Azidocalmodulin derivatives

Activation of, and binding to, three target proteins: aorta myosin light-chain kinase, erythrocyte ($Mg^{2+} + Ca^{2+}$)-dependent ATPase and cardiac sarcoplasmic-reticulum kinase

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Different azidocalmodulin derivatives were synthesized by modification of either one carboxylic acid group or one or several arginine residues and their binding and activation capacity investigated in three target enzyme systems. The systems studied were smooth-muscle myosin light-chain kinase, cardiac sarcoplasmic-reticulum kinase and erythrocyte ($Mg^{2+} + Ca^{2+}$)-dependent ATPase. The results indicated that the activation ability of each calmodulin derivative was different depending on the system studied. Binding studies carried out by the displacement of ^{125}I -calmodulin indicated that the monosubstitutions did not greatly alter the apparent K_d of calmodulin for the enzymes but that the modification of four arginine residues caused a 4–8-fold increase in the apparent K_d in all systems. These results have shown that azidocalmodulin derivatives may have different degrees of usefulness in the study of calmodulin target proteins in different systems, with the behaviour of the derivatives not predictable on the basis of the nature (soluble or membrane-bound) or the type (ATPase or kinase) of enzyme system to be investigated. However, the monosubstituted calmodulin and, in particular, the carboxylic acid-group-modified derivative (where the modification was statistically dispersed over the protein chain) are good candidates for photolabelling calmodulin-binding proteins.

Abbreviations used: DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethane sulphonate; BSA, bovine serum albumin; FNAP, 4-azido-1-fluoro-2-nitrophenyl; EDNAP, *N*-(4-azido-2-nitrophenyl)ethylenediamine; APG, *p*-azidophenylglyoxal; EDC, 3-(3-dimethylamino-propyl)-1-ethylcarbodi-imide hydrochloride; DMF, dimethylformamide; APG₁-calmodulin, monoarginine-substituted calmodulin; APG₄-calmodulin, tetra-arginine-substituted calmodulin; Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-*L*-toluene-*p*-sulphonamidobutan-2-one; SR, sarcoplasmic reticulum; STI, soya-bean trypsin inhibitor; DMSO, dimethyl sulphoxide; DMF, dimethylformamide; TCA, trichloroacetic acid; h.p.l.c., high-pressure liquid chromatography.

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Calmodulin, the most ubiquitous Ca^{2+} -binding protein, is a multifunctional modulator mediating the Ca^{2+} -activation of a huge variety of enzymes (for reviews, see Klee *et al.*, 1980; Carafoli, 1981; Scharff, 1981; Klee & Vanaman, 1982). Obviously, the identification of calmodulin-binding sites is essential in order to understand the calcium regulation of biological systems. In such an investigation, two techniques are available: the gel-overlay technique and photoaffinity labelling. The first synthesis of an azidocalmodulin derivative was described recently by Andreasen *et al.* (1981). This derivative, which was obtained by azidobenzimidate substitution of a lysine residue, is a good tool for the identification of calmodulin target proteins in a number of systems (Hinds & Andreasen, 1981). However, Thiry *et al.* (1980) have shown that the modification of four arginine residues of calmodulin altered adenylate cyclase activation, but had no effect on phosphodiesterase activation. Different proteins may therefore bind to different sites of the calmodulin chain, and consequently any single azido

derivative might not be sufficient for the study of all calmodulin target proteins.

In the present study we synthesized different azidocalmodulin derivatives by the modification of either one carboxylic acid group or one or more arginine residue. The binding and activation capacity of these derivatives have been investigated in three calmodulin target enzyme systems: myosin light-chain kinase, cardiac SR kinase and erythrocyte ($Mg^{2+} + Ca^{2+}$)-dependent ATPase :

Experimental

Materials

[^{32}P]Phosphate and [^{125}I]iodine were purchased from Amersham. [γ - ^{32}P]ATP (sp. radioactivity 400 Ci/mol) was synthesized by the method of Glynn & Chappell (1964). All chemicals were from Merck or were analytical grade. Calmodulin was isolated from ram testis by the method of Autric *et al.* (1980) and was mono-iodinated by the Iodogen technique (Salacinski *et al.*, 1981), without any loss in smooth muscle myosin light-chain kinase activation capacity. Myosin regulatory light chains were obtained from bovine cardiac myofibrils as described previously (Walsh *et al.*, 1980).

