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Proteases in cellular slime mold development: Evidence for their involvement

(cell differentiation/protein degradation/*Dictyostelium discoideum*/amino acid rescue)

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ABSTRACT **Protein degradation appears to be essential for normal differentiation in the cellular slime mold *Dictyostelium discoideum*. Several protease inhibitors block normal differentiation, and in most cases this inhibition can be reversed by addition of amino acids. For example, chloroquine, which inhibits slime mold cathepsin B activity, interfered with development by blocking sorocarp formation, and this inhibition was reversed by the addition of amino acids. Tosyllysyl chloromethyl ketone also blocked development, and this inhibition was reversed by simultaneous additions of amino acids and glutathione. Moreover, the addition of antipain and leupeptin delayed sorocarp formation. These results, together with the finding reported earlier that cathepsin B activity is differentially localized in the prestalk–prespore zones of the migrating slugs, suggest that proteolysis might play a regulatory role in cellular slime mold differentiation.**

In the life cycle of cellular slime molds the growth phase is separate from the differentiation phase. Aggregation begins only after starvation, probably because of amino acid deprivation, as Marin (1) has shown. It has been known since the work of Gregg *et al.* (2) that during the period of differentiation, when no exogenous sources of energy are taken in, proteins are degraded; the products are presumed to be used as an endogenous source of energy (see also refs. 3–6.) Furthermore, a number of workers have provided direct evidence for proteolysis during development (7–12).

Here we provide further evidence that protease activity might be involved in, and even required for, differentiation. In an earlier study (11) it was shown that the proteolytic activity of cathepsin B was higher in the prestalk than in the prespore zone, which opens up the possibility that it might be playing a role in the control of stalk cell–spore differentiation. Here we support such a premise by showing that not only is it possible to inhibit normal development with various protease inhibitors, but the inhibition can, in some instances, be reversed by adding amino acids. From this comes the hypothesis that proteolysis is not just a means of making energy and amino acids available to the starving cell mass, it is an essential component in the normal development of cellular slime molds.

MATERIALS AND METHODS

Strains. Two strains of *Dictyostelium discoideum* were used: strain NC4 of K. B. Raper and a mutant of this strain, A2, that grows axenically (13).

Conditions for Growth. The amoebae of NC4 were grown with *Escherichia coli* B/r on buffered nutrient agar (14). The amoebae of A2 were grown axenically in HL-5 medium in liquid culture (13). Amoebae were harvested during the exponential phase of growth and washed in 100% Bonner's salt solution before use. Cell numbers were estimated by dilution and counting in a hemocytometer.

Conditions for Differentiation. A drum technique was used to study cell differentiation (15, 16). A standard drum consists of two close-fitting plastic cylinders, 4.5 cm in diameter, placed in a 40 × 80 cm crystallizing dish. Between these two cylinders is a dialysis membrane (catalog no. 3787-D52, Arthur H. Thomas), pretreated by boiling for 5 min in 1 mM ethylenediaminetetraacetic acid (EDTA) and then washed in distilled water. Amoebae were placed on the upper side of the dialysis membrane and the dish was filled with buffer solution just up to the lower surface of the membrane. Under these conditions 3×10^7 cells formed a monolayer on the membrane and sorocarps appeared at about 24 hr.

Because of the rich nutrients available in the amino acid "rescue" experiments, all materials and glassware, including the 2% Bonner salt solution for harvesting amoebae, were either autoclaved or filter-sterilized (Millipore SX HA 025 OS sterile Swinnex 0.45 μm, 25 mm) before use. A large drum of 4.5-cm inner diameter contained 4.2×10^7 amoebae, with 35 ml of solution in the glass dish. A smaller drum of 1.2-cm inner diameter contained 3×10^6 amoebae, with 3 ml of solution in the glass dish. (Small drums were used when inhibitors were available only in small quantities.) The dishes were covered with glass covers and autoclaved.

