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EVIDENCE FOR PHOSPHORYL MOIETIES IN A PROTEINASE FROM DICTYOSTELIUM DISCOIDEUM

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SUMMARY A proteinase (called Proteinase I) present in myxamoebae of the cellular slime mold, Dictyostelium discoideum, was labeled in vivo with [^{32}P] by growth of cells on media containing [^{32}P] orthophosphate. The labeled proteinase was purified to apparent homogeneity and characterized by dissociation chromatography and quantitative immune-precipitin analysis. Based upon the results of these studies it was concluded that phosphoryl moieties were tightly associated (presumably covalently bonded) with the polypeptide subunits of Proteinase I.

INTRODUCTION

Differentiation of the cellular slime mold, Dictyostelium discoideum, is accompanied by a rapid turnover and net degradation of intracellular protein (1, 2). One purpose of protein degradation is to provide energy for differentiating cells. In addition, this catabolic process may contribute to the regulation of cellular enzyme levels. The fact that several enzymes accumulate in differentiating cells, against a background of extensive protein utilization, suggests that protein degradation must be a selectively regulated process. Direct support for this view has come from detailed studies (3, 4) of the mechanism of accumulation of one particular enzyme, UDPglucose pyrophosphorylase, during differentiation. However, at present little is known about the factors which provide for the specificity of cellular proteolysis in this differentiating organism.

In order to characterize the protein degrading system of D. discoideum we recently initiated an investigation of the proteinases which are present in growing and differentiating cells. The first such proteinase to be isolated in purified form has been termed Proteinase I. Initial characteriza-

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tions of this enzyme[1] suggested that it was a sulfhydryl endopeptidase, and that it had a rather narrow specificity for degrading other proteins isolated from differentiating cells. Proteinase I activity was maintained at a constant cellular level during the first 6-8 hours of differentiation, but was lost from cells during the final phases of cellular aggregation (8-12 hours of differentiation).

The studies described in this report indicated that Proteinase I had a somewhat novel structure, containing tightly associated phosphoryl moieties.

MATERIALS AND METHODS

Guanidine hydrochloride, Heico's extreme purity grade, was purchased from Heico, Inc.; N-α-carbobenzoxy-L-lysine p-nitrophenyl ester from Sigma Chemical Co.; $H_3{}^{32}PO_4$, carrier free, from ICN Pharmaceuticals, Inc. All other chemicals were reagent grade.

Preparation of [^{32}P]-Labeled Cells: Spores of Dictyostelium discoideum, strain NC-4, were germinated and cultured in association with E. coli on petri plates (8.5 cm diameter) containing 25 ml of nutrient-agar medium. The medium composition was as follows: agar (20 mg/ml), dextrose (10 mg/ml), peptone (10 mg/ml), yeast extract (1 mg/ml), $MgSO_4$ (1 mg/ml), and [^{32}P] P_i (40 μCi/ml - carrier free). Myxamoebae were collected from the petri plates after the bacteria had been depleted but prior to the commencement of aggregation. The labeled cells were washed free of residual bacteria by repeated centrifugation in cold, distilled water (5). In order to generate a sufficient quantity of cells for enzyme purification studies, the labeled cells (obtained from 4 petri plates) were mixed with unlabeled cells at a ratio of approximately 1:40.

Assay of Proteinase I Catalytic Activity: Proteinase I activity was quantified by assaying enzyme fractions for their ability to hydrolyze the synthetic ester substrate, CBZ-lys-ONp[2]. Reaction mixtures contained in a total volume of 1 ml, 180 μmoles potassium phosphate (pH 5.5), 0.5 μmole CBZ-lys-ONp, 5% acetonitrile, and sufficient proteinase to produce an absorbance change of at least 0.04 per min at 340 nm. The change in absorbance was followed continuously using a Coleman-Hitachi Model 124 double beam spectrophotometer equipped with a water-jacketed cuvette holder and a recorder. The temperature of assay mixtures was maintained at 25°. Reaction mixtures lacking proteinase produced an absorbance change of 0.02/min. The rates of absorbance change observed in the presence of proteinase were corrected for this non-specific hydrolysis of ester. One unit of enzyme was defined as that amount catalyzing the synthesis of 1 μmole p-nitrophenol (extinction coefficient, 5600 M^{-1} at pH 5.5) per min.

Assay for Radioactivity: [^{32}P] was quantified by liquid scintillation using a Unilux III Spectrometer (Chicago-Nuclear) and employing Aquasol-2 (New England Nuclear) as a fluor.

Protein Assays: Protein was quantified as described by Lowry et al. (6) with bovine serum albumin as a standard.