Methods

Amino acid analysis of whole-calmodulin derivatives and tryptic peptides was performed with a Beckman analyser after a 24 h hydrolysis in 6 M-HCl under vacuum and using the *o*-phthalaldehyde detection technique as described by Benson & Hare (1975).

Synthesis of calmodulin derivatives. All synthetic work described was carried out in the dark or in a dimly lit room with the reaction vials wrapped in aluminium foil.

Azido-derivative synthesis. FNAP was prepared as described by Fleet *et al.* (1972) by diazotization and azido substitution of 4-fluoro-3-nitroaniline. EDNAP was prepared from FNAP and ethylenediamine in DMSO as described by Chicheportiche *et al.* (1979) (Fig. 1a). APG was synthesized by the method of Vanin *et al.* (1981) or Ngo *et al.* (1981) from *p*-aminoacetophenone by azido synthesis followed by SeO_2 oxidation to the α -dicarbonyl compound (Fig. 1b).

Azidocalmodulin synthesis. EDNAP was coupled to calmodulin via activation of the carboxy groups with a water-soluble carbodi-imide (EDC). Calmodulin (0.25 μ M) was dissolved in 3 ml of water, pH 9 (with NaOH), containing 2 mM- $CaCl_2$ and the pH was then shifted to 4.75 by addition of 0.1 M-HCl, a 500-fold molar excess of EDNAP dissolved in the minimum amount of DMF (0.3 ml) was added and the orange-yellow solution was then

Fig. 1. *Synthesis of azido derivatives*
The synthetic pathway for EDNAP-calmodulin is represented in (a) and for APG₁-calmodulin in (b).

mixed thoroughly. A 40-fold molar excess of EDC (10 μ mol) was then added and after 5 min the reaction was quenched by the addition of 1 M-ammonium acetate. The protein was precipitated by using cold TCA (5%), redissolved and separated by gel filtration on Sephadex G-25 eluted with 65 mM- NH_4HCO_3 /1 mM- $CaCl_2$. The extent of substitution was determined by u.v. spectroscopy assuming an $\epsilon_{470} = 4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for EDNAP dissolved in 50% ethanol (Lee *et al.*, 1979).

APG-calmodulin. Native calmodulin was dissolved in 50 mM-sodium borate buffer, pH 7.9, and 0.2 mM- $CaCl_2$ to a final concentration of 30 μ M. A 200-fold molar excess of APG was then added to the same buffer. After a 2 h incubation at 25°C one-half of the solution containing APG₁-calmodulin was removed and the other half left to incubate for a further 13 h (APG₄-calmodulin). Excess reagent was removed as outlined above and the calmodulin derivatives were kept in 1 mM- $CaCl_2$ /50 mM-sodium borate buffer, pH 7.5. The extent of substitution was estimated by comparison of the arginine content of the derivatives with unmodified calmodulin as determined by amino acid analysis. Before hydrolysis, 20 μ l of thioglycolic acid was added to prevent arginine regeneration, as described by Patthy & Smith (1975).

Characterization of the calmodulin derivatives. Tryptic digest and h.p.l.c. analysis. The azido-calmodulin derivatives (100 μ M) were digested by Tos-Phe- CH_2Cl -trypsin (enzyme/substrate ratio of 0.08) for 2 h at 37°C in 0.1 mM-EGTA, pH 8. The digestion was terminated by the addition of STI and the arylazido groups reduced by the addition of 50 mM-DTT for 30 min at 25°C as described by Staros *et al.* (1978). H.p.l.c. was performed by the method of Autric *et al.* (1980). The protein digest (70–80 nmol) was eluted with a flow rate of 1.5 ml/min for 45 min by a 0–60% acetonitrile linear gradient in 0.1 M-sodium phosphate buffer, pH 2.3. The eluate was simultaneously monitored at 220 nm and either 315 nm or 550 nm, the u.v. absorbance

maxima for APG-calmodulin and EDNAP-calmodulin respectively, after azido reduction to the amine form.

