Calcium-Binding Properties of a Calcium-Dependent Protein Kinase from *Plasmodium falciparum* and the Significance of Individual Calcium-Binding Sites for Kinase Activation†

Yi Zhao,‡,§ Sabine Pokutta,‖ Patrik Maurer,‖ Meinrad Lindt,‡ Richard M. Franklin,*,‡ and Barbara Kappes‡

Department of Structural Biology and Department of Biophysical Chemistry, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Received September 29, 1993; Revised Manuscript Received January 19, 1994®

ABSTRACT: Calcium-dependent protein kinase from *Plasmodium falciparum* (PfCPK) is a multidomain protein composed of an N-terminal kinase domain connected via a linker region to a C-terminal CaM-like calcium-binding domain. The kinase can be activated by Ca^{2+} alone and associates with $^{45}Ca^{2+}$. Here we describe the calcium-binding properties of the kinase and the significance of the individual calcium-binding sites with respect to enzymatic activation, as well as the Ca^{2+}-induced conformational change as detected by circular dichroism. As predicted from the cDNA sequence, the kinase has four EF-hand calcium-binding sites in the C-terminal domain. To understand the roles of the individual calcium-binding sites, two series of mutations were generated at the individual EF-hand motifs. The highly conserved glutamic acid residue at position 12 in each calcium-binding loop was mutated to either lysine or glutamine, and therefore a total of eight mutants were generated. Either of these mutations (to lysine or glutamine) is sufficient to eliminate calcium binding at the mutated site. Sites I and II appear to be crucial for both Ca^{2+}-induced conformational change and enzymatic activation. Whereas mutations at site II almost completely abolish kinase activity, mutations at site I are also deleterious and dramatically reduce the sensitivity of the Ca^{2+}-induced conformational change and the Ca^{2+}-dependent activation. Mutations at sites III and IV have minor effects.

Calcium controls a variety of cellular processes. The calcium signal is transduced into an intracellular response in part by calcium-binding proteins. These proteins are thought to be involved in the regulation of many cellular events such as certain enzyme activities and calcium translocation. Most of the calcium-modulated proteins contain structural features of the EF-hand (Kretsinger & Nockolds, 1973). Among the EF-hand calcium-binding proteins are calmodulin (CaM),[1] troponin C (TnC), parvalbumin, and myosin light chains. All members of this protein family exhibit a common structural motif, and multiple copies of the calcium-binding site are present. Each of these motifs consists of a loop of 12 amino acid residues that is flanked by two α-helices. From the structural information, the putative EF-hand-dependent calcium-binding ability of a protein could be predicted on the basis of the amino acid or cDNA sequence. Most known EF-hand calcium-binding proteins possess two or four copies of the helix–loop–helix motif; two motifs interact with each other, and this seems to enhance the affinity for calcium.

Some enzymes possess EF-hand calcium-binding sequences as a domain distinct from the catalytic domain. These include the Ca^{2+}-dependent protease calpain (Ohno et al., 1984; Ermori et al., 1986) and the diacylglycerol kinase (Sakane et al., 1990). Protein kinases which were activated by Ca^{2+} alone were described in *Paramecium tetrauvelia* (Gundersen & Nelson, 1987; Son et al., 1993) and soybean (Putnam-Evans et al., 1990). Harper and co-workers (1991) have isolated the cDNA corresponding to the soybean Ca^{2+}-dependent protein kinase. The amino acid sequence deduced from the cDNA exhibits an N-terminal kinase catalytic domain and a CaM-like structure at its C-terminus. Recently, we isolated a protein kinase gene from *Plasmodium falciparum* (PfCPK) in which the protein kinase and a CaM-like regulatory element are combined in a single polypeptide (Zhao et al., 1993a) and which has a gene structure similar to that of the soybean Ca^{2+}-dependent protein kinase. The full-length coding region of PfCPK has been expressed in *Escherichia coli* and the recombinant protein has a Ca^{2+}-dependent kinase activity and can associate with $^{45}Ca^{2+}$ (Zhao et al., 1993a,b).

Here we report that, under saturating conditions, the malarial Ca^{2+}-dependent protein kinase can bind four calcium ions per molecule. In order to learn more concerning the roles of the individual calcium-binding sites of the kinase, we prepared a series of mutants in each of which calcium binding was prevented at one specific site. We chose to mutate a highly conserved glutamic acid residue at position 12 of each of the calcium-binding loops. The refinement of the crystal structure of CaM (Babu et al., 1988) has delineated the crucial role of this glutamic acid residue in calcium binding. Among the amino acid residues which coordinate the Ca^{2+} ion, this glutamic acid residue at position 12 provides two carboxylate oxygen ligands to the metal ion. Two alternative substitutions for the glutamic acid residue (lysine and glutamine) have been shown to effectively eliminate Ca^{2+} binding at the mutated site over the Ca^{2+} concentration range at which CaM

† This work was supported in part by funds from the Canton City of Basel, the Swiss National Fund (Grant 31-29969.90), and the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR).

‡ Department of Structural Biology.

§ Present address: Harvard Medical School, Beth Israel Hospital, Division of Endocrinology & Metabolism, 330 Brookline Ave., Boston, MA 02215.

‖ Department of Biophysical Chemistry.

® Abstract published in *Advance ACS Abstracts,* March 1, 1994.

[1] Abbreviations: NTA, nitrilotriacetic acid; IPTG, isopropyl 1-thio-β-D-galactopyranoside; DTT, 1,4-dithio-DL-threitol; PfCPK, a Ca^{2+}-dependent protein kinase from *Plasmodium falciparum*; CaM, calmodulin; TnC, troponin C; MLCK, myosin light chain kinase; CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase.

0006-2960/94/0433-3714$04.50/0

Table 1: Nomenclature and Mutations at Position 12 (E) of the Loops of Individual Calcium-Binding Sites of PfCPK

mutant	mutated site	residue changed	mutagenic oligonucleotide[a]
EK-I	I	E → K	5′ GATAAGAAGAAATTGATTGA 3′
EK-II	II	E → K	5′ GAATATTCAAAGTTTATTTCTGT 3′
EK-III	III	E → K	5′ CGAAGGAAAAATTAGCA 3′
EK-IV	IV	E → K	5′ TGACTTTGATAAATTCGTT 3′
EQ-I	I	E → Q	5′ GATAAGAAGCAATTGATTGA 3′
EQ-II	II	E → Q	5′ GAATATTCACAGTTTATTTCTGT 3′
EQ-III	III	E → Q	5′ CGAAGGAACAATTAGCA 3′
EQ-IV	IV	E → Q	5′ TGACTTTGATCAATTCGTT 3′

[a] The nucleotide G was changed to the underlined nucleotide A or C at each calcium-binding site in order to change the codon for E to the codon for K or Q.

functions (Maune et al., 1992). To study how Ca^{2+} binding to each of the Ca^{2+}- binding sites contributes to the generation of a conformation able to activate the kinase, we used the same two substitutions for each putative calcium-binding site.