Inhibitors and "Rescuers." The buffer to which compounds were added was 16.7 mM Sorensen phosphate, pH 6.0 (KH_2PO_4, 7.94 g; Na_2HPO_4, 1.12 g; H_2O, 1000 ml; then 1:4 dilution with water) containing 2% Bonner's salt solution. Inhibitors were chloroquine (diphosphate salt, Sigma), quinine (hydrochloride, Sigma), tosyllysyl chloromethyl ketone (Tos-LysCH_2Cl; hydrochloride, Sigma), pepstatin, leupeptin, and antipain (the last three from H. Umezawa). Rescue experiments were attempted with the following: L-leucine (Nutritional Biochemicals), glucose (Difco dextrose), glutathione (reduced form, Sigma), and casamino acids (Difco vitamin-free casamino acids; Difco vitamin assay casamino acids). The normal concentration used was 1% (wt/vol) for casamino acids; concentrations for other compounds are noted in *Results*.

Assays for Protein Content and Cathepsin Activities. Cell samples of different developmental stages were harvested from the drums in 2 ml of water, frozen in an ethanol/dry ice bath immediately, and stored at −20°C until use. Thawed samples were sonicated and aliquots were taken to measure the protein content (17) and cathepsin activities. For cathepsin D assay, the reaction mixture contained 0.25 ml of casein (4% wt/vol in 0.05 M citrate buffer, pH 2.5), 0.5 ml of cell extract, and 0.5 ml of 0.1 M citrate buffer, pH 2.5. After 50-min incubation at 37°C, the reaction was terminated by 1 ml of 10% trichloroacetic acid, and the absorbance of acid-soluble products was read at 280 nm. For cathepsin B assay, the reaction mixture contained 0.7 ml of 0.1 M sodium acetate buffer at pH 5.5, 0.1 ml of activators (dithiothreitol and EDTA, 4 mM each), α-*N*-benzoyl-DL-arginine *p*-nitroanilide (Sigma, 10 mg/ml in dimethyl sulfoxide),

Abbreviation: Tos-LysCH_2Cl, tosyllysyl chloromethyl ketone.

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and 0.5 ml of cell extract. After 5-hr incubation at 37°C, samples were boiled and centrifuged, and absorbance at 410 nm was measured.

Assay for Intracellular Amino Acid Pool. NC4 amoebae were put on drums (3×10^7 cells per drum) with and without 2.5 mM chloroquine. At 14 hr of development, 6 drums each from drug treatment and control were harvested into 4 ml of cold water and washed once (7000 rpm, 10 min, Sorvall SS-34 rotor). To each sample 3 ml of cold 5% trichloroacetic acid was added at 4°C for 8 hr. Centrifugation (18,000 rpm, 10 min) gave an acid-soluble fraction. Ether was used to extract the acid. Aqueous fractions were dried on a hot plate (slide warmer, Precision Scientific, Chicago, IL) and assayed for amino acids with an amino acid analyzer (Beckman model 120B) (18).

Assays for Actinomycete Protease Inhibitors. Partially purified proteases from A2 amoebae were used to study inhibitor effects. A hemoglobin-agarose affinity column, made according to Smith and Turk (19), was chosen for cathepsin D; an α-*N*-benzoyl-L-argininamide-agarose column, after Ogino and Nakashima (20), was used for cathepsin B. Details for partial purification are presented in Ref. 21. For the partially purified enzyme fractions from affinity columns, hydrolysis of Azocoll (Calbiochem) at pH 5.5 with dithiothreitol/EDTA in the presence and absence of pepstatin, leupeptin, and antipain was used to test for cathepsin B; hydrolysis of hemoglobin (Worthington) at pH 2.55 was used to test for cathepsin D. Pepstatin was first dissolved in methanol, then transferred to dilute NaOH, and then titrated back to pH 7. Leupeptin and antipain were water soluble.

RESULTS

Effects of Chloroquine on Development. Membrane drums were used to test the effects of protease inhibitors on *D. discoideum* development. The amoebae were deposited on the surface of a dialysis membrane lying over a buffered salt solution. Various compounds were tested for their effects on cell differentiation by placing them in the buffer solution.

Chloroquine was shown previously to inhibit cathepsin B activity *in vitro* in *D. discoideum* (11). In the presence of 2.5 mM chloroquine, amoebae aggregated but further morphogenetic processes were inhibited. At the end of development the control cells differentiated into mature sorocarps (Fig. 1*A*) whereas the drug-treated cells merely formed aggregation clumps (Fig. 1*B*). Chloroquine inhibition of development was stage-specific; its effects were seen only when the cells were exposed to chloroquine before the migration stage. [The fact that there is a slime sheath in the slug acting as a possible permeability barrier should be noted (22).] Moreover, chloroquine inhibition was reversible; after the drug had been removed, sorocarp development continued, following an initial time delay.

