Purification and Preliminary Characterization of dDAP, a Novel Dipeptidylaminopeptidase from *Dictyostelium discoideum*

Paul R. Atkinson, Matthew D. Hilton, and Peter K. Lambooy*

Fermentation Process Development and Biosynthetic Purification Development Departments, Lilly Research Laboratories, Eli Lilly & Company, Lilly Corporate Center, Indianapolis, Indiana 46285

Received January 30, 1995; Revised Manuscript Received June 1, 1995[⊗]

ABSTRACT: We have discovered and purified a novel dipeptidylaminopeptidase (DAP) from the cell-free broth of *Dictyostelium discoideum* Ax3. The enzyme is secreted in parallel with cell growth in axenic broth culture. It shares substrate preferences with both DAP-I and DAP-III enzymes yet is distinct from both in some physical properties. Similar to DAP-I, the *D. discoideum* enzyme is able to cleave a variety of dipeptides from the amino termini of substrates. In addition, it readily cleaves substrate sequences beginning with RR- and KK-, a property of the DAP-III class. The *D. discoideum* enzyme has a pH optimum of 3.5, a subunit molecular mass of 66 000 daltons, and a molecular weight of approximately 225 000 and is not significantly inhibited by cysteine or serine protease inhibitors. It is inhibited by leupeptin and trivalent cations. On the basis of enzymological and other data presented here, we conclude the *D. discoideum* enzyme does not belong to any of the previously reported DAP classes (DAP-I, -II, -III, -IV) and propose that the class DAP-V be established with this *D. discoideum* enzyme as the first member.

Dipeptidylaminopeptidases (DAPs)[1] are a group of complementary enzyme activities that remove dipeptides from the unblocked amino termini of peptides and proteins. There are currently four recognized classes of DAP enzymes: DAP-I, DAP-II, DAP-III, and DAP-IV [reviewed in McDonald and Schwabe (1977)]. These classes are distinguished based primarily on their dipeptidyl substrate profiles and the relative rates of cleavage of those substrates. DAP-I, also known as cathepsin C (EC 3.4.14.1), is a relatively nonspecific cysteine protease that catalyzes the release of most dipeptide combinations from the amino terminus of substrates. DAP-I displays no significant activity toward substrates of the type x-Pro- or Arg-x- (where x is any amino acid). DAP-II (EC 3.4.14.2) shows a marked preference for dipeptide sequences that begin Arg-y- or Lys-y (where y is any amino acid other than Arg or Lys), and DAP-IV (EC 3.4.14.5) exhibits its highest rate of activity toward dipeptide sequences of the form x-Pro-. DAP-III (EC 3.4.14.4), a less well-characterized enzyme, shows a propensity for the dipeptide sequence form Arg-Arg-.

The primary role of the DAP enzymes in nature appears to be digestive, breaking down protein fragments into smaller peptides (McDonald & Schwabe, 1977). However, DAP-IV may also play a role in protein processing by converting inactive precursors (containing a series of x-Pro or x-Ala sequences) to active entities; an example of this is the activation pathway of the antibacterial peptide cecropin (Boman et al., 1989). Since the cellular slime mold *Dictyostelium discoideum* is commonly viewed as a bridge between unicellular microbes and multicellular eukaryotes with developmental cycles, many efforts to isolate proteases and peptidases of *Dictyostelium* have been reported (Chan et al., 1985, 1987; Fong & Rutherford, 1978; Presse et al., 1986; Datta & Firtel, 1987; North et al., 1990). In particular, two recent studies of the presence of DAP-I, -II, and -III activities in *D. discoideum* have appeared (Chan et al., 1985, 1987).

We have isolated and characterized a DAP activity from *D. discoideum* that has properties that distinguish it from the four accepted classes of DAP enzymes, including the DAPs that have been previously reported from *D. discoideum* (Chan et al., 1985, 1987). We initially began our work searching for a DAP-I-like activity from the wild-type strain NC-4. We accumulated evidence for a GF-pNA cleaving activity at pH 5. The NC-4 strain was grown in the presence of bacteria, and this primarily drove us to an evaluation of the Ax3 strain which has no such requirement. The enzyme we subsequently isolated from the Ax3 strain is referred to here as dDAP, where it was found in the extracellular broth of actively growing *D. discoideum* Ax3 amoeba. It does not appear to be developmentally regulated. dDAP cleaved a broad variety of dipeptides from the amino termini of peptide substrates, which initially led us to view it as a member of the DAP-1 class. However, in research to be reported elsewhere, the gene for dDAP has been isolated and the sequence determined and found to lack homology to any known protein, including rat DAP-1, in the GenBank database.[2] In addition, we show in the present report that dDAP cleaves RR- and KK-substrates, a property characteristic of the DAP-III class of enzymes. These observations,

* To whom correspondence should be addressed at Eli Lilly & Company, Lilly Corporate Center, Drop Code 4437, Indianapolis, IN 46285. Phone: (317)276-1092. FAX: (317)276-1969. E-mail: Lambooy_Peter_K@lilly.com.

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1995.

[1] Abbreviations: dDAP, *Dictyostelium discoideum* dipeptidylaminopeptidase; pNA, *p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane; E-64, 1-[N^{α}-(*trans*-epoxysuccinyl)-L-leucylamido]-4-guanidinobutane; PMSF, phenylmethanesulfonyl fluoride; βNA, β-naphthylamide; Ac-, N-terminally acetylated. The one-letter code for amino acids was used: G (Gly), F (Phe), R (Arg), K (Lys), D (Asp), A (Ala), P (Pro), M (Met), S (Ser), E (Glu), W (Trp), S (Ser), H (His), Y (Tyr), V (Val).

[2] C. Hershberger et al., personal communication.

0006-2960/95/0434-10827$09.00/0

plus a low pH optimum (ca. 3.5), insensitivity to all tested cysteine modifying agents and a cysteine protease inhibitor (E-64), and a subunit molecular mass of 66 000 daltons, prove dDAP to be different than previously characterized classes of DAP enzymes, including DAP-I. We propose dDAP is the first of a new class of DAP enzymes.