Electrophoresis of the calmodulin-derivatives (10 μ g) was performed on strips (2.5 cm $\times$ 7.5 cm) of cellulose acetate at room temperature. For EDNAP-calmodulin, the electrophoresis was performed in 0.08 M-cacodylic acid adjusted to pH 5.5 by pyridine and the samples were run at 300 V for 8 min. For APG-calmodulin electrophoresis, the buffer used was 1 M-acetic acid/pyridine, pH 3.5, and the samples were run at 100 V for 24 min. The strips were then stained with Amido Black (0.2%) in acetic acid/methanol (9:1, v/v) and destained using the same solvent.

Preparation of smooth-muscle myosin kinase. Myosin light-chain kinase from bovine aorta was prepared by the method of Vallet *et al.* (1981). After these purification steps, the kinase prepared was 90% homogeneous, as determined by sodium dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis, and contaminated only by traces of actin.

Preparation of SR vesicles. Cardiac SR vesicles were prepared from dog hearts arrested in diastole by pentobarbital injection, essentially by using the protocol described by Le Peuch *et al.* (1979), except that the overnight incubation in Hepes buffer, pH 7.5, containing 0.6 M-KCl, was omitted. After three cycles of washing and sedimentation the vesicles obtained were suspended in Hepes buffer, pH 7.5, at a protein concentration of 15 mg/ml and stored overnight at 4°C.

Preparation of erythrocyte membranes. Human erythrocyte membranes were prepared by osmotic lysis of fresh 0.9% NaCl-washed erythrocytes followed by successive washing of the post-haemolysate residue with solutions containing 1–10 mM EDTA as described previously (Blostein, 1968). The residue was made up to one-half the original packed cell volume of the washed cells and stored at 4°C. The preparation contained approx. 4 mg of protein/ml and was used within 6 days. Before use, the membranes preparations were frozen and thawed twice to ensure complete permeability to substrates.

Enzyme activity assays. Myosin light-chain kinase was measured by the amount of [32 P]phosphate incorporated into 0.36 mg of bovine cardiac myosin regulatory light chains as described previously (Walsh *et al.*, 1980). The assay mixture (total volume 70 μ l) contained 1.6 mM-DTT, 8 mM-magnesium acetate, 1 mM-[γ - 32 P]ATP (50 c.p.m./pmol), 0.1 mM-CaCl₂ in 35 mM-Tris/HCl, pH 7.5. The reaction at 30°C for 5 min was initiated by the addition of 0.5 μ g of enzyme and was terminated by cold TCA (10%) precipitation on Whatman 3MM filter papers.

The Ca²⁺- and calmodulin-dependent SR kinase had been shown to phosphorylate a major endogenous substrate called 'phospholamban' (Le Peuch *et al.*, 1979). SR kinase activity was determined by the amount of [32 P]phosphate incorporated into the membrane proteins by the method of Le Peuch *et al.* (1979). The assay mixture contained 0.05 mM-DTT, 10 mM-NaF, 10 mM-magnesium acetate, 1 mM-[γ - 32 P]ATP (100 c.p.m./pmol) and 0.1 mM-CaCl₂ in 30 mM-sodium phosphate, pH 7.0, in a total volume of 70 μ l. The reaction was initiated by the addition of 45 μ g of SR vesicles and continued at 30°C for 5 min. A portion of the reaction mixture was then withdrawn and pipetted on to a Whatman 3MM filter paper and the filters washed by the method of Corbin & Reimann (1974).