Protein content and protease activities were measured at different time periods for cells on the drums with or without chloroquine. Drug-treated cells consistently contained more protein than the corresponding control cells: chloroquine reduces the rate of protein degradation (Fig. 2*A*). When these cells were tested for cathepsin activities, chloroquine-treated cells had higher cathepsin D (Fig. 2*B*) and cathepsin B (Fig. 2*C*) activities than control cells. These measurements were made by washing the cells, sonicating them, and taking samples for protease assays. Superficially it seems contradictory that chloroquine-treated cells would have higher cathepsin B activity than control cells, because chloroquine inhibits cathepsin B activity *in vitro* (11). However, chloroquine is known to raise the pH of the lysosomes *in vivo* (23), and this may influence the activities of cathepsins and other lysosomal enzymes. Thus one possible explanation would be that the cathepsins themselves, being proteins with catalytic activities, had their own degradation delayed.

If chloroquine really inhibits protein degradation in *D. discoideum* and protein synthesis is always present during development, one would expect exhaustion of the intracellular free amino acid pools in chloroquine-treated cells. As seen in Table 1, when the control reached the slug stage, free amino acid pools were lower in treated samples than in the controls. Chloroquine-treated cells retained only an average of 29% of the free amino acid pools of the control cells.

In order to show that the inhibition of proteolysis is indeed causing the blocking of morphogensis and differentiation, amino acids were added along with chloroquine to see if they would counter the effect of the drug. Cells exposed to 0.75% (wt/vol) casamino acids with chloroquine (2.5 mM) produced normal sorocarps (Fig. 3*B*), as did the controls with casamino acids alone. From this experiment it is apparent that the casamino acids are able to reverse or rescue the amoebae from the inhibitory effects on development of chloroquine.

Apparently it is not just a matter of supplying energy, because the addition of glucose (20 mM) did not reverse the effect of chloroquine (Fig. 3*C*). Similarly, addition of a single amino acid, leucine (10 mM), did not reverse the effect of chloroquine (Fig. 3*D*). Only a mixture of amino acids, which would mimic the products of protein degradation, could restore the inhibition of development of chloroquine.

Effects of Other Protease Inhibitors on Development. In an earlier publication we demonstrated that TosLysCH_2Cl can inhibit cathepsin B activity in crude cell extracts of *D. discoideum* (11). Consequently it would be of interest to check the effects of this inhibitor on development. TosLysCH_2Cl at 3 mM completely blocked development (Fig 4*A*). In contrast to chloroquine-treated cells, TosLysCH_2Cl-treated cells did not even form clumps. When these treated cells were supplied with casamino acids (1%, wt/vol), development continued to cell clumps (Fig. 4*B*). When cells treated with TosLysCH_2Cl and

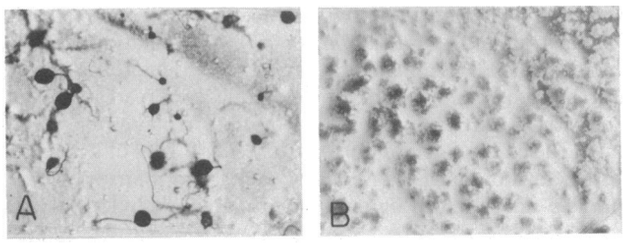

FIG. 1. Inhibition of *D. discoideum* A2 development by chloroquine. (*A*) Buffer control; (*B*) 2.5 mM chloroquine. Photographs were taken after 25 hr of development. (×9.)

Table 1. Free amino acid pools in *D. discoideum*

Amino acid	Pool, nmol/2 × 10^8 cells: Control, 14-hr slugs	Chloroquine, 2.5 mM, 14-hr clumps
Aspartic acid	5.0	2.5
Glutamic acid	26.2	11.2
Glycine	20.1	5.8
Alanine	21.9	4.0
Valine	3.3	Trace
Isoleucine	4.4	0.9
Leucine	3.1	0.6
Phenylalanine	1.5	Trace

Trichloroacetic acid-soluble fractions from drug-treated and control samples were assayed for free amino acid pools.

