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Affinity Chromatography of Brain Cyclic Nucleotide Phosphodiesterase Using 3-(2-Pyridyldithio)propionyl-Substituted Calmodulin Linked to Thiol-Sepharose†

Randall L. Kincaid* and Martha Vaughan

ABSTRACT: [3-(2-Pyridylthio)propionyl]calmodulin (PDP-CaM), an activated thiol derivative of calmodulin (CaM), was synthesized. Preparations of this derivative containing an average of 2.8 mol of substituent/mol of protein activated purified cyclic nucleotide phosphodiesterase in a manner indistinguishable from that of native CaM. PDP-CaM was covalently coupled to free thiol-Sepharose 4B through formation of a stable mixed disulfide bond for use in affinity chromatography. The binding capacity of the disulfide-linked CaM-Sepharose for phosphodiesterase activity was proportional to substituent level up to 4 mg of CaM/mL of gel; the total capacity of the gel for binding phosphodiesterase was 4 times that of CNBr-coupled CaM-Sepharose. Quantitative recovery was achieved by desorption of both ligand and bound proteins with a reducing agent. The thiolated CaM derivative was then separated from phosphodiesterase by rapid gel filtration; the overall recovery of phosphodiesterase activity was greater than 70%. Preparations of homogeneous enzyme in good yield were obtained after a second chromatography step on CaM-Sepharose. Binding and recovery of phosphodiesterase activity were entirely reproducible, since each preparation of affinity gel was used only once. As it permits separation of interacting species in free solution, this general method may be useful with other ligands for increasing yields from affinity chromatography, particularly when dissociation of molecules in their matrix-bound conformation may be difficult to achieve.

Affinity chromatography (Cuatrecasas et al., 1968) depends on the interaction of macromolecules with specific ligands that have been covalently bound to a solid matrix such as agarose. The use of CNBr for coupling of amine-containing ligands to inert supports (Axèn et al., 1967) has enjoyed widespread use, although a variety of other chemical procedures are also available [for a review, see Lowe & Dean (1979)]. These chromatographic media are extremely valuable for many types of selective separations; however, changes in their properties with use can cause problems. Gradual leakage of matrix-bound ligand has been noted (Parikh et al., 1974), and decreases either in functional capacity of the affinity matrix or in recovery of bound proteins occur in some instances. The reliability and reproducibility of purification procedures that depend upon such steps may thus be compromised with repeated usage of the affinity gel. We have observed such changes in the chromatographic behavior of calmodulin (CaM)[1] coupled to CNBr-activated Sepharose (Kincaid &

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[1] Abbreviations: CaM, calmodulin; QAE-Sephadex, [diethyl(2-hydroxypropyl)amino]ethyl-Sephadex; PDP-CaM, [3-(2-pyridyldithio)-propionyl]calmodulin; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl chloride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DEAE, diethylaminoethyl; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate.

Vaughan, 1979). We describe here a new method for preparation and coupling of the 3-(2-pyridyldithio)propionyl (PDP) derivative of CaM to a solid support via a stable disulfide linkage and the use of reducing agents to completely release bound proteins along with the affinity ligand. This disulfide-linked CaM–Sepharose has proven very useful in the purification of calmodulin-activated phosphodiesterase, and the general strategy could be advantageous for affinity chromatography of other macromolecules.

Experimental Procedures

Materials. Tris base, ammonium sulfate, urea, and dithiothreitol were purchased from Bethesda Research Laboratories; DEAE-Bio-Gel A and Affi-Gel 501 were from Bio-Rad; activated thiol-Sepharose 4B, SPDP, and QAE-Sephadex A-25 were from Pharmacia; 2,2′-dipyridyl disulfide, PMSF, and pepstatin A were from Sigma; Ultrogel AcA 54 was from LKB; leupeptin was from Boehringer-Mannheim; BES and CHAPS were from Calbiochem; $NaDodSO_4$ was from Pierce Chemical Co. EGTA (Eastman Kodak) was recrystallized from water prior to use. All other reagents were of the highest grade available.

