The Calmodulin Binding Domain of Nitric Oxide Synthase and Adenylyl Cyclase†

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Received October 21, 1992; Revised Manuscript Received February 2, 1993

ABSTRACT: Peptides corresponding to regions of the calmodulin-activated NO-synthase and of the calmodulin dependent adenylyl cyclase, which could represent the calmodulin binding domains of the two proteins, have been synthesized and tested for calmodulin binding. The chosen peptides were those in the sequence of the two proteins which most closely corresponded to the accepted general properties of the calmodulin binding domains, i.e., a hydrophobic sequence containing basic amino acids. In the case of the NO-synthase, the putative high-affinity calmodulin binding domain was identified by urea gel electrophoresis and fluorescence spectroscopy with dansylcalmodulin as peptide NO-30 (amino acids 725–754). The highest affinity calmodulin binding site of the calmodulin-dependent adenylyl cyclase was assigned to peptide AC-28 (amino acids 495–522) by titration with dansylcalmodulin and by the ability to inhibit the calmodulin-stimulated activity of purified calmodulin-stimulated adenylyl cyclase. The sequence 495–522 is located in the unit protruding into the cytosol from the sixth putative transmembrane domain of the molecule. It has the typical hydrophobic/basic composition of canonical calmodulin binding domains, and also contains, like most calmodulin binding domains, an aromatic amino acid in its N-terminal portion. It also contains two Cys residues in the central portion, which is an unusual feature of the calmodulin binding domain of this enzyme.

The number of proteins modulated by calmodulin is increasing continuously and now includes enzymes involved in very diverse aspects of cell biochemistry. Enzymes modulated by calmodulin normally interact with it with very high affinity, i.e., with a K_d in the low nanomolar range. Calmodulin binding domains have also been described in proteins not normally considered targets of calmodulin regulation (Buschmeier et al., 1987; Li et al., 1991): although their affinity for the modulator is low (Li et al., 1991; Alexander et al., 1988), these proteins may be very abundant (e.g., neuromodulin; Alexander et al., 1988); their calmodulin binding could thus still be physiologically significant.

The high-affinity calmodulin binding domains so far identified in target proteins have a very limited degree of sequence homology. However, their sequence analysis has revealed some general principles that are now assumed to be typical (Blumenthal et al., 1985; Lukas et al., 1986; Cox et al., 1985; James et al., 1988, 1991; O'Neil & DeGrado, 1990) and valid also for calmodulin binding peptide venoms (Anderson & Malencik, 1986; Bennett & Kenneth, 1987). The general backbone of the domain consists of apolar amino acids; some of the residues of the domain (at least four) are basic, mostly arginines; the basic and the hydrophobic residues of the domain are normally sequestered so as to produce an amphiphilic helix; an aromatic residue, frequently a tryptophan, is almost always present in the N-terminal portion of the domain (Vorherr et al., 1990). These general structural rules have exceptions. For example, the calmodulin binding domain of the γ-subunit of phosphorylase kinase appears to consist of two subdomains separated from each other in the protein sequence (Dasgupta et al., 1988). This seems also to be the case for the putative calmodulin binding domain of adenylyl cyclase of *Bordetella pertussis* (Landant, 1988).

Calmodulin binding domains have been conclusively identified only in a few cases, f.i., using specific labels conjugated to calmodulin, site-directed mutagenesis, and proteolysis of proposed calmodulin binding regions [e.g., see James et al. (1988), Pearson et al. (1991), and Ito et al.(1991)]. The solution of the three-dimensional structure of calmodulin binding peptides complexed with calmodulin has also provided strong evidence for calmodulin binding sites (Ikura et al., 1992; Meador et al., 1992). Synthetic peptides corresponding to sequences qualifying as candidates for calmodulin binding domains have also been conveniently used (Vorherr et al., 1990). They have led to the conclusion that the aromatic residue in the N-terminal portion of the domain plays an important role in the reaction with calmodulin (Vorherr et al., 1990).

Adenylyl cyclase is one of the first enzymes which have been shown to be modulated by calmodulin (Wolff & Brostrom, 1987). The first evidence of the existence of calmodulin-sensitive and -insensitive isoforms of the enzyme came from the separation of the two enzymes on calmodulin–Sepharose columns (Westcott et al., 1979). This was confirmed by the isolation of antibodies that distinguish between the two forms of the enzyme (Rosenberg & Storm, 1987; Mollner & Pfeuffer, 1988) and by more recent cloning work (Krupinski et al., 1989; Tang et al., 1991) which has shown that the type I cyclase is calmodulin-sensitive whereas type II is calmodulin-insensitive. Very recently (Choi et al., 1992), a third cyclase isoform (type III; Bakalyar & Reed, 1990) has also been shown to be stimulated by calmodulin, although only in the presence of other effectors and with an affinity for

† This work has been made possible by financial contributions from the Swiss National Science Foundation (Grant 31-30858.91 to E.C.) and the Fonds der Chemischen Industrie (to T.P.). This work was further supported by the Deutsche Forschungsgemeinschaft (SFB 176, Würzburg).

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0006-2960/93/0432-6081$04.00/0

calmodulin which was about 50-fold lower than that of type I cyclase.

Another enzyme, nitric oxide synthase (NO-synthase),[1] is a more recent addition to the list of calmodulin-activated enzymes (Bredt & Snyder, 1990; Bredt et al., 1991). Also, this enzyme exists in several isoforms, only some of which are calmodulin-sensitive. The classical calmodulin-sensitive isoforms are expressed in brain and endothelial cells, whereas the isoform typical of macrophages is not stimulated by added calmodulin (Lowenstein et al., 1992). Interestingly, however, the macrophage isoform has recently been shown to contain calmodulin as a tightly associated subunit (Cho et al., 1992). For both the adenylyl cyclase and the NO-synthase, there is no conclusive indication on the location of the calmodulin binding domain, although a prediction has been made for the brain NO-synthase based on inspection of the sequence (Bredt et al., 1991). As for adenylyl cyclase, no proposals have been put forward. In this paper, the calmodulin binding domains of the calmodulin-dependent NO-synthase and adenylyl cyclase have been investigated using gel electrophoretic, fluorescence, and enzyme inhibition methods. The tools of the study have been synthetic peptides corresponding to sequences in the enzyme that would qualify as calmodulin binding domains. The results obtained have confirmed the predictions on the location of the domain in the brain isoform of NO-synthase (Bredt & Snyder, 1990), and have identified the domain with the highest affinity for calmodulin in type I adenylyl cyclase in the large unit protruding into the cytoplasm from the sixth putative transmembrane domain of the molecule.