EXPERIMENTAL PROCEDURES

Strain and Medium Composition. Research described in this report was performed with *D. discoideum* Ax3 (Accession Number 28368; American Type Culture Collection, Rockville, MD) which is capable of growth axenically. *D. discoideum* was generally grown on ATCC medium 671, composed of (g/L): Difco (Detroit, MI) Bacto Yeast Extract (7.15), Difco Bacto Peptone (14.3), Na_2HPO_4 (0.51), and KH_2PO_4 (0.49). Glucose (10 g/L final) was added aseptically after separate sterilization, and the pH was adjusted to 6.5 ± 0.1 with NaOH or H_2SO_4. Harvests were generally from 10 L working volume vessels (Chemap 2000, Alpha-Laval Corp., S. Plainfield, NJ) fitted with a single Rushton turbine impellor rotating at 150 rpm; temperature was controlled at 22 ± 1 °C, air flow at 1.0 slpm, and back-pressure at 3−5 psi. Some harvests were from larger fermentors with media of similar composition. The pH and dissolved oxygen of all fermentations were monitored continuously with standard probes. Broth supernatants for purification of dDAP were prepared by chilling whole broth to <10 °C and removing cells with a centrifuge.

Fermentation Sample Analyses. Broth samples were periodically taken and analyzed for cell density and dDAP activity. A Petroff-Hauser counting chamber was used to estimate cell densities of (0.5−25) × 10^6/mL. Whole broth supernatants were stored at 4 °C for up to 2 weeks or frozen at −20 °C and later thawed for determination of dDAP activity.

Activity Assays Using Dipeptidyl-pNA Substrates. D. discoideum dipeptidylaminopeptidase (dDAP) and cathepsin c were assayed using the chromogenic substrates GF-pNA, GR-pNA, AA-pNA, and GP-pNA (Sigma, St. Louis, MO). For general use of GF-pNA and for the results for AA-pNA and GP-pNA, solutions of each were prepared at 200 mM concentration in dimethylformamide, diluted to 4 mM into 50 mM acetic acid, and adjusted to pH 3.5 with NaOH or HCl. For estimates of K_M and k_{cat} in Table 3, GF-pNA and GR-pNA were prepared at 20 mM GF-pNA and 10 mM GR-pNA directly into 50 mM acetic acid, pH 2.5, and the pH was adjusted to 3.5. Reactions with dDAP were carried out in the same buffer and in 50 mM acetic acid, 10 mM NaCl, and 2 mM cysteamine, pH 3.5, for cathepsin C. One unit of activity corresponds to the amount of enzyme which produces 1 μmol of pNA (or other product) per minute at pH 3.5 under the conditions used. We used a value for the extinction coefficient of 9.9 for a 1 mM solution of pNA. Typically, around 7−14 μg/mL enzyme was used for these experiments. The GF-pNA activity assay was adapted for use with a Dynatech Laboratories (Chantilly, VA) MR700 plate reader and software to handle the large number of chromatography fractions.

Activity Assays Using Dipeptidyl-β-naphthylamides. Dipeptidyl-β-naphthylamide substrate stocks (Bachem Bioscience, Philadelphia, PA) were prepared in 100 mM acetic acid, adjusted to pH 3.5 with NaOH or HCl, and diluted to 0.5 mM into the same buffer for dDAP and the same buffer supplemented with 10 mM NaCl, 2 mM cysteamine, pH 3.5, for cathepsin C experiments that are contained in Table 2. For the experiments listed in Table 3, the substrates were prepared as 8 mM stocks, and these were used to estimate K_M and k_{cat} for DR-β-naphthylamide, GR-β-naphthylamide, and GF-β-naphthylamide. dDAP and cathepsin C were added to final concentrations of 61 μg/mL and 1.4 μg/mL, respectively, for the data in Table 2 and 1.4 μg/mL each for the data of Table 3. Initial rates of cleavage by dDAP and cathepsin C were determined at room temperature from 0 to 4 min using a Perkin Elmer (Norwalk, CT) LS-3 fluorescence spectrometer using 340 and 410 nm as the excitation and emission wavelengths, respectively (McDonald et al., 1969). Dilutions of authentic β-naphthylamine (Sigma) (for Table 2) as well as fully cleaved DR-β-naphthylamide (used for Table 3) of a known concentration were used to quantify the amount of product. Both methodologies gave similar quantification.

Determination of the pH Optimum for GF-pNA Cleavage. A buffer consisting of 500 mM Tris/sodium phosphate/sodium citrate was prepared, and portions were adjusted with HCl or NaOH to achieve pH values within the range of 3−8; 4 mM GF-pNA was prepared in 2% dimethylformamide, water. dDAP and cathepsin C were diluted into 100 mM cysteamine, 100 mM NaCl, pH 4. Enzymes, substrate, and buffers were combined so that the final substrate and buffer concentrations were 1.8 and 56 mM, respectively. The final concentrations of dDAP and cathepsin C were about 22 and 0.7 μg/mL, respectively. The rates were measured at room temperature, monitoring at 410 nm (plate reader). Several samples were checked to verify pH. Under the conditions used, there was no significant change in absorbance at 410 nm for a cathepsin C/pNA mixture that was adjusted to several pHs within the range tested.

Molecular Weight Estimation by Sedimentation Equilibrium. Purified dDAP, approximately 0.3 mg/mL in 50 mM acetic acid, 0.1 M NaCl, pH 3.5, was run in a Beckman Model E analytical ultracentrifuge at 20 °C for 2 days. The equilibrium speed was 6800 rpm. The solvent density was measured in a pycnometer, and the partial specific volume of the protein was assumed to be 0.73 mL/g. Scans of the cell were done at 28.8 and 50.4 h using the photoelectric scanning optical system at 280 nm. Plots of ln concentration versus radius squared were the same at the two time points, indicating that equilibrium had been reached.

Inhibition of Enzymatic Activity. dDAP and cathepsin C were incubated with protease inhibitors and chemical modifying reagents and then assayed for residual GF-pNA cleaving activity by diluting the enzyme/reagent mixtures (10−20)-fold into 4 mM GF-pNA, 100 mM acetic acid, adjusted to pH 3.5 with NaOH or HCl. Leupeptin and E-64 were from Sigma, and iodoacetamide was from Aldrich (Milwaukee, WI). Incubation conditions were as follows: iodoacetamide (50 mM Tris, adjusted to pH 7 with HCl, or 100 mM acetic acid adjusted to pH 3.5 with NaOH); leupeptin (100 mM acetic acid, 10 mM NaCl, and 2 mM cysteamine, adjusted to pH 3.5 with NaOH or HCl); E-64 and PMSF (100 mM acetic acid, 50 mM NaCl, adjusted to pH 3.5 with NaOH or HCl). Incubations were at room temperature. The concentrations of dDAP and cathepsin C ranged from 0.1 to 0.2 mg/mL and from 0.1 to 0.4 mg/mL, respectively.

