabilizes R30 through interaction with its β-strand-rich C-lobe and provide a novel function for CaM, i.e. structural stabilization of 4.1R80.

Key words: apo-calmodulin, 4.1/ezrin/radixin/moesin domain (FERM domain), protein 4.1R, β-sheet structure, structural stability.

INTRODUCTION

Protein 4.1R is a key membrane skeletal protein in human erythrocytes where it is expressed as an 80 kDa isoform (4.1R80). 4.1R80 comprises four major chymotryptic domains: an N-terminal 30 kDa domain also known as a FERM (4.1/ezrin/radixin/moesin) domain, a 16 kDa domain, a 10 kDa domain and a C-terminal 24 kDa domain [1,2]. The N-terminal domain, which consists of 279 amino acid residues, is the focus of the present study. We refer to it as R30 in the present paper. R30 binds to various transmembrane proteins including band 3 [3], GPC (glycophorin C) [4], CD44 [5] and to the erythrocyte membrane-associated protein p55 [6,7]. The 10 kDa domain of 4.1R80 binds to spectrin and actin filaments [1,2]. Through these multiple interactions, 4.1R80 is a key component for the maintenance of the mechanical stability of human erythrocytes.

CaM (calmodulin), a regulator of cellular signalling, binds to and activates more than 100 known target proteins [8,9]. In human erythrocytes, saturation of CaM with Ca2+ (Ca2+-CaM) destabilizes the mechanical stability of membranes [2]. Although CaM binds to R30 in a Ca2+-independent manner, Ca2+-CaM regulates R30 binding to membrane proteins [2,5,7,10,11] and to the spectrin–actin complex [12,13]. Ca2+-CaM binding to 4.1R80 results in a destabilization of membrane stability. Although the stoichiometry of R30 binding to Ca2+-CaM has been shown to be 1:1 [11], two CaM-binding sites have been identified in R30. The A264KKLWVKCVEHHTFFRL peptide, located in the exon 11-encoded region of R30 (pep11), mediates Ca2+-independent CaM binding. The A151KKLSMYGVDLHKAADLK peptide, located in the exon 9-encoded region of R30 (pep9), is responsible for Ca2+-sensitive CaM binding, with Ser185 being critical for Ca2+-dependency [11]. We have shown previously that the binding affinity of R30 for band 3 and GPC decreases when Ca2+-CaM binds simultaneously to pep11 and to Ser185 [5,7,11], with Ca2+-CaM losing its regulatory effect when Ser185 is mutated to tryptophan or proline [11,14]. In most cases, CaM binding to target proteins strongly depends on Ca2+ saturation of CaM[8,9]. In that respect, the characteristics of CaM binding to R30 are unique. These unique properties raised the question as to why R30 binds to apo-CaM with the same Kd as Ca2+-CaM.

X-ray crystal structure reveals that R30 adopts the shape of a three-lobed clover [15], as depicted in Figure 1 (PDB code 1GG3). The cytoplasmic domains of band 3 and of GPC, and the HOOK domain of p55 bind to the N- (N-terminal),...
α- (α-helix-rich) and C- (C-terminal) lobes respectively [15]. The three-dimensional structure shows that each domain possesses a distinct secondary structure. The C-lobe contains seven β-strands that form three sets of β-sheet structures (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/440/bj4400367add.htm). The CaM-binding pep11 sequence, which adopts an α-helix structure, is located in the C-lobe and the Ca²⁺-sensitive Ser₁₈⁵ is located on a loop structure between the α- and C-lobes [15].

The dynamic binding of R3₀ to multiple proteins suggests that the native structure of R3₀ may be structurally unstable, its free energy being high compared with that of the CaM-bound state. [Ca²⁺]ᵢ (intracellular Ca²⁺ concentration) is maintained at ∼10 nM. In contrast, the equilibrium dissociation constant (Kᵣ) of CaM binding to Ca²⁺ is in the submicromolar range [13]. Since saturation of one molecule of CaM requires four molecules of Ca²⁺, one can predict that nearly all CaM molecules in a cell are in a Ca²⁺-free state, i.e. in an ‘apo-’ state.