MATERIALS AND METHODS

Creatine phosphate and creatine kinase were products of Boehringer AG, Mannheim, Germany; Chaps, Lubrol PX, benzamidine, and benzethonium chloride were purchased from Sigma Chemical Co., Munich, Germany; Pansorbin (*Staphylococcus aureus* cells, Cowan I strain) and the calmodulin binding domain of Ca^{2+}/calmodulin-dependent protein kinase II (residues 290–309) were purchased from Calbiochem, San Diego, CA. Calmodulin was from Pharmacia—LKB Biotechnology Inc., Uppsala, Sweden; aprotinin and Tween 60 were products of Serva, Heidelberg, Germany. Tween 60 was deionized by passage through a mixed-bed ion exchanger (AG501 × 8; BioRad Laboratories GMBH, Munich, Germany). Fresh bovine brains were obtained from a local slaughterhouse. The reagents for peptide synthesis were from Novabiochem, Laeufelfingen, Switzerland. [α-^{32}P]ATP (>400 Ci/mmol) and [^{3}H]cAMP (15–30 Ci/mmol) were from Amersham, Buckinghamshire, England.

Peptide Synthesis. Peptide AC-15 (I-K-P-A-K-R-M-K-F-K-T-V-C-Y-L-NH_2; amino acids 495–509 of the bovine brain calmodulin-dependent adenylyl cyclase) was synthesized by solid-phase peptide chemistry on an Applied Biosystems (Foster City, CA) 430A synthesizer. The following amino acids with protected side chains were used: Bzl for Ser, Thr, Asp, and Glu; pMeBzl for Cys; 2-Br-Z for Tyr; Z for His; MTS for Arg; Met=O for Met; Cl-Z for Lys. Amino acids were coupled as HOBt esters in NMP. Deprotection and cleavage from the resin were done with a trifluoromethanesulfonic acid/thioanisole /ethanedithiol/*m*-cresol/TFA (1:1:0.5:0.5:10, v/v) mixture (Palm et al., 1989). The reaction was performed at 0 °C for 2 h and at 20 °C for 1 h. Reduction of methionine sulfoxide was achieved by adding 30% (v/v) dimethyl sulfide to the mixture. The reaction was allowed to proceed for 1 more h at 20 °C; then the crude peptide was precipitated by ether and purified by gel filtration on Sephadex G10 and preparative reverse-phase HPLC.

The peptides NO-30 [K-R-R-A-I-G-F-K-K-L-A-E-A-V-K-F-S-A-K-L-M-G-Q-A-M-A-K-R-V-K-NH_2, amino acids 725–754 of the rat brain NO-synthase (Bredt et al., 1991)], NO-33 [V-H-K-K-R-V-S-A-A-R-L-L-S-R-Q-N-L-Q-S-P-K-F-S-R-S-T-I-F-V-R-L-H-T-NH_2, amino acids 987–1019 of the rat brain NO-synthase (Bredt et al., 1991)], AC-28 [I-K-P-A-K-R-M-K-F-K-T-V-C-Y-L-L-V-Q-L-M-H-C-R-K-M-F-K-A-NH_2, amino acids 495–522 of the bovine brain calmodulin-dependent adenylyl cyclase (Krupinski et al., 1989)], and AC-24 [V-H-R-L-L-R-R-G-S-Y-R-F-V-C-R-G-K-V-S-V-K-G-K-G-NH_2, amino acids 1024–1050 of the bovine brain calmodulin-dependent adenylyl cyclase (Krupinski et al., 1989)] were synthesized on an Applied Biosystems Model 431 peptide synthesizer using the Fmoc/tBu strategy with 1-methyl-2-pyrrolidone for washing. The following side chain protecting groups were used: tBu for Tyr, Ser, Thr, Asp, and Glu; Trt for His, Cys, Asn, and Gln; Pmc for Arg; Boc for Lys. Activation of the amino acid derivatives was accomplished by TBTU/HOBt/diisopropylethylamine (1:1:2). The peptides were synthesized starting from 0.06 mmol of the Rink resin [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin]. A 9-fold excess of amino acid derivative was used for TBTU/HOBt activation. The cleavage of the peptides was performed in a mixture of 3.5 mL of TFA, 50 μL of dimethyl sulfide, 0.1 mL of ethanedithiol, 230 mg of phenol, 0.1 mL of thioanisole, and 0.1 mL of water for 4 h at room temperature.

Preparative HPLC was performed on a Macherey & Nagel (Oensingen, Switzerland) C8 column using buffers A and B (see below); 143 mg of crude peptide NO-30 yielded 38.6 mg of purified peptide. In the case of peptide NO-33, 9 mg of purified peptide was obtained from 108.5 mg of crude material; 42.5 mg of pure peptide AC-28 was obtained from 134 mg of crude material. Purification of 42 mg of crude peptide AC-24 resulted in 8 mg of purified peptide. The amino acid analysis of the purified peptide agreed with the expected ratios (Cys was not quantitatively determined).

