Communication

Detection of Calcium-dependent Regulatory Protein Binding Components Using $^{125}$I-labeled Calcium-dependent Regulatory Protein*

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SUMMARY

The calcium-dependent regulatory protein (CDR) purified from bovine brain was iodinated with Na$^{[125]}$I using the lactoperoxidase-glucose oxidase system. The iodinated protein retained its ability to stimulate the Ca$^{2+}$-sensitive CDR-depleted cyclic nucleotide phosphodiesterase from bovine heart. Stimulation of the phosphodiesterase by $^{125}$I-CDR was Ca$^{2+}$-dependent and the labeled protein had a $K_m$ for activation of cyclic nucleotide phosphodiesterase that was 4 times greater than unmodified CDR. $^{125}$I-CDR formed a Ca$^{2+}$-dependent complex with the partially purified cyclic nucleotide phosphodiesterase which was detectable by autoradiography following electrophoresis of the complex on nondenaturing gels. This technique was used to detect CDR binding components in crude homogenates prepared from bovine heart and brain.

The calcium-dependent regulatory protein (CDR) was discovered independently by Cheung (1) and Kakiuchi et al. (2). CDR mediates the effects of Ca$^{2+}$ on the activities of a number of different enzymes. Those proteins currently known to interact with CDR include an isozyme of the cyclic nucleotide phosphodiesterase from several tissues (1-3), adenylate cyclase from rat and bovine brain (4, 5), a protein of unknown function from chicken gizzard (10), CDR has been shown to form a complex with the first three of these proteins in the presence of Ca$^{2+}$. These complexes were stable during Sephadex G-200 chromatography, and Sephadex G-150 chromatography. The authenticity of the purified CDR was confirmed by its amino acid analysis (13), ultraviolet spectrum (13), and its ability to stimulate CDR-depleted cyclic nucleotide phosphodiesterase.

$^{125}$I-CDR was prepared using lactoperoxidase and glucose oxidase (15). This reaction mixture contained 100 mM imidazole-HCl at pH 6.9, 50 μM CaCl₂, 0.1 mM PMSF, 5 μg of lactoperoxidase, 20 μg of glucose oxidase, 20 nmol of Na$^{[125]}$I (8.5 x 10⁷ cpm/nmol) and 100 μg of CDR in a volume of 0.4 ml. The reaction was initiated by addition of 200 μg of glucose. After incubation for 60 min at 30°C, the reaction was stopped by addition of 5 μl of β-mercaptoethanol. The reaction mixture was dialyzed overnight against 100 mM imidazole-HCl, pH 6.9, 50 μM CaCl₂, 0.1 mM PMSF, and 1 mM PIFTA. The dialyzed sample was then chromatographed on a DEAE-cellulose column (1.0 x 10 cm) equilibrated with distilled water for elution. The samples were then counted for $^{125}$I and $^{32}$P. Adenosine was used to monitor recovery during the assay procedure. The number of units of CDR per assay was determined by reference to a standard curve produced with unmodified CDR.

$^{125}$I-CDR was iodinated by its ability to stimulate CDR-depleted cyclic nucleotide phosphodiesterase. The assay mixture contained 20 mM Tris·HCl at pH 8.0, 20 mM imidazole, 0.1 mM PMSF, 3 mM MgCl₂, 0.1 mM CaCl₂, 7,000 cpm of $[^{125}]$AMP, and 10 units of cyclic nucleotide phosphodiesterase in a final volume of 0.5 ml. Reaction was initiated by addition of 250 μl of $[^{125}]$AMP (80,000 cpm). Following 15 min incubation at 30°C, the reaction was terminated by boiling for 2 min. After cooling on ice, 50 μl of C. atrox venom (1 mg/ml) was added as a source of 5'-nucleotidase. This reaction mixture was incubated for 15 min at 30°C and then boiled for 2 min. $[^{125}]$I- and $^{125}$I-labeled CDR were then separated from AMP by IRP-58 ion exchange chromatography using distilled water for elution. The samples were then counted for $^{125}$I and $^{32}$P. Adenosine was used to monitor recovery during the assay procedure. The number of units of CDR per assay was determined by reference to a standard curve produced with unmodified CDR.

MATERIALS AND METHODS

$[^{1}]$AMP, $[^{3}]$AMP, and Na$^{[125]}$I were purchased from New England Nuclear. Lactoperoxidase and glucose oxidase were obtained from Calbiochem and Crotilus atrox venom was a product of Sigma. All other chemicals were reagent grade.

CDR-depleted Ca$^{2+}$-sensitive cyclic nucleotide phosphodiesterase was prepared by the method of Wang (3). One unit of cyclic nucleotide phosphodiesterase catalyzed the hydrolysis of 1 nmol of cAMP in 15 min in the absence of added CDR. CDR was purified from bovine brain by a method to be described in detail elsewhere. This procedure employed isoelectric precipitation, DEAE-cellulose chromatography, and Sephadex G-150 chromatography. The authenticity of the purified CDR was confirmed by its amino acid analysis (13), ultraviolet spectrum (13), and its ability to stimulate CDR-depleted cyclic nucleotide phosphodiesterase.

