Cation Binding and Conformation of Human Calmodulin-like Protein†

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ABSTRACT: The Ca^{2+}-binding parameters of recombinant human calmodulin-like protein (CLP), a protein specifically expressed in mammary epithelial cells, were studied by flow dialysis in the absence and presence of 2, 10, and 30 mM $MgCl_2$. In general, the four intrinsic binding constants (K'_{Ca}) are about 8-fold lower than in animal and plant calmodulins. In the absence of Mg^{2+} the K'_{Ca} values of the four binding steps equal 4.0×10^3, 3.3×10^4, 1.0×10^4, and 6.0×10^3 M^{-1}, respectively. They allow us to distinguish two pairs of sites: a higher affinity pair with strong positive cooperativity and a lower affinity pair composed of non-interacting sites with different affinities. Mg^{2+} antagonizes Ca^{2+} binding by decreasing only Ca^{2+}-binding steps 2 and 3, so that at high Mg^{2+} concentrations the positive cooperativity in the high-affinity pair has been lost and that the four K'_{Ca} values are very similar with a mean K'_{Ca} of 4×10^3 M^{-1}. Direct Mg^{2+} binding studies by equilibrium gel filtration indicate that 4–5 Mg^{2+} bind to CLP with a mean K'_{Mg} of 250 M^{-1}. Conformational changes in the unique Tyr138 microenvironment, monitored by fluorimetry and near-UV difference spectrophotometry, indicate that in metal-free CLP this Tyr is shielded from the polar solvent and strongly quenched by a specific chemical group; Ca^{2+} binding induces a shift of Tyr to a more polar environment and removal of the quenching group, but without full exposure to the solvent. Qualitatively this behavior is reminiscent of that of calmodulins which possess one Tyr in a position identical with that of the single Tyr of CLP. Mg^{2+} binding has the same effect as Ca^{2+}, but on a smaller scale. The Tyr-related conformational changes occur upon binding of only two Ca^{2+}, suggesting that the C-terminal domain harbors the high-affinity, strongly interacting pair of sites. The Phe-related conformational changes point to the existence of long-range interactions between the two lobes of CLP. We also monitored a conformational probe in the N-terminal domain, *i.e.*, Cys26, by its reactivity toward 5,5′-dithiobis(2-nitrobenzoic acid). In metal-free CLP the pseudo-first-order rate constant (k_{SH}) is 8- and 20-fold higher than in the Ca^{2+}- and Mg^{2+}-loaded protein, respectively. The profile of k_{SH} during a titration of CLP with Ca^{2+} is biphasic with an increase from 3.9 to 6.7 min^{-1} when two Ca^{2+} are bound, followed by a decrease to 0.5 min^{-1} when the third Ca^{2+} binds to CLP. Our data support the model that binding of Ca^{2+} to the C-terminal domain is directly responsible for Tyr-related conformational changes in this domain, for a modification of the long-range interacting forces between the C- and N-terminal domains, and finally, for the enhancement of the reactivity of the thiol in the N-terminal domain. Subsequent binding of one Ca^{2+} to the N-terminal domain, presumably to site I, leads to a rearrangement of the Cys environment, which strongly reduces its reactivity. In many aspects the interaction of CLP with Ca^{2+} and the ensuing conformational changes are reminiscent of those of CaM, especially of plant CaM. However, in CLP these conformational changes have a higher amplitude than in CaM, which may allow the former to act on unique targets.

Calmodulin (CaM)[1] mediates the Ca^{2+} signal by activating a multitude of different enzyme systems and by reorganizing various components of the cytoskeleton. The protein is highly conserved (Wylie & Vanaman, 1988), and in most cases one single CaM protein exists in a given species, although it may be generated by multiple genes, at least in man and rat (Fischer *et al.*, 1988; Nojima, 1989). In chicken (Stein *et al.*, 1983), rat (Nojima & Sokabe, 1986), and man (Koller & Strehler, 1988) the additional presence of a CaM-like gene has been reported. These intronless genes have retained the potential to encode a protein with a considerable degree of sequence identity (85–87%) to "bona fide" CaM. So far, however, expression of the CaM-like gene has only been reported in humans and seems restricted to specific tissues, *e.g.*, to normal mammary epithelial cells (Yaswen *et al.*, 1990). Intriguingly, its mRNA is drastically reduced in the corresponding tumor cells. Similarly, polyclonal antibodies were able to detect the CaM-like protein (CLP) encoded by this gene in the normal, but not in the transformed, cells. It has thus been proposed that CLP may be involved in the initiation or maintenance of the differentiated state of the cells (Yaswen *et al.*, 1992).

Recently, the protein has been overexpressed in *Escherichia coli* and purified to apparent homogeneity. It binds four Ca^{2+} with an affinity which is approximately 10-fold lower than that of CaM under comparable conditions. Recombinant CLP was able to activate 3′,5′-cyclic nucleotide phosphodiesterase and the erythrocyte plasma membrane Ca^{2+}-ATPase to 100 and 62%, respectively, of the activity obtained with CaM, albeit with a lower affinity. The protein thus represents a true isoform of CaM (Rhyner *et al.*, 1992). The reduced affinities for Ca^{2+} and for the classical CaM targets, together

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[1] Abbreviations: CLP, calmodulin-like protein; CaM, calmodulin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TCA, trichloroacetic acid.