HPLC Analysis and Amino Acid Analysis of the Purified Peptides. Semipreparative and analytical HPLC were carried out using Nucleosil reversed-phase materials packed in columns by Macherey & Nagel. The reverse-phase buffers were as follows: A, 0.1% trifluoroacetic acid in water; B, 0.05% trifluoroacetic acid/50% 1-propanol in water. The peptides were purified on a 250 × 50 mm C_8 column (10 μm, 300 Å) running a linear gradient from A to B. Analytical control was performed on a 120 × 2.1 mm C_{18} column (3 μm, 120 Å) in a solvent system containing 70% acetonitrile/30% water (0.1% TFA) instead of buffer B. Chromatography was carried out using Applied Biosystems equipment, and UV detection

[1] Abbreviations: Boc, tertiary butyloxycarbonyl; 2-Br-Z, 2-bromobenzyloxycarbonyl; Bzl, benzyl; Chaps, 3-[(3-cholamidopropy)dimethylammonio]-1-propanesulfonate; Cl-Z, chlorobenzyloxycarbonyl; DC, dansylcalmodulin; DIPEA, diisopropylethylamine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Met=O, methionine sulfoxide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MTS, mesitylene-2-sulfonyl; NO, nitric oxide; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; pMeBzl, 4-methoxybenzyl; PTC, phenylthiocarbamyl; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; tBu, tertiary butyl; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; Trt, trityl; Z, benzyloxycarbonyl.

was performed at 235 and 210 nm for the preparative and analytical runs, respectively.

An Applied Biosystems 420A derivatizer and on-line PTC detection with the Model 130A Applied Biosystems analyzer were used for the derivatization, separation, and identification of the amino acids.

Gel Electrophoresis in the Presence of Urea. Urea gel electrophoresis was performed according to Erickson-Vitanen and DeGrado (1987). Gels (0.75 mm thick) containing 12.5% acrylamide, 0.33% bis(acrylamide), 4 M urea, 0.375 M Tris-HCl, pH 8.8, and 0.1 mM $CaCl_2$ or 2 mM EGTA were prepared without a stacking gel. Samples containing 3–5 μg of calmodulin and increasing amounts of peptide in 100 mM Tris-HCl, pH 7.2, 4 M urea, and either 0.1 mM $CaCl_2$ or 2 mM EGTA were incubated at room temperature for 2 h. The total volume was 20 μL. Ten microliters of 50% glycerol including the tracer bromophenol blue was added before the gel was loaded. The gels were run at 25-mA constant current. The electrode buffer consisted of 25 mM Tris-HCl, 192 mM glycine, pH 8.3, and 0.1 mM $CaCl_2$ or 2 mM EGTA. The gels were fixed, stained, and destained using the following solutions: 50% methanol/10% acetic acid (0.5 h); 0.025% Coomassie Blue in 10% acetic acid (1 h); 10% acetic acid (overnight).

Fluorescence Measurements. The fluorescence measurements were performed with a SPEX Fluorolog 1680 double spectrometer (Metuchen, NJ) connected to a DM1B coordinator. Quartz cuvettes with a path length of 10 mm and a volume of about 3.3 mL were used for the experiments. Dilution effects were <3%, and the sample temperature was 26 °C; 20 mM Hepes/130 mM KCl, pH 7.2, containing either 0.5–1 mM $CaCl_2$ or 1 mM EGTA was used to buffer the system.

The excitation of the dansyl moiety of dansylated calmodulin was performed at 340 nm, and the peptides were added to the dansylated calmodulin. The latter was prepared as described in Vorherr et al. (1990). The resolution of the excitation monochromator was set at 8 nm. The samples were only stirred upon addition of the peptides dissolved in bidistilled water. The spectra were recorded from 400 to 550 nm. The titrations were performed with the wavelength for fluorescence emission set at 490 nm. One data point corresponded to fluorescence intensities integrated over a total time of 2 s after equilibration of the mixture. The dissociation constants were calculated according to Stinson and Holbrook (1973).

Solubilization of Bovine Brain Adenylyl Cyclase. Bovine brain membranes were prepared as described by Pfeuffer et al. (1985). They were suspended at a concentration of 20 mg/mL, and solubilized by a 30-min incubation at 0 °C in 10 mM MOPS, pH 7.4, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 10 mM benzethonium chloride, and 10 μg/mL aprotinin (buffer A) containing 15 mM Chaps. The solubilized cyclase was obtained following centrifugation at 100000*g* for 30 min at 4 °C.

Preparation of the Monoclonal Antibody BBC-1. The antibody, which reacts against the calmodulin-stimulated adenylyl cyclase from bovine brain cortex, was prepared as described previously (Mollner & Pfeuffer, 1988).

Cross-Linked Pansorbin–BBC-1 Conjugate. This was prepared essentially as published earlier (Mollner et al., 1991).

Preparation of Pansorbin–BBC-1-Coupled Adenylyl Cyclase. One milliliter of Pansorbin–BBC-1 conjugate (10% v/v suspension in buffer A) was washed twice with 3 mL of buffer A/1 mM Tween 60 (buffer B) by centrifugation at 4000*g*. The pellet was resuspended in 1 mL of buffer B, and 250 μL of the solubilized brain adenylyl cyclase was then added. The mixture was gently stirred overnight at 4 °C. The suspension was centrifuged for 10 min at 4000*g* and washed 4 times with 4 mL of buffer B containing 1 mM EGTA, followed by a wash with 4 mL of buffer B without EGTA. The pellet was finally resuspended in 1.4 mL of buffer B and used for the adenylyl cyclase assay. When bound adenylyl cyclase was released by SDS and analyzed with the monoclonal antibody BBC-2 which recognizes an epitope common to all cloned adenylyl cyclases so far, no other species was recognized besides the 115-kDa form. Other cyclases from brain, and those from peripheral tissues containing isoforms II–VI, have higher molecular masses. Among all forms detectable by antibody BBC-2, only the 115-kDa form bound to calmodulin–Sepharose (Mollner & Pfeuffer, 1988).

Adenylyl Cyclase Assay. Adenylyl cyclase was assayed on 10 μL of the enzyme suspension, in the presence of 20 mL of MOPS, pH 7.4, 10 mM creatine phosphate, 50 μg/mL creatine kinase, 5 mM $MgCl_2$, and 0.1 mM [α-^{32}P]ATP (1–2 Bq/pmol). The incubation was performed for 20 min at 30 °C. [^{32}P]cAMP was measured according to Salomon et al. (1974). The basal activity was measured in the presence of 100 μM EGTA. Calmodulin/Ca^{2+} stimulation was determined in the presence of 0.2 μM calmodulin and 30 μM $CaCl_2$. The stimulation of the cyclase with forskolin (100 μM) was used as a positive control. Ethanol was used as the vehicle to a final concentration of 1%. The peptides were dissolved in doubly distilled water. All samples containing calmodulin and the peptides were preincubated with the enzyme for 60 min at 22 °C. The Hill coefficient was deduced from a plot of $\log[v/(V_{max} - v)]$ vs log [peptide].

