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Purification and Characterization of a Calcium-Dependent Protease from Rat Liver†

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ABSTRACT: A calcium-dependent protease, previously identified in rat liver and designated peak II [DeMartino, G. N. (1981) *Arch. Biochem. Biophys. 211*, 253–257], was purified and characterized. The calcium-dependent proteolytic activity was accounted for by an 80 000-dalton protein. Depending on the method of purification, we found that this protease could be associated with a 28 000-dalton subunit, which was devoid of protease activity. The catalytic characteristics of the two different forms of the protease were indistinguishable. Each was half-maximally activated by approximately 250 μM calcium.

Calcium-dependent proteases have been identified in a number of tissues of various species (Murachi et al., 1981; Ishiura, 1981). In most cases, the structure of these enzymes appears to be a heterodimer with subunit molecular weights of 80 000 and 30 000 (Dayton et al., 1981; Mellgren et al., 1982; Hathaway et al., 1982). Some reports, however, describe the enzyme as a monomer of molecular weight approximately 80 000 (Kubota et al., 1981; Ishiura et al., 1978; Suzuki et al., 1981; Croall & DeMartino, 1983). Although in some instances these discrepancies may result from tissue and/or species-specific differences, in other cases the discrepancies exist for enzymes from a single source (Azanza et al., 1979; Tsuji & Imahori, 1981; Mellgren et al., 1982).

Recently, we identified two calcium-dependent proteases in rat liver (DeMartino, 1981). The purpose of the present work was to purify and characterize one of these enzymes, the form which requires high calcium concentrations and is designated peak II. In the course of these studies, we discovered that the subunit composition of the isolated enzyme was critically dependent on the method of purification. These results may provide insight into the reported differences for the protein composition of other calcium-dependent proteases.

Materials and Methods

Preparation of Calcium-Dependent Protease Peak II from Rat Liver. Partially purified calcium-dependent protease peak II was prepared through the diethylaminoethylcellulose (DEAE-cellulose)[1] ion-exchange chromatography step as previously described (DeMartino, 1981). In preparation for the affinity chromatography described below, the activity was dialyzed against 10 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol (DTT), 400 mM KCl, and 0.1 mM EGTA.

Casein–Sepharose Affinity Chromatography of Calcium-Dependent Protease. Casein–Sepharose (15 g, approximately 50 mL of packed gel) was washed and equilibrated with 10 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 400 mM KCl, and 1 mM $CaCl_2$. In order to minimize the time that the protease would spend in the presence of calcium but without substrate, the sample was applied to the column in a batchwise procedure. That is, 50% of the casein–Sepharose was placed in a beaker, and 50% was packed in a 2.5-cm diameter column. To activate the protease, a small volume of 1 M $CaCl_2$ was added to the dialyzed enzyme to achieve a final concentration of 1 mM $CaCl_2$, and this was immediately mixed with the casein–Sepharose in the beaker. This slurry was then applied to the rest of the casein–Sepharose packed in the column. The resin was washed extensively in the buffer described above. The bound protease was eluted in 10 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 400 mM KCl, and 5 mM EGTA. Except where noted, this and all other procedures were carried out at 0–4 °C.

Preparation of Casein–Sepharose 4B. α-Casein (Sigma) was coupled to cyanogen bromide activated Sepharose 4B

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[1] Abbreviations: DEAE, diethylaminoethyl; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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(Sigma or Pharmacia) in 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl by a procedure recommended by the manufacturer (Pharmacia Fine Chemicals, "Affinity Chromatography Principles and Methods"). The resin normally contained 3–5 mg of casein/mL of gel.

Measurement of Protease Activity. Protease activity was measured by the hydrolysis of radioactively labeled protein substrates to acid-soluble peptides as described previously (DeMartino, 1981). Substrates included [*methyl*-^{14}C]casein and [*methyl*-^{14}C]globin.