Our goal is to explain our previous observation that the kinetic parameters for binding of apo-CaM to R3₀ are the same as those for binding of Ca²⁺–CaM to R3₀ [11] and to determine whether apo-CaM binding confers on R3₀ the structural stability on the basis of RMD (resonant mirror detection) and FTIR (Fourier-transform IR) spectroscopy analyses. The results of the present study clearly indicate that apo-CaM stabilizes the β-sheet-rich C-lobe of R3₀ by binding to the pep₁₁ sequence and unveil a novel function for apo-CaM in stabilizing the structure of proteins with which it interacts, such as R3₀.

EXPERIMENTAL

Materials

pGEX-4T2 bacterial expression vector, glutathione–Sepharose CL-6B, heparin–Sepharose, phenyl-Sepharose 4B, Sephacryl S-200 and Akta Prime Plus® were purchased from GE Healthcare. All other reagents were purchased from Wako Pure Chemicals and Sigma, unless noted otherwise. IAsys® cuvettes coated with aminosilane were obtained from Affinity Sensors.

Figure 1 Three-dimensional structure of R3₀

In the three-dimensional structure of R3₀ (PDB code 1GG3), the Ca²⁺-insensitive CaM-binding sequence (pep₁₁), and the Ca²⁺-sensitive site (Ser¹₈⁵ appearing as sphere) are labelled. The side chains in pep₁₁ are presented as a stick model. The cytoplasmic domains of band 3 and GPC, and p₅₅ bind to the N-, α- and C-lobe respectively (reviewed in [2]).

Figure 2 Purification of R3₀ and CaM

(A) Elution profile of R3₀ loaded on to a Sephacryl S-200™ size-exclusion chromatography column. Arrows indicate elution position of marker proteins: a, Blue Dextran (2000 kDa); b, BSA (66 kDa); c, ovalbumin (43 kDa); d, chymotrypsinogen (25 kDa). (B) SDS/PAGE assessment of R3₀ purity on a 15 % gel. Proteins were stained with Gelcode Blue®. Molecular masses are indicated in kDa.

Synthesis and purification of recombinant proteins

Recombinant R3₀ was expressed as a GST (glutathione transferase)-fusion protein in BL21 bacteria. Following sonication, the bacterial lysate was loaded on to a glutathione-affinity column and further purified on a heparin-Sepharose column to remove contaminants and breakdown products. Finally, R3₀ was loaded on to a Sephacryl S-200 size-exclusion chromatography column equilibrated with 50 mM Tris/HCl (pH7.5) containing 0.5 M NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM benzamidine, 0.1 % glycerol and 2 mM NaF (Figures 2A and 2B). Preparation of the cytoplasmic domains of band 3 and GPC, and p₅₅ was conducted as described previously [5, 7, 11]. Protein purity was assessed by SDS/PAGE (12.5 % gel). Proteins were stained with Gelcode Blue®.

Purification of CaM

CaM was purified from bovine brain by phenyl-Sepharose affinity chromatography with slight modifications, as described.
previously [11]. The purity of CaM was assessed by TOF (time-of-flight)-MS (Figure 2C) and SDS/PAGE (15% gel shown in Figure 2D). For SDS/PAGE analysis, 5 μg of CaM in 50 mM Tris/HCl (pH 7.5) containing 0.15 M NaCl and 1 mM EDTA was loaded on to the gel. The CaM concentration was calculated based on the absorbance at 280 nm and an E\textsuperscript{1%/1} of 1.6 for CaM.