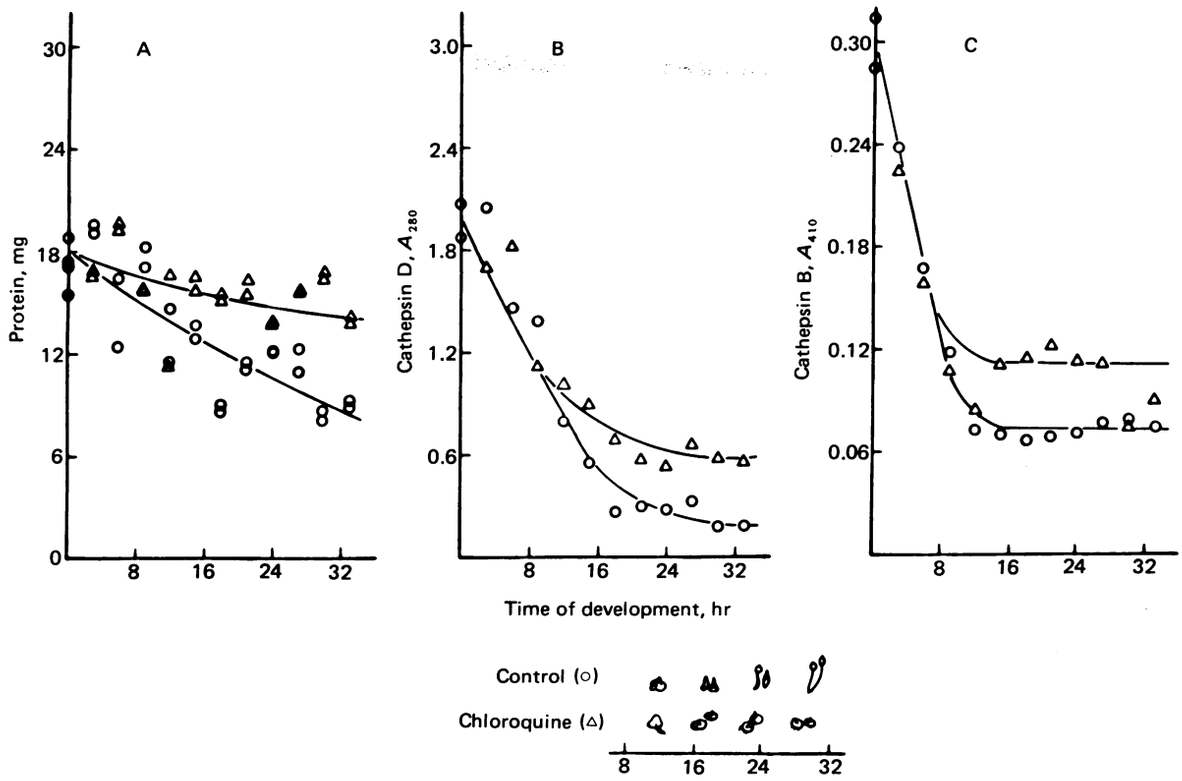

FIG. 2. Changes in protein and cathepsin activities in *D. discoideum* NC4 in the presence of 1 mM chloroquine (△) or its absence (○). All assays are given per 3×10^7 cells. (*A*) Protein content, assayed by the Lowry method (17); (*B*) cathepsin D activity, assayed by casein hydrolysis as A_{280}; (*C*) cathepsin B activity, assayed by benzoylarginine *p*-nitroanilide hydrolysis as A_{410}. The *x* axis is time of cell differentiation; the corresponding morphologies have also been sketched.

casamino acids were, in addition, exposed to glutathione (2 mM), they differentiated into normal fruiting bodies (Fig. 4*D*). The controls showed that glutathione itself had no effect on myxamoebae differentiation (data not shown), nor did it alter the inhibitory effect of TosLysCH$_2$Cl (Fig. 4*C*). It is possible, as suggested by Penn *et al.* (24), that TosLysCH$_2$Cl is acting intracellularly by alkylating compounds with free —SH groups such as reduced glutathione.

Quinine is a compound related to chloroquine, and its effect on *Dictyostelium* development is similar. Cells formed clumps and tiny slugs in the presence of 2.5 mM quinine, and this developmental inhibition could be reversed by the addition of 1% (wt/vol) casamino acids (data not shown).