Nondenaturing Polyacrylamide Gel Electrophoresis. Nondenaturing polyacrylamide gel electrophoresis was performed in 8% acrylamide gels (8 cm × 0.7 cm diameter). Separating gels were buffered with 85 mM Tris-HCl, pH 7.5 at 4 °C, and contained 0.1 mM EDTA and 0.1 mM DTT. Stacking gels (3.5%) were prepared with an acrylamide to bis(acrylamide) ratio of 4:1 and buffered with 50 mM Tris–sodium phosphate, pH 6.1 at 4 °C. Stacking gels also contained 0.1 mM EDTA and 0.1 mM DTT. Electrophoresis was performed at 3 mA per gel at 4 °C in 35 mM Tris–cacodylate buffer or 35 mM Tris–barbital buffer, pH 7.0, which contained EDTA and DTT at 0.1 mM each. Electrophoresis was completed in approximately 5 h. Prior to electrophoresis, samples were dialyzed against 50 mM Tris–sodium phosphate, pH 6.1 at 4 °C, 0.5 mM DTT, and 0.5 mM EDTA. Gels were fixed in 10% trichloroacetic acid–20% methanol and stained with either Coomassie Blue or silver (Wray et al., 1981). Protease activity was extracted from 0.25- or 0.5-cm slices by homogenization in 0.5 mL of 5 mM Tris-HCl, pH 7.5 at 4 °C, 0.5 mM DTT, and 0.5 mM EGTA. Gel fragments were removed by low-speed centrifugation, and the extracts were assayed for protease activity. Recovery of applied protease activity ranged from 40 to 75% in various experiments.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Polyacrylamide slab gels (10%) (14 × 14 × 0.3 cm) were prepared and run essentially according to the method of Laemmli (1970). Gels were stained with 0.25% Coomassie Blue R250 in 50% methanol–7% acetic acid or with silver by using the Bio-Rad silver-staining kit. Standards of known molecular weights were electrophoresed on each gel: RNA polymerase subunits (M_r 39 000, 155 000, and 165 000); bovine serum albumin (M_r 68 000); soybean trypsin inhibitor (M_r 21 500).

Gel Filtration Column Chromatography on Sephacryl S-200. Gel filtration chromatography was performed with Sephacryl S-200 equilibrated with 50 mM Tris-HCl, pH 7.6, 8 mM KCl, 0.5 mM DTT, and 0.5 mM EGTA and packed in a 95 × 2.5 column. The column was calibrated with proteins of known molecular weight (Figure 4).

Protein Determinations. Protein was determined by the method of Bradford (1976) using premixed reagent purchased from Bio-Rad Laboratories.

Results and Discussion

Purification of Calcium-Dependent Protease Peak II by Casein–Sepharose Affinity Chromatography. Calcium-dependent protease peak II was isolated from soluble extracts of rat liver (DeMartino, 1981) and subjected to affinity chromatography on casein–Sepharose. The protease activity bound to the affinity resin in the presence of calcium and was released from the column by either EGTA or EDTA. The bulk of nonprotease protein passed directly through the column (Figure 1).

The protease activity isolated by this method was characterized by polyacrylamide gel electrophoresis (PAGE). In the presence of SDS, three protein bands were identified with

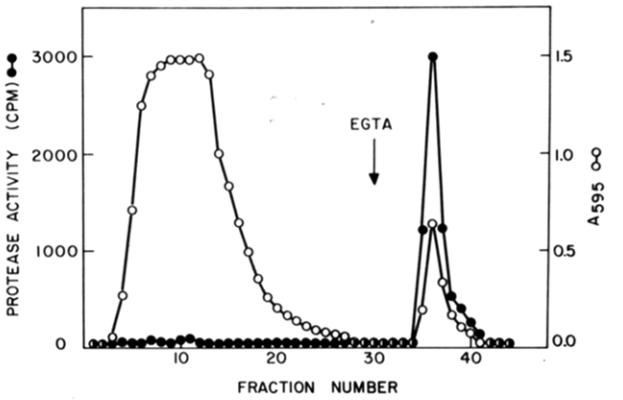

FIGURE 1: Casein–Sepharose affinity chromatography of calcium-dependent protease. Partially purified calcium-dependent protease, peak II, was chromatographed on casein–Sepharose as described in the text. (●) Protease activity; (○) protein.