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with the rather low concentration of CLP in particular tissues, suggest that CLP does not compete with CaM *in vivo*. If this is the case, it may have its own specific or preferred targets (Yaswen *et al.*, 1990) and should possess particular Ca^{2+}-induced conformational changes. A specific, Ca^{2+}-dependent interaction of CLP with two proteins has indeed been observed recently (J. A. Rhyner, unpublished results).

In this report we characterized in detail the Ca^{2+}- and Mg^{2+}-binding properties and the Ca^{2+}–Mg^{2+} antagonism of recombinant CLP under physiological conditions. We also monitored by difference spectrophotometry and fluorimetry the Ca^{2+}- and Mg^{2+}-dependent changes in the environment of the unique Tyr138[2] residue and studied the Ca^{2+} and Mg^{2+} dependence of the thiol reactivity of the unique Cys26 residue. Both residues occupy positions which are identical in plant calmodulins, where the Ca^{2+}-dependent changes in their microenvironment have already been studied (Maune *et al.*, 1992a, b; Yoshida *et al.*, 1983; Yazawa *et al.*, 1984; Anderson *et al.*, 1980). The conformational data show that CLP displays many of the characteristics found in plant CaM, but that it also has some intriguing particularities.

MATERIALS AND METHODS

Expression and Purification of CLP. Human CLP was expressed and purified as previously described (Rhyner *et al.*, 1992).

Direct Ca^{2+} and Mg^{2+} Binding Studies. For removal of contaminating metal ions and for complete equilibration of the protein in the assay buffer, CLP was precipitated 2–3 times with 3% trichloroacetic acid (TCA) and then passed through a 1 × 40 cm Sephadex G-25 column equilibrated in the assay buffer. TCA-treated CLP shows the same electrophoretic mobility and potency of enzyme activation as the EDTA-treated protein (J. A. Rhyner, unpublished results). The buffers were freed of contaminating metals by passage over a column of EDTA–agarose (Haner *et al.*, 1984). Ca^{2+} contamination was always less than 0.1 mol/mol of protein. Total Ca^{2+} concentrations were determined with a Perkin-Elmer Cetus Instruments 2380 atomic absorption spectrophotometer. For the atomic absorption measurements EDTA up to 1 mM was added to all solutions including the standards, in order to normalize the quenching effects. The protein concentration was determined from the UV absorption spectrum using a molar extinction coefficient of 1350 M^{-1} cm^{-1} at 280 nm for metal-free CLP.

Ca^{2+} binding to CLP in the absence and presence of 2, 10, and 30 mM Mg^{2+} was measured at 25 °C by the flow dialysis method of Colowick and Womack (1968) in 50 mM Tris buffer, pH 7.5, 150 mM KCl, and 7.5 mM mercaptoethanol (buffer A). In order to maintain a constant ionic strength in buffers with differing Mg^{2+} concentrations, the concentration of KCl was lowered so as to compensate for the contribution of Mg^{2+}; *e.g.*, at 30 mM $MgCl_2$ only 60 mM KCl was present. The protein concentrations were 40–60 μM. Treatment of the raw data was as described by Cox *et al.* (1990).

Mg^{2+} binding to CLP in the absence of Ca^{2+} was measured by the equilibrium gel filtration method of Hummel and Dryer (1962). A Sephadex G-25 column (0.7 × 35 cm) was equilibrated in buffer A containing 50 or 100 μM EGTA and the indicated concentrations of $MgCl_2$. One milliliter of 100–800 μM metal-free CLP was applied to the column. In the eluant, Mg^{2+} concentrations were determined by atomic absorption, and protein concentrations, by UV absorption.

The binding data were analyzed by means of the Scatchard plot for simple isotherms (Mg^{2+} binding) or by the Adair (1925) equation for four binding sites, and the stoichiometric binding constants (K) were calculated with a curve-fitting procedure, *i.e.*, Simplex in the Matlab environment. The intrinsic association constants (K') were obtained from the stoichiometric constants by applying the statistical factors of the respective binding steps (Cornish-Bowden & Koshland, 1975).

Optical Methods To Probe the Tyr Environment. Emission fluorescence spectra were taken with a Perkin-Elmer LS-5B spectrofluorimeter interfaced with a computer. The measurements were carried out on 20 μM TCA-treated metal-free CLP at room temperature with excitation and emission slits of 5 nm. Fifty micromolar EGTA, 15 mM $MgCl_2$, or 1 mM $CaCl_2$ was added subsequently to obtain the metal-free, Mg^{2+} and Ca^{2+} form, respectively. The Ca^{2+} titration of the fluorescence change was done by additions of increments up to 6 times the protein concentration, which was 1.05 mM, so as to approach a stoichiometric titration. Given the high concentration of CLP, a raster filter with a cutoff of 96.5% of the light was inserted in the excitation beam.

UV absorption and difference spectra were measured with a Perkin-Elmer Cetus Instruments λ 5 UV/vis spectrophotometer at room temperature. The metal-free protein was dissolved in buffer A. Difference spectra were taken on solutions with an optical density at 280 nm of 1.5, corrected for the blank and for dilutions and normalized to molar absorption differences. The UV spectrophotometer is interfaced with a data processor, thus allowing arithmetic operations on the spectra such as correction for dilution and base-line adjustment. Ca^{2+} titrations were done with 1.04 mM CLP in buffer A. In order to monitor changes in both the Tyr and the Phe environment, spectra were taken after every Ca^{2+} increment.