Ca^{2+}-ATPase Activity Assay. The plasma membrane Ca^{2+}-ATPase activity was measured by following the release of inorganic phosphate by the colorimetric method of Lanzetta et al. (1979). The purified Ca^{2+}-ATPase (8 pmol) was incubated with calmodulin (120 pmol) and/or the NO-synthase or adenylyl cyclase peptides in 20 mM Hepes, pH 7.2, 100 mM KCl, 1 mM $MgCl_2$, 0.65 mM $CaCl_2$, and 0.432 mM EGTA. The ATPase activity was measured at 660 nm after 20-min incubation at 37 °C in the presence of 1 mM ATP. The ATPase was isolated from human erythrocytes by CaM affinity chromatography as described by Niggli et al. (1979).

RESULTS

The primary sequences of the rat brain, calmodulin-dependent NO-synthase and of the bovine brain calmodulin-sensitive adenylyl cyclase (type I; Krupinski et al., 1989) contain hydrophobic stretches with some basic residues which would qualify as candidates for calmodulin binding sites. Two such domains were chosen for each enzyme, and the corresponding carboxamide peptides were prepared by solid-phase peptide synthesis followed by preparative HPLC (see Materials and Methods).

Calmodulin Binding Domain of NO-Synthase. In the case of the NO-synthase peptide NO-30 (K-R-R-A-I-G-F-K-K-L-A-E-A-V-K-F-S-A-K-L-M-G-Q-A-M-A-K-R-V-K-NH_2, amino acids 725–754 of the rat brain enzyme), the sequence corresponded to a region which had been suggested to be involved in calmodulin binding (Bredt et al., 1991). Peptide NO-33 (V-H-K-K-R-V-S-A-A-R-L-L-S-R-Q-N-L-Q-S-P-K-F-S-R-S-T-I-F-V-R-L-H-T-NH_2) also had properties that made it a plausible calmodulin binding domain, although it did not contain an aromatic residue next to its N-terminal. Gel electrophoresis in the presence of urea, which has been successfully used to monitor complex formation

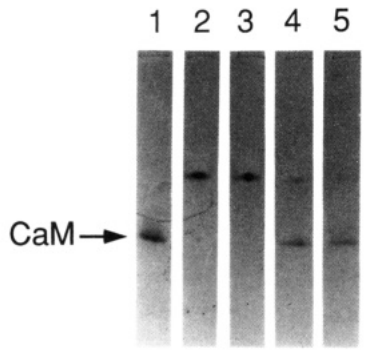

FIGURE 1: Ca^{2+}-dependent complex formation of calmodulin with peptide NO-30. The complex formation was monitored by polyacrylamide gel electrophoresis in the presence of 4 M urea and 1 mM $CaCl_2$. The calmodulin concentration was 10 μM. As indicated under Materials and Methods, staining was performed with 0.025% Coomassie Blue in 10% acetic acid. Lane 1, calmodulin; lane 2, peptide NO-30/calmodulin (1:1); lane 3, peptide NO-30/calmodulin (10:1); lane 4, peptide NO-33/calmodulin (1:1); lane 5, peptide NO-33/calmodulin (10:1). The thick arrow indicates the calmodulin band; the remaining bands are the slower migrating complex bands.

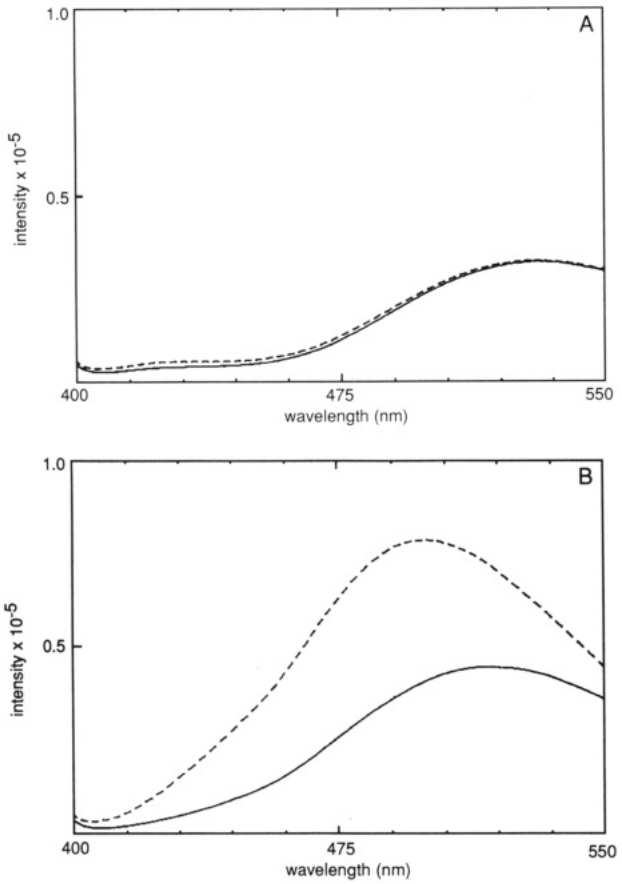

FIGURE 2: (A) Emission spectrum of dansylcalmodulin with peptide NO-30 in 1 mM EGTA solution. The figure shows the emission spectrum of dansylcalmodulin (—) and of the peptide NO-30/dansylcalmodulin mixture (- - -). The solution contained 200 nM dansylcalmodulin and 400 nM peptide NO-30. (B) Emission spectrum of dansylcalmodulin after complex formation with peptide NO-30 in the presence of 1 mM Ca^{2+}. The figure shows the emission spectrum of dansylcalmodulin (—) and of the peptide NO-30/dansylcalmodulin complex (- - -). The solution contained 200 nM dansylcalmodulin and 240 nM peptide NO-30. Excitation was performed at 340 nm.