Cleavage of MRMYFVNQHL, MDFPAMSL, and KKFPAMSL by dDAP. The synthetic peptides MRMYFVNQHL, KKFPAMSL, and RRFPAMSL were prepared by Harlan Long (Eli Lilly & Co., Indianapolis, IN). MDFPAMSL and N-terminally acetylated MDFPAMSL were custom-synthesized by Bachem California (Torrance, CA). MD, MR, and FV dipeptides were obtained from Bachem Bioscience and along with the MY were used as identity references; 10 mM MRMYFVNQHL was prepared in 0.01 M HCl. The MRMYFVNQHL was diluted to 2 mM into 100 mM glycine and adjusted to pH 3.5 with NaOH or HCl. dDAP was added to a level of 0.1 milliunit/mL. The reaction was monitored by reversed-phase HPLC. This chromatographic assay used a Zorbax 150 A, C-8 column (4.6 mm × 25 cm; Mac-Mod, Chadds Ford, PA) and a buffer with final concentrations of 100 mM phosphoric acid, 10 mM octanesulfonic acid (Kodak, Rochester, NY), adjusted to pH 2.9 with NaOH (buffer A), and the same buffer containing 50% acetonitrile (buffer B). pH adjustment for buffer B was made prior to addition of the acetonitrile. The chromatography was run at room temperature, 1 mL/min, using either a Beckman Gold (Fullerton, CA) or a Hewlett Packard HP1090 (Palo Alto, CA) HPLC system. Absorbance was monitored at 210 or 214 nm. A typical gradient consisted of 10% B/5 min, 10−40% B/10 min, 40−51% B/10 min, 51% B/20 min, and 10% B/10 min. Modifications to the gradient were made to optimize the retention region of interest. MD dipeptide was obtained from Bachem Bioscience. MDFPAMSL and KKFPAMSL were prepared at 20 mg/mL in 100 mM acetic acid, 10 mM NaCl, and 2 mM cysteamine, adjusted to pH 3.5 with NaOH. dDAP and cathepsin C were added to 4.8 μg/mL final concentrations. Incubations were quenched by diluting 10-fold into 100 mM ammonium phosphate, 7 M urea, pH 7. The samples were analyzed by HPLC using a Spherisorb ODS-2, 4.6 mm × 10 cm column, in a 40 °C column oven, and a buffer system consisting of 0.1 M dibasic ammonium phosphate adjusted to pH 7.0 with phosphoric acid, 21% acetonitrile. The flow rate was 1 mL/min, and it was run isocratically. The absorbance of the effluent was monitored at 258 nm. K_M and k_{cat} of dDAP for MDFPAMSL were estimated from a Eadie plot. Approximately 6 μg/mL dDAP was added to a range of MDFPAMSL concentrations (1.25−15 mM), and the initial rate of FPAMSL product formation was captured using a reversed-phase HPLC system (for this experiment, a TFA/ACN-based system was used on a Vydac Protein C4 column, column oven 55 °C, 1 mL/min, 13% ACN isocratically). FPAMSL production was quantified from a standard curve using authentic FPAMSL.

Purification of Cathepsin C. Cathepsin C was purified in a fashion similar to that reported by Metrione et al. (1966), up through the size exclusion step.

RESULTS

Dictyostelium discoideum Fermentation. Our initial research on the *D. discoideum* Ax3 strain examined whole cell extracts for hydrolysis of GF-pNA as we had done for the NC-4 strain. However, it quickly became apparent that the majority of the GF-pNA cleaving activity was present in the extracellular broth. We do not know if the GF-pNA cleaving activity we initially observed in the NC-4 strain was predominantly a secreted activity since we had focused on whole cell extracts. The extracellular GF-pNA activity (dDAP activity) accumulated in parallel with the growth of

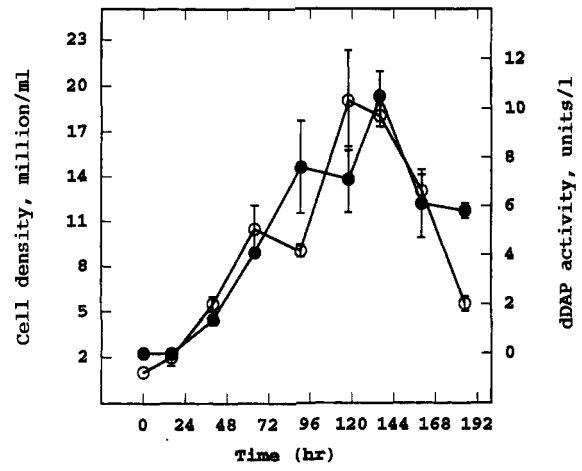

FIGURE 1: Stirred liquid cultivation of *Dictyostelium discoideum*. Secreted dDAP (GF-pNA) activity (●) and cell growth (○) during cultivation of *Dictyostelium discoideum* Ax3 in a 10 L stirred axenic culture. Error bars represent the standard deviation on duplicate (activity) and quadruplicate (cell density) determinations of time point samples.

D. discoideum Ax3 in stirred liquid culture, with peak activity coincident with peak cell density (Figure 1). Cell densities up to 2 × 10^7 mL^{-1} and doubling times between 1 and 5 days were observed. Dissolved oxygen concentration decreased steadily during cell growth to 0% of saturation and then increased rapidly when cell density decreased (data not shown). Broth pH remained steady near 6.5 for about 3 days and then increased progressively. Harvest of subsequent broth supernatants for purification of dDAP was timed to be coincident with peak cell density which was typically around the fifth day. After this time, the cell count and activity concentration decreased.