**RMD binding assays**

Kinetic analysis

Interactions of R30 with apo-CaM were examined using the IAsys® RMD system following the manufacturer’s instructions (Affinity Sensors) [17]. The protein immobilized on the cuvette is referred to as the ‘ligand’, whereas the protein added to the cuvette in solution is referred to as the ‘analyte’. CaM was immobilized on aminosilane cuvettes as described previously [11]. Binding assays were conducted at temperatures ranging from 9 to 39 °C with constant stirring. R30 was dissolved in 50 mM Tris/HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA and 1 mM 2-mercaptoethanol (buffer A1) and with 4 mM (final concentration) CaCl2 (buffer A2) and used at concentrations ranging from 50 nM to 1 μM. Kinetic analysis of analyte binding to ligand was conducted using equations reported previously [5,11]. Dissociation constants at equilibrium (termed K\textsubscript{d}) were calculated using eqn (1):

$$K_d = k_d / k_a \quad (1)$$

where k\textsubscript{i} is the association rate constant, and k\textsubscript{d} is the dissociation rate constant. K\textsubscript{d} was obtained from the means of three to five measurements for k\textsubscript{i} and k\textsubscript{d}. K\textsubscript{d} was confirmed by Scatchard plotting using maximum binding ($B_{max}$) and molar concentrations of analyte [11,18]. The $B_{max}$ was calculated from binding characteristics using the software package FASTfit®, version 2.1.

R30 was pre-incubated in buffer A1 at various temperatures (5–50 °C) for 30 min before binding assays with immobilized cytoplasmic domains of band 3 and GPC, or p55. Binding assays using IAsys® were carried out at 25 °C. The cuvettes were reused after cleaning with 20 mM HCl. Original binding curves could be replicated after HCl washing, indicating that the washing did not denature the bound ligands. R30 (0.4 μM) in buffer A1 or buffer A2 was incubated for 30 min at temperatures ranging from 5 °C to 50 °C with or without CaM (4.4 μM) before binding assays with immobilized p55. The maximum response expressed as $B_{eq}$ (represented by ‘arc second’) was estimated from the binding profile using the software package FASTfit®, version 2.1. The $B_{eq}$ for R30 binding to each binding partner at 5 °C was 100% and the binding ratio at each temperature was calculated. Temperatures resulting in 50% binding corresponded to the $B_{eq}$ value.

**Thermodynamic analysis**

Change in standard Gibbs free energy ($\Delta G^0$) as a result of binding was determined using eqn (2):

$$\Delta G^0 = RT \cdot \ln K_d = \Delta H - T \Delta S^0 \quad (2)$$

where R is the gas constant, 8.314 J · K\textsuperscript{-1} · mol\textsuperscript{-1}, T is the absolute temperature, $K_d$ is the average value for the dissociation constant of two to five measurements from IAsys®, as indicated above, $\Delta H$ is the change in enthalpy, and $\Delta S^0$ is the change in standard entropy. Change in heat capacity ($\Delta C_p$) was determined by fitting to eqn (3), which reflects the correlation between temperature and $\Delta G$ [19]. Curve fitting was performed using the SALS software package [20].

$$\Delta G^0(T) = -\Delta C_p \cdot T \ln \left( \frac{T}{T_0} \right) + \left[ \Delta H(T_0) - T_0 \Delta C_p \right] \left( 1 - \frac{T}{T_0} \right) + \Delta G^0(T_0) \frac{T}{T_0} \quad (3)$$

$\Delta H$ is shown as a function of temperature in eqn (4), where $T_0$ is 300.15 K:

$$\Delta H(T) = \Delta C_p (T - T_0) + \Delta H(T_0) \quad (4)$$

In order to determine the thermodynamic parameters of the transition state of R30 upon binding to CaM, the Eyring equation (5) was used [21]:

$$\Delta G^a = -RT \cdot \ln(k_h / k_0T) \quad (5)$$

where $R$ is the gas constant, 8.314 J · K\textsuperscript{-1} · mol\textsuperscript{-1}, $k_a$ is the association rate constant from eqn (1), $h$ is the Planck constant, $6.63 \times 10^{-34}$ J · s, and $k_0$ is the Boltzmann constant, $1.38 \times 10^{-23}$ J · K\textsuperscript{-1}. $\Delta H^a$ and $-T \Delta S^a$ were calculated as described above, using plots of $\Delta G^a$ with temperature.