(Erikson-Vitanen & DeGrado, 1987), was used for the qualitative characterization of calmodulin binding to the two NO-synthase domains. The certified calmodulin binding domain of the plasma membrane Ca^{2+} pump (peptide C20W, L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K; James et al., 1988; Vorherr et al., 1990) and an acidic domain located N-terminally to it (peptide A18; Verma et al., 1988) were employed in the gels as positive and negative controls, respectively. Two molar ratios of peptide to calmodulin were tested (Figure 1): as expected, a strict Ca^{2+} dependence was observed for the mobility shifts (not shown); i.e., no complex formation occurred when 2 mM EGTA was added. An upward shift of the calmodulin band indicated complex formation at a molar ratio of 1:1 for peptide NO-30, without further mobility changes occurring when the molar ratio of peptide to calmodulin was increased to 10. By contrast, only traces of calmodulin complex formation were detected in the case of peptide NO-33. The controls showed the expected complex formation with the plasma membrane Ca^{2+}-ATPase peptide C20W, and no complex with the acidic peptide A18 (not shown).

Fluorescence titration experiments were then performed to determine the affinity of peptide NO-30 for calmodulin. In the absence of Ca^{2+}, no significant fluorescence enhancement around 500 nm upon addition of peptide NO-30 to a solution of 200 nM dansylated calmodulin was observed (Figure 2A). By contrast, in the presence of 1 mM Ca^{2+}, a 1.9-fold increase in fluorescence intensity and a shift of the maximum of emission toward 490 nm demonstrated complex formation (Figure 2B). Peptide NO-33 showed no significant fluorescence enhancement in similar experiments, even when used in micromolar concentrations (not shown). A titration experiment with peptide NO30 was then carried out, yielding about 80% saturation at a peptide/dansylcalmodulin ratio of 1:1 (Figure 3A). The data points of one representative experiment were used to calculate the K_d of the interaction according to Stinson and Holbrook (1973) (Figure 3B). The calculated K_d was 1.8 nM with a margin of error of ±1 nM.

Calmodulin Binding Domain of Adenylyl Cyclase. Fluorescence enhancement indicating the formation of a calmodulin complex was established in experiments using dansylated calmodulin and peptide AC-28 (495–522) (Figure 4). By contrast, peptide AC-24 (1024–1050) showed no significant fluorescence enhancement even when employed in much higher concentrations (not shown). Since peptide AC-24 was highly soluble (more so, actually, than peptide AC-28), the possibility that the formation of aggregates precluded favorable calmodulin binding could be ruled out. As was the case for peptide NO-30, the effect of peptide AC-28 was entirely Ca^{2+}-dependent; i.e., no significant changes in the spectrum of dansylcalmodulin were observed when the peptides were added in the presence of EGTA (data not shown). In the presence of Ca^{2+}, the enhancement factor was 2.55 at the maximum of 492 nm (Figure 4). Interestingly, peptide AC-15 (495–509), a shorter version of peptide AC-28, also showed Ca^{2+}-dependent fluorescence enhancement. Titration experiments at 490 nm were then performed using increasing amounts of peptides AC-15 and AC-28. In the case of peptide AC-15, the end point of the titration could not be determined since no plateau was reached during the course of the titration (Figure 5A). On the other hand, the titration with peptide AC-28 was completed in the nanomolar concentration range (Figure 5B). The K_d for peptide AC-15 was calculated to be 750 ± 80 nM (Figure 6A), whereas that for peptide AC-28 was 2.5 ± 0.8 nM (Figure 6B).

To confirm the findings from fluorescence measurements, the effects of the peptides on the calmodulin stimulation of purified adenylate cyclase were studied, using Pansorbin–BBC-1-coupled calmodulin-dependent adenylyl cyclase (Fig-

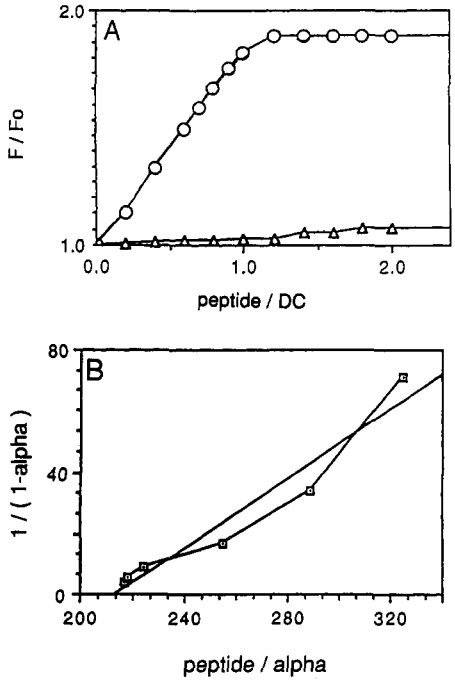

FIGURE 3: (A) Titration of dansylcalmodulin with peptide NO-30. Dansylcalmodulin (200 nM) was titrated with the peptide in the presence (O) and absence (Δ) of 1 mM Ca^{2+}. Excitation was performed at 340 nm. The relative fluorescence intensities are plotted against the ratio between the total concentration of peptide NO-30 and the total concentration of dansylcalmodulin, as given by one representative titration experiment. The data points were recorded as outlined under *Materials and Methods*. (B) *Determination of the* affinity constant of peptide NO-30 for dansylcalmodulin. The data points for the titration of dansylcalmodulin with peptide NO-30 in the presence of 1 mM Ca^{2+} were recorded, and the fractional degree of saturation of dansylcalmodulin (alpha) was calculated as outlined under Materials and Methods. The plot of $1/(1 - \text{alpha})$ against the total concentration of peptide (nM) divided by alpha results in a straight line if a 1:1 complex is formed and the titration end point is correctly estimated. The zero intercept on the x axis refers to the total dansylcalmodulin concentration (200 nM), and serves as a control. The reciprocal of the slope gives the affinity constant. The plot describes a representative experiment. The calculated K_d value for the peptide was 1.8 nM, and an r value of 0.970 was calculated for the curve.