Assay of Phosphodiesterase Activity. Phosphodiesterase activity was assayed as described (Kincaid et al., 1979) with 0.5 μM [^{3}H]cGMP as substrate. One unit of activity is defined as the hydrolysis of 1 μmol of cGMP/min under these assay conditions. Assays of CaM-stimulated activity contained 0.33 mM $CaCl_2$ and 0.25 μg of CaM (50 nM). The concentration of CaM was determined spectrophotometrically by using a value of 0.18 at 277 nm for a solution of 1.0 mg/mL.

Purification of Calmodulin. CaM was purified from bovine brain by an unpublished method (R. Kincaid, C. Odya, V. Manganiello, and M. Vaughan, unpublished observations), which involves extraction of whole brain tissue with 6 M urea, batchwise adsorption of supernatant proteins with QAE-Sephadex A-25, ammonium sulfate precipitation, and finally ion-exchange chromatography on QAE-Sephadex A-25. The purified CaM was homogeneous by sodium dodecyl sulfate electrophoresis and had ultraviolet and circular dichroic spectra identical with those published (Watterson et al., 1976). CaM was stored as a lyophilized powder after exhaustive dialysis against distilled water.

Preparation of [3-(2-Pyridyldithio)propionyl]calmodulin (PDP-CaM). PDP-CaM was prepared by dropwise addition of 0.1 volume of 30 mM *N*-succinimidyl 3-(2-pyridylthio)-propionate (Pharmacia) in absolute ethanol to a gently stirring solution of CaM (5–7 mg/mL) in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl. After incubation for 45 min at 25 °C, the mixture was placed on ice and then dialyzed vs. 100 volumes of 0.1 M sodium acetate buffer, pH 5.3, containing 0.1 M NaCl or desalted in the same buffer on a column of Bio-Gel P-6 (Bio-Rad). Desalting with buffer of pH 4.6 as recommended by the manufacturer resulted in precipitation of the modified protein and skewed elution profiles. There was no detectable loss of pyridyl protecting groups from PDP-CaM stored at pH 5.3 for at least 2 months.

The degree of substitution of PDP-CaM was determined essentially as described by the manufacturer. Briefly, samples (25–100 μL) of the dialyzed protein solution were reduced by incubation with 100 μL of 50 mM dithiothreitol for 5 min, and after addition of distilled water to 1 mL, the absorbance of each sample at 343 nm was measured. The number of moles of pyridine-2-thione released was then calculated by using a molar extinction coefficient of 8.08×10^3 and related to the amount of protein in the sample.

Production of Disulfide-Linked CaM–Sepharose. Activated thiol-Sepharose 4B was converted to the free thiol form by reduction with 70 mM dithiothreitol in buffer containing 0.1 M sodium bicarbonate (pH 8.3) and 1 mM EDTA as described by the manufacturer and stored in 0.1 M acetic acid containing 0.5 M NaCl until use. (Reduced gels were stable for several months in this form.) Immediately before conjugation, the reduced gel (10 mL) was washed with 50 mL of 20 mM Tris, pH 8.0, containing 5 mM $MgCl_2$, 0.1 mM EGTA, and 0.25 M NaCl, suspended in 10 mL of the same buffer, and added to 8 mL of PDP-CaM (5 mg/mL) to which had been added 1 mL of 1 M Tris-HCl, pH 8.0. So that substitution of less than 4 mg of PDP-CaM/mL of gel could be achieved, the concentration of PDP-CaM was decreased for some preparations. The suspension was mixed end-over-end for 18 h at 4 °C, after which the gel was thoroughly washed with the above buffer and stored at 4 °C. The efficiency of coupling to the gel was estimated by measurement of the amount of protein recovered in the washes by the G-250 dye-binding assay (Bio-Rad) with purified CaM as a standard.