Effects of Microbial Protease Inhibitors on Partially Purified *Dictyostelium* Proteases *In Vitro*. Umezawa and his associates have isolated protease inhibitors from culture filtrates of actinomycetes and have used these compounds to characterize the proteolytic enzymes (25). We had previously found two cathepsin activities in *D. discoideum* crude cell extracts

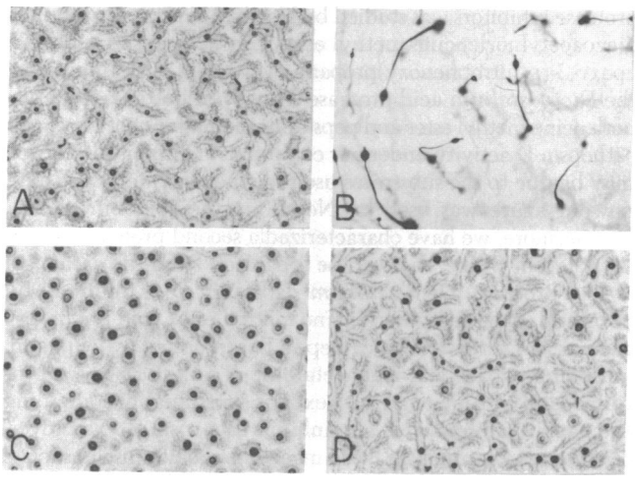

FIG. 3. "Rescue" attempts with chloroquine inhibition of *D. discoideum* NC4 differentiation. (*A*) Chloroquine at 2.5 mM; (*B*) chloroquine and 0.75% casamino acids; (*C*) chloroquine and 20 mM glucose; (*D*) chloroquine and 10 mM leucine. (×8.)

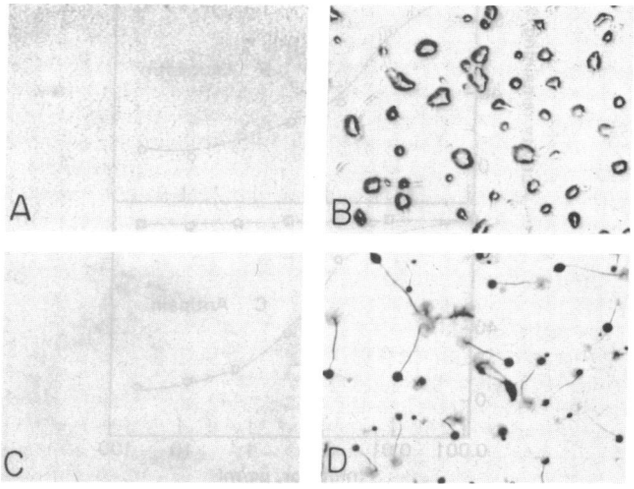

FIG. 4. Inhibition of *D. discoideum* NC4 by TosLysCH$_2$Cl. (*A*) TosLysCH$_2$Cl at 3 mM; (*B*) TosLysCH$_2$Cl and 1% casamino acids; (*C*) TosLysCH$_2$Cl and 2 mM glutathione; (*D*) TosLysCH$_2$Cl and casamino acids and glutathione. All three compounds were mixed in the buffer before the addition of amoebae. All were 28-hr samples. (×8.)

(11), and we extended our studies by using microbial protease inhibitors together with partially purified myxamoebae enzyme fractions. As shown in Fig. 5A, the activity of the cathepsin D-like enzyme eluted from a hemoglobin-Sepharose column was inhibited up to about 60% by pepstatin. Moreover, the activity of the residual enzyme was unaffected by pepstatin even though the concentration of inhibitor was increased by three orders of magnitude. This suggests that a pepstatin-insensitive acid protease may be present together with the cathepsin D-like protease in our partially purified enzyme fraction. Furthermore, both leupeptin and antipain had no effect on this cathepsin D-like activity (Fig. 5 *B* and *C*). On the other hand, both leupeptin and antipain decreased by 90% the cathepsin B-like enzyme activity eluted from a benzoylarginineamide-Sepharose column, whereas pepstatin had no effect on activity of this enzyme (Fig. 5). Thus we have evidence that both cathepsin D and cathepsin B are found in *D. discoideum* and that these enzymes can be separated from one another by affinity chromatography.