Purification and Properties of Dictyostelium discoideum dDAP. Purification of dDAP from *D. discoideum* fermentation broth began with cell removal by centrifugation followed by anion exchange chromatography (Figure 2A). All steps were performed at approximately 4−8 °C. Cell-free broth (adjusted to pH 7 with NaOH) containing about 1 unit of dDAP activity was loaded onto a column containing Pharmacia (Piscataway, NJ) Q Sepharose Fast Flow resin (30 mL, 1.6 cm × 15 cm) equilibrated in 50 mM Tris-HCl, pH 7. The column was eluted with a 10 column volume, linear gradient of 0−0.5 M NaCl in the same buffer. A linear flow rate of about 50 cm/h was used. Figure 2A shows the profile of relative GF-pNA cleaving activity (peak fraction of activity defined as 100%) and the absorbance at 280 nm multiplied times 10 as well as the conductivity in millisiemens. Fractions containing dDAP activity as determined by GF-pNA activity were pooled. The anion exchange pool was adjusted to pH 3.5 with HCl and supplemented with 140 g/L ammonium sulfate. This material was then loaded onto a 12 mL Pharmacia phenyl-Sepharose column (1 cm × 15 cm) equilibrated in 50 mM citrate, 140 g/L ammonium sulfate, pH 3.5 (Figure 2B). About 3 mg of protein was loaded per milliliter of resin. The linear flow rate was about 40 cm/h. The column was then eluted with a 10 column volume decreasing linear gradient from 140 g/L ammonium sulfate to 0 g/L ammonium sulfate in the same buffer. In Figure 2B, the absorbance at 280 nm has been multiplied by 100. The dDAP activity (GF-pNA activity) eluted as a single peak. The phenyl-Sepharose-purified material was concentrated using an Amicon (Beverly, MA) YM-10

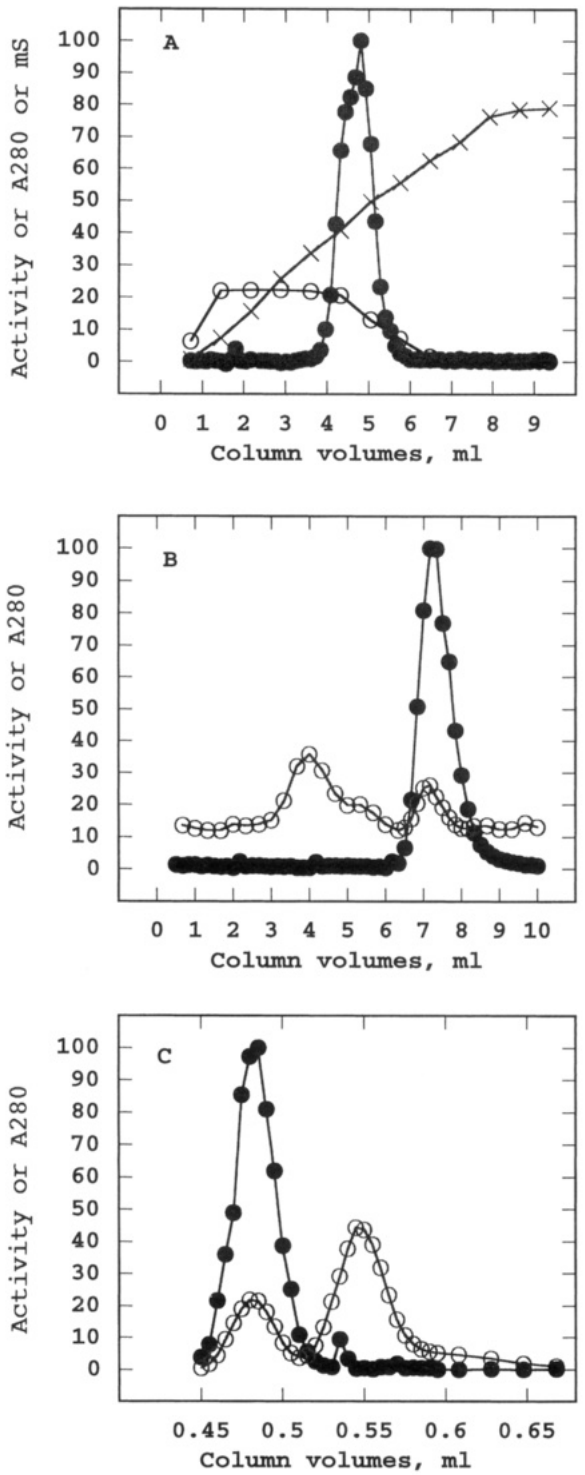

FIGURE 2: Purification of dDAP from the cell-free broth of *Dictyostelium discoideum* Ax3. dDAP was purified using Q-Sepharose fast flow anion exchange (1 column volume equals 30 mL) (A), phenyl-Sepharose hydrophobic interaction (1 column volume equals 12 mL) (B), and Sephacryl S-200HR size exclusion chromatographies (1 column volume equals 320 mL) (C). Relative rate of dDAP activity as monitored by GF-pNA (individual fractions relative to fraction containing the most activity) (●), absorbance at 280 nm multiplied times 10 for the Q column, 100 for the phenyl column, and 25 for the S200HR column (○), and conductivity (×) in millisiemens. See Experimental Procedures for further details.

membrane and applied (2−5 mL of sample/100 mL resin) to a column (1.6 cm × 160 cm) of Pharmacia Sephacryl S-200HR size exclusion resin equilibrated in 50 mM acetic acid, 500 mM sodium chloride, pH 3.5 (Figure 2C). The linear flow rate was 8 cm/h. The applied material was eluted

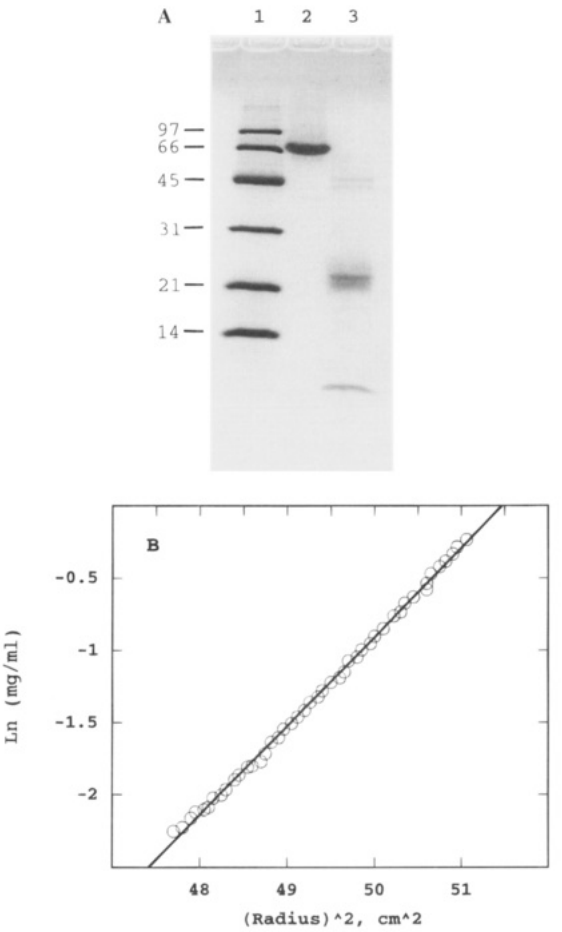

FIGURE 3: Analysis of dDAP by SDS−polyacrylamide gel electrophoresis and analytical ultracentrifugation. (A) Samples containing approximately 20 μg of dDAP (lane 2) and cathepsin C (lane 3) were prepared under reducing conditions and loaded onto an 8−16% polyacrylamide gradient gel which was run in the presence of 0.1% SDS. The gel was stained with Coomassie blue and destained using 10% acetic acid, 30−50% methanol. Lane 1 contains molecular mass markers, including phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). (B) Approximately 0.3 mg/mL dDAP in 50 mM acetic acid, 0.1 M NaCl, pH 3.5, was spun in a Beckman Model E analytical ultracentrifuge for 2 days at 20 °C. The equilibrium speed was 6800 rpm. Scans were performed at 28.8 and 50.4 h using the photoelectric scanning system at 280 nm.

