FEBS Letters 577 (2004) 284–288 FEBS 28946

Interaction of calmodulin with the phosphofructokinase target sequence

Stephen R. Martin[a], Rodolfo R. Biekofsky[b], Murray A. Skinner[a], Remo Guerrini[c], Severo Salvadori[c], James Feeney[b], Peter M. Bayley[a,*]

[a]*Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK*
[b]*Division of Molecular Structure, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK*
[c]*Department of Pharmaceutical Sciences, University of Ferrara, Italy*

Received 16 September 2004; accepted 4 October 2004

Available online 21 October 2004

Edited by Christian Griesinger

Abstract **$Ca_4 \cdot$calmodulin ($Ca_4 \cdot$CaM) inhibits the glycolytic enzyme phosphofructokinase, by preventing formation of its active tetramer. Fluorescence titrations show that the affinity of complex formation of $Ca_4 \cdot$CaM with the key 21-residue target peptide increases 1000-fold from pH 9.0 to 4.8, suggesting the involvement of histidine and carboxylic acid residues. ^{1}H NMR pH titration indicates a marked increase in pK_a of the peptide histidine on complex formation and HSQC spectra show related pH-dependent changes in the conformation of the complex. This unusually strong sensitivity of a CaM–target complex to pH suggests a potential functional role for $Ca_4 \cdot$CaM in regulation of the glycolytic pathway.**

Keywords: Calmodulin; Phosphofructokinase; Target sequences; Fluorescence; Histidine; Nuclear magnetic resonance

1. Introduction

Mammalian phosphofructokinase (PFK) plays a key role in the regulation of glycolysis and muscle PFK is a calmodulin (CaM)-binding protein [1]. CaM atypically acts as a Ca^{2+}-dependent inhibitor of the active tetrameric PFK enzyme by stabilizing it in an inactive dimeric form, binding in the central region linking two domains of PFK [3]. The PFK target sequence, $P_{1–21}$, has relatively few charged residues, but includes Glu12 and a conserved His19. We examine here the effects of pH on the target peptide affinity with CaM, the kinetics of dissociation, and the conformation of the complex, in comparison with properties of analogous complexes from other CaM-dependent kinases.

2. Materials and methods

Recombinant *D. melanogaster* wild-type and uniformly labeled ^{15}N CaM were expressed and characterized as described [4,5]. Peptides $P_{1–21}$, $P_{1–21}$(E12Q), P_{9-21}, WFFu, FFFu, and FFFp (University of Bristol), and $P_{1–26}$ (University of Ferrara) were characterized by HPLC and mass spectra. Fluorescence spectra and binding analyses were performed as described [6]. NMR spectroscopy was performed using VARIAN Inova 500 and 600 spectrometers, for histidine titrations [7] and multi-dimensional techniques [8].

3. Results and discussion

3.1. Peptide binding affinity as a function of pH

Initial studies with the P_{9-26} peptide showed that the strength of the interaction with Ca_4CaM is strongly pH-dependent, with K_d varying from 0.8 to 300 nM for pH 5.0–8.5 (Skinner, PhD thesis, University of London, 1996). For quantitation of the high affinity 1:1 complex formed with peptide $P_{1–21}$ at lower pH values, competition titrations were performed, using spectroscopically "silent" peptides (FFFu, $K_d = 750 \pm 120$ pM and FFFp, $K_d = 45 \pm 5$pM [2]), whose affinity was established to be pH-independent in titrations of the Ca_4CaM–WFFu complex as a function of pH (Fig. 1A). Analysis of these curves with the K_d for FFFp = 45 pM gave K_d values for WFFu of 165 ± 45 pM (pH 8.03), 110 ± 30 pM (pH 6.67), and 140 ± 25 pM (pH 5.43). In addition, the rate of dissociation of peptide from the Ca_4CaM–WFFu complex measured following the addition of a large excess of FFFp (Fig. 1B) ($\sim$0.05 s^{-1}) is effectively constant over the pH range 4.8–8.5, consistent with an association rate constant of $\sim 5 \times 10^8$ $M^{-1}\,s^{-1}$, close to the diffusion-controlled limit. Thus, the affinity of WFFu for Ca_4CaM is essentially pH-independent, as is that of the displacing peptides FFFu and FFFp. The corresponding competition titrations of Ca_4CaM–$P_{1–21}$ (Fig. 1C) give K_d values varying by $\sim$1000-fold for pH 9.0–4.8, with consistent results being obtained with FFFu and FFFp in the limited pH range where either peptide may be used. Direct fluorescence titrations gave $K_d = 4.530.29$ nM (pH 8.53) and 1.25 ± 0.32 nM (pH 7.21) (data not shown). $P_{1–21}$ displacement induced by large excess of FFFp (Fig. 1D) shows that the dissociation rate constant is dramatically reduced at low pH, i.e., >1 s^{-1} (pH 8.4), $\sim$0.08 s^{-1} (pH 6.5) and $\sim$0.002 s^{-1} (pH 4.95). Combining the results, the association rate constant remains essentially constant (in the range $3–6 \times 10^8$ $M^{-1}\,s^{-1}$) over the whole pH range. Fig. 2A shows that the affinity of peptide $P_{1–21}$ for $Ca_4 \cdot$CaM varies by several orders of magnitude with pH, in contrast to the pH-independent affinity of the typical kinase peptides such as that of skeletal muscle myosin light chain kinase (WFFu, see above). This effect