EXPERIMENTAL PROCEDURES

Plasmid Reconstruction and Site-Directed Mutagenesis. Restriction enzyme digestion, ligation for plasmid reconstruction, and other recombinant DNA techniques were carried out according to standard protocols (Ausubel et al., 1987; Sambrook et al., 1989). All mutants were derived from the previously described construct (Zhao et al., 1993a), in which the full-length coding region of PfCPK cDNA was inserted in the *Hin*dIII/*Eco*RI site of pBluescript SK(+) (Stratagene) with a PCR-generated fragment containing a *Hin*dIII and a *Bam*HI site directly upstream of the initiation codon. For mutagenesis, the full-length coding region was released from pBluescript by restriction enzymes *Bam*HI and *Pst*I and then subcloned into the phagemid pAlter-1 (Promega), which contains a gene for an inactivated ampicillin resistance. Single-stranded DNA (ssDNA) templates for mutagenesis were produced with helper phage R408. The ampicillin repair oligonucleotide plus one of the mutagenic oligonucleotides (Table 1) were annealed to the ssDNA template, and subsequent synthesis and ligation of the mutant strand linked the two oligonucleotides. The resultant DNA was transformed into a repair minus strain of *E. coli* (BMH71-18 mut S), and then the cells were grown in the presence of ampicillin. A second round of transformation in JM109 was carried out to ensure proper segregation of mutant and wild-type plasmids. All mutations were verified by dideoxy sequencing using Sequenase, version 2 (United States Biochemical).

Expression and Purification of Wild-Type and Mutant PfCPK. After mutagenesis, the mutated cDNA of PfCPK was transferred into an expression vector (pDS56/RBSII, 6xHis) (Stueber et al., 1990) and expressed in *E. coli* strain SG13009 (Gottesmann et al., 1981), with induction by isopropyl 1-thio-β-D-galactopyranoside (IPTG), as previously described (Zhao et al., 1993a). Five- to ten-liter fermentations were carried out for each mutant. The recombinant proteins were purified by affinity chromatography on nickel–NTA agarose and then gel filtration on a Sephacryl S-300 column, as described (Zhao et al., 1993b). For all of the mutant PfCPK, proteins were purified to homogeneity as judged by SDS gel electrophoresis. Glycerol was added to the purified proteins to a final concentration of 10%, and samples were divided into aliquots and stored at −70 °C after rapid freezing in liquid nitrogen.

Protein Kinase Assays. Protein kinase activity was determined by measuring the incorporation of the ^{32}P of [γ-^{32}P]ATP into casein as substrate. The assays were carried out in a final volume of 35 μL of a mixture of 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM [γ-^{32}P]-ATP (about 1.2×10^3 cpm/pmol), 0.6 mg/mL casein (Sigma), and 100–200 ng of enzyme, containing either 0.1 mM EGTA or a variable concentration of Ca^{2+}. The reactions were initiated by the addition of [γ-^{32}P]ATP, incubated for 5 min, and stopped by pipetting 25-μL aliquots of the incubation mixtures onto 3-cm^2 pieces of P-81 phosphocellulose paper (Whatman). After five washes in 150 mM phosphoric acid, the ^{32}P radioactivity absorbed on the paper was determined by liquid scintillation counting. Variable Ca^{2+} concentrations were used in the kinase assays, as previously described (Zhao et al., 1993b).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Proteins were analyzed by SDS–PAGE using the system described by Laemmli (1970). Samples were boiled for 3–4 min in sample buffer containing either 5 mM $CaCl_2$ or 5 mM EGTA before being loaded onto the gels. No Ca^{2+} or EGTA was added in the gels or the tray buffer.

Protein Concentration Determinations. Protein concentrations were measured by a modified Lowry method (Sandermann & Strominger, 1972), with bovine serum albumin as standard, or calculated by optical density at 280 nm using the extinction coefficient 52 860 M^{-1} cm^{-1}. The resulting values obtained by both methods were almost identical.

Ca^{2+} Determinations. Levels of free Ca^{2+} ion in solution were determined by using the fluorescent dye Fluo-3 (Molecular Probes Inc.) as an indicator. Fluorescence was measured with a Jasco FP-777 (Jasco Co., Tokyo) spectrophotometer. Fluorescence titrations and evaluation of the data were done according to Eberhard and Erne (1991).

Removal of Contaminating Divalent Metal Ions. Plasticware, dialysis membranes, and the buffer solutions for use in the experiments involving quantitative studies of Ca^{2+} binding were treated according to published procedures (Potter et al., 1983; Bazzi & Nelsestuen, 1990). Calcium was removed from buffers and protein solutions by passing them through a Chelex 100 column (Bio-Rad) or an EDTA–agarose column (Eberhard & Erne, 1991). The contaminating calcium in buffers and protein solutions used in equilibrium dialysis experiments was lower than 0.5 μM.

Calcium-Binding Studies. Calcium binding was determined by equilibrium dialysis using a microdialysis system with a chamber volume of 250 μL (Englund et al., 1969). One hundred fifty microliter protein aliquots (10–60 μM) was dialyzed for 24 h at 23 °C against the same volume of standard buffer (50 mM Tris-HCl and 150 mM NaCl, pH 7.4) containing $^{45}Ca^{2+}$ (0.14 μCi mL^{-1}) and varying concentrations (up to 2000 μM) of unlabeled Ca^{2+}. The enzyme did not lose activity during dialysis. At the end of the dialysis period, 100-μL aliquots were removed from each side of the dialysis membrane and used to determine the radioactivity in a liquid scintillation spectrometer. With the protein concentration and the initial calcium concentration known, the moles of bound calcium per mole of protein and the concentration of unbound calcium could be evaluated.

The calcium-binding data were analyzed with the following theoretical model of ligand binding to multiple classes of binding sites:

$$B = \sum_i \frac{n_i[Ca^{2+}]/K_{d_i}}{1 + [Ca^{2+}]/K_{d_i}}$$

where B is the moles of bound Ca^{2+} per mole of protein, n is

A:

B:

Ca^{2+}-binding loop	Position	Sequence
I	385-396	D K N G D G Q L D K K E
II	429-440	D F D K N G Y I E Y S E
III	465-476	D T D K S G K I T K E E
IV	499-510	D K N K D N M I D F D E
		x y z -y -x -z

FIGURE 1: (A) The domain structure and (B) the positions and amino acid sequences of the loop regions of calcium-binding sites I, II, III, and IV of PfCPK.