with the same buffer. There was distinct separation of two major absorbance peaks at 280 nm (absorbance multiplied by 25). dDAP activity was coincident with the first peak. The second peak corresponded to a protein having an apparent subunit molecular mass of approximately 34 000 daltons as visualized by SDS−polyacrylamide gel electrophoresis. The final dDAP preparation was held at −20 °C in the presence of 51% (v/v) glycerol without significant loss of activity. The concentration of dDAP was determined by its absorbance at 280 nm where it has an extinction coefficient of around 1.0−1.2 for a 1 mg/mL solution.

Table 1 summarizes the purification process of one preparation and indicates a purification of about 213-fold. The specific activity of this final preparation was about 1.6 units/mg as determined at 37 °C with 4 mM GF-pNA, 50 mM acetic acid adjusted to pH 3.5 with HCl or NaOH. The overall purification yield was approximately 30%. Figure 3A is a photograph of a SDS−polyacrylamide gel (reducing conditions) of a final dDAP preparation showing a single

Table 1: Purification of Dipeptidylaminopeptidase (dDAP) from *Dictyostelium discoideum*[a]

purification step	volume (mL)	protein (mg)	total act. (units)	sp act. (units/mg)	cumulative yield (%)	cumulative purification
cell-free broth	81	622	4.7	0.0075	100	1
Q-Sepharose	52	28	4.5	0.16	96	21
phenyl-Sepharose	67	7.3	2.0	0.27	43	36
concentration	1.5	2.2	2.0	0.87	43	116
S-200HR	16	1.0	1.6	1.6	34	213

[a] dDAP activity was estimated using 4 mM GF-pNA at pH 3.5 in 50 mM acetic acid. Activity values were determined at 37 °C. Estimation of protein content was by the Pierce Micro BCA Protein Assay (Rockford, IL) using bovine serum albumin as standard.

strongly stained band. The apparent subunit molecular mass of dDAP (Figure 3A, lane 2) is about 66 000 daltons, compared to the subunit molecular mass of about 24 000 daltons for a preparation of cathepsin C (Figure 3A, lane 3). This apparent subunit molecular mass of dDAP is unchanged when analyzed by SDS−PAGE under nonreducing conditions. Analytical ultracentrifugation was employed to estimate the native molecular weight of the enzyme by sedimentation equilibrium (Figure 3B). The plot of ln concentration versus radius squared is linear with the exception of a small amount of curvature near the meniscus. The molecular weight was calculated to be 225 000. Analytical size exclusion chromatography using a Pharmacia Superose 12 size exclusion column was also employed to estimate the native size of dDAP and to compare it with bovine cathepsin C (data not shown). dDAP eluted earlier than cathepsin C: an estimated 270 000 daltons by use of calibrated standards. A Stoke's radius of approximately 6.6 nm was calculated. Assuming a molecular weight of 225 000 from the sedimentation data, the radius of an unhydrated sphere can be estimated (approximately 4 nm), and thus a frictional ratio of 1.6 can be calculated (assumed partial specific volume of 0.73 mL/g and a viscosity of 1.0). Cathepsin C is known to be an oligomeric protein composed of eight subunits, associated noncovalently, having a native molecular weight of about 200 000−210 000 (Metrione et al., 1966, 1970). The SDS gel and centrifugation data likewise suggest that dDAP is an oligomeric protein, possibly a homotetramer or homotrimer.

pH Optimum of dDAP Activity. The pH optimum for dDAP activity against the substrate GF-pNA is approximately 3.5 (Figure 4). The activity decreased sharply at higher pH. For comparison, the pH optimum for bovine spleen cathepsin C is relatively broad and is about 4−6 (McDonald et al., 1969, and Figure 4). Other experiments indicated that purified dDAP has a pH optimum of about 3−4 for GR-pNA, GR-βNA, RR-βNA, MDFPAMSL, MR-human proinsulin, and MD-human growth hormone (data not shown).

Effect of Protease Inhibitors and Modifying Agents on the Activities of dDAP and Cathepsin C. Purified dDAP and cathepsin C were assayed for GF-pNA cleaving activity at pH 3.5 after prior incubation of the enzymes with a variety of reagents. Leupeptin (0.1 mM) inhibited dDAP activity about 60% after 2 h of incubation and 8% for cathepsin C over the same time frame. Incubation of dDAP with 5 mM iodoacetamide at pH 7 and pH 3.5 for 30 min and with 1 mM E-64 at pH 3.5 for 1 h resulted in 0%, 6%, and 3% inhibition, respectively, in contrast to that observed for cathepsin C, which was inhibited greater than 96% for the same three conditions mentioned above. Other cysteine modifying agents, *N*-ethylmaleimide and potassium tetrathionate, inhibited cathepsin C but not dDAP. This provides

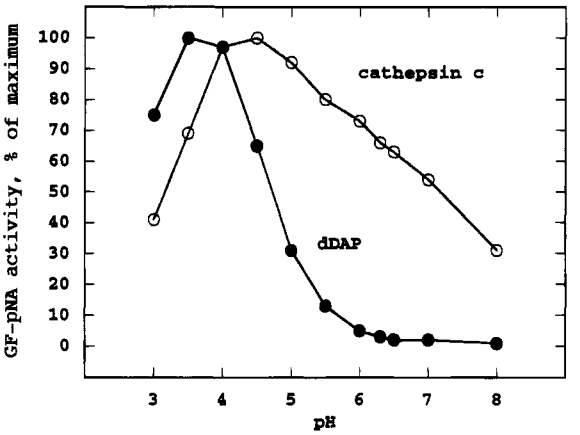

FIGURE 4: Effect of pH on the activities of dDAP and cathepsin C. dDAP and cathepsin C were assayed for cleavage of GF-pNA at several pHs as described under Experimental Procedures. Rates are expressed relative to the pH maximum for the individual enzyme. dDAP (●) and cathepsin c (○).