Activation of cyclic nucleotide phosphodiesterase by CDR had an absolute requirement for Ca$^{2+}$. Half-maximal stimulation of 10 units of cyclic nucleotide phosphodiesterase was achieved with 1.0 ng of CDR/assay. The purified CDR ran as a single band on 12.5% DAVIS Gels (14). One unit of CDR produced half-maximal activation of 10 units of cyclic nucleotide phosphodiesterase. The activation constant, $K_a$, is the concentration of purified CDR required to produce half-maximal activation of 10 units of cyclic nucleotide phosphodiesterase.

CDR was assayed by its ability to stimulate CDR-depleted cyclic nucleotide phosphodiesterase. The assay mixture contained 20 mM Tris·HCl at pH 8.0, 20 mM imidazole, 0.1 mM PMSF, 3 mM MgCl₂, 0.1 mM CaCl₂, 7,000 cpm of $[^{125}]$AMP, and 10 units of cyclic nucleotide phosphodiesterase in a final volume of 0.5 ml. Reaction was initiated by addition of 250 μl of $[^{125}]$AMP (80,000 cpm). Following 15 min incubation at 30°C, the reaction was terminated by boiling for 2 min. After cooling on ice, 50 μl of C. atrox venom (1 mg/ml) was added as a source of 5'-nucleotidase. This reaction mixture was incubated for 15 min at 30°C and then boiled for 2 min. $[^{125}]$I- and $^{125}$I-labeled CDR were then separated from AMP by IRP-58 ion exchange chromatography using distilled water for elution. The samples were then counted for $^{125}$I and $^{32}$P. Adenosine was used to monitor recovery during the assay procedure. The number of units of CDR per assay was determined by reference to a standard curve produced with unmodified CDR.

$^{125}$I-CDR was prepared using lactoperoxidase and glucose oxidase (15). This reaction mixture contained 100 mM imidazole-HCl at pH 6.9, 50 μM CaCl₂, 0.1 mM PMSF, 5 μg of lactoperoxidase, 15 μg of cyclic nucleotide phosphodiesterase, 20 nmol of Na$^{[125]}$I (8.5 x 10⁷ cpm/nmol) and 100 μg of CDR in a volume of 0.4 ml. The reaction was initiated by addition of 100 μg of glucose. After incubation for 60 min at 30°C, the reaction was stopped by addition of 5 μl of β-mercaptoethanol. The reaction mixture was dialyzed overnight against 100 mM imidazole-HCl, pH 6.9, 50 μM CaCl₂, 0.1 mM PMSF, and 1 mM PIFTA. It was then washed with distilled water and then dialyzed against 25 mM sodium succinate, pH 6.9, and 1 mM PIFTA. The dialyzed sample was then chromatographed on a DEAE-cellulose column (1.0 x 10 cm) equilibrated with the same buffer used for dialysis. This column was then washed with 20 ml of the loading buffer followed by 20 ml of the same buffer containing 0.15 M NaCl. $^{125}$I-CDR was eluted with a linear NaCl gradient (0.15 to 0.3 M). The pooled fractions were then eluted from a 4% to 7% PAGE. CDR prepared by the procedure outlined above will be referred to as "high specific activity $^{125}$I-CDR." CDR was also iodinated by the procedure given above with the following changes in reagents: 15 μg of lactoperoxidase, 45 μg of glucose oxidase, 840 nmol of NaI (1.2 x 10⁶ cpm/nmol), 125 μg of CDR, and 200 μg of glucose. $^{125}$I-CDR detection of CDR binding components in crude mixtures of proteins which should be useful for identifying other CDR binding proteins which have not yet been discovered.

* The abbreviations used are: CDR, calcium-dependent regulatory protein; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl)ether,N,N'-tetracetic acid. 

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preparations were prepared with these modifications will be referred to as "low specific activity \(^{125}\)I-CDR."

The number of CDR binding components present in crude homogenates was estimated as described below. Tissue samples were homogenized in 1 volume of a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM PMSF. The homogenate was then centrifuged for 1 h at 100,000 × g, after which the supernatant was filtered through glass wool. \(^{125}\)I-CDR was added to the filtrate and dialyzed against 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM \(\beta\)-mercaptoethanol for 4 h at 4°C. The sample was then dialyzed against the same buffer containing 0.1 mM CaCl\(_2\), with no EDTA present and finally against 5 mM Tris-HCl, pH 8.3, 38 mM glycerol, 1 mM \(\beta\)-mercaptoethanol, 0.1 mM CaCl\(_2\), and 10% sucrose. After addition of 0.005% bromphenol blue, the dialyzed preparation was electrophoresed on polyacrylamide gels by the Davis method (14). The running and stacking gel buffers contained 0.2 mM and 0.05 mM CaCl\(_2\), respectively. The samples were electrophoresed under the conditions for tracking dye and free \(^{125}\)I-CDR ran off the end of the gel. The gel was dried onto filter paper and submitted to autoradiography for various periods of time. Polyacrylamide gels were stained for cyclic nucleotide phosphodiesterase activity by the method of Harpehak et al. (16). Protein was determined by the Lowry (17) method using bovine serum albumin as a standard with the exception of \(^{125}\)I-CDR which was determined by the method of Bradford (18) using purified CDR as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli using 10% acrylamide gels (19).