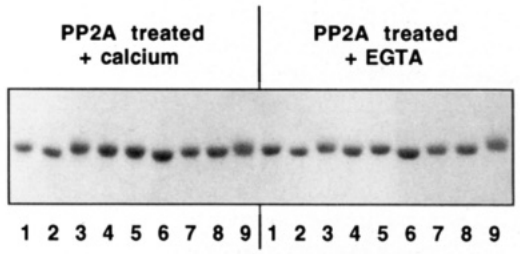

FIGURE 2: Effect of Ca^{2+} and EGTA on the electrophoretic mobility of the wild-type and mutant PfCPK in SDS–PAGE. The wild-type and mutant PfCPK proteins were analyzed after PP2A treatment. Lanes: 1, EK-I; 2, EK-II; 3, EK-III; 4, EK-IV; 5, EQ-I; 6, EQ-II; 7, EQ-III; 8, EQ-IV; 9, wild-type. Samples (3 μg per lane) were electrophoresed on an 8% SDS–PAGE gel. Either 5 mM $CaCl_2$ (PP2A treated + calcium) or 5 mM EGTA (PP2A treated + EGTA) was included in the electrophoresis sample buffer.

the number of Ca^{2+} binding sites of each class, $[Ca^{2+}]$ is the concentration of free calcium, and K_{d_i} is the dissociation constant of the i^{th} class. The equation was fitted to the experimental data by using a Marquart nonlinear least-squares fit procedure with the computer program Cosy (Eberhard, 1990). The 95% confidence limit of χ^2 was used as the goodness of fit criterion.

Circular Dichroism Measurements. Circular dichroism spectra were recorded at 25 °C using a Jasco J720 spectropolarimeter. Spectra were recorded in a 1-mm quartz cell at a protein concentration of 10 μM. Calcium titrations at 222 nm were carried out in a 5-mm cell at a protein concentration of 2 μM in 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl. Aliquots of a calcium stock solution were added to the decalcified protein, and measurements were made after a 5-min interval for equilibration. Three to six independent titrations were done for each protein studied. The molar ellipticity $[\Theta]$ (expressed in deg cm^2 $dmol^{-1}$) was calculated on the basis of a mean residue molecular mass of 110 Da. The calcium-induced change $\Delta[\Theta]_{222}$ was calculated according to $\Delta[\Theta]_{222} = ([\Theta](\text{Ca bound}) - [\Theta](\text{Ca free})) / [\Theta](\text{Ca bound})$.

RESULTS

Mutation and Purification of the Mutant PfCPK. The domain structure of PfCPK and the sequences of the 12 amino acid residues that form the calcium-binding loops, which are located at the C-terminus of the Ca^{2+}-dependent protein kinase, are shown in Figure 1. The highly conserved glutamic acid residue at position 12, which provides two carboxylate oxygen ligands to Ca^{2+} and is known to be crucial in calcium binding, was chosen for site-directed mutagenesis. We substituted (i) lysine (K) or (ii) glutamine (Q) for this glutamic

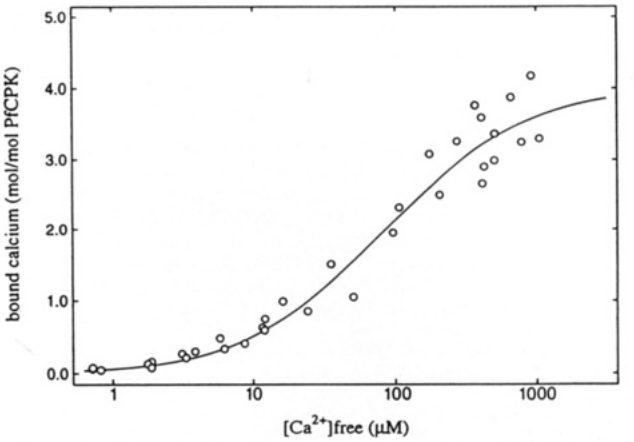

FIGURE 3: Ca^{2+} binding to recombinant wild-type PfCPK. The calcium-binding data were obtained by equilibrium dialysis. The line represents the best fit to these data using a model of ligand binding to a single class of binding sites.

acid residue at each calcium-binding site. The mutations made using synthetic oligonucleotides at each calcium-binding site and the nomenclature of each mutant used in this paper are shown in Table 1. Purification of all of these mutant proteins was achieved by affinity chromatography on a nickel–NTA column followed by gel filtration on Sephacryl S-300, as described previously (Zhao et al., 1993b). Affinity chromatography on a nickel–NTA column is based on the affinity to the six histidines at the N-terminus of these mutant proteins. Most of the mutant proteins have an affinity to the nickel–NTA column similar to that of the wild-type protein, but mutants EK-I and EK-II have a weaker affinity than the wild-type protein. Lower yields of these two mutants were obtained under the same conditions as for the purification of wild-type protein (33 mM imidazole in the washing step). The optimal concentration of imidazole for the washing step of these mutants was 28 mM. All of the mutant PfCPKs were purified to homogeneity, as judged by Coomassie blue stained SDS–PAGE.

Effect of Ca^{2+} on the Electrophoretic Mobility of the Wild-Type and Mutant PfCPKs on SDS–PAGE. CaM and a Ca^{2+}-dependent protein kinase from soybean have an increased mobility on SDS–PAGE when they are denatured in the presence of Ca^{2+} relative to that when denatured in the presence of EGTA (Klee et al., 1979; Harmon et al., 1987). This effect is not understood [cf. Maune et al. (1992)]; it might be due to the fact that some proteins still bind calcium in the presence of SDS and thereby assume a more compact form and a higher mobility (Burgess et al., 1980). The recombinant PfCPK becomes autophosphorylated during bacterial expression, and the autophosphorylated kinase migrates slower than the kinase treated with the catalytic subunit of protein phosphatase 2A (PP2A) (Zhao et al., 1993b). Since most of the mutants have a reduced kinase activity (see below), which could influence their autophosphorylation status and thereby their electrophoretic mobility, we treated all mutant proteins and the wild-type protein with the PP2A catalytic subunit. Whereas differences in electrophoretic mobility on SDS–PAGE were observed with the untreated recombinant proteins (data not shown), these proteins migrated at slightly different rates after PP2A treatment. In order to see whether Ca^{2+} affects the electrophoretic mobility of PfCPK and the mutant proteins, PP2A-treated wild-type protein and mutants of PfCPK were denatured in the presence of 5 mM Ca^{2+} or 5 mM EGTA prior to analysis by SDS–PAGE . No differences in electrophoretic mobility were observed for the individual proteins under the two conditions of denaturation (Figure 2).