* Corresponding author. Fax: +44-20-8906-4477.
E-mail address: pbayley@nimr.mrc.ac.uk (P.M. Bayley).

Abbreviations: PFK, 6-phosphofructokinase; PFK peptides [1], $P_{1–26}$ KLRGRSFMNNWEVYKLLAHIRPPAPK, (rabbit muscle PFK, residues 371–396) and the corresponding peptides $P_{9–21}$, $P_{1–21}$ and $P_{1–21}$(E12Q) sequences; Other peptides, see [2]; CaM, calmodulin

0014-5793/$22.00
doi:10.1016/j.febslet.2004.10.023

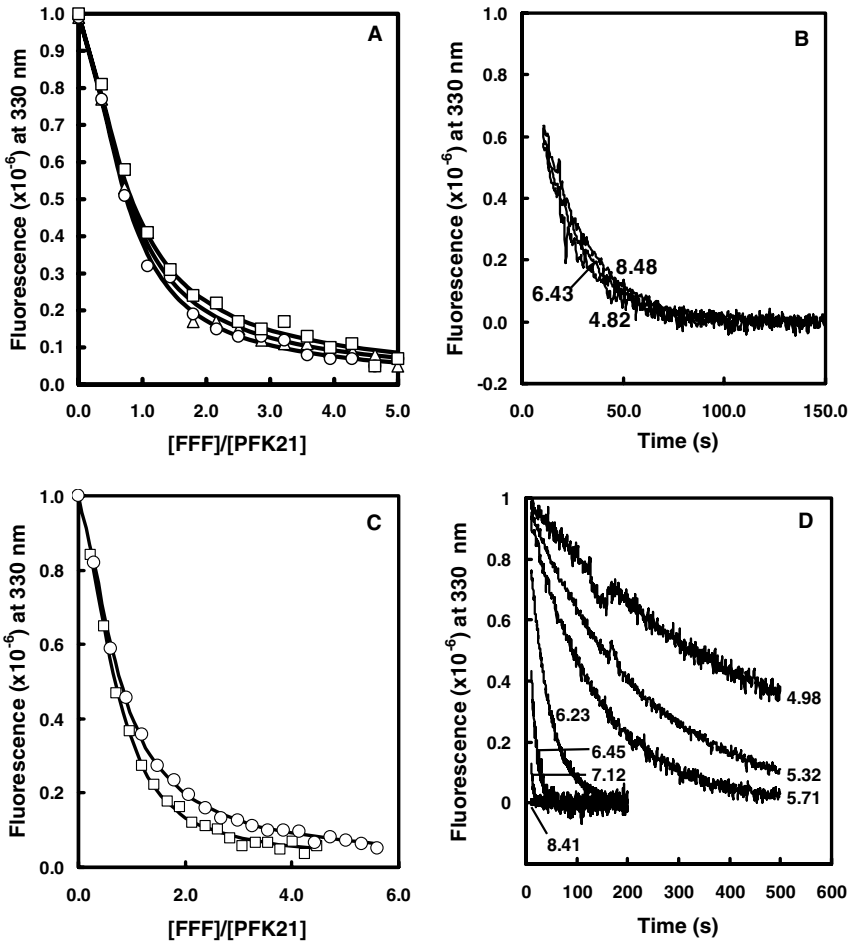

Fig. 1. Interaction of CaM with peptides WFFu and $P_{1–21}$ as a function of pH. (A) Fluorescence competition titrations: 0.5 μM WFFu + 0.55 μM CaM was titrated with FFFp at pH 8.03 (□), 6.67 (○), and 5.43 (△). (B) Dissociation kinetics: 20 μM FFFp was added to a solution containing 0.5 μM WFFu + 0.6 μM CaM. (C) Fluorescence competition titrations: 0.5 μM $P_{1–21}$ + 0.55 μM CaM was titrated with FFFu at pH 8.53 (□) and with FFFp at pH 6.32 (○). (D) Dissociation kinetics: 20 μM FFFp was added to a solution containing 0.5 μM $P_{1–21}$ + 0.6 μM CaM. Buffer: 25 mM Tris/bis-Tris mixtures with 100 mM KCl and 1 mM $CaCl_2$. Fluorescence changes have been normalized for comparison; solid lines in A and C are computed best fits.

evidently derives almost exclusively from changes in the peptide dissociation rate constant. Since the replacement of the acidic glutamate with glutamine in peptide $P_{1–21}$(E12Q) shows a similar strong pH dependent affinity, (Fig. 2A), this variation is not attributable to the presence of the γ-carboxylate group in $P_{1–21}$.

3.2. Calcium binding studies

Peptide binding increases the effective Ca^{2+} affinity of CaM, analysis of which can provide information on the role of different domains in complex formation. Stoichiometric Ca^{2+} association constants were measured using an indicator method [9]. The chromophoric chelator, 5,5′-Br_2BAPTA, was titrated with Ca^{2+} in the presence of apo-CaM alone or with a 5-fold