Purification of Phosphodiesterase. Partially purified CaM-activated phosphodiesterase from 2 kg of bovine brain was prepared by method I (Kincaid et al., 1981) with slight modifications (Kincaid et al., 1982). The fraction eluted from DEAE-Bio-Gel A at pH 5.45 was immediately adjusted to pH 7.5 with 1 M Tris-HCl, pH 8.0, and rapidly concentrated with a Pellicon cassette system (Millipore Corp., PTGC membrane, 10–12 L/h). This fraction (~200 mL) was further concentrated by ultrafiltration (Amicon, PM-10 membrane, 60 psi) to a volume of ~40 mL (protein concentration ~20 mg/mL). After addition of $CaCl_2$ to a final concentration of 1 mM, the sample was applied to a column (4.2 × 2.5 cm) of CNBr-coupled CaM–Sepharose 4B (~0.8 of CaM/mL of swollen gel) prepared as previously described (Kincaid & Vaughan, 1979). This column quantitatively bound the CaM-binding protein calcineurin, which was present in the eluate of the DEAE-Bio-Gel A column (see also the legend to Table I).[2] After the first 15 mL of effluent was discarded, the succeeding effluent and wash (20 mL of 25 mM BES buffer, pH 7.0, containing 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 250 mM NaCl) were combined and added to 2.5 mL of disulfide-linked CaM–Sepharose 4B. After being gently mixed for 5 h at 4 °C, followed by centrifugation (2 min, 2000*g*), the gel was suspended in a minimal volume of the above washing buffer and poured into a column (1-cm diameter). The gel was washed with 5 mL and then with 15 mL of 20 mM Tris buffer, pH 8.5, containing 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 250 mM NaCl; 1.5 mL of the same buffer minus $CaCl_2$ and containing 2 mM EGTA, 20 mM dithiothreitol, and 10% glycerol was then added. Thirty minutes later, the gel was eluted with a total of 7.0 mL of the thiol-containing buffer. The eluate was immediately applied to a gel filtration column as described in the legend to Figure 4. All buffers used in the affinity chromatography steps contained PMSF, 50 μg/mL, pepstatin A, 10 μg/mL, and leupeptin, 10 μg/mL.

Sodium Dodecyl Sulfate Gel Electrophoresis. Electrophoresis under denaturing conditions was carried out essentially as described by Laemmli (1970) except that $NaDodSO_4$

[2] The removal of calcineurin on the first calmodulin–Sepharose column was not dependent on the particular type of calmodulin affinity gel used, since CNBr-coupled affinity gels of different substituent concentrations or those prepared by the present procedure worked similarly as long as this first affinity step removed ~15–20% of the phosphodiesterase activity (Kincaid et al., 1982). This preferential binding of calcineurin under conditions of limited availability of calmodulin sites suggests that it has a greater operational affinity for matrix-bound calmodulin than phosphodiesterase.

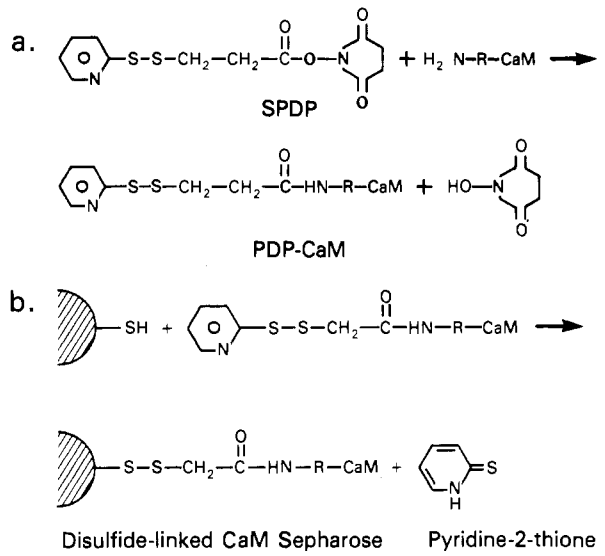

FIGURE 1: (a) Reaction of *N*-succinimidyl 3-(2-pyridyldithio)-propionate with calmodulin. (b) Coupling of PDP-CaM to a matrix of free thiol-Sepharose 4B.