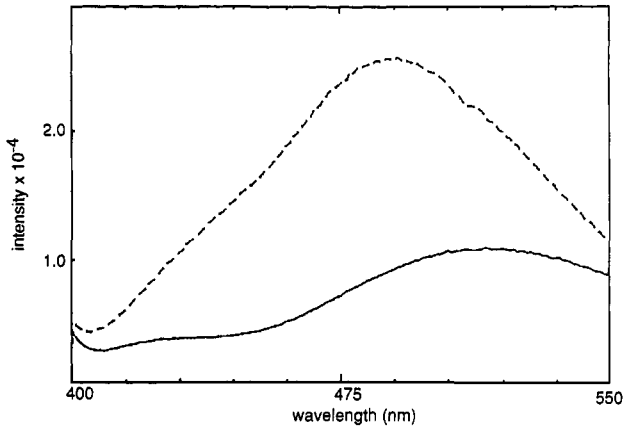

FIGURE 4: Emission spectrum of dansylcalmodulin after complex formation with peptide AC-28 in the presence of Ca^{2+}. Excitation was performed at 340 nm. The figure shows the emission spectrum of Ca^{2+}-bound dansylcalmodulin (—) and of the peptide AC-28/dansylcalmodulin mixture (complex) in the presence of 1 mM Ca^{2+} (- - -). The solution contained 90 nM dansylcalmodulin and 200 nM peptide AC-28.

ure 7, see Materials and Methods). BBC-1 is a monoclonal antibody which had previously been raised against the CaM-sensitive adenylyl cyclase purified from bovine brain cortex

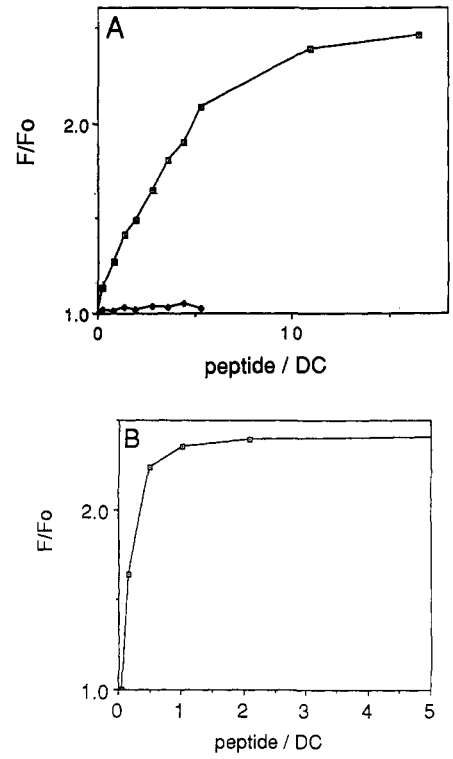

FIGURE 5: Titration of dansylcalmodulin with peptides AC-15 and AC-28. (A) Dansylcalmodulin (90 nM) was titrated with the calmodulin binding peptide AC-15 in the presence (□) and absence (■) of 1 mM Ca^{2+}. Excitation was performed at 340 nm. (B) Dansylcalmodulin (90 nM) was titrated with the calmodulin binding peptide AC-28 in the presence of 1 mM Ca^{2+}. Excitation was performed at 340 nm. The relative fluorescence intensities were plotted against the ratio between the total concentration of the peptide and the total concentration of dansylcalmodulin, as given by one representative titration experiment. The data points were recorded as outlined under Materials and Methods.

(Mollner & Pfeuffer, 1988; Pfeuffer et al., 1985). BBC-1 was found to be directed against the extreme C-terminus of bovine brain type I cyclase, since a peptide synthesized according to the published sequence prevented its recognition. This sequence is unique to type I since this antibody failed to detect types II–VI adenylyl cyclases in bovine tissues like brain, lung, heart, kidney, and olfactory cilia. Addition of saturating concentrations of calmodulin (2 μM) to the Pansorbin–BBC-1-coupled complex in the presence of 30 μM Ca^{2+} produced a 9-fold stimulation of the activity seen in the presence of EGTA (data not shown). Half-maximal stimulation was observed at a concentration of 15 nM (not shown). The peptides were then tested for their ability to inhibit the stimulation of the cyclase by calmodulin (0.2 μM). When peptide AC-24 was used, concentrations of 2–3 μM were required for half-maximal inhibition of the stimulation. Peptide AC-15 exhibited an IC_{50} at similar concentrations (3–5 μM). In the case of peptide AC-28, much lower concentrations were necessary for inhibition (IC_{50}: 400–500 nM) (Figure 7). It deserves to be mentioned that the shorter version of peptide AC-28, peptide AC-15, exhibited normal Michaelis–Menten inhibition ($n = 1$), while peptide AC-28 inhibited calmodulin-stimulated activity in a highly cooperative manner ($n \gg 5$). The inhibitory effects were dependent on the calmodulin concentration, and the basal and forskolin-stimulated activities were not affected. The effects of AC-28 were more pronounced than those of the calmodulin binding domain of the plasma membrane Ca^{2+} pump (peptide C20W), which was used as a control (IC_{50}: 1 μM). A sample of the calmodulin binding domain of the CaM-dependent protein

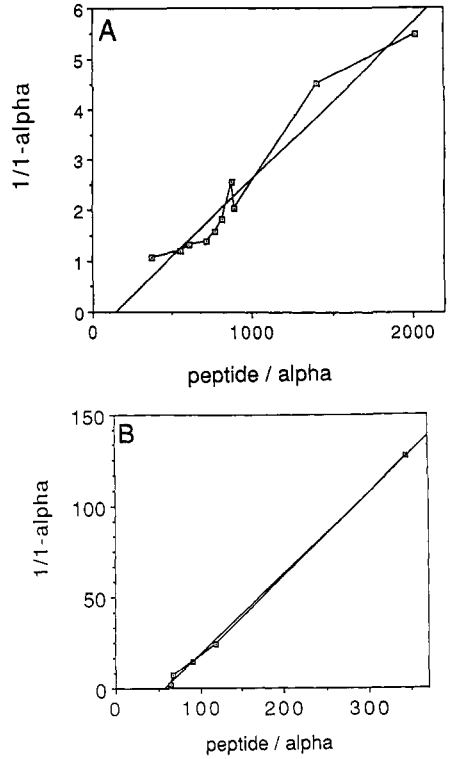

FIGURE 6: Determination of the affinity constant of peptides AC-15 and AC-28 for dansylcalmodulin in the presence of Ca^{2+}. The data points were recorded, and the fractional degree of saturation of dansylcalmodulin (alpha) was calculated as outlined under Materials and Methods and in Figure 3. (A) Titration curve of peptide AC-15 in the presence of 1 mM Ca^{2+}. 90 nM dansylcalmodulin was titrated with the peptide. The plots describe one representative experiment. The calculated K_d value was 752 nM, and an r value of 0.969 was calculated for the curve. (B) Titration curve of peptide AC-28 in the presence of 1 mM Ca^{2+}. 90 nM dansylcalmodulin was titrated with the peptide. The plot describes one representative experiment. The calculated K_d value for the peptide was 2.5 nM.