molar excess of $P_{1–21}$ at pH 8.35 and 6.71 (Fig. 2B). Control titrations showed that the calcium binding constant of 5,5′-Br_2BAPTA (5.7×10^5 M^{-1}; [10]) is independent of pH over this range. The results are given in Table 1 as products of the stoichiometric constants, K_1K_2, and K_3K_4, which are better determined than individual K_i values [11]. Ca^{2+} binding to CaM alone is effectively the same at both pH values. In the presence of the peptide the total Ca^{2+} affinity is increased at pH 6.7, due primarily to the higher value of K_3K_4. This increase is consistent with the corresponding ~10-fold higher peptide affinity over this pH range (Fig. 2A). For both pH values, the relative magnitude of $K_1K_2 > K_3K_4$ observed with CaM alone is maintained in the presence of $P_{1–21}$. When the C-terminal pair of calcium binding sites has much greater Ca^{2+} affinity than the N-terminal pair, the values of K_1K_2 and K_3K_4 then reflect the Ca^{2+} affinities of the C- and N-terminal domains, respectively [11]. The peptide-induced increase in K_1K_2 is much greater than that in K_3K_4, suggesting that this peptide interacts more strongly with the C-domain of CaM than with the N-domain at high pH. At lower pH, the observed increase in the affinity of $Ca^{2+}$for the N-domain sites is significantly greater. This would be consistent with increased interaction of the peptide with the N-domain at lower pH, although this interpretation is tentative, since it depends on the unquantified effect of pH on the affinity of the peptide for apo-CaM.

3.3. Determination of histidine pK_a values

The pH dependence of the affinity of $P_{1–21}$ with Ca_4CaM (as compared to the pH-independent binding of WFFu, FFFu and FFFp) suggests a possible role for the unusual His residue of the PFK target sequence. ^{1}H NMR methods have been used to determine the pH sensitivity of His19 in free $P_{1–26}$, His107 in