Thiol Reactivity. The influence of cations on the thiol reactivity was assayed on a CLP sample which was previously reduced by overnight incubation with 50 mM dithiotreitol and removal of the excess reducing agent by gel filtration on a Sephadex G-25 column (0.7 × 35 cm) equilibrated in nitrogen-saturated buffer A. The thiol reactivity was assayed by monitoring spectrophotometrically at 412 nm the kinetics of the reduction of Ellman's reagent according to Riddles *et al.* (1983). The solutions contained 30 μM CLP and varying concentrations of Ca^{2+}. The reaction was initiated by mixing the sample solution with 0.3 mM DTNB; the optical densities were measured every second for 10–15 min and stored in computer files. The pseudo-first-order rate constant (k_{SH}) was calculated with the Guggenheim equation, $\ln(x' - x) = -k_{SH}t + \ln[X(1 - e^{-k}SH^{\Delta t})]$, where x, x', and X are the optical densities at time t, time $(t + \Delta t)$, and infinite time, respectively. This way of analysis, which was computer programmed by Dr. B. Schwendimann (Department of Biochemistry, Geneva), is independent of the evaluation of X. In Ca^{2+}-titration experiments CLP solutions were incubated with the desired total Ca^{2+} concentration before addition of DTNB. At all Ca^{2+} concentrations the reaction obeyed the pseudo-first-order rate equation with a correlation coefficient of at least 0.996. The free Ca^{2+} concentration was calculated from the total Ca^{2+} and protein concentrations and the binding constants using the speciation program of Perrin and Sayce (1967).

[2] Amino acid numbering is as in Rhyner *et al.* (1992), *i.e.*, Met at the N-terminus is not counted.

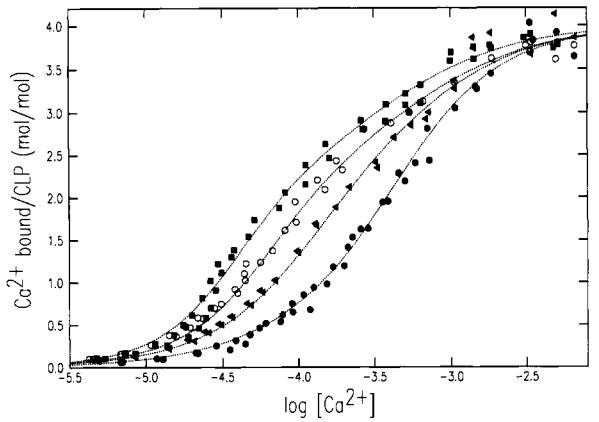

FIGURE 1: Ca^{2+} binding to CLP (40–60 μM) in the absence (■) and presence of 2 (○), 10 (▲), and 30 mM Mg^{2+} (●). Ca^{2+} binding was monitored by the flow dialysis method at 25 °C. The lines represent the isotherms calculated with the Adair equation using the stoichiometric constants of Table I.

Table I: Stoichiometric (K_n) and Intrinsic (K'_n) Ca^{2+}-Binding Constants of CLP, Calculated with an Iterative Program from the Data of Figure 1

	no Mg^{2+}	2 mM Mg^{2+}	10 mM Mg^{2+}	30 mM Mg^{2+}
K_1	1.6×10^4	1.5×10^4	1.5×10^4	1.0×10^4
K_2	5.0×10^4	2.3×10^4	9.0×10^3	3.0×10^3
K_3	7.0×10^3	5.0×10^3	3.4×10^3	2.0×10^3
K_4	1.5×10^3	1.0×10^3	1.0×10^3	1.0×10^3
K'_1	4.0×10^3	3.8×10^3	3.8×10^3	2.5×10^3
K'_2	3.3×10^4	1.5×10^4	6.0×10^3	2.0×10^3
K'_3	1.0×10^4	7.5×10^3	5.1×10^3	3.0×10^3
K'_4	6.0×10^3	4.0×10^3	4.0×10^3	4.0×10^3

RESULTS

Direct Ca^{2+}-Binding Studies. A detailed examination of the Ca^{2+}-binding isotherms measured in the presence of 0, 2, 10, and 30 mM Mg^{2+} (Figure 1) reveals a rather complex pattern of Ca^{2+} binding and Mg^{2+} antagonism. Analysis according to the Adair equation with a curve-fitting program yields the stoichiometric binding constants (K_{Ca}) reported in Table I. The intrinsic constants (K'_{Ca}), which differ from the K_{Ca} values by the factors 1/4, 2/3, 3/2, and 4 (Cornish-Bowden & Koshland, 1975) and reflect more directly the affinity of each site for Ca^{2+}, are also presented. This analysis reveals the features which are common to CLP and vertebrate CaM (for which a similar study has been carried out (Milos *et al.*, 1986)) and those which differentiate the two proteins. Resemblance to CaM includes (i) the pronounced positive cooperativity in the second binding step ($K'_{Ca2} >> K'_{Ca1}$) at low Mg^{2+} concentration and (ii) the shift of the midpoint (increase of the mean dissociation constant) of the Ca^{2+}-binding isotherm to the right at higher Mg^{2+} concentrations to a very similar extent as observed with CaM; for example, at 10 mM Mg^{2+} the mean affinity is 2.5-fold lower than in the absence of Mg^{2+}, compared to 3-fold for CaM (Milos *et al.*, 1986). The differences between CLP and vertebrate CaM, however, are more striking: (i) The midpoints of the Ca^{2+}-binding isotherms of CLP are 8 times higher than those of CaM. (ii) In the absence of Mg^{2+} the mean intrinsic affinity constant of the first pair of sites is 1.4-fold higher than that of the last pair; the last step displays an intrinsic affinity constant nearly 2-fold lower than the third binding step. In bovine brain CaM the four intrinsic constants are very similar, except for the cooperative binding at the first two sites. (iii) Mg^{2+} almost exclusively decreases the affinity constants of the second and third steps, the second Ca^{2+}-binding steps being 4 to 5 times more antagonized than the third. In vertebrate CaM, Mg^{2+} antagonizes the four binding steps to the same extent. (iv) The binding of 4 Ca^{2+} at 10 mM free Mg^{2+} is non-cooperative in appearance, whereas at 30 mM Mg^{2+} the intrinsic constants of the last two steps are slightly higher than those of the first two steps. No such cooperativity between the pairs of sites is observed in the case of CaM.