Effects of Microbial Protease Inhibitors on Development. We then tested leupeptin and antipain for their effects on cell differentiation. Pepstatin was not attempted because of its poor solubility in aqueous buffer and we needed to use concentrations that were orders of magnitude greater than required for the inhibition of enzyme activities *in vitro*. At 2.5 mM, both leupeptin and antipain delayed sorocarp development. When sorocarps appeared in the control, leupeptin-treated cells showed slugs and early culminating cell masses (data not shown). Antipain-treated cells differentiated even more slowly and were at late aggregation and early slug stage (Fig. 6*B*) when the controls became sorocarps (Fig. 6*A*). However, after the delays, sorocarps were formed in these leupeptin- and antipain-treated samples. These effects of actinomycete protease inhibitors on *Dictyostelium* development also suggested that protease activity is required for differentiation, at least in the sense of finishing the program within a normal period of time.

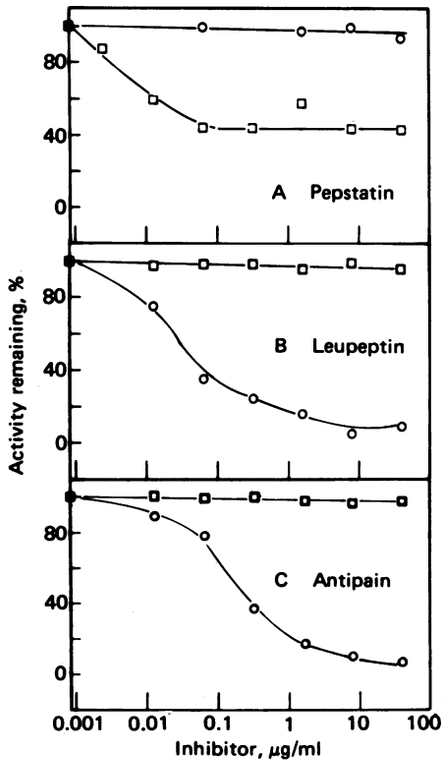

FIG. 5. Inhibition of *D. discoideum* protease activities by actinomycete protease inhibitors. (*A*) Pepstatin; (*B*) leupeptin; (*C*) antipain. The cathepsin D (□) assay was hemoglobin hydrolysis at pH 2.55; the cathepsin B (○) assay was Azocoll hydrolysis at pH 5.5 in the presence of dithiothreitol and EDTA. At zero inhibitor concentration, cathepsin is considered to possess 100% enzymatic activity.

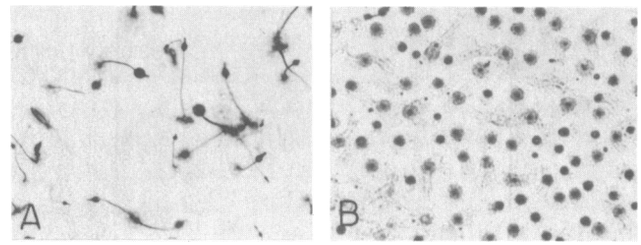

FIG. 6. Delay of *D. discoideum* NC4 cell differentiation by antipain. (*A*) Buffer control and (*B*) 2.5 mM antipain, both at 24 hr of development. (×8.)

DISCUSSION

We have shown that protease inhibitors can delay or inhibit cell differentiation in *D. discoideum*. In a number of cases the inhibitions can be reversed by the simultaneous addition of amino acid mixtures. Because a large number of new enzymes and other proteins are synthesized during development (26) in spite of the net loss in total protein content (4), the most direct effect of protease inhibitors may be in reducing the amino acids required for protein synthesis *de novo*. Our results are consistent with the view that proteolysis is a necessary step in the differentiation of the cellular slime mold and that the responsible proteases are cathepsin-like. Before returning to this main point, let us briefly review the previous work on proteolysis in the cellular slime molds and show how it relates to the work presented here.

Evidence for Protease Activity. Sussman and Sussman (7) found acid protease activity in *D. discoideum*, and Wiener and Ashworth (8) reported its possible localization in the lysosomes. Gustafson and Thon (12) suggested the presence of phosphoryl moieties in a *Dictyostelium* protease that they characterized and named proteinase I. Evidence was presented by Rossomando *et al.* (10) for the secretion of acid protease activity into the medium during the aggregation stage. The appearance of one new protease band at the culmination stage was detected electrophoretically by North and Harwood (27).