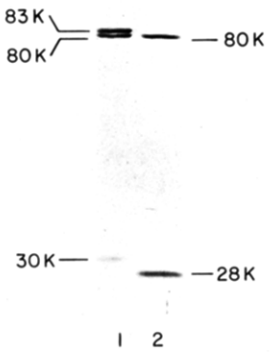

FIGURE 2: SDS–polyacrylamide gel electrophoresis of the peak protease fractions from the casein–Sepharose column (lane 1) and from the reactive red agarose column (lane 2). The gel was stained with silver.

molecular weights of 83 000, 80 000, and 30 000 (Figure 2). Three protein bands were also observed by PAGE under nondenaturing conditions. Each of these bands (designated B1, B2, and B3, respectively; Figure 3) represented one of the peptides identified by SDS–PAGE as demonstrated by excising the individual bands from the nondenaturing gel and reelectrophoresing them in the presence of SDS (Figure 3).

In order to determine the relationship of these proteins to protease activity, we assayed extracts of gel slices after nondenaturing electrophoresis. The B1 protein (M_r 30 000 by SDS–PAGE) had no detectable protease activity. Calcium-dependent proteolytic activity was found in the region where proteins B2 and B3 migrated (Figure 3), but because these proteins were not well separated, we could not determine whether both or only one protein accounted for the activity. To clarify this point, we chromatographed the casein–Sepharose-purified protease activity by gel filtration on Sephacryl S-200. Each column fraction was assayed for protease activity and analyzed on SDS–PAGE. Although the elution profiles for the B2 and B3 proteins overlapped, they were clearly distinct, and only the B3 protein (M_r 80 000 by SDS–PAGE) was coincident with protease activity (data now shown). By gel filtration, the apparent molecular weight of the protease was 85 000 (Figure 4). Surprisingly the B1 protein, which migrated on SDS–PAGE with an M_r of 30 000, eluted from the Sephacryl S-200 column with an apparent molecular weight of 120 000. Similar estimates of molecular weight for

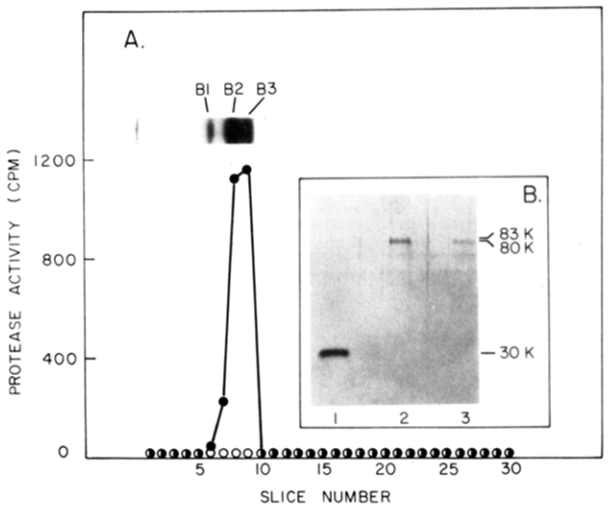

FIGURE 3: Nondenaturing polyacrylamide gel electrophoresis of casein–Sepharose-isolated calcium-dependent protease. (A) The protease activity isolated by casein–Sepharose chromatography (Figure 1) was electrophoresed under nondenaturing conditions as described under Materials and Methods. The gels were either stained for protein or assayed for protease activity. (●) Protease activity with calcium; (○) protease activity without calcium. (B) The three proteins isolated by the nondenaturing PAGE (A) were excised, treated with SDS, and electrophoresed in the presence of SDS as described under Materials and Methods. Lane 1, protein B1; lane 2, protein B2; lane 3, protein B3. The gel shown was stained with silver.