**FTIR spectroscopy of the ATR (attenuated total reflection) spectrum**

IR spectra of solutions of proteins R30, CaM or a 1:1 (molar ratio) mixture of R30 and CaM dissolved in 50 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA and 1 mM 2-mercaptoethanol (buffer B) were recorded with a Tensor27 spectrometer (Bruker Optik). Protein samples were prepared in a BioATR celli II (Harrick Scientific Products), connected to a thermostat (DC30-K20, Thermo Scientific Haake Products). The BioATR sample cell was used to analyse protein samples in solution. For each spectrum, a 64 scan interferogram was obtained at a single beam mode at 4 cm\textsuperscript{-1} resolution. Reference spectra for buffer B alone in the cell were recorded under similar conditions. Recorded and evaluated IR spectra were analysed with the Opus 6.5 software (Bruker Optik). The temperature interval was 2 °C and the temperature range was 20–54 °C. Second-derivative amide I spectra were determined using nine smoothing points according to the Savitzky–Golay algorithms [22].

**DLS (dynamic light scattering) analysis**

The apparent molecular mass of CaM was calculated from the molecular diameter determined by DLS [23] using a Zetasizer NanoZS (Malvern Instruments). R30, CaM and the complex (1:1 in molar ratio) were dissolved in buffer B. Cell temperature during measurement was strictly controlled by the system. Z-Average diameter was determined by Cumulant analysis (ISO13321). All samples were filtered through a 0.22 μm pore-size membrane following dialysis and degassed before analysis.

**Visualization of R30 and apo-CaM and plotting of B-factor of R30**

Three-dimensional structures of R30 (PDB code 1GG3) could be visualized as a ribbon structure and a surface model respectively using the MolFeat Ver. 4.6 (FiatLux) and PyMOL software packages (http://www.pymol.org). B-factors of R30 were obtained from the RSCB PDB (http://www.rcsb.org/pdb/). Each ribbon was assigned a particular colour in accordance to the temperature factor ramped from cold-blue to hot-red for B-factors ≤20 to ≥80 Å\textsuperscript{2} (1 Å = 0.1 nm) respectively.

© The Authors Journal compilation © 2011 Biochemical Society
RESULTS

Structurally unstable sites in R30

The site-specific structural stability of R30 was assessed by binding analysis at various temperatures using the IAsys® system. Following R30 incubation at each temperature for 30 min, the binding activity was computed as $B_{eq, \text{half}}$ (the 50% binding ratio). The $B_{eq, \text{half}}$ for GPC and band 3 occurred at ~40°C (Figure 3A). Although $B_{eq, \text{half}}$ of R30 binding to p55 occurred at 34°C, R30 was still able to bind to p55 at 44°C in the presence of apo-CaM (Figure 3B). This suggested that the p55-binding site, located in the C-lobe of R30, might be structurally unstable and that it was stabilized in the presence of apo-CaM. We hypothesized that this stabilization would result primarily from the interaction of apo-CaM with the β-sheet in the C-lobe of R30 (Figure 1 and Supplementary Figure S1).

It has been reported previously that the structural stability of a peptide derived from MLCK (myosin light-chain kinase) complex is significantly higher in an apo-CaM-bound state than in a Ca^{2+}-CaM-bound state [23–25]. We therefore investigated the effects of Ca^{2+}-CaM on the binding properties of R30 at various temperatures. Surprisingly, binding of R30 to p55 was very similar at 34, 44 and 50°C in the presence or absence of Ca^{2+}-CaM. As shown in Figure 3(C), R30 binding to p55 was already observed at 34°C and was comparable in the presence or absence of Ca^{2+}-CaM. Although binding of R30 to p55 was still observed at 44°C, R30 could no longer bind to p55 at 50°C either in the presence or absence of Ca^{2+}-CaM. These results indicated that the structural stabilization of R30 mediated by CaM was not altered by Ca^{2+}. Thus R30 appears to adopt a unique behaviour with respect to Ca^{2+}-dependency of regulatory properties mediated by CaM.