RESULTS AND DISCUSSION

Purified CDR was iodinated under two different conditions which resulted in differing degrees of iodine incorporation. High specific activity \(^{125}\)I-CDR contained 0.5 mol of iodine/mol of CDR and this preparation was used for the electrophoretic experiments described below. Low specific activity \(^{125}\)I-CDR contained 3.0 mol of iodine/mol of CDR and it was examined for its ability to stimulate the activity of cyclic nucleotide phosphodiesterase partially purified from bovine heart.

Iodinated CDR preparations were submitted to Sephadex G-150 chromatography and DEAE-cellulose ion exchange chromatography primarily to remove lactoperoxidase and glucose oxidase. The Sephadex G-150 elution profile exhibited two \(^{125}\)I-labeled peaks (Fig. 1). The first peak ran in the void volume of the column and contained no CDR activity. The second included peak, contained CDR activity and unmodified CDR was found to elute in an identical position. The \(^{131}\)I-CDR obtained from Sephadex G-150 chromatography was applied to a DEAE-cellulose column and eluted as a single symmetrical peak (data not shown). The \(^{131}\)I-CDR preparations were homogeneous and did not appear to be degraded during the iodination procedure. The major peak obtained with Sephadex G-150 chromatography was symmetrical with CDR activity and \(^{131}\)I label eluting at a position identical with unmodified CDR (Fig. 1). When \(^{125}\)I CDR was electrophoresed on nondenaturating gels, in the absence of CDR binding activities, no other bands were observed (see Fig. 3D). In addition, both iodinated CDR samples were electrophoresed on sodium dodecyl sulfate gels, autoradiographed, and stained for protein with Coomassie blue. In both cases, a single band having an \(R_s\) of 0.7 was observed which corresponded to the mobility of unmodified CDR on the same gel system. \(^{125}\)I-CDR retained its ability to stimulate CDR-depleted cyclic nucleotide phosphodiesterase (Fig. 2). Low specific activity CDR and high specific activity CDR had apparent \(K_m\) values for activation of cyclic nucleotide phosphodiesterase of 8.0 and 2.6 ng/ml, respectively. The \(K_m\) for unmodified CDR was 2.0 ng/ml. The extent of stimulation by both iodinated CDR preparations was, however, comparable to that obtained with unmodified CDR. It should be emphasized that these are apparent activation constants because the \(^{125}\)I-CDR preparations almost certainly contained a mixture of the various iodinated species. In addition, high specific activity iodinated CDR only contained an average of 0.5 mol of iodine/mol of CDR and it necessarily contained significant amounts of unmodified CDR.

It might be argued that the stimulation observed with \(^{125}\)I-CDR was due to unmodified CDR present in the preparation. However, stimulation of cyclic nucleotide phosphodiesterase activity was observed with low specific activity \(^{125}\)I-CDR which contained 3 mol of iodine/mol of CDR. CDR contains 2 tyrosine residues and could in principle accumulate a maximum of 4 mol of iodine/mol of CDR. Iodination of tyrosine residues...
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Fig. 3. Determination of the number of CDR binding components in bovine heart extract. Sample preparation and electrophoresis were done as described under "Materials and Methods." A, total protein of the crude extract stained with Coomassie brilliant blue; B, autoradiogram of 250 µg of crude heart extract + 0.2 µg of ¹²⁵I-CDR (2 x 10⁶ cpm), exposed for 150 h; C, autoradiogram of 100 units of partially purified cyclic nucleotide phosphodiesterase (PDE) + 0.2 µg of ¹²⁵I-CDR (2 x 10⁶ cpm), exposed for 12 h; D, autoradiogram of 0.2 µg of ¹²⁵I-CDR (2 x 10⁶ cpm) run without other proteins, exposed for 150 h. The arrows indicate the position at which cyclic nucleotide phosphodiesterase activity was observed with crude heart and brain preparations was not a gel artifact due to aggregation of iodinated CDR. In addition, weak complexes might dissociate during electrophoresis and the procedure obviously requires exchange between iodinated CDR and endogenous CDR.

When ¹²⁵I-CDR was submitted to this electrophoresis system, it ran with the dye front (Rf = 1.0) and no other bands were observed. Therefore, the occurrence of the various bands observed with crude heart and brain preparations was not a gel artifact due to aggregation of iodinated CDR. In addition, the substitution of 200 µM EGTA for Ca²⁺ in the electrophoresis buffers eliminated binding of ¹²⁵I-CDR to any protein in heart or brain extracts. Therefore, binding of ¹²⁵I-CDR to the various proteins described above was, in all cases, Ca²⁺-dependent.

The data presented in this study demonstrates that CDR can be iodinated without eliminating its calcium-dependent affinity for various CDR binding proteins or its capacity to stimulate cyclic nucleotide phosphodiesterase in the presence of Ca²⁺. The availability of radioactively labeled CDR should provide a useful tool for detecting CDR binding proteins in heterogeneous preparations.

REFERENCES

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