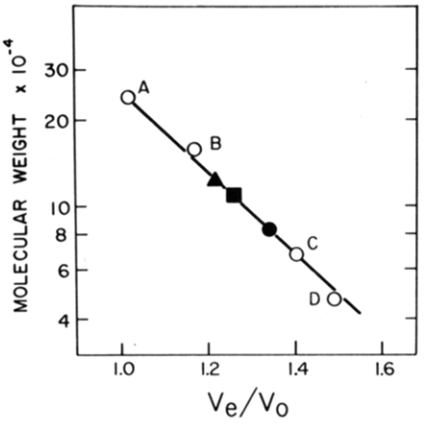

FIGURE 4: Estimation of the molecular weight of calcium-dependent proteases by gel filtration chromatography. Calcium-dependent protease purified either by casein–Sepharose chromatography (●) or by reactive red agarose chromatography (■) was chromatographed on Sephacryl S-200 as described under Materials and Methods. The elution position of the B1 protein (Figure 2) is also indicated (▲). Calibration proteins: (A) catalase, M_r 240 000; (B) aldolase, M_r 158 000; (C) bovine serum albumin, M_r 68 000; (D) hen egg albumin, M_r 48 000.

the protease (M_r 82 000) and B1 protein (M_r 120 000) were obtained by electrophoresis under nondenaturing conditions (data not shown) using the method of Hedrick & Smith (1968).

These results indicated that the calcium-dependent protease from rat liver was isolated as a monomer of M_r 80 000 by casein–Sepharose affinity chromatography. Thus, it appears similar to the composition reported for the protease from some sources but lacks the 30 000-dalton subunit described for the protease from other sources. Although we did isolate a 30 000-dalton protein by casein–Sepharose chromatography, it was an apparently unrelated protein with an M_r of 120 000 under nondenaturing conditions. The biochemical basis for the isolation of this and the 83 000-dalton protein by casein–Sepharose chromatography is unclear but appears unrelated

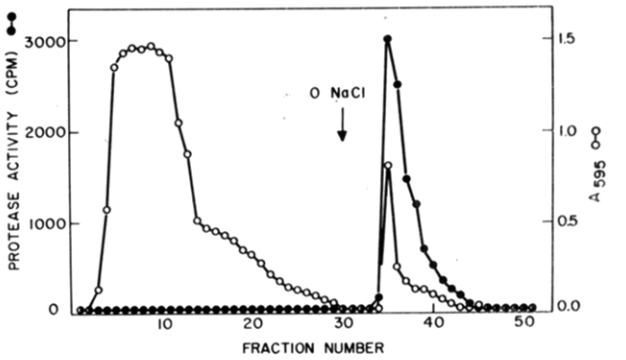

FIGURE 5: Reactive red agarose chromatography of calcium-dependent protease peak II. Partially purified calcium-dependent protease peak II was chromatographed on reactive red agarose as described in the text. Protease activity (●); protein (○).

to possible artifacts caused by autoproteolysis during the affinity chromatography. For example, because each contaminant had a native molecular weight greater than the protease, neither could be an autoproteolytic fragment. In fact, our evidence indicates that autoproteolysis was very low during this chromatography. In separate experiments, we have shown that substrates such as casein prevent autoproteolysis, and presumably, the Sepharose-bound casein affords similar protection. The three isolated proteins can be rechromatographed on casein–Sepharose and are recovered with unaltered molecular weights, in similar amounts (90–95% recovery) and in the same relative proportions compared to the original isolation (data not shown).

We concluded that the 83 000- and 30 000-dalton proteins are retained on the column in a calcium-dependent manner either directly, by interacting with casein, or indirectly, by interacting with the protease. In either case, the presence of these contaminants necessitated additional purification of the protease because Sephacryl S200 chromatography as described above was not adaptable for adequate preparative purification.