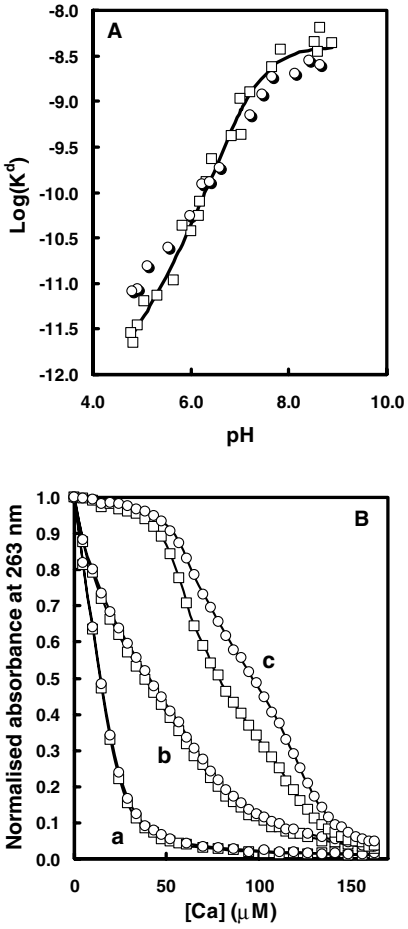

Fig. 2. Dissociation constants and calcium binding as a function of pH. (A) K_d values for P_{1-21} (□) and P_{1-21}(E12Q) (○) as a function of pH from competition titrations with FFFp or FFFu. The curve for the P_{1-21} data shows the least-squares fit to a simple binding scheme for P_{1-21} with two protonation steps giving the low pH limit of K_d<2 pM (see text). (B) Calcium titrations of 25 μM 5,5′-Br_2BAPTA alone (a) and in the presence of 25 μM CaM (b) or 25 μM CaM + 25 μM P_{1-21} (c). Titrations were performed at 20 °C in 10 mM Tris, 100 mM KCl at pH 8.35 (□) and at pH 6.71 (○). Solid lines are the computed best fits to the normalized absorbance.

CaM alone, and both residues in the Ca_4CaM–P_{1-26} complex. The pK_a values were determined in D_2O by measuring the ^1H chemical shift of the histidine imidazole ring $H^{\varepsilon 1}$ protons as a function of pH from 1D ^1H NMR spectra. (The ^1H and ^{15}N signals for the His107 $H^{\varepsilon 1}$ proton were assigned from 2D ^1H–^{15}N HSQC spectra in H_2O of a ^{15}N labeled Ca_4CaM sample complexed with unlabeled P_{1-21}. In this case, $^1H^{\varepsilon 1}$–$^{15}N^{\delta 1}$ two-bond coupling crosspeaks were detected in the spectra). The pH titrations (Fig. 3A) show an increase in pK_a for peptide His19 from 6.35 (free) to 7.25 (complex), indicating that the protonated form is stabilized in the complex with Ca_4CaM. In contrast, the pK_a for His107 in Ca_4CaM decreases by a smaller amount from 6.15 to 5.85 on complex formation with the peptide.

3.4. Modeling the pH dependence of peptide binding

The effect of His19 protonation on binding peptide P_{1-21} to Ca_4CaM can, in principle, be represented by the following scheme:

$$\begin{array}{ccc} & \overset{K_1}{\leftrightarrow} & \\ & \pm H^+ & \\ P_{1\text{-}21} & & HP_{1\text{-}2} \\ (\pm Ca_4CaM) \quad \updownarrow K_L & & \updownarrow K_{HL} \quad (\pm Ca_4CaM) \\ & \overset{K_1^*}{\leftrightarrow} & \\ & \pm H^+ & \\ Ca_4CaM\text{-}P_{1\text{-}21} & & Ca_4CaM\text{-}HP_{1\text{-}21} \end{array}$$