Erythrocyte (Mg²⁺ + Ca²⁺)-dependent ATPase activity was determined by the amount of [32 P]P_i released from [γ - 32 P]ATP as described previously (Katz & Blostein, 1975). The assay mixture contained 2.5 mM-magnesium acetate, 100 mM-KCl, 0.1 mM-DTT, 1 mM-[γ - 32 P]ATP (100 c.p.m./pmol) and 25 μ M-CaCl₂ in 20 mM-Tris/HCl, pH 7.5, in a total volume of 200 μ l. The reaction was carried out at 37°C for 10 min and initiated by the addition of 30 μ g of membranes. [32 P]P_i was precipitated on to Whatman 31 ET filter papers presoaked in TCA (5.7%), ammonium heptamolybdate (2.9%) and triethylamine (29 mM) by the method of Reimann & Umfleet (1978).

The assays described were performed in the dark with various concentrations of the calmodulin derivatives. In each biological system, the activation ability of the derivatives was estimated by comparison with the standard activation curve obtained with unmodified calmodulin.

Calmodulin-binding determination. The assay was carried out at room temperature in 100 mM-KCl/2.5 mM-magnesium acetate/100 μ M-CaCl₂/20 mM-Tris/HCl, pH 7.4. Approx. 150 μ g of SR or erythrocyte membranes or 6 μ g of myosin kinase (in the presence of 150 μ g of BSA) were incubated with 0.1 μ M-¹²⁵I-calmodulin (3.5 $\times$ 10⁶ total c.p.m.). After a 10 min incubation, an equal volume of 20% (w/v) polyethylene glycol 20000 was added to precipitate the bound calmodulin. These manipulations were performed in Eppendorf microfuge tubes (1.5 ml). After a further 5 min incubation the mixture was centrifuged for 5 min in a Beckman Microfuge B. The supernatant was removed by aspiration and the residual film soaked up by a cotton applicator. The pellet remaining was then counted for radioactivity in an MR 252 gamma counter. Non-specific binding of ¹²⁵I-calmodulin, appreciable at higher concentrations, was estimated by the inclusion of a 50-fold excess of unlabelled calmodulin in the assay mixture.

The binding capacity was estimated by the

displacement of ^{125}I -calmodulin by various concentrations of non-labelled derivatives or unmodified calmodulin. These studies were performed as described above.

Miscellaneous methods. Protein concentrations were determined by the Coomassie Blue technique (Spector, 1978). Concentrations of modified and unmodified calmodulin were determined by amino acid analysis of stock solutions.

Results

Characterization of the calmodulin derivatives

The EDNAP-modified-amino acid/calmodulin ratio was determined to be 1:1 by u.v. determination.

H.p.l.c. analysis of the EDNAP-calmodulin tryptic digest revealed no single major peak detected at 550nm and thus a random distribution over several tryptic peptides (results not shown). This result indicated that the substitution was statistically distributed over different carboxylic acid groups along the protein chain. This was also confirmed by electrophoresis on cellulose acetate plates: the EDNAP-calmodulin derivative was detected as a smear below the unmodified calmodulin spot, indicating a heterogeneous derivative population less acidic than the native calmodulin (Fig. 2). The electrophoresis pattern clearly indicated that no unmodified calmodulin remained after the 5 min incubation period.

APG₁-calmodulin differed from native calmodulin by one arginine residue modification as determined by amino acid analysis. No significant modification of other amino acids was noted (Table 1). H.p.l.c.

analysis of APG₁-calmodulin tryptic peptides revealed a major peak by detection at 315 nm as shown in Fig. 3. The values obtained for the amino acid analysis of this peptide as reported in Table 1 are in good agreement with those of the (Glu-31-Arg-74)-peptide according to the calmodulin sequence established by Grand *et al.* (1981). It can be assumed from these results that the Arg-37 residue was protected from tryptic attack by the derivative-formation procedure. The electrophoretic behaviour of APG₁-calmodulin at pH 3.5 is illustrated in Fig. 2. No remaining traces of unmodified calmodulin were

Fig. 2. Electrophoresis on cellulose acetate
The electrophoretic behaviour of the calmodulin derivatives (2) was compared with that of unmodified calmodulin (1). APG₁-calmodulin migration (a) was carried out in the presence of acetic acid, pH 3.5, for 24 min at 100 V. EDNAP-calmodulin migration was performed in cacodylate, pH 5.5, for 8 min at 300 V. The arrows indicate the point of deposit of the compounds on the plates.