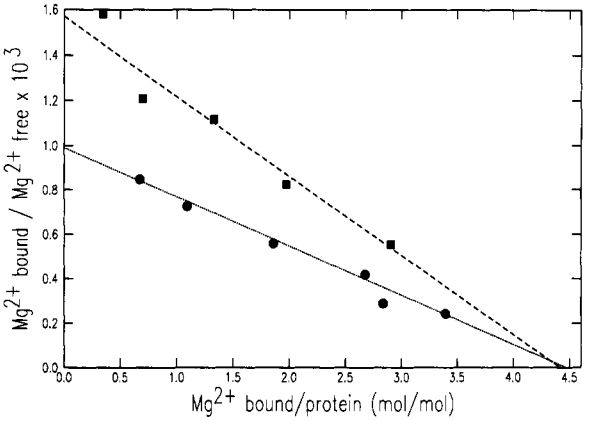

FIGURE 2: Scatchard plot of Mg^{2+} binding to CLP in the absence of Ca^{2+} as monitored by equilibrium gel filtration at room temperature in buffer A containing 50 (●) or 100 μM EGTA (■). The contaminating Ca^{2+} concentrations were below 5 μ M.

Direct Mg^{2+}-Binding Studies. Mg^{2+}-binding data, assayed by Hummel–Dryer gel filtration, were analyzed with a Scatchard plot (Figure 2). CLP binds 4–5 Mg^{2+} with an equal intrinsic affinity constant. The value of the constant is somewhat dependent on the concentration of EGTA: it amounts to 222 and 356 M^{-1} in the presence of 50 and 100 μ M EGTA, respectively. The EGTA dependence, which is not due to differences in Ca^{2+} contamination, is presently not understood, but may be related to weak binding of the chelator (Chiancone *et al.*, 1986). If one assumes that Mg^{2+} antagonizes Ca^{2+} through straight competition, the intrinsic K_{Mg} values, calculated with the competition equation from the K'_{Ca} values of Table I, amount to 15, 83, 490, and 0 M^{-1} for the first, second, third, and fourth binding step, respectively. The discrepancies of these values with the experimental ones suggest that the Mg^{2+} antagonism is probably indirect, *i.e.*, the Mg^{2+}-binding sites are different from the Ca^{2+}-binding sites, but their occupancy antagonizes the Ca^{2+}-binding sites. Such a behavior has also been observed in the case of calmodulin where all four Ca^{2+} binding constants are equally affected by Mg^{2+} [for review, see Cox (1988)]. Two isolated Hummel–Dryer experiments in the presence of 12 mM Mg^{2+} + 4 mM Ca^{2+} and 5 mM Mg^{2+} + 1.5 mM Ca^{2+}, respectively, revealed that when Ca^{2+} binding is maximal, less than 0.2 mol of Mg^{2+} was bound per mol of CLP (data not shown). Although this behavior suggests that the Mg^{2+}–Ca^{2+} antagonism is direct, it can not be excluded that bound Ca^{2+} lowers the affinity of the auxiliary Mg^{2+} sites to such an extent that under the experimental conditions Mg^{2+} binding is not perceived.

Conformational Changes in the Tyr Microenvironment. CLP contains one single Tyr residue at position 138, *i.e.*, at position 10 of the Ca^{2+}-binding loop of the fourth EF-hand [for code, see Strynadka and James (1989)], and it is anticipated that changes in the environment of Tyr reflect binding of Ca^{2+} to the C-terminal half of CLP. Figure 3 shows the fluorescence spectra of guanidine hydrochloride-denatured CLP, of the native metal-free form, and of the Ca^{2+}- or Mg^{2+}-loaded protein after excitation at 280 nm. In the native metal-free CLP Tyr138 displays an unusually low fluorescence yield; the denaturation by guanidine hydrochlo-

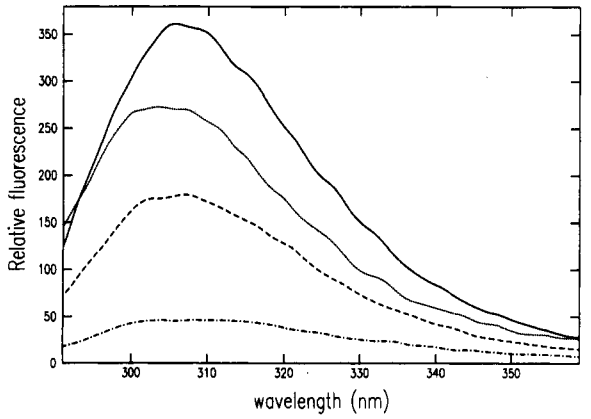

FIGURE 3: Tyrosine fluorescence spectra of CLP (20 μ M) in the absence of metals (···) or in the presence of 15 mM $MgCl_2$ (---), 2 mM $CaCl_2$ (—), or 3 M guanidine hydrochloride (···). The spectra were corrected for the buffer contribution.