where K_1 and K_1^* are protonation constants (e.g., K_1 = [H][L]/[HL]) and K_L and K_{HL} are dissociation constants for CaM binding (e.g., K_L = [Ca_4CaM][P_{1-21}]/[Ca_4CaM–P_{1-21}]). Taking $K_L = 4$ nM (the high pH limit of the observed $K_{d,obs}$), $K_1 = 4.5 \times 10^{-7}$M (pK_1 = 6.35), and $K_1^* = 5.6 \times 10^{-8}$M (p$K_1^*$ = 7.25) gives $K_{HL} = 0.5$ nM (as $K_1^* \cdot K_L/K_1$). The pH dependence of K_d would be given by $K_{d,obs} = (1+[H]/K_1)/(1/K_L+[H]/K_1K_{HL})$ (Eq. 1). However, the predicted limiting affinity at low pH would then be given by $K_{d,obs} = K_{HL} = 0.5$ nM, much higher than the experimental value (<2 pM). The observed pH dependence therefore requires additional protonation steps, involving potentially a multiplicity of protonation states for peptide, CaM and the complex. Including these steps formally requires expansion of Eq. 1 as the ratio of two polynomial expressions involving higher powers of [H], and multiple protonation constants, whose relative importance would be model-dependent, giving a highly degenerate solution. For the limiting case involving only one additional group (in either CaM or peptide), the apparent perturbation of the second pK on complex formation would be of the order of 2.4 log units to account for the high affinity at low pH. Realistically, this affinity could derive from several protonation steps with relatively small changes in their pK values, but these are not resolvable experimentally. We note that $K_{d,obs}$ = {CaM}·{peptide}/{Cam·peptide complexes}, where {} denotes summation over all protonation states of these species. Thus, inflections in the plot of Log($K_{d,obs}$) vs. pH (Fig. 2A) do not correspond to individual pK values of the system, but result from the summation of the coupled, pH-dependent multiple equilibria.

3.5. Monitoring the effect of pH effects on Ca_4CaM and $Ca_4CaM \cdot P_{1-21}$

To assess possible structural effects of protonation, changes in 2D ^1H–^{15}N HSQC NMR spectra of ^{15}N labeled $Ca_4CaM \cdot P_{1-21}$ complex were monitored as a function of pH. Fig. 3B shows the superimposition of a series of spectra for the complex and for Ca_4CaM alone. The HN cross-peak chemical shift changes with pH occur in fast exchange on the NMR time scale, leading to averaged signals. Backbone assignments for individual residues of the Ca_4CaM–P_{1-21} complex were obtained using triple resonance methods. In contrast to what is observed for Ca_4CaM alone, the ^1H–^{15}N HSQC spectra for the complex show that peaks corresponding to several residues, notably Gly113, Gly33, and Val55, shift progressively with pH. The signal changes for those residues showing the largest chemical shift difference are plotted as a function of pH (Fig. 3C). The pH titration curves (10% D_2O) are consistent with an exchange between two states, with an average midpoint at pH 7.05.