Purification of Calcium-Dependent Protease Peak II by Reactive Red Agarose Chromatography. While our work was in progress, others reported the purification of a calcium-dependent protease from smooth muscle by chromatography on reactive red agarose (Hathaway et al., 1982). We tested whether this technique would be a useful alternative or addition to casein–Sepharose chromatography in the purification of the rat liver protease. The peak II protease, isolated by DEAE-cellulose ion-exchange chromatography as described previously, was chromatographed on reactive red agarose (Figure 5). The protease activity bound to the column in buffer containing 0.5 M NaCl and was eluted in buffer without NaCl. SDS–PAGE analysis demonstrated two protein bands (M_r 80 000 and 28 000) were present in the protease fraction (Figure 2). The 80 000-dalton protein was indistinguishable from the 80 000-dalton protein isolated by casein–Sepharose chromatography. The 28 000-dalton protein, however, was clearly different from the 30 000-dalton protein we described earlier. Furthermore, the two proteins isolated by reactive red agarose chromatography migrated as a single band, coincident with protease activity on nondenaturing PAGE (Figure 6). The two proteins also coeluted on Sephacryl S-200 gel filtration chromatography. They were coincident with protease activity (data not shown) and eluted at an M_r of 110 000 (Figure 4). A similar estimate, M_r 109 000, was obtained by analysis of mobility on nondenaturing PAGE (data not shown). Thus, in contrast to the results obtained by casein–Sepharose, the protease isolated by reactive red agarose chromatography was composed of two subunits with molecular weights of 80 000 and 28 000.

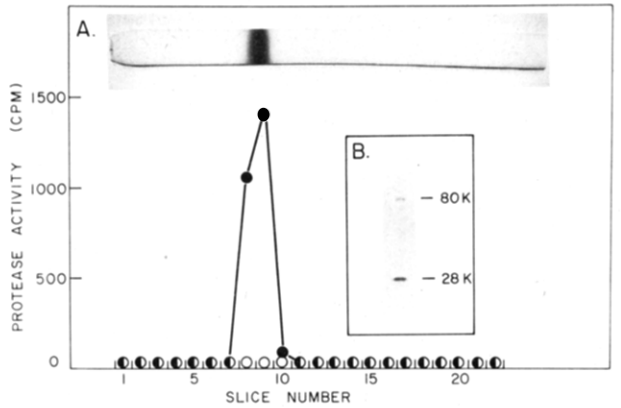

FIGURE 6: Nondenaturing polyacrylamide gel electrophoresis of the reactive red agarose isolated calcium-dependent protease. (A) The protease activity isolated by reactive red agarose chromatography was electrophoresed under nondenaturing conditions as described under Materials and Methods. The gels were either stained with Coomassie Blue R250 or assayed for protease activity in the presence (●) or absence (○) of calcium. (B) The isolated protease band in panel A was excised, treated with SDS, electrophoresed in the presence of SDS as described under Materials and Methods, and stained with silver.

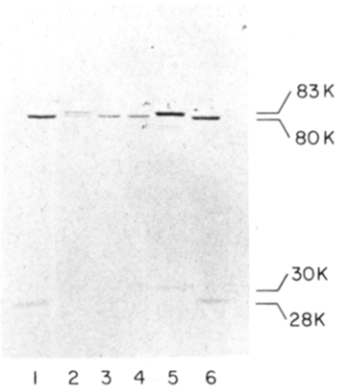

FIGURE 7: SDS–PAGE analysis of purified peak II calcium-dependent proteases. Peak II proteases prepared by chromatography on reactive red agarose (lanes 1 and 6) and casein–Sepharose (lane 2), by sequential chromatography on casein–Sepharose and reactive red agarose (lane 3), or by sequential chromatography on reactive red agarose and casein–Sepharose (lane 4) were boiled in SDS and electrophoresed on a 10% acrylamide slab gel as described under Materials and Methods. The gel shown was stained with Coomassie Blue R250. Lane 5 shows the proteins which are isolated by casein–Sepharose chromatography of the proteins which do not bind to reactive red agarose. A silver stain of this gel enhances the 30 000-dalton protein in lanes 2 and 5, but no other qualitative or quantitative differences were observed.