(0.1%) was present in the upper reservoir buffer only. Before electrophoresis, samples were precipitated with cold trichloroacetic acid (10% w/v final concentration). After centrifugation (10 min, 10000*g*), the pellet was solubilized in gel buffer containing 1% $NaDodSO_4$, 5% β-mercaptoethanol, 10% glycerol, and 0.0015% bromophenol blue. Total concentration of acrylamide (% T) in resolving gel was 10% and the cross-linking agent, bis(acrylamide), was 2.5% of the total acrylamide (% C) (Chrambach et al., 1976). Stacking gel concentrations were 5% T/15% C with *N,N'*-diallyltartardiamide as the cross-linking agent. Gel mixtures containing the catalysts potassium persulfate (65 μg/mL for resolving gel, 250 μg/mL for stacking gel) and riboflavin 5'-phosphate (6.5 μg/mL) were deaerated for 5 min after which *N,N,N',N'*-tetramethylenediamide (0.0004% v/v for resolving gel, 0.001% v/v for stacking gel) was added. Photopolymerization for 20 min was used in all cases. Electrophoresis was carried out at a constant power of 2 W/gel (0.075 × 9 × 14 cm). All reagents were "electrophoresis grade" and were purchased from Bio-Rad Laboratories.

Results

Characterization of PDP-CaM. Incubation of CaM with SPDP (7.5 mol/mol of CaM) resulted in covalent incorporation of ~2.8 mol of substituent/mol of CaM with >90% recovery of protein. This reaction (Figure 1) involves nucleophilic attack by α- or ϵ-amino groups of CaM on the *N*-hydroxysuccinimide ester with production of a stable amide bond and release of *N*-hydroxysuccinimide (Anderson et al., 1964). As expected, modification with a pyridyl substituent produced a dramatic alteration in the ultraviolet spectrum of CaM wiith a 5-fold increase in the absorbance at 280 nm (Figure 2). The absorbance (1-cm light path) of the non-reduced derivative (1 mg/mL) at this wavelength was 0.88. The solubility of PDP-CaM at low ionic strength (0.1 M NaCl) was dramatically lower than that of the unmodified molecule; it was quantitatively but reversibly precipitated at pH 4.5 (data not shown).

The reduced (thiolated) form of PDP-CaM was as active as the parent protein in stimulating a homogeneous preparation of CaM-activated phosphodiesterase (Figure 3). Half-maximal activation was produced by ~2 nM of either, and maximal activation was ca. 10-fold.[3] In assays with 50 mM

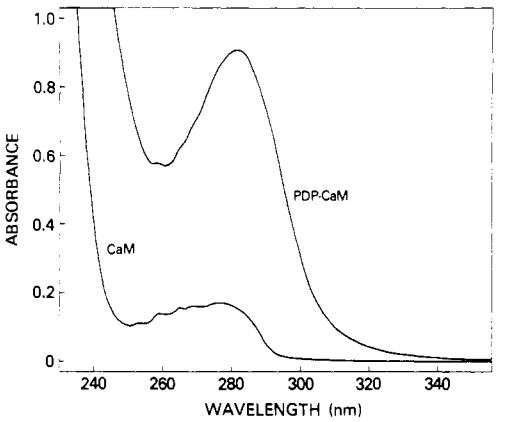

FIGURE 2: Ultraviolet spectra of native and PDP-CaM. After exhaustive dialysis of the proteins against 20 mM Tris, pH 8.0, containing 200 mM NaCl, 0.1 mM EGTA, and 5 mM $MgCl_2$, the concentrations of both samples were adjusted to 1.0 mg/mL with the same buffer and spectra recorded on an Acta III spectrophotometer.

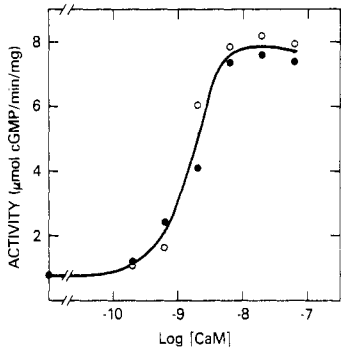

FIGURE 3: Activation of purified phosphodiesterase by native and PDP-CaM. The activity of purified phosphodiesterase (2 ng) was assayed in a total volume of 300 μL containing 0.2 mM dithiothreitol as described under Experimental Procedures. Native CaM (●) or PDP-CaM (○) was present at the indicated molar concentration. The concentration of native CaM was determined spectrophotometrically and that of PDP-CaM by protein measurement with native CaM as standard.