Table 1. Amino acid composition of calmodulin derivatives and APG₁-tryptic peptide

Data in parentheses are from Autric *et al.* (1980), except for those for the content of the (31-74)-peptide, which are from Grand *et al.* (1981). The yield after h.p.l.c. separation was 18 nmol, compared with 27 nmol of calmodulin before tryptic attack.

Residue	Amino acid content (residues/mol)				
	Initial calmodulin	EDNAP-calmodulin	APG ₁ -calmodulin	APG ₄ -calmodulin	APG ₁ -(31-74)-peptide
Asx	24 (23)	23	23	24	6.6 (7)
Thr	10.5 (12)	11	11	11	3.8 (4)
Ser	4.8 (4)	4.9	5.5	4.3	1.1 (1)
Glx	30.1 (30)	29.1	28.8	29.2	7.4 (7)
Gly	11.5 (11)	11.6	11.5	11.7	3.6 (4)
Ala	11.6 (11)	11.9	11.6	11.9	3.3 (3)
Val	5.8 (7)	6.8	5.5	5.5	1.6 (2)
Met	6.1 (9)	5.8	7.9	8.3	3.5 (4)
Ile	6.2 (7)	6.6	5.9	5.6	2.3 (2)
Leu	9.5 (9)	10	9.1	10	4.3 (4)
Tyr	1.8 (2)	1.5	1.8	1.9	—
Phe	7.5 (8)	8.2	7.4	7.1	2.6 (2)
His	1.2 (1)	1.2	1.1	1.2	—
Lys	7.4 (7)	7.2	7.2	6.6	—
Arg	5.6 (6)	5.9	4.5	2.1	1.3 (2)

detected. The APG₁-calmodulin appeared as a single spot corresponding to a more acidic protein than calmodulin.

APG₄-calmodulin was derived from native calmodulin by the same treatment as produced APG₁-calmodulin except that the reaction was continued for a further 13h (see under 'Methods'). Four arginine residues were determined to be modified, as indicated in Table 1. The high-pressure liquid chromatogram of the tryptic fragments of this derivative was too complex to be analysed; the modified residues could therefore not be localized with certainty.

Fig. 3. H.p.l.c. peptide map of APG₁-calmodulin after tryptic digestion

The upper trace indicates the absorbance of the eluate at 220nm. The lower trace indicates the absorbance of the eluate at 315nm. Lower and upper traces were obtained by simultaneous detection.

Activation of calmodulin-target enzymes

The dose-effect curves for the activation of myosin kinase, SR kinase and erythrocyte (Mg²⁺ + Ca²⁺)-dependent ATPase by the different calmodulin derivatives are illustrated in Fig. 4. The activation ability of each calmodulin derivative differed depending on the system studied. APG₄-calmodulin was found to be a poor activator of myosin kinase with a 6-fold increase in the apparent K_m compared with native calmodulin. APG₁- and EDNAP-calmodulin activated myosin kinase to a greater extent, with apparent K_m values approaching those of the unmodified calmodulin. The APG₁-calmodulin activation curve was biphasic, indicating contamination by small amounts (less than 1 part per 100) of a more potent derivative form. All three calmodulin derivatives were found to be good activators of SR kinase; APG₁-calmodulin was slightly more efficient than unmodified calmodulin, whereas APG₄-calmodulin was less efficient, with a large shift in the apparent K_m value observed. The results obtained in the erythrocyte (Mg²⁺ + Ca²⁺)-dependent ATPase system more closely approached those obtained in the myosin kinase system, except for the surprising activation level observed in the presence of APG₄-calmodulin.