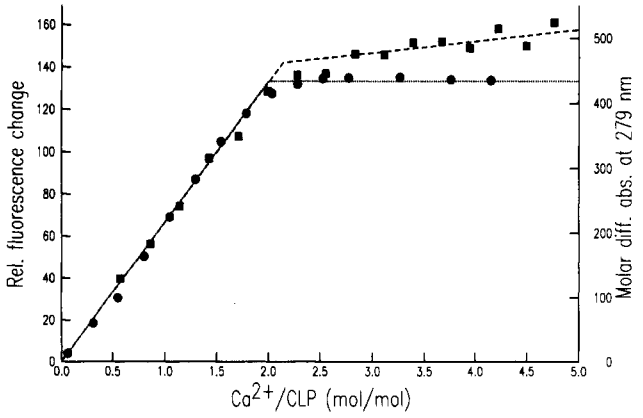

FIGURE 4: Conformational titration of 1.0–1.1 mM CLP with Ca^{2+}. The Tyr conformational change was followed by fluorimetry (■) with excitation and emission at 280 and 308 nm, respectively, or by difference spectroscopy (●) at 279 nm.

ride increases the fluorescence yield 6-fold with a concomitant slight blue-shift. These data suggest that in metal-free CLP the Tyr is not solvent-exposed, but is strongly quenched by an electronegative group or by hydrogen bonding of the phenol group (Lakowicz, 1983). The quench effect is specifically linked to the conformation since it is relieved by denaturation. Ca^{2+} binding provokes an increase of fluorescence intensity at 307 nm of 8 times that of native metal-free CLP and of 1.3 times that of denatured protein, indicating that the Tyr residue becomes shielded from the specific quenching group (as occurs in metal-free CLP) *and* also partly shielded from the solvent (as occurs in the denatured protein). Further addition of Mg^{2+} does not lead to any fluorescence increase. Addition of 15 mM Mg^{2+} to the metal-free protein leads to a 2.4-fold increase of fluorescence intensity; subsequent Ca^{2+} addition yields the same spectrum as Ca^{2+} alone. The Ca^{2+} dependence of the fluorescence change in the absence of Mg^{2+} is shown in Figure 4. Because of the high protein concentration used, this titration is stoichiometric, *i.e.*, virtually all added Ca^{2+} is bound. The curve shows an inflection point at a molar ratio of 2.14 Ca^{2+}/CLP with a small additional increase upon binding of Ca^{2+} to the low-affinity pair of sites.

Figure 5 shows UV difference spectra of CLP induced by 3 mM Ca^{2+} or 15 mM Mg^{2+}. Saturation by Ca^{2+} provokes quite important perturbations in the microenvironment of aromatic residues. The negative twin peak with minima at 286 and 279 nm is due to the unique Tyr residue, whereas the marked peaks at 257, 263, 250, and 267 nm are due to Phe. According to reference spectra of Tyr and Tyr-rich proteins

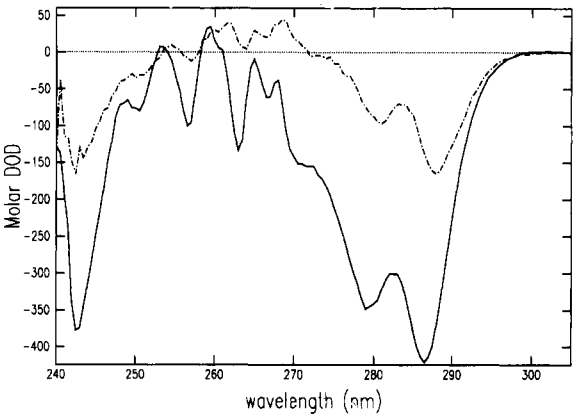

FIGURE 5: Ca^{2+} or/and Mg^{2+} induced difference spectra of CLP (1.1 mM) in buffer A at room temperature. Ca^{2+} or Mg^{2+} were added up to 2.5 and 15 mM, respectively: Ca^{2+} form – apo form (—); Mg^{2+} form – apo form (···).

(Bailey *et al.*, 1968; Donovan, 1973), the appearance of negative peaks at 279 and 286 nm points to a shift of Tyr from a hydrophobic to a polar environment upon Ca^{2+} binding. Mg^{2+} binding leads to a perturbation in the Tyr environment which is of the same nature as that induced by Ca^{2+}, but much less pronounced; the Phe environment apparently is not much affected by Mg^{2+}. The Ca^{2+} dependence of the difference spectral change at 279 nm is shown in Figure 4. The protein concentration and affinity for Ca^{2+} were such that the titration was stoichiometric. As for the Tyr fluorescence titration, the curve shows a sharp inflection point at a molar ratio of 2 Ca^{2+}/CLP, indicating that the conformational change in the environment of Tyr is linear up to 2 Ca^{2+}. Interestingly, the conformational changes in the environment of the Phe residues (negative peaks at 257 and 263 nm) are also complete after binding of 2 Ca^{2+} to the pair of high-affinity sites (data not shown).