presumptive evidence that the catalytic centers of dDAP and cathepsin C are different; however, in the case of dDAP, the mechanism is not yet fully understood. The serine protease inhibitor PMSF did not inhibit dDAP when present at 1 mM at pH 3.5 for 1 h. Aprotinin, pepstatin A, EDTA, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1,-10-phenanthroline, bestatin, $ZnCl_2$, $MgCl_2$, $CaCl_2$, $CoCl_2$, $MnCl_2$, $NiCl_2$, $FeCl_3$, and $AlCl_3$ were also evaluated for inhibition of dDAP activity (data not shown). From this group, only the trivalent cations, Fe^{3+} and Al^{3+}, caused a reduction in activity. However, precipitation was observed in some incubations,[3] suggesting that this apparent inhibition may not be specific to the catalytic center. The above list includes reagents or protease inhibitors known to affect cysteine-, serine-, aspartic-, and metallo-based enzymes.

Activity of dDAP against Dipeptidyl-β-naphthylamide, Dipeptidyl-pNA, Peptide, and Protein Substrates. Since we had found evidence that dDAP was similar to DAP-I but was different in its monomeric size, pH optimum, and apparent lack of an active site cysteine or serine, and because the four classes of DAP enzymes are classified primarily on the basis of relative cleavage rates and types of substrates (McDonald & Schwabe, 1977), we measured the activity of dDAP against a panel of dipeptidyl-β-naphthylamide substrates typically used to assist in the classification of DAP enzymes (Table 2). Bovine cathepsin C was evaluated at pH 3.5 in parallel with results conforming to those previously published (McDonald et al., 1969). dDAP cleaved a variety of the dipeptidyl-β-naphthylamide substrates. Similar to cathepsin C, dDAP did not cleave GP-βNA (a characteristic

[3] J. Ivancic, personal communication.

Table 2: Rates of Dipeptidyl-β-naphthylamide Cleavage by dDAP and Cathepsin C[a]

	sp act. (μmol min^{-1} mg^{-1})		rel act. (%)	
substrate	dDAP	cathepsin C	dDAP	cathepsin C
GW-	0.004	12	0.8	32
AA-	*nd*	6.2	*nd*	16
GF-	0.006	7.4	1.2	19
SM-	0.084	7.6	16	20
EH-	0.14	14	27	37
HS-	*nd*	2.9	*nd*	7.6
LA-	*nd*	3.7	*nd*	9.7
DA-	*nd*	0.9	*nd*	2.4
GR-	0.048	38	9.2	100
DR-	0.52	16	100	42
AR-	0.34	33	65	87
RR-	0.25	*nd*	48	*nd*
ZRR-	*nd*	*nd*	*nd*	*nd*
SY-	0.11	5.1	21	13
KK-	0.21	*nd*	40	*nd*
K-	*nd*	*nd*	*nd*	*nd*
KA-	*nd*	*nd*	*nd*	*nd*
GP-	*nd*	*nd*	*nd*	*nd*

[a] 0.5 mM substrates were incubated with dDAP and cathepsin C at pH 3.5 as described under Experimental Procedures. The relative activities were normalized with respect to DR- and GR- for dDAP and cathepsin C, respectively. "*nd*", no detectable fluorescence increase over a period of 3 min.

Table 3: K_M and k_{cat} Values for dDAP and Cathepsin C against a Set of Substrates at pH 3.5[a]

enzyme	substrate	K_M (M × 10^{-3})	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1}, M^{-1})
dDAP	GF-pNA	1.8	5.3	2944
	GR-pNA	0.18	2.1	11666
	DR-βNA	0.7	6.4	9143
	MDFPAMSL	0.55	78	141818
cathepsin C	GF-pNA	3.9	1.8	462
	DR-βNA	0.21	43	204761
	GR-βNA	0.6	134	222333
	GF-βNA	0.14	16.6	118571

[a] Enzymes and substrates were incubated at pH 3.5 at room temperature as described under Experimental Procedures. HPLC (octapeptide), absorbance (pNA), and fluorescence (βNA) were used to quantify product. Reactions were carried out at room temperature. K_M and k_{cat} were calculated from Eadie plots of the data. A molecular weight of 225 000 was assumed for dDAP and 210 000 was assumed for cathepsin C.

DAP-IV substrate) or KA-βNA (a traditional DAP-II substrate) under these conditions. It was somewhat surprising that dDAP did not significantly cleave the GF-βNA or GR-βNA substrates since dDAP did cleave the equivalent GF-pNA and GR-pNA substrates (see Table 3). dDAP did not cleave GP-pNA and AA-pNA. However, dDAP readily cleaved the substrates RR-βNA and KK-βNA (dipeptide release for RR-βNA was verified by HPLC). This was a surprise and interesting since RR-βNA is an accepted DAP-III substrate and is not cleaved by cathepsin C (McDonald & Schwabe, 1977; McDonald et al., 1969; and Table 2). dDAP did not cleave the N-blocked RR-βNA derivative, benzyloxycarbonyl-RR-βNA, demonstrating that the cleavage of RR-βNA was via a dipeptidylaminopeptidase activity. Table 2 also indicates that for those β-naphthylamide substrates tested and readily cleaved by both enzymes under the specified conditions, the specific activity of dDAP was substantially lower (10−100-fold) than the specific activity of cathepsin C for those same -βNA substrates. The reason

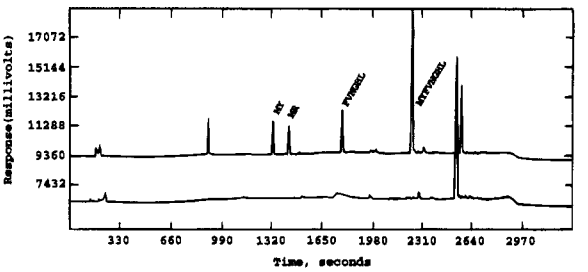

FIGURE 5: Cleavage of MRMYFVNQHL by dDAP. dDAP was incubated with MRMYFVNQHL at pH 3.5 as described under Experimental Procedures. Lower trace, MRMYFVNQHL; upper trace, MRMYFVNQHL incubated with 0.1 milliunit/mL dDAP for 8 h. Peaks are identified based on comparisons with references. The peak at about 900 s is an artifact of the HPLC system.