Temperature-induced secondary-structural change in R30

The second derivative (d²A/Δν²) in the ATR analysis of R30 in the presence or absence of apo-CaM is shown in Figure 4A (original data are shown in Supplementary Figure S2 at http://www.BiochemJ.org/bj/440/bj4400367add.htm). Amide I bands ranged from 1720 to 1600 cm⁻¹. The downward band centred at 1628 cm⁻¹ indicated that the β-sheet structure had undergone structural changes with temperature. Of particular note, this band was not as pronounced in the presence of apo-CaM (Figure 4B), indicating that the β-strand structure of R30 was more structurally stable in the presence of apo-CaM. In contrast, the α-helix structure of R30, detected at 1652 cm⁻¹, did not change up to 55°C, in either the presence or absence of apo-CaM (Figure 4B). This suggested a specific effect of apo-CaM on the stabilization of the β-sheet structure of R30. We could not detect any significant change in the secondary structure of apo-CaM in the range of temperatures tested (Figure 4A).

In order to investigate further the effect of apo-CaM on R30 stability, we compared the aggregation state of R30 at 35°C, in the presence or absence of apo-CaM, by DLS. DLS enables the estimation of polydispersity or the percentage of width (nm) to diameter (nm) of a protein, an indicator of the heterogeneity of its tertiary structure [23]. Restriction of molecular fluctuation of a protein in response to the binding of specific ligands will result in a decrease in polydispersity. The molecular size of R30 in the presence or absence of apo-CaM was ~5–6 nm and ~8–9 nm respectively. Polydispersity of R30 at 35°C, in the presence or absence of apo-CaM, was 38.6% and 23.9% respectively, indicating stabilization of R30 in the presence of apo-CaM. The increase in β-sheet structure was not due to aggregation (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/440/bj4400367add.htm). Indeed,
FTIR measurements enabled the visualization of the second derivative of change in the Amide I region (1720–1600 cm$^{-1}$). Second derivative of change in the Amide I region of R30 (top panel) and of R30 bound to apo-CaM (middle panel) as a function of temperature is shown (A). The derivative of apo-CaM alone is shown as control (bottom panel). Changes in absorbance for specific regions of R30 (1628 cm$^{-1}$ for $\alpha$-helix and 1652 cm$^{-1}$ for $\beta$-sheet structure) in the presence or absence of apo-CaM as a function of temperature are shown (B). Small and large circles are presented as raw data and mean values respectively. $/H17034$, R30; $/H17033$, R30–apo-CaM complex. The same $d^2A/dx^2$ scale (y-axis) is used for both 1628 cm$^{-1}$ and 1652 cm$^{-1}$.

DLS measurements showed no aggregation at 39°C for R30 and 40°C for R30 in the presence of apo-CaM. The sensitivity of the apparatus used in the present study was not sufficient to detect smaller fluctuations in temperature (<1°C) for R30 with and without apo-CaM.

### Thermodynamic parameters of CaM binding to R30

The IAsys® system can be used at different temperatures, thus enabling thermodynamic analysis of interactions. The $K_d$ for R30 binding to apo-CaM changed considerably with temperature. The minimum $K_d$ was detected at 25°C. The temperature-dependence of $K_d$ was due primarily to that of $k_a$ with $k_d$ showing little change with temperature (Figure 5A). In order to characterize the mechanism for the temperature-dependence of R30 binding to apo-CaM, the thermodynamic parameters of R30 binding to apo-CaM were inferred from these results.