Table 2: Biophysical and Biochemical Parameters of Wild-Type and Mutant PfCPK

	calcium binding		kinase activity	circular dichroism	
protein	no. of Ca^{2+}-binding sites[a]	K_d [μM][a]	$K_{0.5}$[b] [μM] for Ca^{2+}	$\Delta[\Theta]_{222}$ [%][c]	$K_{0.5}$ [μM][d]
wild type	3.9	80	15	16	25 ± 15
EK-I	2.3	330	350	16	600 ± 200
EK-II	2.2	210	*e*	17	400 ± 200
EK-III	2.9	40	15	14	40 ± 15
EK-IV	3.2	120	77	15	35 ± 15
EQ-I	1.9	90	260	19	1200 ± 300
EQ-II	3.4	210	>1000	19	30 ± 15
EQ-III	2.7	35	15	17	20 ± 15
EQ-IV	2.7	110	41	20	30 ± 15

[a] Binding parameters are obtained from best fits of the experimental data. The data analyses were based on the model of non-cooperative binding to equivalent binding sites and a single class binding site model. [b] The kinase activity obtained for each protein in the presence of 1 mM Ca^{2+} was set as full activity. $K_{0.5}$ is the concentration of Ca^{2+} required for half-maximal kinase activity. Since mutants EK-II and EQ-II have very low kinase activity and no apparent Ca^{2+}-dependent elevated activity in the presence of up to 1 mM Ca^{2+}, the exact $K_{0.5}$ for these two mutants was not determined. [c] $\Delta[\Theta]_{222}$ was calculated from the intensities of the CD band at 222 nm in the presence (5 mM) and absence of calcium. The error in $\Delta[\Theta]_{222}$ is about ±3%. [d] $K_{0.5}$ is the concentration of calcium required for a half-maximal change of ellipticity. [e] This mutant has no kinase activity in the presence (up to 5 mM) or absence of Ca^{2+}.

Ca^{2+}-Binding Properties of Wild-Type and Mutant PfCPK. Ca^{2+} binding to recombinant wild-type and mutant PfCPK was determined by equilibrium dialysis. The binding parameters were evaluated by fitting the model for ligand binding to a single class of binding sites to the experimental data. Although models which assume two or more classes of binding sites fit the data with the same goodness of fit, the quality of our data does not allow us to discriminate between the different binding models. Therefore we chose the model with the least assumptions.

The wild-type protein binds 4 mol of Ca^{2+} per mole of protein with a K_d of 80 μM (Figure 3). The K_d was obtained by assuming a model of one type of binding site. Addition of Mg^{2+} (10 mM) had only a minor effect on the stoichiometry of Ca^{2+} binding to the wild-type protein (data not shown), indicating that all four sites are calcium specific. The calcium-binding data of the mutant proteins were also analyzed with the single class binding site model (Table 2; Figure 4). Mutation of the glutamic acid residue in position 12 of the calcium-binding loops to lysine or glutamine always eliminates one binding site. But in the cases of EK-I, EQ-I, and EK-II, the binding capacity was reduced to about 2 mol of calcium per mole of protein at the free calcium concentration of 1 mM (Table 2; Figure 4). The K_d values of calcium binding to the mutant proteins vary between 35 and 330 μM, depending on the mutant. Whereas there are quantitatively similar changes of dissociation constants for the E to K and E to Q mutations at sites II–IV, at site I the affinity depended on the mutation (Table 2).

Kinase Activities and Ca^{2+}-Dependent Activations of the Mutant PfCPK. The effects of mutation of the individual calcium-binding sites on the kinase activity and the Ca^{2+}-dependent enzymatic activation were investigated. In all cases Ca^{2+} is an absolute requirement for activating the kinase. Except for mutants EK-III and EQ-I, all mutants have a lower maximal kinase activity than that of wild-type PfCPK and mutations at site II almost completely abolish activity of the kinase to phosphorylate exogenous substrate at concentrations of Ca^{2+} up to 1 mM. Both mutations at the individual calcium-binding sites had a similar influence on activation of the Ca^{2+}-dependent kinase (Figure 5). The mutants EK-II and EQ-II were enzymatically inactive at Ca^{2+} concentrations up to 1 mM, and exact values of $K_{0.5}$ for Ca^{2+} could not be determined (Table 2). Even at higher Ca^{2+} concentrations (2–5 mM) in the kinase assay buffer, both mutants were only slightly activated (kinase activity < 5% of that of the wild-type protein). Mutations at site I strongly reduce the sensitivity of the enzyme to Ca^{2+}. Mutations at site IV have only a slight effect, and

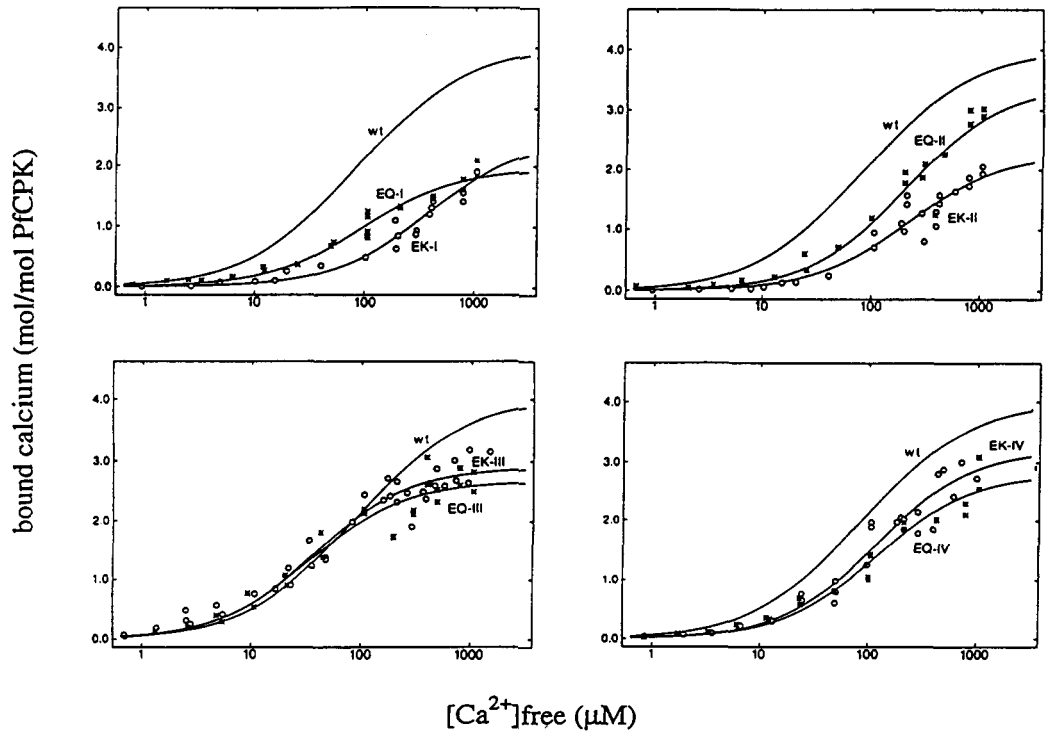

FIGURE 4: Calcium binding to mutant PfCPK. Calcium binding was determined by equilibrium dialysis and analyzed in the same way as described for the wild-type protein. For comparison, the fitted curve of the wild-type protein is shown in each graph. Open circles represent the calcium binding for the E to K mutations, and stars represent the data obtained for the E to Q mutations.