Changes in the Thiol Reactivity Induced by Ca^{2+} and Mg^{2+}. CLP contains one Cys residue at position 26 (Y in the Ca^{2+}-binding loop of site I),[3] which corresponds to the position of the unique Cys residue in plant calmodulins. The pseudo-first-order rate constants (k_{SH}) of the reaction of the thiol with DTNB in the presence of 100 μM EDTA, 2 mM $CaCl_2$, or 15 mM $MgCl_2$ (78% of the Mg conformation) are 3.9, 0.5, and 0.2 min^{-1}, respectively (data not shown). The variation of k as a function of the Ca^{2+} concentration is shown in Figure 6. The k_{SH} value increases 1.6-fold at low Ca^{2+} concentration and subsequently undergoes a dramatic 12-fold decrease at high Ca^{2+} concentration. The comparison of this profile with the distribution curves of the CLP·Ca_n species indicates that the phase of increase corresponds to binding of the second Ca^{2+}, whereas the decrease is concomitant with binding of the third and/or fourth Ca^{2+}.

A more quantitative interpretation of the variation of k_{SH} as a function of Ca^{2+} concentration was carried out on the assumption that each CLP·Ca_n species has its well-defined rate constant, k_{nCa}, and that in the mixture of species these rate constants do not change in the initial phase of the reaction. Then the following polynomial can be applied (Burger *et al.*, 1984):

$$k_{SH} = k_{0Ca} + k_{1Ca}[CLP{\cdot}Ca_1] + k_{2Ca}[CLP{\cdot}Ca_2] + k_{3Ca}[CLP{\cdot}Ca_3] + k_{4Ca}[CLP{\cdot}Ca_4] \quad (1)$$

A curve-fitting program was used to evaluate the k_{nCa} values

[3] Numbering of the Ca^{2+}-binding sites by roman characters is from the N- to the C-terminus.

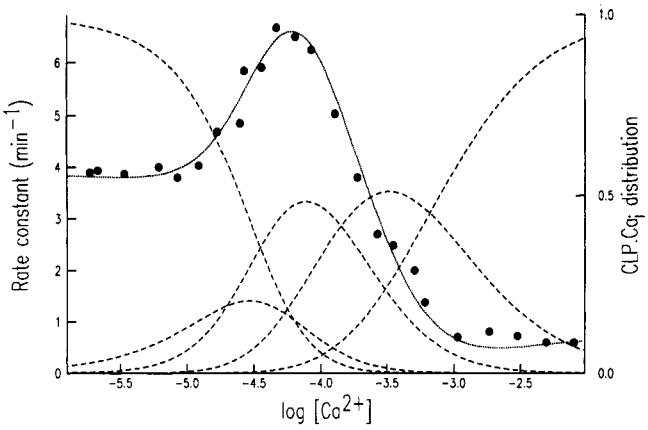

FIGURE 6: Reactivity of Cys26 toward DTNB as a function of the free Ca^{2+} concentration. The CLP concentration was 30 μM. The broken lines show the statistical distribution of the CLP·Ca_i complexes, calculated with the Adair equation using the constants listed in Table I, with i going from 0 to 4 from left to right. The dotted line corresponds to the theoretical profile calculated with eq 1 with values of 3.9, 1.2, 12.3, 0.15, and 0.5 min^{-1} for k_{0Ca}, k_{1Ca}, k_{2Ca}, k_{3Ca}, and k_{4Ca}, respectively.

(and uncertainty level) that best fit the experimental data and yielded 3.9 ± 0.2, 1.2 ± 1.7, 12.3 ± 1.5, 0.45 ± 0.45, and 0.55 ± 0.55 min^{-1} for k_{0Ca}, k_{1Ca}, k_{2Ca}, k_{3Ca}, and k_{4Ca}, respectively. Although the precision of these kinetic constants is understandably limited, one can safely conclude that binding of Ca^{2+} to the high-affinity pair provokes a transient exposure of Cys26, whereas binding to the pair of independent sites very efficiently decreases the reactivity (for CLP·Ca_2, $t_{1/2}$ is 3.4 s; for CLP·Ca_3, 92 s).

DISCUSSION

At low ionic strength (Rhyner *et al.*, 1992) as well as under physiological ionic strength conditions (this study) the mean Ca^{2+} affinity of CLP is 8-fold lower than that of vertebrate CaM. A priori this is quite surprising since only sites II and IV display significant amino acid differences in CaM and CLP. However, recent site-directed mutagenesis studies on classical CaMs revealed that the decrease of affinity of one site has profound effects on the affinity of all the remaining sites, especially when mutations occur in the C-terminal half of CaM (Maune *et al.*, 1992a). A close look at the intrinsic affinity constants in the absence of Mg^{2+} (Table I) allows one to subdivide the four sites of CLP into two pairs: one strongly interacting pair of high-affinity sites ($K'_1 << K'_2$) and one pair of non-interacting sites, with one of the latter having a 2-fold higher affinity for Ca^{2+} than the remaining one ($K'_3 > K'_4$). The pronounced positive cooperativity of the second step and the 3.3-fold lower value of K'_3 with respect to K'_2 indicates that CLP·Ca_2 is a stable and abundant intermediate species during titrations (see species distribution in Figure 6). Interestingly, wheat CaM follows a very similar ($K'_1 << K'_2$) $>> K'_3 > K'_4$ pattern, albeit with higher values for the affinity constants (K'_i values are 5×10^4, 1.7×10^5, 3.8×10^4 and 9.6×10^3 M^{-1}, respectively) (Yoshida *et al.*, 1983). Mg^{2+} antagonizes Ca^{2+} binding to the high-affinity, interacting pair by strongly decreasing K'_2 with a minor effect on the first constant. As a result, Mg^{2+} diminishes and even abolishes ($K'_1 = K'_2$ at 30 mM Mg^{2+}; Table I) the positive cooperativity in the first pair. In the pair with nonequal independent sites Mg^{2+} decreases K'_3, but with a 3-fold lower efficiency than it influences K'_2, and is without effect on the last step. As a result the intrinsic constants K'_3 and K'_4 become nearly equal at high Mg^{2+} concentrations. At this time it is not possible to distinguish if the antagonistic effect of Mg^{2+} is due to straight competition with Ca^{2+} at these sites or if the effect is allosterically due to the presence of auxiliary metal binding sites (Milos *et al.*, 1986). A final interesting observation in Table I is that at 30 mM Mg^{2+} the K'_1 and K'_2 values are smaller than K'_3 and K'_4, leading to positive cooperativity (n_H = 1.2) in the global isotherm. This suggests that Ca^{2+} binding to CLP always occurs according to a well-defined pathway: first to the interacting pair, then to the pair of independent sites.