for this difference is not known but might be related to the βNA portion of the substrate and how it interacts with the dDAP active site at pH 3.5 (see also Table 3). Starkey and Barrett (1976) showed that elastase was 15-fold more reactive toward a nitrophenyl ester substrate relative to the equivalent naphthyl ester. To gain some additional information as to what might be the cause for lack of cleavage of GF-βNA and GR-βNA (since dDAP cleaves GR-pNA and GF-pNA), an experiment was carried out where dDAP was incubated with various levels of DR-βNA containing a fixed level of 0.5 mM GF-βNA (data not shown). The cleavage of DR-βNA by dDAP was inhibited. At 2 mM DR-βNA, the initial rate of the reaction in the presence of 0.5 mM GF-βNA was only 66% of the rate in the absence of GF-βNA. An Eadie plot of the data indicates a slight increase in K_M from 0.9 to 1.4 mM and a slight decrease in V_{max} from 1.8 to 1.4 μmol min^{-1} mg^{-1} for DR-βNA. This indicates that GF-βNA might be acting as a competitive inhibitor or via some mixed mode of inhibition. The K_M and k_{cat} for dDAP were estimated for a few of the substrates used throughout this report (Table 3). Table 3 indicates that for DR-βNA the difference between the reactivity of dDAP and cathepsin C is partly related to both K_M and k_{cat} (k_{cat}/K_M for cathepsin C is about 22 times higher than for dDAP).

dDAP cleaved the peptides MDFPAMSL, RRFPAMSL, and KKFPAMSL at similar rates, resulting in the release of the MD, RR, or KK dipeptides and the FPAMSL hexapeptide (data not shown). dDAP had similar specific activities toward MDFPAMSL and KKFPAMSL (about 13−26 μmol min^{-1} mg^{-1}), suggesting that the same activity is responsible for the cleavage of both substrates (see also Table 3 for estimate of K_M and k_{cat} for MDFPAMSL). Cathepsin C did not cleave KKFPAMSL and had a specific activity of 0.8 μmol min^{-1} mg^{-1} for MDFPAMSL. dDAP had a specific activity of 0.24 μmol min^{-1} mg^{-1} and 2.1 μmol min^{-1} mg^{-1} for the protein substrates MD-human growth hormone and MR-human proinsulin, respectively. Incubation of the peptide MRMYFVNQHL with dDAP resulted in the release of MR dipeptide, MY dipeptide, MYFVNQHL, and FVNQHL as determined by retention times compared to authentic peptides in reversed-phase HPLC (Figure 5).

DISCUSSION

We have provided extensive evidence for a previously unreported and novel dipeptidylaminopeptidase isolated from the extracellular broth of the slime mold *D. discoideum* Ax3. We developed a purification regimen and performed pre-

liminary physical and enzymatic characterization of the isolated preparation. We believe that the dDAP preparation contains a single activity based on the variety of chromatographies used for purification, based on visual estimates of purity by SDS−polyacrylamide gel electrophoresis (Figure 3A), and based on the absence of other observed proteolytic activities when dDAP preparations have been incubated with human growth hormone and human proinsulin. The dDAP activity was proven to be a dipeptidylaminopeptidase by its ability to hydrolyze several classes of peptide substrates having an unblocked amino terminus (Tables 2, 3; Figures 4, 5) with the release of dipeptides and by its inability to hydrolyze peptide substrates having a blocked amino terminus (Table 2). We conclude that dDAP is different from DAP-I and previously reported *Dictyostelium* DAPs. The reported DAP-I from *D. discoideum* NC-4 cell extracts has a pH optimum above 7 (Chan et al., 1985), where dDAP has no activity (Figure 4). In addition, the apparent DAP-I activity observed by Chan and co-workers (Chan et al., 1985) was minimal during growth and increased during aggregation and culmination of the *D. discoideum*. dDAP, however, appeared to be growth-associated (Figure 1). DAP-III of *D. discoideum* Ax3 has a pH optimum of 9.5 (Chan et al., 1987; Huang et al., 1992) and is present in cell extracts of Ax3 during both the vegetative and developmental phases.

The role of dDAP and other DAP enzymes in the biology of *D. discoideum* is not clear. The two general possibilities for DAP enzymes are, first, they may have a role in processing a precursor peptide or protein as has been reported for DAP-IV processing of cecropins (Boman et al., 1989), or, second, they may simply be digestive, degrading proteins to provide nutrients for *D. discoideum* growth (McDonald & Schwabe, 1977). It has been speculated that DAP enzymes might have a role in *D. discoideum* development (Chan et al., 1987). We found dDAP predominantly in the extracellular matrix, as might be expected for a molecule mediating cell−cell communication. However, the research in this report was done with an axenic mutant, Ax3. The mutation allowing axenic growth has been shown to result in abnormal membrane processing, including gratuitous pinocytosis [reviewed in Clarke and Kayman (1987)]. This raises the possibility that secretion is an abnormality due to the mutation, and further studies comparing the wild-type and Ax3 strains might be useful to better understand this question. *D. discoideum* will not grow in the pH range where dDAP is active; hence, we favor the hypothesis that dDAP is a lysosomal enzyme with a role in digestion and its secretion into the extracellular broth is incidental. The secretion of dDAP in parallel with growth appears consistent with that reported for other *d. discoideum* Ax3 lysosomal enzymes (Dimond et al., 1981). We have also found that dDAP binds to concanavalin A, suggesting that dDAP is glycosylated as is common for *Dictyostelium* lysosomal proteins (Freeze, 1986). The broad substrate specificity of dDAP (Table 2) coupled with its ability to cleave RR- or KK-substrates enables dDAP to potentially fulfill the roles of two classes of DAP enzymes (DAP-I and -III) at a single pH (as opposed to different pH optima for the individual DAP-I and DAP-III enzymes).