Dissociation Chromatography: Purified [^{32}P]-Proteinase I (6 mg/ml) in 10 mM Tricine-5mM DTT was adjusted to contain approximately 6 M GuHCl by addition of 0.81 g of the solid salt to 0.8 ml enzyme solution. The resulting

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[2]Abbreviations used are: CBZ-lys-ONp, N-α-carbobenzoxy-L-lysine p-nitrophenyl ester; GuHCl, guanidine hydrochloride; and DTT, dithiothreitol.

solution was incubated 2h at 37° and dialyzed overnight against 6M GuHCl-1mM DTT. This denatured preparation of Proteinase I was then chromatographed on a column of Sepharose 6B, previously equilibrated with 6 M GuHCl-1 mM DTT, according to the general procedure described by Fish et al. (7).

Preparation of Proteinase I Antibody: Antisera obtained from rabbits immunized with purified Proteinase I[1] was fractionated as described by Livingston (8). The purified IgG fraction obtained from this treatment was utilized for all immunological studies.

Determination of the Phosphorous Content of Proteinase I: Purified Proteinase I was ashed in the presence of $MgNO_3$, and the resulting residue analyzed for inorganic phosphate according to the procedures of Ames (9).

RESULTS AND DISCUSSION

Purification of [^{32}P]-Proteinase I: Myxamoebae of Dictyostelium discoideum were uniformly labeled with isotope by growing them for several generations in association with bacteria on media containing [^{32}P] P_i as a sole source of phosphorous. Proteinase I was isolated from labeled cells as described elsewhere[1]. Briefly, the stepwise purification was performed as follows: (1) Proteinase I was precipitated from crude extracts as a complex with protamine sulfate and nucleic acids; (2) enzyme was extracted from the precipitated complex in the presence of NaCl and subjected to an ammonium sulfate fractionation; (3) salt-fractionated enzyme was chromatographed on a column of DEAE cellulose, developed with a linear gradient of NaCl; and, (4) enzyme purification was completed by gel filtration on a column of G200 Sephadex. The purified enzyme had a specific activity of 34 units/mg protein. Immunological characterizations, employing Proteinase I-specific antisera, have indicated that this purification procedure yields a relatively homogeneous preparation of the enzyme[1].

Fig. 1 shows that a significant quantity of [^{32}P] eluted with Proteinase I in the final gel filtration step of enzyme purification. The specific radioactivity (determined both as cpm/mg protein, and as cpm/unit enzyme activity) was constant throughout the peak of eluted enzyme. From a chemical analysis of purified Proteinase I for phosphorous (see "Methods") it was determined that the purified enzyme contained approximately 0.7 μmole phosphorous/mg protein. Assuming that the protein assay yielded a reasonably accurate value for Proteinase I protein, and that an amino acyl residue in the enzyme has a molecu-

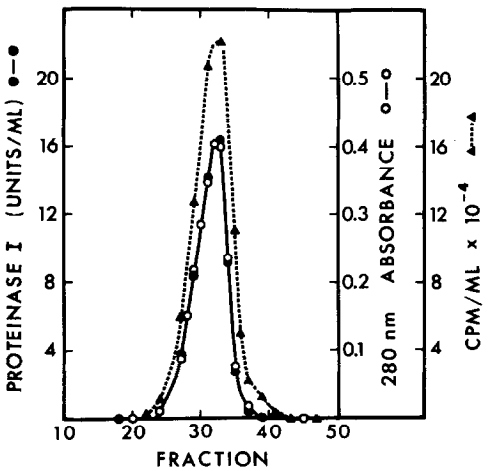

Fig. 1. Gel filtration of [^{32}P]-Proteinase I. Enzyme obtained from the DEAE cellulose chromatography step of the purification procedure was applied (in a total volume of 1 ml buffer) to a 1.7 x 46 cm column of G200 Sephadex. The column was developed with 10 mM Tricine (pH 8) -1 mM DTT, and 1.6 ml fractions were collected. The void volume of the column was 32 ml, corresponding to fraction #20.

lar weight of 120, the above value would correspond to the presence of one phosphoryl moiety per 12 amino acyl residues.