The near-UV difference spectrum of the Ca^{2+} form versus the metal-free form is, as far as the Tyr region is concerned, quite similar to that of CaM from *Drosophila* (Maune *et al.*, 1992a), wheat (Yoshida *et al.*, 1983), or mushroom CaM (Nakamara *et al.*, 1984), which all have one single Tyr in position 138. The intensity ratio 279/286 nm of CLP corresponds best to that of mutants in the C-terminal half of *Drosophila* CaM. For these CaMs it has been proposed that Ca^{2+} promotes the burying and immobilization of Tyr138 [for discussion, see Maune *et al.* (1992a,b)]. On the other hand, however, Kilhoffer *et al.* (1981) previously noticed that the unique Tyr138 in octopus CaM is definitely more exposed to the solvent in the Ca^{2+} than in the metal-free conformation. Later, the elucidation of the crystal structure of Ca^{2+}-saturated vertebrate (Babu *et al.*, 1988) and *Drosophila* CaM (Taylor *et al.*, 1991) confirmed this relative exposure: the solvent-accessible surface of the phenol group of Tyr138 is 32 A^2 compared to 69 A^2 for the fully solvent-accessible Tyr99 in vertebrate CaM. The phenol oxygen, however, is buried in both CaMs. The model of Ca^{2+}-induced partial exposure of Tyr138 also better explains the Tyr fluorescence changes of CLP and CaM's possessing only one Tyr. In metal-free octopus CaM the quantum yield is about 1/10 that of free Tyr in water (Kilhoffer *et al.*, 1981), likely due to strong quenching by CONH or COO^- groups or by hydrogen-bond formation of the phenol group (Lakowicz, 1983). This quenching is fully relieved by denaturation and partly by the binding of Ca^{2+} (3-fold increase in fluorescence intensity in octopus CaM). Similar enhancements were reported for *Neurospora* (Cox *et al.*, 1982) and plant CaM (Anderson *et al.*, 1980). It must be stressed that there are important quantitative differences in the fluorescence properties of CLP and these CaMs, since CLP shows an 8-fold increase. Thus Ca^{2+} removes much more quenching in CLP than in all other CaMs. This quantitative difference is specific for Ca^{2+}; 5 mM Mg^{2+} provokes the same 2-fold increase in the quantum yield in both octopus CaM (Kilhoffer *et al.*, 1981) and CLP (data not shown).

The single thiol of CLP is more accessible to DTNB in the metal-free form than in the Ca^{2+} form, and Mg^{2+} allows even a better protection than Ca^{2+}. The Ca^{2+} dependency of the thiol reactivity is opposite to that found in wheat CaM (Yoshida *et al.*, 1983), where the pseudo-first-order constant increases from 0.05 to 0.27 min^{-1} upon binding of Ca^{2+}. Remarkably, the thiol reactivity of metal-free wheat CaM is 80-fold lower than that of CLP, pointing to an important conformational difference between the two proteins.

The Tyr-linked conformational changes and the thiol reactivity as a function of free Ca^{2+} concentration reveal interesting details on the ordered filling of the four Ca^{2+}-binding sites and on long-distance interactions between the N- and C-terminal lobes. The fluorescence change and the difference absorption of the Tyr in CLP are linear up to two bound Ca^{2+} and do not significantly change with occupation

of the last two sites. Since Ca^{2+} binding to the first two sites is very cooperative and Tyr-related conformational changes are concomitant, it is likely that the C-terminal pair of sites is the one which is titrated first. Predominant changes in the C-terminal lobe upon binding of the first two Ca^{2+} have been reported in most of the CaM species and mutants studied to date, and have been interpreted as reflecting preferred binding of these two Ca^{2+} to the C-terminal lobe (Starovasnik *et al.*, 1992, and references within). The assignment of the C-terminal lobe of CLP as possessing the high-affinity pair of sites is therefore appealing, but by no means settled, especially since we clearly observed long-range effects between the two lobes. A first indication of long-range interactions comes from the signal of the Phe residues, which is complete when two Ca^{2+} are bound, although the nine Phe residues are equally distributed in the two lobes. This phenomenon was also observed in some mutants of *Drosophila* CaM (Maune *et al.*, 1992a). The clearest indication for long-range effects comes however from the increase of k_{SH} upon binding of Ca^{2+} to the high-affinity pair of sites followed by a large decrease of k_{SH} upon binding to the low-affinity pair. In wheat CaM, too, it has been shown that the thiol reactivity, after an increase upon binding of up to two Ca^{2+}, decreases upon binding of the third Ca^{2+}, although not as dramatically as in CLP (Yoshida *et al.*, 1983). However, conformational changes around Cys, monitored by ^{13}C-NMR downfield shift after cyanylation, occur at Ca^{2+}/CaM ratios between 2 and 4, indicating that the N-terminal domain contains the low-affinity sites (Yazawa *et al.*, 1984).