KCl (Davis & Daly, 1978) or 10 μM spermine (Kincaid et al., 1979), basal activity was diminished, and stimulation was ≥30-fold (data not shown).

Coupling of PDP-CaM to Reduced Sulfhydryl Gels. When different amounts of PDP-CaM were incubated with the reduced form of activated thiol-Sepharose 4B, coupling was virtually quantitative up to ~4.2 mg of protein/mL of swollen gel, and the amount of phosphodiesterase activity bound to each preparation of disulfide-linked CaM–Sepharose was proportional to its CaM content, indicating that functional capacity was not diminished, e.g., because of steric hindrance, with increasing degree of substitution (data provided to reviewers). By measurement of the increase in absorbance of the coupling solution due to the released pyridine-2-thione, the time course of ligand attachment was followed (Figure 1). Coupling was usually complete in 6 h (data not shown) with release of pyridine-2-thione equivalent to the amount of PDP (linked to CaM) that was added; i.e., there were no PDP groups remaining on CaM that might react with proteins applied to the affinity gel.

[3] When incubated with PDP-CaM at higher concentrations (10^{-8} M) and then assayed in the absence of reducing agent, purified phosphodiesterase [(0.5–1) × 10^{-10} M] was activated with stoichiometric amounts of PDP-CaM, indicating the formation of a fully active cross-linked species (R. L. Kincaid and M. Vaughan unpublished observations).

Table I: Purification of Calmodulin-Activated Phosphodiesterase by Affinity Chromatography on Disulfide-Linked Calmodulin–Sepharose

	vol (mL)	protein[a] (mg)	act. (milli-units/mg)	recovery (%)
applied	45	1000	23.2	100
not bound	45	986	2.1	7
bound and eluted with thiol	7	8[b]	2300	79

[a] Determined by the G-250 dye-binding assay (Bio-Rad) with bovine serum albumin as standard. [b] Non-calmodulin protein present in eluate. The fraction applied to 2.5 mL of disulfide-linked CaM–Sepharose was prepared as follows: supernatant (6.3 L) from 2 kg of bovine brain was applied to 1.6 L (bed vol) of DEAE-Bio-Gel A, and after being washed with 40 mM sodium acetate buffer (pH 5.95) containing 2 mM $MgCl_2$, 1 mM NaN_3, and 0.2 mM EGTA, phosphodiesterase was eluted with 5 L of the above buffer (containing 10% glycerol) that had been adjusted to pH 5.45 with acetic acid. After adjustment of the pH of the eluate to 7.5 and subsequent concentration, it was depleted of calcineurin with CaM–Sepharose as described under Experimental Procedures. The phosphodiesterase activity not retained on the first affinity column (~85% of that applied), which was purified 10.5-fold from supernatant (27% recovery), was then added to disulfide-linked CaM–Sepharose.

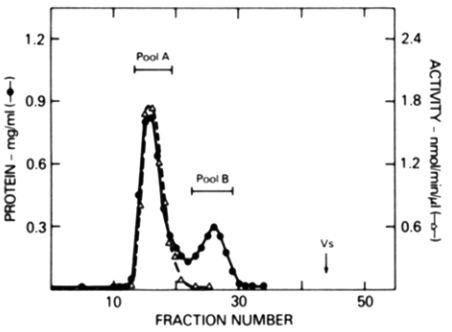

FIGURE 4: Separation of phosphodiesterase and thiolated CaM by gel filtration after release from disulfide-linked CaM–Sepharose with dithiothreitol. The eluate (6.8 mL) from the affinity column was applied to a column (2.6 × 25 cm) of Ultrogel AcA 54 and eluted with 25 mM BES, pH 7.0, containing 250 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM EDTA, leupeptin (10 μg/mL), pepstatin A (10 μg/mL), and PMSF (50 μg/mL) (45 mL/h). Fractions (3 mL) were collected and samples assayed for protein (●) and phosphodiesterase (△). The fractions containing phosphodiesterase activity (pool A) and those containing reduced CaM (pool B) were pooled separately. V_s was monitored by the appearance of dithiothreitol by UV absorption and was equal to 1 column volume.