Although it shares some substrate preferences with DAP-I and DAP-III, dDAP is different in physical and enzymatic properties from previously characterized mammalian and microbial DAP enzymes. Traditional DAP enzymes have been placed in one of four classes based primarily on their ability to catalyze the release of specific dipeptide pairs if they are present at the amino terminus of a peptide. First detection of dDAP was by hydrolysis of GF-pNA, a substrate for mammalian DAP-I, and, therefore, we initially viewed dDAP as similar to DAP-I. This view was supported by the additional observation that dDAP shares with DAP-I enzymes the ability to cleave a variety of amino-terminal dipeptides but did not cleave when proline was in the penultimate position. However, we subsequently discovered that dDAP cleaved RR- and KK-dipeptidyl and octapeptide substrates (Table 2), an activity of DAP-III (McDonald & Schwabe, 1977), with comparable specific activities (Table 2 and text). Conversely, bovine spleen DAP-I (cathepsin C) readily cleaved AA-, LA-, and HS-dipeptidyl β-naphthylamide substrates while dDAP did not (Table 2), nor the substrate AA-pNA. From Table 3, it might also be speculated that the dDAP/substrate binding site, unlike cathepsin C, is most catalytically efficient when the substrate is larger than a dipeptidyl substrate. There is a greater than 10-fold increase in k_{cat}/K_M going from the -pNA and -βNA derivatives to the octapeptide substrate. Additional distinctions between dDAP and DAP-I were revealed. The dDAP subunit mass is about 66 000 daltons compared to 24 000 daltons for cathepsin C (Metrione et al., 1970; and Figure 3A). dDAP has a pH optimum near 3.5 whereas the optimum for DAP-I is broader and slightly higher (McDonald et al., 1969; and Figure 4), and DAP-III enzymes have optimal activity at pH 8−10 (Huang et al., 1992). dDAP was able to catalyze release of MR dipeptide from MR-human proinsulin when the methionine had been oxidized to methionyl sulfoxide while DAP-I did not (data not shown). dDAP does not appear to have an active site cysteine while DAP-I does (McDonald & Schwabe, 1977). DAP-I (e.g., cathepsin C) and DAP-III enzymes are inhibited by sulfhydryl modifying reagents (Chan et al., 1987; Huang et al., 1992; Fruton & Mycek, 1956; Mycek, 1970; Hameed & Haard, 1985; Watanabe et al., 1990) which has led to the conclusion that these proteases have critical cysteines in their active sites. By contrast, dDAP was purified and used without added reducing agents, and its activity was not significantly affected by several sulfhydryl modifying reagents or the cysteine protease inhibitor E-64. This leads us to conclude that dDAP does not have a cysteine residue at its active site that is required for activity. Likewise, the lack of inhibition by PMSF would indicate that dDAP is not a serine class protease. We do not currently know what amino acid residues are critical for activity or how leupeptin or the trivalent cations mediate inhibition.

We conclude that dDAP is a DAP and that it is at least as different from each of the historical four classes of DAP enzymes as they are from one another. This conclusion is partly based on the same type of substrate preference and inhibition criteria as have been historically used to define DAP-I, -II, -III, and -IV, and based on evidence supporting significant differences in size of the monomeric species. On the basis of the available evidence for the uniqueness of dDAP, we propose that dDAP represents a new and distinct class of dipeptidylaminopeptidase, and recommend that members to this class be referred to as DAP-V enzymes. The broad specificity for substrates, the reactivity against several DAP-I and DAP-III substrates, and the low pH optimum may make this enzyme and those in its class useful

in processing appropriate precursors of pharmaceutical proteins, such as human growth hormone and human proinsulin, produced by recombinant DNA technology. In addition, the dDAP enzyme is readily isolated from a microbial organism as opposed to animal-derived tissues and in this particular case from the cell-free broth itself.

ACKNOWLEDGMENT

We thank Mr. Allen Pekar for the centrifugation work, Ms. Susan Bushrod and Mr. Ron Foster for technical support, and Drs. Gary Smith and John Ivancic for valuable discussions. We also thank Mr. Paul Rosteck and Drs. Ray Kaiser and Charles Hershberger for sharing with us the results of their dDAP protein and DNA sequencing work prior to publication.

REFERENCES

Boman, H. G., Boman, I. A., Andreu, D., Li, Z., Merrifield, R. B., Schlenstedt, G., & Zimmermann, R. (1989) *J. Biol. Chem. 264*, 5852−5860.

Chan, S. A. T., Toursarkissian, K., Sweeney, J. P., & Jones, T. H. D. (1985) *Biochem. Biophys. Res. Commun. 127*, 962−968.

Chan, S. A. T., Jones, T. H. D., Sweeney, J. P., & Toursarkissian, K. (1987) *Exp. Mycol. 11*, 27−35.

Clarke, M., & Kayman, S. C. (1987) *Methods Cell Biol. 28*, 157−176.

Datta, S., & Firtel, R. A. (1987) *Mol. Cell. Biol. 7*, 149−159.

Dimond, R. L., Burns, R. A., & Jordan, K. B. (1981) *J. Biol. Chem. 256*, 6565−6572.

Fong, D., & Rutherford, C. L. (1978) *J. Bacteriol. 134*, 521−527.

Freeze, H. H. (1986) *Mol. Cell. Biochem. 72*, 47−65.

Fruton, J. S., & Mycek, M. (1956) *Arch. Biochem. Biophys. 65*, 11−20.

Hameed, K. S., & Haard, N. F. (1985) *Comp. Biochem. Physiol. 82B*, 241−246.

Huang, J., Kim, J., Ramamurthy, P., & Jones, T. (1992) *Exp. Mycol. 16*, 102−109.

McDonald, J. K., & Schwabe, C. (1977) in *Proteinases in Mammalian Cells and Tissue* (Barrett, A. J., Ed.) Chapter 8, Elsevier/North-Holland Biomedical Press, Amsterdam, The Netherlands.

McDonald, J. K., Zeitman, B. B., Reilly, T. J., & Ellis, J. (1969) *J. Biol. Chem. 244*, 2693−2709.

Metrione, R. M., Neves, A. G., & Fruton, J. S. (1966) *Biochemistry 5*, 1597−1604.

Metrione, R. M., Okuda, Y., & Fairclough, G. F., Jr. (1970) *Biochemistry 9*, 2427−2432.

Mycek, M. J. (1970) *Methods Enzymol. 19*, 285−309.

North, M. J., Cotter, D. A., & Franek, K. J. (1990) *J. Gen. Microbiol. 136*, 835−840.

Presse, F., Bogdanovski-Sequeval, D., Mathieu, M., & Felenbok, B. (1986) *Mol. Gen. Genet. 203*, 324−332.

Starkey, P. M., & Barrett, A. J. (1976) *Biochem. J. 155*, 265−271.

Watanabe, Y., Kumagai, Y., & Fujimoto, Y. (1990) *Chem. Pharm. Bull. 38*, 246−248.

BI950197L