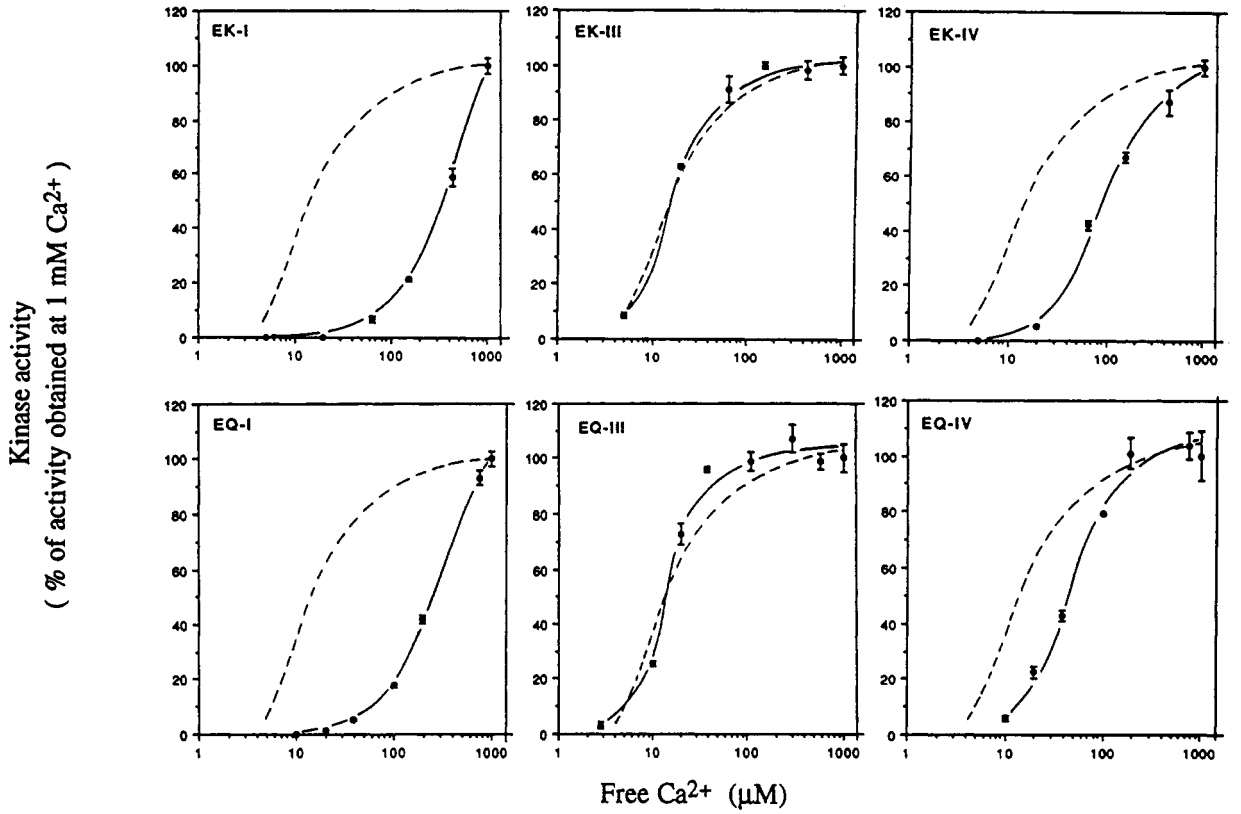

FIGURE 5: Ca^{2+}-dependent activation of the mutant PfCPK. Kinase assays were done at pH 7.4 using casein as substrate. Kinase activity is calculated as a percentage of the activity obtained at 1 mM Ca^{2+}. The specific activities obtained at 1 mM Ca^{2+} for each protein are shown in Table 2. Each point is the mean ± SE of 3–6 determinations. Since the mutations at calcium-binding site II (EK-II and EQ-II) almost completely abolish the kinase activity up to 1 mM Ca^{2+}, the Ca^{2+}-dependent activation is not shown for these two mutants. The dashed curves show the Ca^{2+}-dependent activation for the wild-type protein.

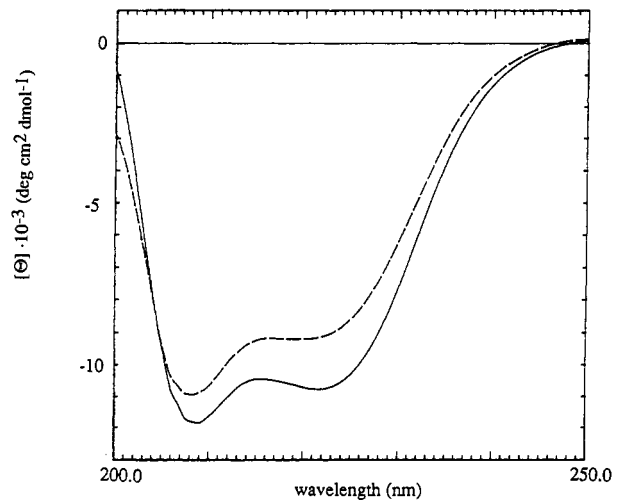

FIGURE 6: Circular dichroism spectra of PfCPK in the presence and absence of calcium. Spectra were recorded in a 1-mm quartz cell at a protein concentration of 10 μM in 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl for calcium-depleted (solid line) and calcium-saturated (1 mM, dashed line) PfCPK.

those at site III have no effect on the Ca^{2+}-dependent kinase activation.

Circular Dichroism Measurements. The far-UV CD spectra of wild-type PfCPK are characterized by bands at 209 and 222 nm (Figure 6). The α-helical contribution can be roughly estimated from the intensity of the latter band to be about 34% (Chen et al., 1972). Calcium induces a 16% decrease of the mean molar ellipticity. This change was fully reversible when the calcium-loaded form of PfCPK was decalcified again on the EDTA–agarose column (data not shown). The calcium-induced decrease of α-helicity is not reversible by addition of excess EDTA or EGTA, due perhaps to slow dissociation kinetics or binding of the chelators to the protein. The CD spectra of all 8 mutants are very similar to that of wild-type protein (data not shown). In addition, the calcium-induced changes of the mean molar ellipticity at 222 nm are indistinguishable from that of the wild-type protein (Table 2). Thus the mutants have a secondary structure similar to that of the wild-type protein in both the calcium-free and the calcium-loaded forms, and the effect of the mutations on the overall backbone structure is rather small.

The signal at 222 nm was used to evaluate the calcium dependence of the conformational change (Figure 7). The mutations at sites III and IV and the EQ-II mutation have no effect on the sensitivity of the conformational change toward calcium. The half-maximal change ($K_{0.5}$) is induced by about 30 μM calcium, as was the case for the wild-type protein. In contrast, a 20-fold decrease in the apparent affinity resulted from the E to K mutations at sites I and II, and a 40-fold decrease was observed in the EQ-I mutant (Table 2). For all proteins except EQ-I, about 85% (±10%) of the conformational change is completed when two calcium ions are bound.