kinase II (L-K-K-F-N-A-R-R-K-G-A-I-L-T-T-M-L-A-OH) was also used as a control: it started to inhibit at a lower concentration than peptide AC-28 (IC_{50}: 200 nM); however, complete inhibition was obtained in the same concentration range (Figure 7).

Effect of the Putative Calmodulin Binding Peptide of Adenylyl Cyclase and NO-Synthase on the Stimulation of the Plasma Membrane Ca^{2+} Pump by Calmodulin. The possibility that the proposed calmodulin binding peptides of the synthase and the cyclase had secondary effects on each respective enzyme (i.e., direct inhibiton of the active sites) was tested by measuring their effects on the stimulation by calmodulin of an unrelated calmodulin-modulated enzyme. The enzyme chosen was the plasma membrane Ca^{2+}-ATPase, which was purified from human erythrocytes. Figure 8 shows that none of the four peptides tested had any effect on the basal activity of the ATPase. The stimulation by calmodulin, however, was affected much in the same way as observed for the synthase and the cyclase. Essentially, complete removal of the stimulation was observed when peptides NO-30 and AC-28 were used in 5-fold molar excess over calmodulin. Intermediate levels of inhibition were observed at lower molar ratios. As expected, peptide NO-33 had no effect, whereas a small effect was seen with peptide AC-24.

DISCUSSION

Despite the lack of sequence similarities, calmodulin binding domains share some general properties, and could thus in

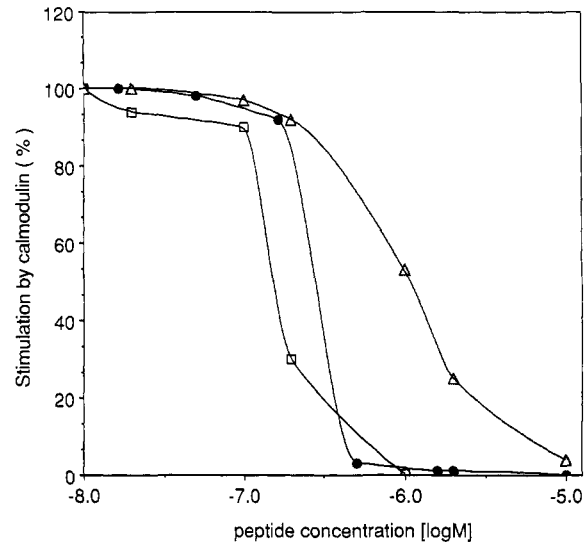

FIGURE 7: Inhibition of the calmodulin-stimulated activity of adenylyl cyclase by calmodulin binding peptides. The stimulation by calmodulin (0.2 μM) of adenylyl cyclase coupled to Pansorbin–BBC-1 antibody was measured in the presence of the indicated peptides: AC-28 (●), the Ca^{2+}-pump peptide C20W (△), and the peptide corresponding to the calmodulin binding domain of CAM-dependent kinase II (□). Adenylyl cyclase measurements were performed as described under Materials and Methods. The results are representative of eight identically conducted experiments, starting with crude Chaps-solubilized bovine brain membranes.

principle be predicted from analysis of the sequence of calmodulin-modulated proteins. However, despite these general properties (see the introduction), a safe identification is not generally possible: many proteins contain basic–hydrophobic stretches, often as a repeat feature, yet only a handful of them are calmodulin-modulated. A case in point is the adenylyl cyclase type I analyzed in the present paper: sequence analysis would have indicated as the best candidate for the calmodulin binding domain the sequence here defined as peptide AC-24. It had the required hydrophobic character, it contained five Arg and three Lys, and it had an aromatic amino acid close to the N-terminus. In addition, the sequence was located C-terminally, a property frequently found for calmodulin binding domains [e.g., see Lukas et al. (1986) and James et al. (1988)]. Yet, peptide AC-24 evidently was not the calmodulin binding domain of the cyclase, given its very poor affinity for calmodulin. The adenylyl cyclase domain, which proved to be the most plausible calmodulin binding domain, was the sequence here defined as AC-28. This peptide indeed had the appropriate high calmodulin affinity when tested directly, and, furthermore, it efficiently interfered with the stimulation of adenylyl cyclase by calmodulin. AC-28 had the expected hydrophobic character; it contained six Lys and two Arg, and had a Phe in position 9. When plotted according to the helix wheel model, it formed an amphipathic helix at the very N- and C-terminal portions. Rather unusually, however, the domain is located centrally in the cyclase molecule rather than terminally. Interestingly, the corresponding sequence is entirely different in adenylyl cyclase type II, which does not bind calmodulin (Feinstein et al., 1991), and even in adenylyl cyclase type III, which reacts with calmodulin with very low affinity (Choi et al., 1992). The rather high affinity of peptide AC-28 for calmodulin (2 nM) is at variance with that of the enzyme whose K_d for calmodulin has been determined to be 15 nM. A similar value (20 nM) has been obtained for the enzyme expressed in Sf9 cells (Tang-Wei et al., 1991). The reason for this discrepancy is not known.