Binding to calmodulin-target enzymes

Displacement of ¹²⁵I-calmodulin was performed using increasing concentrations of modified or unmodified cold calmodulin. The radioactivity monitored in the pellet as described under 'Methods' revealed a non-specific binding component of less than 5%. No modification of the apparent K_d was observed after iodo-substitution. The apparent K_d

Fig. 4. The effect of increasing concentrations of calmodulin derivatives on aortic myosin light-chain kinase (a), cardiac SR kinase (b) and erythrocyte (Mg²⁺ + Ca²⁺)-dependent ATPase (c) activities

The assays were performed in duplicate under the conditions described under 'Methods' in the presence of various amounts of unmodified calmodulin (●), APG₁-calmodulin, (□), APG₄-calmodulin (■) or EDNAP-calmodulin (○). pCAM = $-\log[\text{calmodulin}]$.

Table 2. Comparison of the activation and the binding ability of the calmodulin derivatives in three biological systems

Activation values represent the relative potency (efficiency) expressed as a percentage of the control value obtained with native calmodulin. Full activation was regarded as the first activation plateau in the biphasic curves. The apparent K_m of activation is the concentration required for half-maximal stimulation of the enzyme. The percentage of activation and apparent K_m of activation are derived from data in Fig. 3. K_d values were determined by displacement of unmodified ^{125}I -calmodulin by different amounts of unlabelled derivatives as described under 'Methods'. K_d of displacement is the concentration producing half displacement of ^{125}I -calmodulin.

Enzyme	Calmodulin derivative	Activation (%)	K_m (nM)	K_d (nM)
Myosin light-chain kinase	Normal	100	6	15
	APG ₁	42	6	27
	APG ₄	15	39	63
	EDNAP	31	18	21
SR kinase	Normal	100	13	50*
	APG ₁	108	22	40*
	APG ₄	81	160	56*
	EDNAP	98	36	32*
Erythrocyte ATPase	Normal	100	20	10
	APG ₁	34	13	16
	APG ₄	71	100	79
	EDNAP	41	25	32

* These values represent the binding to all the target proteins present in the SR vesicles.

values for the binding of the derivatives were determined in the same three biological systems. Table 2 shows these apparent K_d values and the apparent K_m values and activation capacity of the calmodulin derivatives compared with native calmodulin. The binding data indicate that the mono-substitutions (EDNAP-calmodulin and APG₁-calmodulin) did not greatly alter the apparent K_d of the calmodulin for the enzyme. The modification of four arginine residues (APG₄-calmodulin), though, caused a 4–8-fold increase in the apparent K_d in the myosin kinase and erythrocyte ($\text{Mg}^{2+} + \text{Ca}^{2+}$)-dependent ATPase systems. Whatever the modification, the K_d value for SR vesicles was similar; it must be noted, though, that this value encompasses data for all SR calmodulin target proteins.

Discussion

The present study was carried out in order to determine the azidocalmodulin derivative most suitable for the study of different calmodulin target systems. Three biological systems were chosen as

models for the determination of the activation and binding capacities of the azidocalmodulin derivatives synthesized. These included two kinase activities, one soluble (myosin light-chain kinase) and the other membrane-bound (cardiac SR kinase) and the ($\text{Mg}^{2+} + \text{Ca}^{2+}$)-dependent ATPase from EDTA-washed erythrocyte membranes. It must be noted that the activation data (percentage activation and apparent K_m) represent a single enzyme activity in all cases. On the other hand, the K_d values obtained for the cardiac sarcoplasmic reticulum preparation reflect all the high- and low-affinity calmodulin target proteins present; at least seven calmodulin-binding sites have been identified in these preparations using the calmodulin overlay technique (Molla *et al.*, 1983b). The two other systems are less complex to analyse, as the enzyme studied was the sole calmodulin target protein present. Myosin light-chain kinase was purified to 90% purity and was contaminated only by traces of actin. It was also previously shown that the ($\text{Mg}^{2+} + \text{Ca}^{2+}$)-dependent ATPase was the sole calmodulin-binding protein in erythrocyte membranes (Hinds & Andreassen, 1981). This was confirmed, in our hands, using the calmodulin overlay technique (results not shown).