A change in the association rate constant with temperature is an indication of the activation energy in the transition state of R30 upon binding to apo-CaM. This energy was determined from Eyring plots [21]. In the transition state, the change in activation Gibbs free energy ($\Delta G^*$) for R30 bound to apo-CaM was 49 kJ·mol$^{-1}$ at 37°C (310.15 K). The activation energy of binding was derived from the entropic effect, as $-T\Delta S^*$, 153.1 kJ·mol$^{-1}$, whereas activation enthalpy, $\Delta H^*$, was negative, $-104.1$ kJ·mol$^{-1}$ at 37°C (310.15 K). The negative activation heat capacity, $\Delta C_p^*$ ($-8.0$ kJ·K$^{-1}$·mol$^{-1}$) reflected a decrease in hydrophobic hydration and/or conformational change (partial folding) of R30 upon CaM binding.
The $\Delta G$ upon CaM binding at equilibrium was derived from eqn 2. $\Delta C_r$ was obtained by non-linear least-squares fitting of eqn 3 to temperature as a factor of $\Delta G$. Characteristics of R30 binding to apo-CaM at 300.15 K (27°C) were as follows: $\Delta C_r$, $-10$ kJ·K$^{-1}$·mol$^{-1}$; $\Delta G^\circ$, $-39.1$ kJ·mol$^{-1}$; $\Delta H^\circ$, $-21.3$ kJ·mol$^{-1}$; and $-T \Delta S^\circ$, $-17.8$ kJ·mol$^{-1}$ (Figure 5B and Table 1). The magnitude of the negative heat capacity change $\Delta C_r$, $-10$ kJ·K$^{-1}$·mol$^{-1}$, observed in the present study is typical of that observed during protein folding/unfolding [26]. This observation once again strongly supports the notion that the structure of R30 is dramatically changed by apo-CaM binding. Changes in structure could involve a decrease in the hydrophobic hydration and/or a conformational change of R30 upon apo-CaM binding.

Importantly, there were no significant differences for thermodynamic parameters between R30 binding to apo-CaM or Ca$^{2+}$-CaM, and thus in both transient and stable states (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/440/bj4400367add.htm). For example, $\Delta C_r$ values were very similar when R30 was bound to either Ca$^{2+}$-CaM or apo-CaM ($\sim -10$ kJ·K$^{-1}$·mol$^{-1}$).

### DISCUSSION

In the present study, we have found strong evidence for the structure of R30 being stabilized upon apo-CaM binding. Our conclusion is based on the results of a set of sophisticated biophysical and biochemical analyses. Highly significant findings regarding apo-CaM binding to R30 include: (i) stabilization of the $\beta$-sheet-rich C-lobe of R30 by apo-CaM as shown by site-specific binding assays and FTIR analysis; and (ii) folding of R30 upon apo-CaM binding as documented by the thermodynamic parameters derived from the kinetic analysis.

The thermodynamic parameters support the notion that apo-CaM may induce conformational changes in R30. The structural analysis of various complexes consisting of apo-CaM and target peptides has been reported [27–29]. The N- and C-lobes of apo-CaM have been shown to wrap the target peptide as in the case of the Ca$^{2+}$-CaM complex [30]. We hypothesize that, in the case of R30, apo-CaM may interact with the surface of R30 and pull out the pep11 helix usually masked in the non-complexed structure as shown in Figure 6. A flexible loop sequence, L$^{380}$PS, upstream of pep11 [15] could act as a hinge during this process. Thermodynamic parameters, particularly the negative value for $\Delta C_r$ ($\sim -10$ kJ·K$^{-1}$·mol$^{-1}$) have indicated that R30 undergoes conformational changes subsequent to binding to apo-CaM. On the basis of the location of specific residues (shown in bold in the following sequence), the sequence of pep11, AKKLWKVCVEHHTFFRL, has been classified as a 1-5-10 motif [31]. A 1-5-10 motif has been described to act as a Ca$^{2+}$-dependent CaM-binding site in CaMKI (Ca$^{2+}$/CaM-dependent protein kinase I) and in Hsp90 (heat-shock protein 90) [31]. In that respect, R30 pep11 is an exception as it constitutes a Ca$^{2+}$-independent CaM-binding site. Although pep11 is a Ca$^{2+}$-independent CaM-binding sequence, it does not contain the IQ motif [29]. Further investigation, such as X-ray diffraction analysis of crystals of the R30–apo-CaM complex, should enable us to elucidate the unique structural properties of this complex.