Dissociation Chromatography: Native Proteinase I is a polymeric enzyme consisting of three types of immunologically cross-reactive subunits which can be dissociated and resolved from one another by chromatography on a 6% agarose column in the presence of 6 M GuHCl[1]. Fig. 2 shows the results obtained when purified, [^{32}P]-labeled Proteinase I was subjected to dissociation chromatography. All of the [^{32}P] which eluted in association with Proteinase I during gel filtration with G200 Sephadex (Fig. 1) remained associated with Proteinase I subunits during dissociation chromatography. The majority of isotope eluted in the peak corresponding to the largest subunit (designated A in Fig. 2). However, subunits B and C also contained some radioactivity. As previously discussed by Mann and Fish (10), all proteins which have been carefully studied have been shown to possess no residual non-covalent structure in the presence of high concentrations of GuHCl. Thus, the results of dissociation chromato-

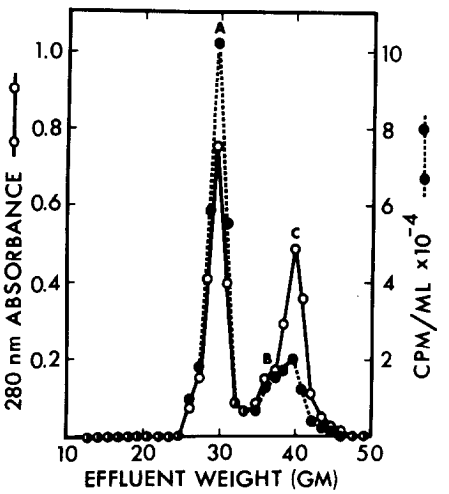

Fig. 2. Dissociation chromatography of [^{32}P]-Proteinase I. Denatured enzyme (see "Methods") was chromatographed on a 1.3 x 45.5 cm column of Sepharose 6B in the presence of 6 M GuHCl -1 mM DTT. Fractions (40 drops each) were collected and evaluated for effluent weight, 280 nm absorbance, and radioactivity.

graphy of labeled Proteinase I strongly suggested that the phosphoryl moieties associated with purified enzyme were covalently bonded to its subunits.

Immunological Studies: Quantitative precipitin analysis (Fig. 3) with purified [^{32}P]-Proteinase I and Proteinase I-specific antibody also indicated that the catalytic and isotopic activities associated with purified enzyme resided in a common macromolecule. The point in the immune titration at which Proteinase I catalytic activity appeared in the soluble fraction corresponded with the point of maximum [^{32}P] precipitation and was immediately preceded by the point where maximum precipitation of protein was observed. In the region of maximum protein precipitation greater than 90% of the isotope added to reaction mixtures was recovered in the immune precipitate. The specific radioactivity of the precipitate (cpm/mg protein) increased throughout the titration, undoubtedly reflecting an increase in the antigen:antibody ratio of the immune precipitate. Based on the above observations it was concluded that the quantity of [^{32}P] precipitated at each point in the titration was reflective of the quantity of Proteinase I protein precipitated.

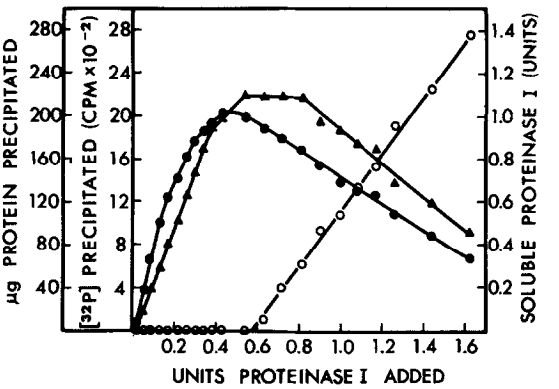

Fig. 3. Quantitative precipitin reaction. Reaction mixtures contained 0.25 ml Proteinase I antibody, specified quantities of purified, [^{32}P]-Proteinase I, and sufficient buffer (10 mM potassium phosphate - 0.85% NaCl, pH 8) to give a volume of 0.5 ml. Each mixture was incubated 4h at 25° and then centrifuged (1000 xg, 30 min). The supernatant fluids were decanted and assayed for Proteinase I activity (o-o). The precipitates were washed with 5 ml of chilled buffer and then analyzed for protein (●-●) and radioactivity (▲-▲). A similar experiment with gamma globulin from nonimmune serum yielded no visible precipitate and no loss of Proteinase I activity.

In view of the apparently novel structure of Proteinase I, this enzyme should provide an interesting model for studying the regulation of cellular proteolysis. As an extension of our investigations of Proteinase I, we are currently attempting to determine: (a) the chemical composition of its phosphoryl moieties, (b) whether these moieties influence the catalytic specificity of Proteinase I for degrading other cellular proteins, and (c) whether covalent modifications of the conjugated enzyme (eg., an *in vivo* hydrolytic removal of the phosphoryl moieties) may contribute to modulation in cellular Proteinase I activity during differentiation. In relation to these studies we have recently isolated two additional proteinases from crude extracts of *D. discoideum* which are immunologically cross-reactive with Proteinase I, but differ from Proteinase I in apparent molecular weights and phosphorous contents. Comparisons of the catalytic and structural properties of these additional proteinase species with Proteinase I, and analyses of how their levels vary during differentiation, may contribute information regarding the above cited issues.

ACKNOWLEDGEMENT

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