Purification of Phosphodiesterase with Disulfide-Linked CaM–Sepharose. Disulfide-linked CaM–Sepharose containing ~4 mg of CaM/mL of gel was used to isolate phosphodiesterase from a partially purified enzyme fraction; approximately 2.5 mL of gel was required to bind >90% of the activity of this fraction from 2 kg of bovine brain (Table I). Greater than 95% of the applied protein did not bind to the gel, and after being washed, the enzyme was eluted with Tris buffer, pH 8.5, containing 20 mM dithiothreitol and 2 mM EGTA in 80% yield with ~100-fold purification. (Recoveries of enzyme activity were >90% from disulfide-linked CaM–Sepharose prepared by derivatization of the reduced sulfhydryl gel after its initial use.)

Separation of Phosphodiesterase from Thiolated CaM. Separation of the reduced CaM derivative from the phosphodiesterase was achieved by gel filtration immediately after elution from the affinity gel. The sample (up to 6% of total bed volume) was applied to a short column of Ultrogel AcA 54 (Figure 4), and the column was eluted rapidly [~10 mL/cm²·h)]. Separation from the CaM derivative was complete in ~2 h; the active phosphodiesterase fractions were pooled and made 20% in glycerol for storage. Under the conditions described here for use of the disulfide-linked CaM–Sepharose, significant amounts of nonspecifically bound contaminants were eluted along with the phosphodiesterase.[4] So that a homogeneous preparation of phosphodiesterase could be obtained, the pooled gel filtration fractions were adjusted to 2 mM Ca^{2+} and chromatographed on CNBr-coupled CaM–Sepharose; after being washed, the column was eluted with 2 mM EGTA and concentrated on a column of organomercurial agarose from which phosphodiesterase was subsequently eluted. This increased the specific activity of the phosphodiesterase 5–6-fold and yielded an enzyme of ~95% purity on the basis of gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 5). Gel filtration fractions containing thiolated CaM were combined and subjected to anion-exchange chromatography to eliminate any contaminating proteins.

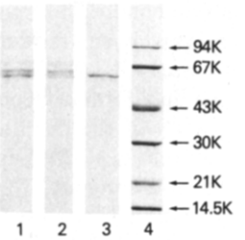

FIGURE 5: Sodium dodecyl sulfate gel electrophoresis of phosphodiesterase fractions. After dialysis against gel filtration buffer containing 20% glycerol, half of the phosphodiesterase fraction from the AcA 54 step (pool A in Figure 4) was made 2 mM in $CaCl_2$ and applied to a small column (0.8 × 4.5 cm) of CaM–Sepharose 4B (3.2 mg of CaM/mL of gel). After being washed with Ca^{2+}-containing buffer, the column was eluted with 6 mL of a buffer made 2 mM in EGTA and concentrated on a column (0.5 × 2 cm) of organomercurial agarose (Affi-Gel 501, Bio-Rad). Phosphodiesterase was eluted from this column with buffer containing 20% glycerol and 10 mM dithiothreitol. Samples of fractions from each step were assayed for enzyme activity or subjected to electrophoresis as described under Experimental Procedures: (lane 1) gel filtration pool A (10 μg); (lane 2) material not retained on CaM–Sepharose 4B (10 μg); (lane 3) eluate from organomercurial agarose (4 μg); (lane 4) protein standards (3–4 μg each), phosphorylase *a*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin (Pharmacia). Activity in the eluate from organomercurial agarose was 70% of that applied (pool A); specific activity was 9500 milliunits/mg of protein, representing a 4300-fold purification from the brain supernatant.