DLS is a sensitive method to detect changes in the hydrodynamic diameter of proteins with exposed amino acid hydrophobic side chains upon heat denaturation [23]. Using this technique, we could not detect any difference in the distribution of hydrodynamic diameters for R30 in the presence or absence of apo-CaM when varying the temperature (see Supplementary Figure S3). The aggregation of R30 may involve a portion of the hydrophobic surface that does not mediate the binding of R30 to apo-CaM. Alternatively, the apparent distribution of the hydrophobic surface of R30 may not change upon interaction with apo-CaM. If so, and since FTIR measurements and Iasys® binding assays indicate a potential change in the $\beta$-sheet structure of R30 with temperature, this would indicate that the region of the $\beta$-sheet structure affected by temperature is not situated at the surface of R30. The region of the $\beta$-sheet structure of R30 that is stabilized by apo-CaM still remains to be identified. Mapping of this region will require additional studies of the structure of the R30–apo-CaM complex.

Examination of chain mobility may lead to a better understanding of the mechanism for stabilization. $B$-factors, or ‘temperature factors’, are related to the amplitude of the motion of atoms, with greater $B$-factors indicating more extensive atom disorder [32,33]. Smith et al. [34] have studied the mobility of the ezrin FERM domain and we have characterized that of R30 using the same method. As is evident from Supplementary Figures S5(A) and S5(B)

### Table 1 Thermodynamic parameters of R30 binding to apo-CaM

<table>
<thead>
<tr>
<th>Transition state</th>
<th>Temperature (K)</th>
<th>$\Delta G^\circ$ (kJ·mol$^{-1}$)</th>
<th>$\Delta H^\circ$ (kJ·mol$^{-1}$)</th>
<th>$-T \Delta S^\circ$ (kJ·mol$^{-1}$)</th>
<th>$\Delta C_r$ (kJ·K$^{-1}$·mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>300.15</td>
<td>45.4</td>
<td>-24.1</td>
<td>69.5</td>
<td>-8.0</td>
</tr>
<tr>
<td></td>
<td>310.15</td>
<td>49.0</td>
<td>-104.1</td>
<td>153.1</td>
<td>-8.0</td>
</tr>
</tbody>
</table>

The unstable C-lobe through which pep11 passes diagonally and the stable N- and $\alpha$-lobes are shown. X-ray crystallization of R30 reveals that pep11 is part of a compact $\alpha$-helix in the C-lobe. Apo-CaM pulls this $\alpha$-helix out to form a more structurally stable complex than R30.

The unstable C-lobe through which pep11 passes diagonally and the stable N- and $\alpha$-lobes are shown. X-ray crystallization of R30 reveals that pep11 is part of a compact $\alpha$-helix in the C-lobe. Apo-CaM pulls this $\alpha$-helix out to form a more structurally stable complex than R30.

Figure 6  Proposed model for the structure of the R30–apo-CaM complex

The unstable C-lobe through which pep11 passes diagonally and the stable N- and $\alpha$-lobes are shown. X-ray crystallization of R30 reveals that pep11 is part of a compact $\alpha$-helix in the C-lobe. Apo-CaM pulls this $\alpha$-helix out to form a more structurally stable complex than R30.
Apo-calmodulin stabilizes 4.1R FERM domain

Nakamura and Shin-ichi Kidokoro analysed the thermodynamic parameters and edited the paper before submission. Yuichi Takakuwa contributed to editing of the paper before submission.

ACKNOWLEDGMENTS

We thank Dr Philippe Gascard (Department of Pathology, University of California, San Francisco, San Francisco, CA, U.S.A.) for a critical reading and editing of the paper before submission.

FUNDING

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education Culture, Sport, Science and Technology of Japan [grant number 15570123 (to W.N.)].