Protease Inhibitors. The inhibition of acid protease by protease inhibitors was studied by North (28), who showed that diazoacetylnorleucine methyl ester, but not pepstatin or 1,2-epoxy-3-(*p*-nitrophenoxy)propane, was effective in inhibiting the *Dictyostelium* acid protease. However, both diazoacetylnorleucine methyl ester and pepstatin can inhibit *Dictyostelium* cathepsin D activity under our conditions (11). This difference may be due to the substrates used for the enzyme assay (hide powder azure was used by North and hemoglobin by us). Furthermore, we have characterized a second protease, which is cathepsin B-like, that can be separated from the cathepsin D-like protease. *Dictyostelium* cathepsin D is inhibited by pepstatin, diazoacetylnorleucine methyl ester, and phenylpyruvic acid; *Dictyostelium* cathepsin B is inhibited by leupeptin, antipain, iodoacetate, iodoacetamide, TosLysCH$_2$Cl, chloroquine, and a crude egg white extract (presumably containing the papain inhibitor that also inhibits cathepsin B). Moreover, *Dictyostelium* cathepsin B can inactivate rabbit muscle aldolase at pH 6 whereas cathepsin D cannot (unpublished data), and this aldolase inactivation has been reported as a characteristic for cathepsin B (29).

Rescue Experiments. The fact that we were able to reverse

the effects of certain protease inhibitors by adding a mixture of amino acids seems, at first glance, to conflict with the experiments of Marin (1), who showed that amino acid starvation was the specific stimulus in initiating development in *D. discoideum*. Yet our experiments showed normal development in the presence of vitamin-free casamino acids (1%, wt/vol) in buffer. This discrepancy can probably be accounted for by differences in concentration. With 2% (wt/vol) casamino acids the amoebae did not differentiate (data not shown), thus giving results similar to those of Marin. (He used a concentration of 0.5 mg/ml for each of his amino acids, but it should be noted that his conditions for differentiation were very different from ours because he used submerged cultures.)

Role of Proteolytic Activity in Development. It had always been assumed from the early work of Gregg *et al.* (2) and others that the sole purpose of the degradation of protein was to supply energy for the developing, starved cells. This was thought to be the natural result of separating the growth phase from the developmental phase of the life cycle. Hames and Ashworth (5) questioned this assumption and reported the same rate of proteolysis regardless of whether or not the amoebae were starved of glucose. In the light of more recent work, including the experiments presented here, we can now interpret Hames and Ashworth's pioneering finding by assuming that the proteolysis may be entirely or partially involved in specific developmental or differentiation processes.

In some cases previously described it has been suggested that the proteolysis itself is important to achieve a specific developmental step. For instance, O'Day (9) proposed that the acid proteases in a related species, *Polysphondylium pallidum*, played a specific role in the removal of microcyst wall during its germination. In *D. discoideum*, Rossomando *et al.* (10) postulated that extracellular proteolytic activity may release cell membrane components to facilitate amoebae migration and aggregation. The curbing of proteases has been suggested by Wright and Thomas (30) to be the cause of developmental accumulation of enzymes at the stage of culmination. Evidence supporting this view has come from DeToma *et al.* (31), who found that the *in vitro* stability of the enzyme UDP-glucose pyrophosphorylase was increased by the protease inhibitor TosLysCH$_2$Cl.

Another way in which proteolysis might play a significant role is in the control of development. The fact that there is a differential distribution of a particular protease in prespore and prestalk cells (11), and the fact reported here that protease inhibitors that retard or block development can be reversed with mixtures of amino acids, suggest that in some interesting way proteases may be playing a key role in differentiation. (This hypothesis is crudely analogous to the situation in vertebrates, in which cell death plays a central role in certain stages of development, such as in digit formation.) It is not clear how proteolysis might control differentiation, although certainly one possibility is that it has to do with the supply (perhaps a differential supply) of amino acids. Even the end product of protein and amino acid degradation, ammonia, may play a regulatory role in development, as Sussman and Schindler (32) and others have suggested.

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