DISCUSSION

The carboxy-terminal domain of PfCPK has a 32–38% amino acid identity with CaMs and TnCs. Four EF-hand calcium-binding motifs have been identified (Zhao et al., 1993a). The kinase requires Ca^{2+} for enzymatic activation and associates with $^{45}Ca^{2+}$ (Zhao et al., 1993b). The purpose of this study was to investigate the Ca^{2+}-binding properties of PfCPK and the influence of the individual sites on kinase activation.

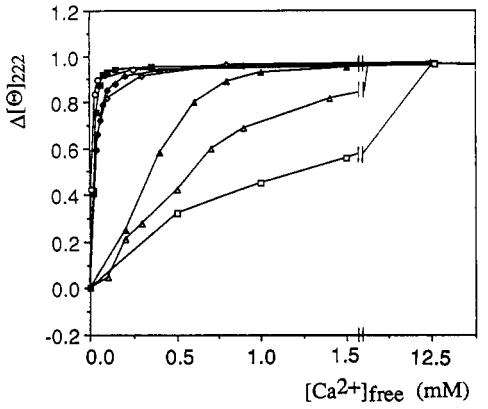

FIGURE 7: Ca^{2+} binding to PfCPK as monitored by circular dichroism. The fraction $\Delta[\Theta]_{222}$ of the total change was determined by titration with Ca^{2+} at 222 nm for wild-type PfCPK (■), EK-I (▲), EQ-I (□), EK-II (▲), EQ-II (◇), EQ-III (○), and EQ-IV (◆). Mutants EK-III and EK-IV are not included because the curves are nearly identical to the wild-type curve.

Our data for the wild-type protein demonstrate that one molecule of PfCPK binds four calcium ions under saturation conditions. The mean calcium dissociation constant for CaM is 4 μM (Kilhoffer et al., 1992a), and the Ca^{2+} affinity is even increased in the presence of target proteins (Olwin & Storm, 1985; Haiech et al., 1991; Yazawa et al., 1992). The mean $K_d(Ca^{2+})$ for PfCPK is about 80 μM, and this is significantly different from CaM since in PfCPK the CaM domain is always connected to its substrate, the kinase domain. Although the K_d of *P. falciparum* CaM is not known, the lower affinity of PfCPK suggests the possibility of a calcium regulation of PfCPK independent from the CaM pathway.

The kinase activity is strongly calcium dependent and saturates at a calcium concentration of about 150 μM, corresponding to two calcium ions bound per mole of PfCPK (Figure 8). The calcium dependence of the enzymatic activity is roughly parallel to the conformational change, which reaches 85% of the total change when two calcium ions are bound.

In order to investigate the characteristics of the individual sites, we prepared two series of mutant proteins, in each of which calcium binding at one specific site was prevented by changing the highly conserved glutamic acid residue at position 12 of the calcium-coordinating loop to either lysine or glutamine. Both the mutation to lysine and the more conservative change to glutamine are effective in eliminating at least one calcium-binding site, a result comparable to that achieved with the same site-directed mutations of CaM (Maune et al., 1992). Haiech et al. (1991) have specifically replaced the E at the same position in the EF-hand motifs of CaM with A. The mutation in one EF-hand motif reduced the binding affinity for two of the Ca^{2+} ions 60–300-fold, but the affinity could be recovered to wild-type values in the presence of a peptide corresponding to the CaM-binding region of myosin light chain kinase, MLCK. The mechanism of this effect remains to be elucidated (Kilhoffer et al., 1992b). In contrast to these results with A mutations in CaM, the reduced affinities of the mutated sites of PfCPK are not recoverable.

The mutations at sites III and IV of PfCPK have very similar effects, namely, the elimination of one binding site and only a slight influence on the calcium dependence of kinase activation and the calcium-induced conformational change. Nonetheless the effect on calcium affinity is different for mutations at sites III and IV. While both site III mutants have an increased mean affinity, the mutations at site IV decrease it slightly. Furthermore, in the mutants EK-I, EK-II, and EQ-I, the affinity of a second site is so far reduced that it cannot be saturated with 1 mM Ca^{2+}. Numerous studies on the mechanism of calcium binding to CaM have demonstrated cooperative interactions between the paired motifs of one domain [e.g., Linse et al. 1991)] and at least for some cases also between the two domains [e.g., Yazawa et al. (1992) and Starovasnik et al. (1993)]. Thus interactions of the calcium-binding motifs presumably also influence the affinities of one or several other site(s) in PfCPK. Although the equilibrium dialysis data do not allow us to determine the individual calcium dissociation constants of the four sites, a comparison of the properties of the different mutants enables us to elucidate the importance of individual sites in the calcium activation of PfCPK.

Mutations at site I drastically reduce the sensitivity of the kinase activity for calcium. Both site II mutants are completly inactive. The mutants at sites I and II are still able to undergo a structural rearrangement upon calcium binding, however, but at much higher calcium concentrations than those required for the wild-type protein. The EQ-II mutant behavior differs from that of the other mutants at sites I and II. It still binds three calcium ions, and the calcium-dependent conformational change is similar to that of the wild-type protein. Perhaps interaction of the mutated site at EQ-II with some other site is not disturbed, and therefore this mutant behaves like the wild-type protein with respect to the conformational change. The other mutations at sites I and II affect the correct interactions between two calcium-binding motifs, thus de-

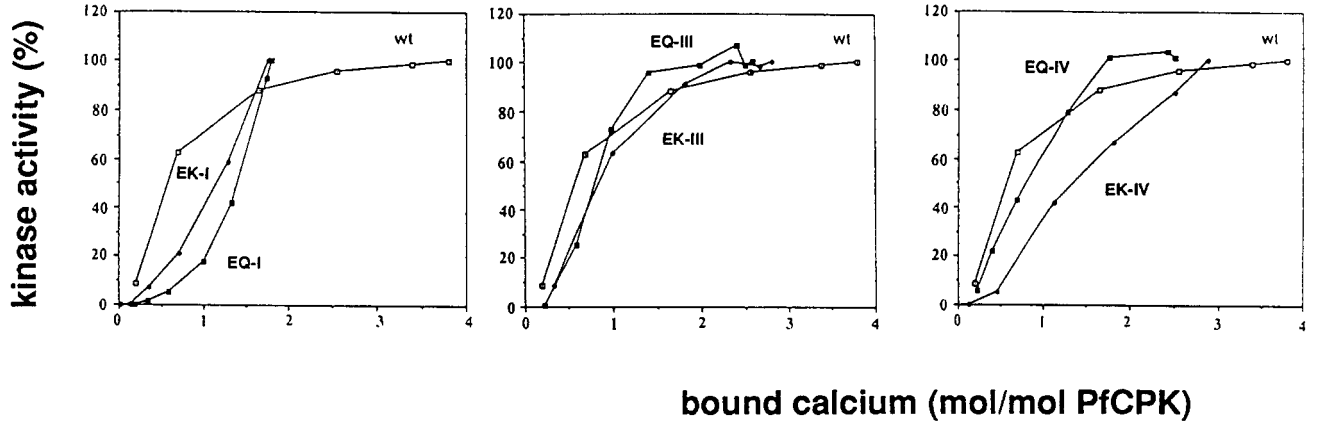

FIGURE 8: Correlation between the kinase activity and the number of calcium ions bound to PfCPK. The number of calcium ions bound was determined by equilibrium dialysis; the kinase activity was measured as described under Experimental Procedures. For every mutation the curve for the wild-type protein is shown for comparison (□).