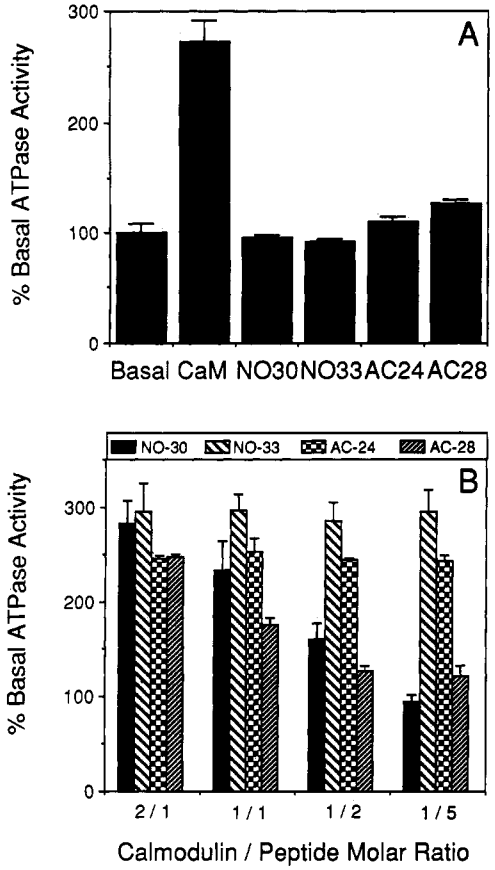

FIGURE 8: Effect of the NO-synthase and adenylyl cyclase peptides on stimulation of the plasma membrane Ca^{2+}-ATPase by calmodulin. Ca^{2+}-ATPase activity (average of two experiments) plotted as the percentage of the activity measured in the absence of CaM and peptides. (A) From right to left: basal activity (8 pmol of purified plasma membrane Ca^{2+}-ATPase), maximal calmodulin stimulation (120 pmol of CaM), and ATPase activity in the presence of the maximal amount of the peptides used for the competition experiment shown in (B) (600 pmol). (B) Effect of the peptides on calmodulin stimulation: different amounts of peptides NO-30, NO-33, AC-24, and AC-28 were added to 120 pmol of calmodulin, and the Ca^{2+}-ATPase activity was measured as described under Materials and Methods. The error bars indicate the variation in the two experiments performed.

As for the calmodulin-stimulated brain NO-synthase, the domain that could be predicted to be the best candidate from the analysis of the sequence (Bredt et al., 1991) was indeed the domain with the highest calmodulin affinity. The domain showed Ca^{2+}-dependent complex formation in urea gels and had the appropriate high affinity for calmodulin in the fluorescence titration experiments. Furthermore, the sequence had the expected hydrophobic character, contained seven Lys and three Arg, and had a Phe in position 7. In the helix wheel representation, the sequence could be predicted to form an amphipathic helix. The other tested domain of the synthase, here defined as peptide NO-33, also appeared to be a candidate in principle, except for the fact that it did not contain an N-terminally located aromatic amino acid: however, it failed to bind calmodulin in the urea gel experiments. One interesting point on the NO-synthase has emerged from the sequence alignment of the brain (calmodulin-stimulated) and the macrophage enzymes. The sequence 725–754 (peptide NO-30) in the former is replaced in the latter by the sequence 504–532: 16 out of 29 residues are different in the 2 sequences (5 are conservative replacements), yet the latter sequence contains 9 basically charged residues in a background of hydrophobic residues and a phenylalanine in position 6. It also thus nicely qualifies as a calmodulin binding domain. Recent work (Cho et al., 1992) has indeed shown that the macrophage enzyme contains very tightly bound calmodulin as a subunit: interestingly, the binding does not require the elevation of the concentration of Ca^{2+} above the level found in resting cells, i.e., to 400–1100 nM (Olwin et al., 1984; Olwin & Storm, 1985), as typically demanded by conventional calmodulin–enzyme complexes: in this case, association occurs at 39 nM. The macrophage synthase is thus one of the only four enzymes known so far to bind calmodulin in an apparently Ca^{2+}-independent way: the other three are the γ-subunit of phosphorylase kinase (Cohen et al., 1978; Picton et al., 1980), a cyclic nucleotide phosphodiesterase (Sharma & Wang, 1986), and the adenylyl cyclase of *Bordetella pertussis* (Landant, 1988). Apparently, the affinity of the macrophage synthase domain for calmodulin is unusually high: it will now be interesting to prepare the putative calmodulin binding peptide of the inducible macrophage synthase and to test it for calmodulin binding and affinity.

At least two problems could be considered at this point. One is the possibility that domains that do not have very high affinity for calmodulin (e.g., K_d's higher than 1–5 μM) could still bind calmodulin in vivo. This is a permissible question, since the concentration of calmodulin in mammalian tissues varies between 2 and 30 μM (Carafoli, 1987). Although no precise information is available on the percentage of the total mammalian cell calmodulin that is free, the possibility of a role for binding sites having K_d's in the high nanomolar range cannot be ruled out. The recent work on the peptide inhibition of the heart plasma membrane Na^+/Ca^{2+} exchanger (Li et al., 1991), a protein not normally known to be calmodulin-regulated, certainly goes in this direction. Another factor to be considered (see the introduction) is the abundance of the calmodulin binding protein, which could make even a low-affinity interaction physiologically significant. While this is probably not the case for low-abundance proteins like the Na^+/Ca^{2+} exchanger, it may well be the case for other proteins (Alexander et al., 1988).

The other problem is that of the molecular significance of the general structural properties of the domains for the binding to calmodulin. According to general consensus, hydrophobic interactions appear to play a role in the interaction of calmodulin with targets, and mutations in the basic amino acids of the binding domains in target proteins lead to the impairment of the binding of calmodulin. Recent work by Ikura et al. (1992) has shed light on the role of the hydrophobic backbone and of the basic amino acids of the binding domain for the case of myosin light chain kinase. Very interestingly, it has also provided a structural rationale for the importance of the N-terminally located aromatic residue. Although caution in making this type of extrapolation is necessary, the presence of an N-terminal located aromatic residue may be a necessary feature of calmodulin binding domains.

ACKNOWLEDGMENT

We are indebted to Prof. Palm for the synthesis of peptide AC-15. The expert technical help of Mrs. E. Pfeuffer is gratefully acknowledged.

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