Rechromatography of the Purified Calcium-Dependent Proteases. These observations suggest that some of the disparate conclusions reported for the subunit composition of calcium-dependent proteases (see above) may result from differences in the methodologies used for purification.

In order to elucidate the basis for the isolation of the different forms of the protease in our own work, the protease activity which was originally purified by casein–Sepharose chromatography was subsequently chromatographed on reactive red agarose, and the protease which was originally purified by reactive red agarose was chromatographed on casein–Sepharose. In each case, the protease activity bound to and eluted from the columns as described above. However, each reisolated protease activity was associated with only a single 80 000-dalton protein, indistinguishable from the 80 000-dalton protein in each original preparation and from each other (Figure 7). Thus, casein–Sepharose chromatography resulted in the separation of the 28 000-dalton subunit from the catalytic activity while reactive red agarose separated the 83 000- and 30 000-dalton proteins from the 80 000-dalton casein–Sepharose-purified protease. We have subsequently identified the 83 000- and 30 000-dalton proteins in the fractions which did not bind to reactive red agarose (i.e., fractions 5–15, Figure 5). Rechromatography of these protease-free proteins on casein-Sepharose demonstrated that both proteins bound to the resin in a calcium-dependent manner (Figure 7, lane 5). These results suggest that the 83 000- and 30 000-dalton peptides (1) bind directly to the casein–Sepharose and not via an interaction with protease and (2) do not originate by autoproteolysis during casein–Sepharose chromatography. The functional basis for calcium-dependent retention of these proteins on casein-Sepharose is unknown.

We are uncertain of the reason for the loss of the 28 000-dalton subunit during casein–Sepharose affinity chromatography. This protein was identified in the fractions which did not bind to the casein–Sepharose, but recovery was not quantitative. Thus, unlike the 80 000-dalton subunit, it is possible that some of the 28 000-dalton subunit was selectively degraded during the affinity chromatography. This, however, does not readily account for the separation of the apparently unaltered 28 000-dalton subunit from the catalytic subunit. Recently, Melloni et al. (1982) have provided evidence that calcium per se can promote the dissociation of the 80 000- and 28 000-dalton subunits of a protease from human erythrocytes. These workers irreversibly inactivated the protease with iodoacetate prior to treatment with calcium in order to eliminate the possibility of autoproteolysis. In the presence of calcium, the subunits of the inactive heterodimer dissociated and were separated by gel filtration chromatography. Our attempts to perform similar experiments with the rat liver peak II protease have been unsuccessful because the iodoacetate- or *N*-ethylmaleimide-inactivated enzyme becomes insoluble. Nevertheless, the results of Melloni et al. may explain our failure to isolate the heterodimer by affinity chromatography and would predict that any form of affinity chromatography requiring the active enzyme (i.e., protease in the presence of calcium) would isolate the monomeric form. In fact, other reports of purification using active protease do isolate the protease as a monomer (Kubota et al., 1981; Cottin et al., 1981; Croall & DeMartino, 1983).

The function of the 28 000-dalton subunit is unclear. We compared various catalytic properties of the two forms of the protease (i.e., the monomer and the heterodimer) and have been unable to detect any significant differences. For example, each had a pH optimum of 7.5; each was inhibited by sulfhydryl reagents such as *N*-ethylmaleimide and iodoacetate and by leupeptin, antipain, and the endogenous protein inhibitor of calcium-dependent proteases (Waxman & Krebs, 1978). Each form of the protease was totally dependent on calcium for activity and required similar concentrations of calcium for activation, 250 μM calcium for half-maximal activity and approximately 700 μM calcium for maximal activity (Figure 8). In the absence of exogenous substrates, each form of the protease rapidly autoproteolyzed. Finally, the two forms of the protease had similar specific activities on the basis of calculations using respective molecular weights of 110 000 and 80 000. Additional work will be required to learn the role of the 28 000-dalton subunit.