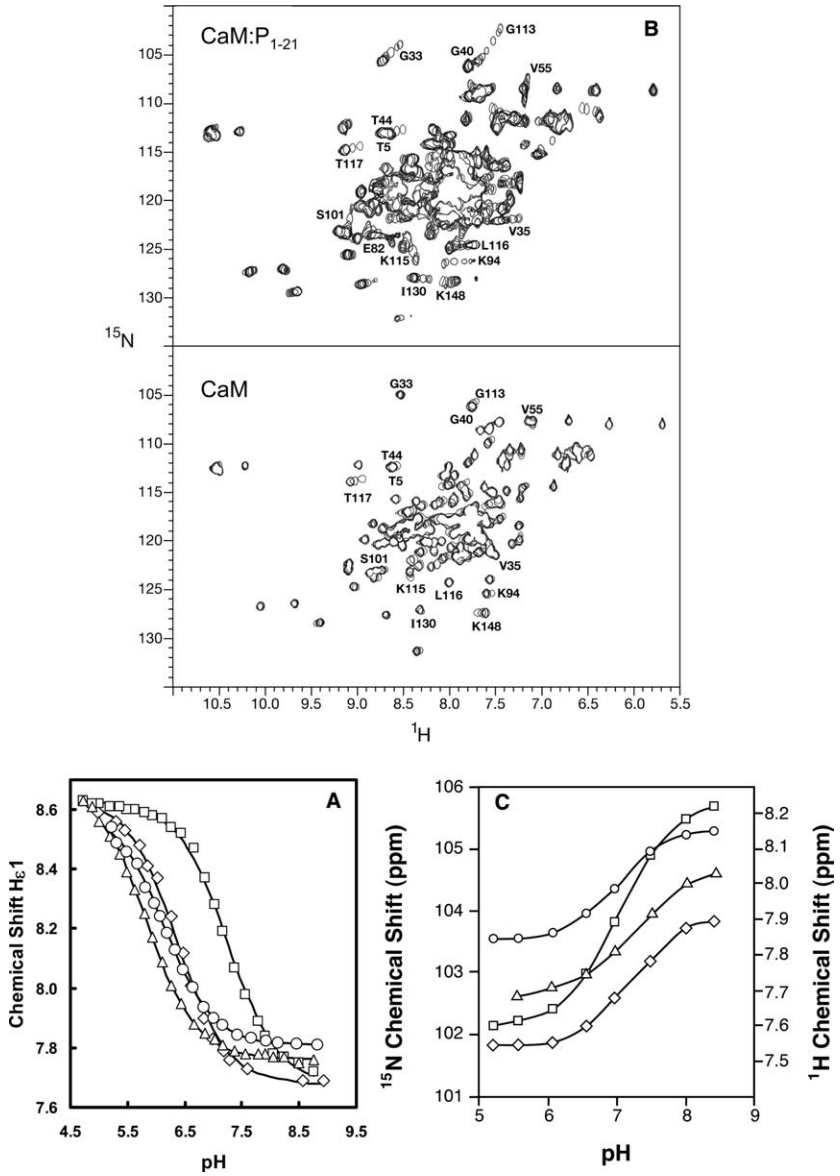

Fig. 3. The pH dependence of the $Ca_4CaM \cdot P_{1–26}$ complex studied by 1- and 2-D NMR spectroscopy. (A) pH titration curve of the ^1H chemical shift of the histidine imidazole ring $H^{\varepsilon 1}$ protons as a function of pH (pH in D_2O at 35 °C) for $Ca_4CaM \cdot P_{1–26}$ complex (CaM His107 (△) and peptide His19 (□)), Ca_4CaM alone (His107 (○)) and $P_{1–26}$ peptide alone (His19 (◇)). (B) ^1H–^{15}N HSQC spectra for the $Ca_4CaM \cdot P_{1–21}$ complex and for Ca_4CaM at pH 5.25–9.0, showing pH dependent shifts for the complex. (C) pH dependence of the ^{15}N signal for some CaM residues in the Ca_4Cam–$P_{1–21}$ complex: Gly113 (□); Gly 33 (○); and Val 55 (◇) offset by −5 ppm, and ^1H signal, Lys94 (△).

4. General discussion

The above scheme rationalizes the increase of peptide affinity with decreasing pH initially in terms of an interaction between the peptide His residue and CaM, resulting in a shift in the His pK_a from 6.35 to 7.25 on forming the complex. The midpoint for the change in chemical shift (Fig. 3C) at ~pH 7.0 suggests that the fast exchange process correlates with structural effects essentially associated with this first protonation. At lower pH, the further 100-fold increase in affinity is asso-

Table 1
Stoichiometric calcium binding constants for CaM and the $CaM \cdot P_{1–21}$ complex (10 mM Tris, 100 mM KCl, 20 °C)

	pH	$\text{Log}(K_1K_2)$	$\text{Log}(K_3K_4)$	$\text{Log}(K_1K_2K_3K_4)$
CaM	8.35	11.60 (0.15)	9.65 (0.15)	21.25 (0.30)
CaM	6.71	11.65 (0.20)	9.55 (0.20)	21.20 (0.30)
CaM + $P_{1–21}$	8.35	14.45 (0.25)	11.20 (0.15)	25.65 (0.40)
CaM + $P_{1–21}$	6.71	14.65 (0.20)	11.75 (0.15)	26.40 (0.35)

The constants are expressed as K_1K_2 and K_3K_4, with their product $K_1K_2K_3K_4$ (all ± S.D., see text).

ciated with additional side-chains whose pK_a is increased in the region, pH 5–7, due to complex formation. These probably include carboxylate groups, but exclude the γ-carboxylate of Glu12 in view of the similar pH dependence of affinity of $P_{1–21}$ and $P_{1–21}$(E12Q).

The crystal structure of the complex of $Ca_4 \cdot CaM$ with the PFK target peptide [12] conforms most closely to that of the $Ca_4 \cdot CaM$–CamKII–peptide complex (1cdm.pdb), with the C-domain of CaM interacting with Trp 11 and the N-domain with Ile 20. Both these structures (with 1:10 hydrophobic motifs) are incomplete in the linker sequence. By analogy with 1cdm.pdb, His19 (preceding the second key hydrophobic residue of the PFK peptide) is oriented towards the interdomain linker containing the negatively charged residues Asp78, Asp80, Glu82, Glu83 and Glu84.