The distinct differences in Ca^{2+} and Mg^{2+} binding properties of CLP when compared to vertebrate CaM and the pronounced effects of metal binding on the conformation of CLP indicate that this CaM "isoform" performs a specialized function clearly separated from that of bona fide CaM in the restricted cell types where it is expressed.

REFERENCES

Adair, G. S. (1925) *J. Biol. Chem. 63*, 529–545.

Anderson, J. M., Charbonneau, H., Jones, H. P., McCann, R. O., & Cormier, M. J. (1980) *Biochemistry 19*, 3113–3120.

Babu, Y. S., Bugg, C. E., & Cook, W. J. (1988) *J. Mol. Biol. 204*, 191–204.

Bailey, J. E., Beaven, G. H., Chignell, D. A., & Gratzer, W. B. (1968) *Eur. J. Biochem. 7*, 5–14.

Burger, D., Cox, J. A., Comte, M., & Stein, E. A. (1984) *Biochemistry 23*, 1966–1971.

Chiancone, E., Thulin, E., Boffi, A., Forsén, S., & Brunori, M. (1986) *J. Biol. Chem. 261*, 16306–16308.

Colowick, S. P., & Womack, F. C. (1969) *J. Biol. Chem. 244*, 774–777.

Cornish-Bowden, A., & Koshland, D. E. (1975) *J. Mol. Biol. 95*, 201–212.

Cox, J. A. (1988) *Biochem. J. 249*, 621–629.

Cox, J. A., Ferraz, C., Demaille, J. G., Perez, R. O., Van Tuinen, D., & Marmé, D. (1982) *J. Biol. Chem. 257*, 10694–10700.

Cox, J. A., Milos, M., & MacManus, J. P. (1990) *J. Biol. Chem. 265*, 6633–6637.

Donovan, J. W. (1973) *Methods Enzymol. 27*, 497–525.

Fischer, R., Koller, M., Flura, M., Mathews, S., Strehler-Page, M.-A., Krebs, J., Penniston, J. T., Carafoli, E., & Strehler, E. E. (1988) *J. Biol. Chem. 263*, 17055–17062.

Haner, M., Henzl, M. T., Raissouni, B., & Birnbaum, E. R. (1984) *Anal. Biochem. 138*, 229–234.

Hummel, J. P., & Dryer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.

Kilhoffer, M.-C., Demaille, J. G., & Gérard, D. (1981) *Biochemistry 20*, 4407–4414.

Koller, M., & Strehler, E. E. (1988) *FEBS Lett. 239*, 121–128.

Lakowicz, J. R. (1983) in *Principles of fluorescence spectroscopy*, pp 342–381, Plenum Press, New York.

Luan-Rilliet, Y., Milos, M., & Cox, J. A. (1992) *Eur. J. Biochem. 208*, 133–138.

Maune, J. F., Klee, C. B., & Beckingham, K. (1992a) *J. Biol. Chem. 267*, 5286–5295.

Maune, J. F., Beckingham, K., Martin, S. R., & Bayley, P. M. (1992b) *Biochemistry 31*, 7779–7786.

Milos, M., Schaer, J.-J., Comte, M., & Cox, J. A. (1986) *Biochemistry 25*, 6279–6287.

Nakamura, T., Fujita, K., Eguchi, Y., & Yazawa, M. (1984) *J. Biochem 95*, 1551–1557.

Nojima, H. (1989) *J. Mol. Biol. 208*, 269–282.

Nojima, H., & Sokabe, H. (1986) *J. Mol. Biol. 190*, 391–400.

Perrin, D. D., & Sayce, I. G. (1967) *Talanta 14*, 833–842.

Rhyner, J. A., Koller, M., Durussel-Gerber, I., Cox, J. A., & Strehler, E. E. (1992) *Biochemistry 31*, 12826–12832.

Riddles, P. W., Blakeley, R. L., & Zerner, B. (1983) *Methods Enzymol. 91*, 49–60.

Starovasnik, M. A., Su, D.-R., Beckingham, K., & Klevit, R. E. (1992) *Protein Sci. 1*, 245–253.

Stein, J. P., Munjaal, R. P., Lagacé, L., Lai, E. C., O'Malley, B. W., & Means, A. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6485–6489.

Strynadka, N. C. J., & James, M. N. G. (1989) *Annu. Rev. Biochem. 58*, 951–998.

Taylor, D. A., Sacks, J. S., Maune, J. F., Beckingham, K., & Quiocho, F. A. (1991) *J. Biol. Chem. 266*, 21375–21380.

Wylie, D. C., & Vanaman, T. C. (1988) in *Molecular aspects of cell regulation: Calmodulin* (Cohen, P., & Klee, C. B., Eds.) Vol. 5, pp 1–15, Elsevier, Amsterdam, New York, Oxford.

Yaswen, P., Smoll, A., Peehl, D. M., Trask, D. K., Sager, R., & Stampfer, M. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7360–7364.

Yaswen, P., Smoll, A., Hosoda, J., Parry, G., & Stampfer, M. R. (1992) *Cell Growth Differ. 3*, 335–345.

Yazawa, M., Kawamura, E., Minowa, O., Yagi, K., Ikura, M., & Hikichi, K. (1984) *J. Biochem. 95*, 443–446.

Yoshida, M., Minowa, O., & Yagi, K. (1983) *J. Biochem. 94*, 1925–1933.