Liu & Cheung (1976) have found that up to five carboxy groups of calmodulin are very easily accessible and can be modified without a noticeable loss in calmodulin activation of phosphodiesterase. Consequently we synthesized a calmodulin derivative by modification of the carboxy groups. The EDNAP-calmodulin derivative thus obtained was monosubstituted, and the azido modification was dispersed along the calmodulin chain, confirming the accessibility of several carboxy groups. This EDNAP-calmodulin was as potent for SR kinase activation as unmodified calmodulin, although a 3-fold shift in the K_m value was observed. The same decrease in the binding capacity was observed in the two other systems, but was accompanied by a loss of more than 50% in the activity noted in the presence of unmodified calmodulin.

The two other azidocalmodulin derivatives studied were synthesized by substitution of arginine residues. Arg-37 appears to be the most reactive arginine residue towards the α -dicarbonyl reagent. APG₁-calmodulin is a good photolabelling derivative, since the K_d values obtained were close to that of unmodified calmodulin. We had thought that poly-substitution by azido groups would increase the possibility of binding to different target proteins. The synthesis of APG₄-calmodulin with four unidentified arginine residues modified ruled out this supposition, as a 10-fold shift in the K_d value of APG₄-calmodulin was observed in all the three enzyme systems studied.

These studies with azido derivatives provide, as

well, some information on the binding and enzyme activation sites of calmodulin. In contrast with the two other systems used, phospholamban SR kinase activation was not affected by the modification of the carboxy groups and arginine residues. These data indicate that these calmodulin residues are not implicated in the SR kinase activation. However, a large increase in the K_d value was observed with APG₄-calmodulin, suggesting either a direct involvement of arginine residues in the binding or a sterical hindrance of the arylazido groups near the binding site. The present study provides further evidence that the site of calmodulin interaction with different enzymes is different. However, as far as calmodulin binding and activation are concerned, myosin kinase and erythrocyte ATPase appear to react in similar ways. Previously it had been observed that none of these enzyme systems was activated by skeletal-muscle troponin C (Walsh & Stevens, 1977; Jarrett & Penniston, 1977). The α -helix B of domain I of calmodulin appears to be important in the activation of these two enzymes, since a loss of more than 50% activation was observed with APG₁-calmodulin. The arginine residues or their immediate environment appear to be essential in the binding of target enzymes, since APG₄-calmodulin binding affinity was 5–10 times lower than unmodified calmodulin. The biphasic activation curves obtained with APG₁-calmodulin in these systems suggested the presence of traces of a more potent activator than the APG₁-calmodulin derivative, possibly residual calmodulin or di- or tri-substituted derivatives. The surprising activation of erythrocyte ATPase by APG₄-calmodulin favours the hypothesis of a more efficient di- or tri-substitution. The localization of this supplementary modification will thus be important in the determination of the calmodulin interaction site of these two proteins.

In conclusion, these studies have shown that azidocalmodulin derivatives may have different degrees of usefulness in the determination of the target proteins in different systems. In addition the behaviour of the derivatives in a system cannot be predicted on the basis of the nature (cytoplasmic or membrane-bound) or of the type (ATPase or kinase) of enzyme investigated. The two kinases studied react in a very different manner. Conversely, the erythrocyte ATPase and myosin kinase behave in the same way.

The polysubstituted azido-derivatives proved not to be an efficient tool for the photolabelling of calmodulin-binding proteins. It was found to be preferable to synthesize monosubstituted derivatives, which conserve a binding capacity similar to that of unmodified calmodulin. APG₁- and EDNAP-calmodulin are thus good candidates for photoaffinity-labelling studies. In complex systems, containing several calmodulin-binding proteins, it would be

more preferable to use derivatives with azido groups at different positions in order to increase the cross-linking probability. EDNAP-calmodulin, which has a random distribution of azido-group modification along the calmodulin chain, was therefore the most useful derivative studied.

Further photolabelling experiments performed with the ¹²⁵I-labelled derivatives confirmed the results of the present study. APG₁- and EDNAP-calmodulin were found to be useful photoaffinity labelling derivatives in the identification of target proteins in cardiac SR and in *Xenopus* oocytes (Molla *et al.*, 1983a).

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