creasing the affinity of the other site(s) and the sensitivity of the calcium-induced conformational change. Since the circular dichroism signal at 222 nm monitors only an overall secondary structure, the calcium induced change of ellipticity cannot be assigned to certain domains of PfCPK or to the binding of calcium to an individual EF-hand motif. The EQ-II mutant is completely inactive despite the conformational change, which is apparently the same as for the wild-type protein. Assuming the secondary structures of the calcium-free and calcium-bound forms of EQ-II and wild-type protein to be the same, one can conclude that the conformational change alone is not sufficient for kinase activation. This has also been shown for mutant CaMs able to bind to, but not to activate, target enzymes (George et al., 1990; VanBerkum & Means, 1991). Irrespective of this, the data clearly indicate that a bound calcium at site II is essential for kinase activation.

The recent elucidation of the three-dimensional structure of CaM bound to a peptide corresponding to the CaM-binding region of MLCK provides a molecular basis for understanding the interaction of CaM with its target enzymes (Ikura et al., 1992; Meador et al., 1992). The target peptide is α-helical, and the central helix of CaM is bent and twisted, allowing the globular domains to engulf the target peptide. The complex is stabilized through multiple hydrophobic contacts as well as hydrogen bonds and salt bridges (Ikura et al., 1992; Meador et al., 1992). This structure explains the results of a variety of mutagenesis studies on essential features of CaM in the interaction with its targets. These features include the importance of methionines (Guerini et al., 1987), charge clusters (Craig et al., 1987; Weber et al., 1989; Shoemaker et al., 1990; Farrar et al., 1993), residues on the external surface of CaM (VanBerkum & Means, 1991), and the central helix (Putkey et al., 1988; Persechini et al., 1989; VanBerkum et al., 1990).

None of the amino acids located in the Ca^{2+}-binding loops are involved in the contacts between CaM and the target peptide. Nevertheless, mutations in the Ca^{2+}-binding loops differentially affect the activation of target enzymes. The activation of MLCK with CaM is not influenced by E to A mutations in position 12 of the EF-hand motifs (Haiech et al., 1991; Kilhoffer et al., 1992b). Upon interaction with this target these mutants recover Ca^{2+}-binding activity (see above) and seem to be capable of forming a complex with a conformation very similar to that of the wild-type CaM. A different result was obtained when the same CaM mutants were tested with respect to regulation of erythrocyte Ca-ATPase (Bzdega & Kosh-Kosicka, 1992). All mutants were able to stimulate Ca-ATPase activity to the same level as wild-type CaM. The concentrations of mutant CaM required for half-maximal activation were significantly higher than for unmodulated CaM and were strongly dependent on the domain in which the mutated E was located. Mutations in the C-terminal EF-hand motifs had more pronounced effects than the mutations in the N-terminal motifs, in contrast to our results on PfCPK.

A site-dependent activation and different effects on several enzymes (smooth and skeletal MLCK, adenylylcyclase, Ca-ATPase) have also been found for a series of *Drosophila* CaM mutants with the same E to K and E to Q substitutions as used in our study (Gao et al., 1993). In general, as with the PfCPK mutants, both mutations at a given site had similar effects on a given enzyme and all mutants had reduced affinity for, and reduced ability to activate, the four enzymes investigated. The *Drosophila* CaM EK-I mutant was exceptional since it possessed a wild-type-like activation. For three of the enzymes (smooth and skeletal MLCK, adenylylcyclase) the activation patterns with the two mutant series were very similar. Mutations of site IV were most deleterious, then site II, site III, and finally site I, a ranking also observed in Ca^{2+}-binding and Ca^{2+}-induced conformational studies of these enzymes (Maune et al., 1992; Gao et al., 1993; Mukherjea & Beckingham, 1993).

Studies on TnC/CaM hybrids have established the influence of EF-hand motif I of CaM on the activation of different targets (George et al., 1990). One chimera (TaM), in which the EF-hand motif I of CaM was exchanged by the non-Ca^{2+}-binding first motif of TnC, did fully activate 3′,5′-cyclic nucleotide phosphodiesterase, but it activated MLCK and CaM kinase II only poorly. Thus certain enzymes can be activated by a CaM with at least one nonfunctional Ca^{2+}-binding site, a conclusion also drawn from the maximal activation of Ca-ATPase by the isolated C-terminal fragment of CaM (Guerini et al., 1984). The poor activation of MLCK and CaM kinase II with TaM could not be reversed when Ca^{2+} binding to site I was restored by site-directed mutagenesis. The resulting mutant was actually a potent antagonist of CaM activation (George et al., 1990). Thus the failure of TaM to activate both enzymes was not a result of the lack of Ca^{2+} binding and led to the identification of three amino acids of domain I (not involved in Ca^{2+} binding) essential for the activation of MLCK (VanBerkum & Means, 1991).

The different effects in these and our studies show that the activation of target enzymes by CaM is a complex process controlled by multiple factors, which makes simplistic interpretations and generalizations from one system to another difficult.

Nevertheless, we propose the following mechanism of the calcium-dependent activation of PfCPK. PfCPK binds a maximum of four calcium ions. Enzymatic activity is induced on the binding of two calcium ions, one of which is at site II. Presumably this signal is transduced via a conformational change to the kinase domain. The linker region between the catalytic domain and the CaM-like domain contains a segment showing homology to the regulatory domain of the CaM kinase II. The regulatory domain of this CaM kinase II comprises an autoinhibitory and a CaM-binding domain. The autoinhibitory segment is positioned in the active site, blocking access to its substrates by steric hindrance. In a model put forward by Schulmann, calcium-activated CaM binding displaces this segment and thereby activates the enzyme (Schulman, 1993). A similar control mechanism for kinase activation may exist for PfCPK with the difference that the CaM-like structure is an integral part of the protein.

ACKNOWLEDGMENT

We thank Prof. Jürgen Engel for giving us the opportunity to carry out part of the work in his laboratory and Dr. Marc Eberhard for providing the EDTA–agarose column.

REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1987) *Current Protocols in Molecular Biology*, Greene Publishing Associates, Inc., New York.

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

Bazzi, M. D., & Nelsestuen, G. L. (1990) *Biochemistry 29*, 7624–7630.
Burgess, W. H., Jemiolo, D. K., & Kretsinger, R. H. (1980) *Biochim. Biophys. Acta 623*, 257–270.
Bzdega, T., & Kosk-Kosicka, D. (1992) *J. Biol. Chem. 267*, 4394–4397.
Chen, Y.-H., Yang, J. T., & Martinez, H. M. (1972) *Biochemistry 11*, 4120–4131.
Craig, T. A., Watterson, D. M., Prendergast, F. G., Haiech, J., & Roberts, D. M. (1987) *J. Biol. Chem. 262*, 3278–3284.
Eberhard, M. (1990) *Comput. Appl. Biosci. 6*, 213–221.
Eberhard, M., & Erne, P. (1991) *Eur. J. Biochem. 202*, 1333–1338.
Englund, P. T., Huberman, J. A., Jovin, T. M., & Kronberg, A. (1969) *J. Biol. Chem. 244*, 3038–3044.
Ermori, Y., Kawasaki, H., Imajoh, S., Kawashima, S., & Suzuki, K. (1986) *J. Biol. Chem. 261*, 9472–9476.
Farrar, Y. J. K., Lukas, T. J., Craig, T. A., Watterson, D. M., & Carlson, G. M. (1993) *J. Biol. Chem. 268*, 4120–4125.
Gao, Z. H., Krebs, J., VanBerkum, M. F. A., Tang, W.-J., Maune, J. F., Means, A. R., Stull, J. T., & Beckingham, K. (1993) *J. Biol. Chem. 268*, 20096–20104.
George, S. E., VanBerkum, M. F. A., Ono, T., Cook, R., Hanley, R. M., Putkey, J. A., & Means, A. R. (1990) *J. Biol. Chem. 265*, 9228–9235.
Gottesmann, S., Halpern, E., & Trisler, P. (1981) *J. Bacteriol. 148*, 265–273.
Guerini, D., Krebs, J., & Carafoli, E. (1984) *J. Biol. Chem. 259*, 15172–15177.
Guerini, D., Krebs, J., & Carafoli, E. (1987) *Eur. J. Biochem. 170*, 35–42.
Gundersen, R. E., & Nelson, D. L. (1987) *J. Biol. Chem. 262*, 4602–4609.
Haiech, J., Kilhoffer, M.-C., Lukas, T. J., Craig, T. A., Roberts, D. M., & Watterson, D. M. (1991) *J. Biol. Chem. 266*, 3427–3431.
Harmon, A. C., Putnam-Evans, C., & Cormier, M. J. (1987) *Plant Physiol. 83*, 830–837.
Harper, J. F., Sussman, M. R., Schaller, G. E., Putnam-Evans, C., Charbonneau, H., & Harmon, A. C. (1991) *Science 252*, 951–954.
Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., & Bax, A. (1992) *Science 256*, 632–638.
Kilhoffer, M.-C., Kubina, M., Travers, F., & Haiech, J. (1992a) *Biochemistry 31*, 8098–8106.
Kilhoffer, M.-C., Lukas, T. J., Watterson, D. M., & Haiech, J. (1992b) *Biochim. Biophys. Acta 1160*, 8–15.
Klee, C. B., Crouch, T. H., & Krinks, M. H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6270–6273.
Kretsinger, R. H., & Nockolds, C. E. (1973) *J. Biol. Chem. 248*, 3313–3326.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Linse, S., Helmersson, A., & Forsén, S. (1991) *J. Biol. Chem. 266*, 8050–8054.
Maune, J. F., Klee, C. B., & Beckingham, K. (1992) *J. Biol. Chem. 267*, 5286–5295.
Meador, W. E., Means, A. R., & Quiocho, F. A. (1992) *Science 257*, 1251–1255.
Mukherjea, P., & Beckingham, K. (1993) *Biochem. Mol. Biol. Int. 29*, 555–563.
Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M., & Suzuki, K. (1984) *Nature 312*, 566–570.
Olwin, B. B., & Storm, D. R. (1985) *Biochemistry 24*, 8081–8086.
Persechini, A., Blumenthal, D. K., Jarrett, H. W., Klee, C. B., Hardy, D. O., & Kretsinger, R. H. (1989) *J. Biol. Chem. 264*, 8052–8058.
Potter, J. D., Strang-Brown, P., Walker, P. L., & Iida, S. (1983) *Methods Enzymol. 102*, 135–143.
Putkey, J. A., Ono, T., VanBerkum, M. F. A., & Means, A. R. (1988) *J. Biol. Chem. 263*, 11242–11249.
Putnam-Evans, C. L., Harmon, A. C., & Cormier, M. J. (1990) *Biochemistry 29*, 2488–2495.
Sakane, F., Yamada, K., Kanoh, H., Yokoyama, C., & Tanabe, T. (1990) *Nature 344*, 345–348.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sandermann, H., & Strominger, J. L. (1972) *J. Biol. Chem. 247*, 5123–5131.
Schulman, H. (1993) *Curr. Opin. Cell Biol. 5*, 247–253.
Shoemaker, M. O., Lau, W., Shattuck, R. L., Kwiatkowski, A. P., Matrisian, P. E., Guerra-Santos, L., Wilson, E., Lukas, T. J., Van Eldik, L. J., & Watterson, D. M. (1990) *J. Cell Biol. 111*, 1107–1125.
Son, M., Gundersen, R. E., & Nelson, D. L. (1993) *J. Biol. Chem. 268*, 5940–5948.
Starovasnik, M. A., Davis, T. N., & Klevit, R. E. (1993) *Biochemistry 32*, 3261–3270.
Stueber, D., Matile, H., & Garotta, G. (1990) in *Immunological Methods* (Lefkovits, I., & Pernis, B., Eds.) Vol. IV, pp 121–152, Academic Press, New York.
VanBerkum, M. F. A., & Means, A. R. (1991) *J. Biol. Chem. 266*, 21488–21495.
VanBerkum, M. F. A., George, S. E., & Means, A. R. (1990) *J. Biol. Chem. 265*, 3750–3756.
Weber, P. C., Lukas, T. J., Craig, T. A., Wilson, E., King, M. M., Kwiatkowski, A. P., & Watterson, D. M. (1989) *Proteins 6*, 70–85.
Yazawa, M., Vorherr, T., James, P., Carafoli, E., & Yagi, K. (1992) *Biochemistry 31*, 3171–3176.
Zhao, Y., Kappes, B., & Franklin, R. M. (1993a) *J. Biol. Chem. 268*, 4347–4354.
Zhao, Y., Kappes, B., & Franklin, R. M. (1993b) *Mol. Biochem. Parasitol.* (submitted for publication).