Added in Proof

Since the submission of the manuscript, we have demonstrated that the 83 000-dalton nonprotease protein isolated by casein–Sepharose chromatography is a calcium-dependent

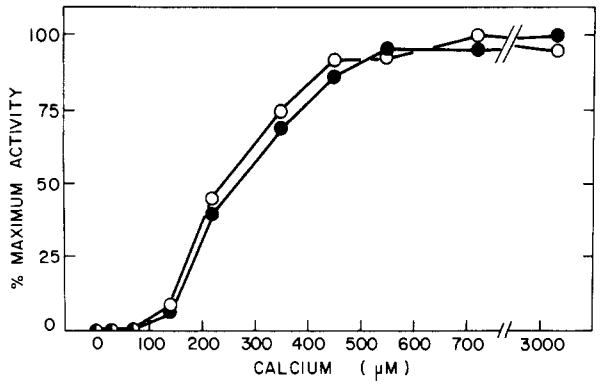

FIGURE 8: Effect of calcium concentration on the activity of calcium-dependent proteases. The calcium-dependent protease isolated either by reactive red agarose chromatography (●) or by reactive red agarose chromatography followed by casein–Sepharose affinity chromatography (○) as described in the text was assayed in the presence of the indicated concentrations of free calcium. For each protease, the activity was expressed as a percentage of the maximum activity.

transglutaminase (D. E. Croall and G. N. DeMartino, unpublished results).

Acknowledgments

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Registry No. Ca, 7440-70-2; proteinase, 9001-92-7; transglutaminase, 80146-85-6.

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Effects of Guanine Nucleotides on Cholera Toxin Catalyzed ADP-Ribosylation in Rat Adipocyte Plasma Membranes†

C. Bruce Graves, Nancy B. Klaven, and Jay M. McDonald*

ABSTRACT: ADP-ribosylation of rat adipocyte plasma membrane proteins was investigated following incubation of membranes with [α-^{32}P]NAD and cholera toxin in the presence and absence of various guanine nucleotides. In membranes incubated without guanine nucleotides, cholera toxin induced incorporation of ^{32}P into three discrete proteins of 48, 45, and 41 kDa. In membranes containing 100 μM GTP or GDP, toxin-catalyzed incorporation of ^{32}P into the 41-kDa protein was inhibited. GMP and Gpp(NH)p (100 μM) allowed moderate incorporation of ^{32}P into the 41-kDa protein. Toxin-catalyzed labeling of all proteins was rapid, reaching maximal levels between 5 and 10 min. Toxin-catalyzed ADP-ribosylation of the 48- and 45-kDa proteins was stimulated by GTP, reaching maximal levels at 10^{-5} M GTP. Inhibition of toxin-dependent labeling of the 41-kDa protein required GTP concentrations above 10^{-7} M with complete inhibition occurring between 10^{-5} and 10^{-4} M GTP. Cholera toxin catalyzed ADP-ribosylation was increased up to 2-fold in membranes supplemented with adipocyte cytosol. These results indicate that cholera toxin catalyzes ADP-ribosylation of three distinct adipocyte plasma membrane proteins, each of which is regulated by the amount and type of added guanine nucleotides.

Posttranslational covalent modification of proteins plays a pivotal role in the activation and inactivation of enzymes which act at key control points in the regulation of cellular metabolism. Although proteins undergoing reversible phosphorylation have been the most actively studied models in this area, bacterial exotoxin catalyzed modification of proteins has been the focus of a number of recent publications (Vaughan & Moss, 1981; Ueda et al., 1982). Cholera toxin, an enterotoxin of *Vibrio cholerae*, catalyzes the transfer of ADP-ribose from NAD to a variety of proteins and peptides (Gill, 1982). Most

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