REFERENCES

19 Horn, J. R., Russell, D., Lewis, E. A. and Murphy, K. P. (2001) Van’t Hoff and calorimetric enthalpies from isothermal titration calorimetry: are there significant discrepancies? Biochemistry \textbf{40}, 1774–1778


SUPPLEMENTARY ONLINE DATA

Structural stabilization of protein 4.1R FERM domain upon binding to apo-calmodulin: novel insights into the biological significance of the calcium-independent binding of calmodulin to protein 4.1R

Wataru NUNOMURA*1, Daisuke SASAKURA†2, Kohei SHIBA‡, Shigeyoshi NAKAMURA§, Shun-ichi KIDOKORO§ and Yuichi TAKAKUWA*

*Department of Biochemistry, Tokyo Women’s Medical University, Kawada 8-1, Shinjuku, Tokyo 162-8666, Japan, †Bruker Optics K.K., Taitou 1-6-4-6F, Taitou, Tokyo 110-0016, Japan, ‡Sysmex Corporation, Takatsukadai 4-4-4, Nishiku, Kobe 651-2271, Japan, and §Department of Bioengineering, Nagaoka University of Technology, Kamitomioka 1603-1, Nagaoka, Niigata 940-2188, Japan

Figure S1 Secondary structure of R30

The secondary structure of R30 is derived from the three-dimensional structure (PDB code 1GG3). Nomenclature for each secondary structure is as follows: N, A and C refer to the N-, α- and C-lobes respectively; α and β refer to α-helices and β-strands respectively. Numbers following α and β refer to the numbering of each secondary-structure type in a given lobe. The Figure was downloaded from PDBj (http://www.pdbj.org/).

1 To whom correspondence should be addressed at the present address: Center for Geo-Environmental Science, Graduate School of Engineering and Resource Science, Akita University, Tegata-gakuın 1-1, Akita, 010-8501, Japan (email nunomura@gipc.akita-u.ac.jp).
2 Present address: Malvern Instruments Ltd, Kanda-tsukasa 2-6-2F, Chiyoda, Tokyo 101-0048, Japan.
Figure S2 Changes of secondary structure of R30 in the presence or absence of apo-CaM as a function of temperature

FTIR measurements enable to visualize changes in the Amide I region. The raw data of changes in the Amide I region (1720–1600 cm$^{-1}$) for R30 (upper panel) or R30 bound to apo-CaM (lower panel) as a function of temperature are shown.

Figure S3 Changes in the hydrodynamic diameter of R30 in the presence or absence of apo-CaM as a function of temperature

The hydrodynamic diameter of R30 (upper panel) and R30–apo-CaM (lower panel) was measured by DLS. Coloured lines indicate hydrodynamic diameter profiles at each temperature tested. The colour code for each temperature tested is shown on the right-hand side.
Figure S4  Thermodynamic characterization of the interaction between R30 and apo-CaM (●) or Ca$^{2+}$–CaM (∅).

M, T and S refer to mixture, transition state and stable state respectively.
Figure S5  Mobility of FERM domains

(A) Distribution of $B$-factor on three-dimensional structures of R30 (PDB code 1GG3). Ribbon diagram were generated using the PyMOL program (http://www.pymol.org). Ribbon diagram of R30 showing the compact globular cloverleaf-shaped structure with three lobes: N-lobe (residues 1–82), α-lobe (residues 83–180) and C-lobe (residues 181–279). The ribbon is coloured according to temperature factor ramped from cold blue to hot red for $B$-factors from $\leq 20$ to $\geq 80\,\text{Å}^2$ respectively. Images on the right and left show the two sides of R30. (B) $B$-factor plots as a function of residue numbering of R30. (C) Average $B$-factor (shown as $<B\text{-factor}>$) for each lobe for the native structure of R30. Details of $B$-factor variations within each lobe are shown in (B).

Received 18 April 2011/9 August 2011; accepted 17 August 2011
Published as BJ Immediate Publication 17 August 2011, doi:10.1042/BJ20110676