Discussion

Continued use of CaM–Sepharose prepared by a CNBr coupling procedure (Watterson & Vanaman, 1976; Miyaki et al., 1977) resulted in decreasing capacity of the gel and variable recovery of phosphodiesterase activity (R. L. Kincaid, V. C. Manganiello, and M. Vaughan, unpublished observations). It was, therefore, important to find an affinity chromatography stategy that would provide more reproducible results. Since phosphodiesterase can be quantitatively eluted from sulfhydryl affinity gels (Kincaid et al., 1982) with no detectable change in its properties, we attempted to immobilize CaM in a form that could be released by a reducing agent. As CaM itself has no free sulfhydryl groups (Teo et al., 1973; Watterson et al., 1976), it was necessary to prepare the activated sulfhydryl derivative PDP-CaM, which was then attached to thiol–Sepharose 4B (reduced–SH form) through

[4] Contaminants could be substantially reduced in amount by washing with buffer containing 0.1 mM $CaCl_2$ and 0.5% CHAPS, a zwitterionic detergent (Hjelmeland, 1980), before elution of the enzyme. Although this did not greatly reduce the yield at this step, it resulted in some loss of activity during the subsequent gel filtration.

formation of a disulfide bond. This approach also obviated the need to reduce selectively any residual reactive groups as would have been necessary if coupling of thiolated affinity ligand to the activated thiol form of Sepharose 4B were used. The operational capacity of the disulfide-linked CaM–Sepharose was 4–5 times that of CaM–Sepharose prepared by CNBr coupling. On the average, CaM–Sepharose (3.2 mg of CaM/mg of gel) that had been used 3 of 4 times had a capacity of ~1.7 units of DEAE-purified phosphodiesterase/mL,[5] whereas the disulfide-linked CaM–Sepharose (~4 mg of CaM/mL of gel) bound at least 8 units. As might be expected with covalent sulfhydryl chromatography, release of the disulfide-linked CaM and bound proteins by reducing agent in the absence of Ca^{2+} was virtually quantitative. These were rapidly separated by gel filtration (a step that also permits rapid removal of reducing agent, which could be important for thiol-sensitive proteins).

SPDP has several advantages over other kinds of cross-linking agents for preparation of this type of affinity matrix. At first described by Carlsson et al. (1978), the derivatization procedure is selective for primary amino groups, thereby limiting the heterogeneity of cross-linked adducts, and is quite gentle, preserving a large degree of native structure. Indeed, the biological activity of the reduced derivative appeared identical with that of native CaM. The degree of substitution of the thiolated ligand can be directly quantified by the amount of pyridine-2-thione released and thus controlled. Since linkage to the solid matrix occurs only via the substituent, the number of bound ligand configurations is limited, and an optimal degree of substitution that is compatible with both attachment and affinity interaction can be systematically established. Further, after desorption, the reduced form of the ligand can be easily reactivated with 2,2′-dipyridyl disulfide to yield the original activated species, e.g., PDP-CaM, particularly advantageous if reuse of the affinity ligand is important. The glutathione moiety present on activated thiol-Sepharose 4B may, in addition, provide a useful hydrophilic spacer for interactions that require some distance between ligand and matrix but are not optimal in presence of certain spacer groups. In this regard, the use of succinylated (aminopropyl)agarose for coupling CaM was reported to yield an ineffective affinity matrix (Miyaki et al., 1977).

One general advantage of the strategy described here is that compounds lacking a thiol group (small molecules or proteins) could be derivatized and used as desorbable affinity ligands. Since dilution of protein during elution may lead to inactivation, this approach could improve yields simply by permitting rapid release of the interacting species in a relatively small volume. This may be particularly important if the interaction of a protein with matrix-bound ligand is difficult to reverse.[6] Presumably, after release from the solid support, the ligand–protein complex in solution would be readily dissociable, permitting separation of the components. It is not known to what extent the matrix-bound conformation of the ligand may contribute to problems of recovery and lowered capacities with cumulative usage; however, one can infer that this occurs with conventional CaM–Sepharose, since accumulation of matrix-bound assayable phosphodiesterase activity has been noted (Kincaid & Vaughan, 1979). It is hoped that the approach described here may be useful for situations where similar problems have been recognized.

[5] Capacities of such gels on first use were at least twice this value.

[6] Sweet & Adair (1975) earlier proposed the use of a desorbable ligand approach for affinity chromatography to improve recovery of labile proteins.

Acknowledgments

We thank D. Marie Sherwood for expert secretarial assistance.

Registry No. SPDP, 68181-17-9; cyclic 3′,5′-nucleotide phosphodiesterase, 9040-59-9; thiol-Sepharose 4B, 69552-82-5.

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