We therefore propose that the initial protonation of His19 induces an electrostatic interaction between its protonated imidazole side-chain and one (or more) of the carboxylates in the linker, producing 10-fold enhancement of peptide affinity and creating two states in a pH dependent, fast exchange process. This interaction appears to cause significant changes in a number of residues in both domains of CaM and at residues apparently well removed from either His19 or the linker region. Peptide binding may also bring adjacent carboxylates of the linker into potentially close proximity with one another, so that the effective carboxylate pK is significantly increased compared to CaM alone. A similar effect could result from pH dependent target-induced ordering in the corresponding negatively charged N-terminal sequence that is necessary for activation of several kinases, implying a possible interaction within the vicinity of the kinase target sequence [13]. Protonation and H-bonding interactions appear possible within numerous CaM residues Asp2, Glu6, Glu7, Glu11, and C-terminal Lys 148 and potentially also the hydrophilic Glu/Gln12 peptide reside.

The results of this study indicate that pH sensitivity is yet another potential degree of versatility in CaM–target interactions. While the incidence of histidine within CaM target sequences is uncommon, there is a high frequency for a basic residue (Arg/Lys) preceding the second key hydrophobic residue of the common 1:14 target sequence interacting with CaM–Glu84 (see the Calmodulin Target Database: http://calcium.uhnres.utoronto.ca/ctdb/flash.htm). Our results predict that, when this residue is His, the target affinity may be strongly pH sensitive in the physiological range, pH 5–8. Although Ca–CaM interactions with enzymes can be significantly weaker than with the corresponding target sequence (e.g., calmodulin dependent protein kinase I, [2]), it seems most likely that the pH-dependence in affinity of $Ca_4 \cdot CaM$ for the PFK target peptide sequence would also be found with PFK itself. This could have functional significance, for example under conditions of stress where lowered pH and elevated [Ca^{2+}] prevail, by providing a Ca^{2+}-dependent and pH-dependent inhibitory mechanism with a possible role in regulation of glycolysis [14].

Acknowledgements: We thank John McCormick for CaM samples, and Annalisa Pastore and Berry Birdsall for helpful support and advice.

References

[1] Mayr, G.W. and Heilmeyer, L.M.G. (1983) FEBS Lett. 195, 51–57.
[2] Clapperton, J.A., Martin, S.R., Smerdon, S.J., Gamblin, S.J. and Bayley, P.M. (2002) Biochemistry 41, 14669–14679.
[3] Buschmeier, B., Meyer, H.E. and Mayr, G.W. (1987) J. Biol. Chem. 262, 9452–9462.
[4] Biekofsky, R.R., Martin, S.R., Browne, J.P., Bayley, P.M. and Feeney, J. (1998) Biochemistry 37, 7617–7629.
[5] Browne, J.P., Strom, M., Martin, S.R. and Bayley, P.M. (1997) Biochemistry 36, 9550–9561.
[6] Martin, S.R. and Bayley, P.M. (2002) Protein Sci. 11, 2909–2923.
[7] Gronenborn, A.M., Birdsall, B., Hyde, E.I., Roberts, G.C.K., Feeney, J. and Burgen, A.S. (1981) Biochemistry 31, 1717–1722.
[8] Cavanagh, J., Fairbrother, W.J., Palmer, A.G. and Skelton, N.J. (1996) Academic Press, New York.
[9] Linse, S., Brodin, P., Johansson, C., Thulin, E., Grundström, T. and Forsén, S. (1988) Nature 335, 651–652.
[10] Martin, S.R., Bayley, P.M., Brown, S.E., Porumb, T., Zhang, M. and Ikura, M. (1996) Biochemistry 35, 3508–3517.
[11] Linse, S., Helmersson, A. and Forsén, S. (1991) J. Biol. Chem. 266, 8050–8054.
[12] Bayley, P.M., Skordalakes, E., Haire, L., Gamblin, S., Smerdon, S., Zanini, F., Browne, P., Skinner, M., Martin, S.R. and Dodson, G. (2000) Biophys. J. 78, 151A.
[13] Persechini, A., Gansz, K.J. and Paresi, R.J. (1996) J. Biol. Chem. 271, 19279–19282.
[14] Mayr, G.W. (1984) Eur. J. Biochem. 143, 521–529.