Developmentally regulated enzymes and cyclic AMP-binding sites in *Dictyostelium discoideum* cells blocked during development by α -chymotrypsin

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When cells of the slime mould *Dictyostelium discoideum* are allowed to starve in the presence of α -chymotrypsin, they are blocked in development at the stage where tight aggregates form tips. Analysis of developmentally regulated enzymes has shown that α -mannosidase, β -N-acetylglucosaminidase, threonine deaminase, tyrosine aminotransferase, β -glucosidase and the carbohydrate-binding protein discoidin are unaffected, but enzymes that show an increase in specific activity during post-aggregative development, namely glycogen phosphorylase, UDP-glucose pyrophosphorylase, UDP-galactose 4-epimerase, UDP-galactose polysaccharide transferase and alkaline phosphatase, did not show the characteristic increase when development was blocked by α -chymotrypsin. Recovery of cells from the effects of α -chymotrypsin was accompanied by the formation of fruiting bodies and a concomitant increase in the specific activity of UDP-glucose pyrophosphorylase. Uptake or efflux of $^{45}\text{Ca}^{2+}$ was not altered in the presence of α -chymotrypsin. Cells allowed to develop in α -chymotrypsin, or treated with the enzyme for 15 min, had a markedly reduced ability to bind cyclic AMP with low affinity; high-affinity binding was unaffected. Pronase had a similar effect on cyclic AMP binding, but trypsin, which does not alter developmental processes, has no effect on cyclic AMP binding to *D. discoideum* cells.

The cellular slime mould *Dictyostelium discoideum* is a convenient organism for the study of eukaryotic cellular differentiation (Loomis, 1975). Myxamoebae of *D. discoideum* will grow and divide in the presence of a bacterial food source, and in this vegetative state the amoebae are solitary. Starvation causes the induction of a developmental phase of the life cycle in which amoebae aggregate into a multicellular structure within which individual amoebae differentiate into either spore or stalk cells.

There is particular interest in the transition from the unicellular to multicellular states, which is accompanied by increased cohesiveness of the cells that is resistant to EDTA (Gerisch, 1968), and the appearance of several membrane proteins thought to be involved in cell–cell cohesion; these are contact sites A (Beug *et al.*, 1973), discoidin (Rosen *et al.*, 1973) and two unrelated glycoproteins of mol.wts. 150 000 (Geltosky *et al.*, 1979) and 95 000 (Steine-

mann & Parish, 1980). Although these cohesive interactions are thought to be important in development, their precise role is not yet clear. Mutants which do not express contact sites A (Beug *et al.*, 1973) or functional discoidin I (Ray *et al.*, 1979) fail to complete development and are blocked at the aggregation stage, suggesting that cell–cell interactions have a crucial function. There are major changes in the populations of mRNA molecules (Alton & Lodish, 1977a) and proteins (Alton & Lodish, 1977b) at this stage of development, and cell–cell cohesion is thought to be necessary for initiation of these changes in gene expression, although continued synthesis of the proteins is dependent on cyclic AMP (Landfear & Lodish, 1980). However, multicellularity does not seem to be required for the development of certain mutants that express post-aggregative enzymes in response to cyclic AMP when this is added to cells shaken in suspension (Town & Gross, 1978) or in amoebae of sporogenous mutants that can develop into spores without making contact with any other cells (Kay & Trevan, 1981).

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We have previously shown that the normal development of *D. discoideum* can be inhibited at two distinct points by α -chymotrypsin (Schmidt *et al.*, 1978; Kilpatrick *et al.*, 1980). Amoebae exposed to the proteinase from the onset of starvation aggregate normally and develop EDTA-resistant cohesion, but are blocked at the stage where tight aggregates form tips. If normal cell aggregates are exposed to α -chymotrypsin, fruiting bodies are formed but the spores are not properly encapsulated by the spore coat and remain immature (Kilpatrick *et al.*, 1980). Developmentally regulated enzymes are regarded as indicators of certain stages of the developmental cycle, and, by analysing enzyme levels in a series of eleven morphological mutants, Loomis *et al.* (1976) have outlined a nine-step-dependent sequence during development. In the present paper we have analysed the specific activities of developmentally regulated marker enzymes during exposure of *D. discoideum* to α -chymotrypsin in an attempt to pinpoint the biochemical timing of the developmental block that occurs after aggregation. In view of the recent reports linking post-aggregative development with cyclic AMP signalling (Town & Gross, 1978; Landfear & Lodish, 1980), we have analysed the effect of α -chymotrypsin on cell-surface cyclic AMP binding.

Experimental

Materials

4-Methylumbelliferyl glycosides and *p*-nitrophenyl phosphate were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. UDP-glucose, α -D-glucose 1-phosphate, NAD, NADP, alkaline phosphatase (phosphodiesterase-free) glucose 6-phosphate dehydrogenase, phosphoglucomutase, 6-phosphogluconate dehydrogenase, threonine, Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine}, Bicine [*NN*-bis-(2-hydroxyethyl)glycine] sodium pyrophosphate and α -chymotrypsin (type II) were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Oyster glycogen and dithiothreitol were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Pronase was from Calbiochem, Bishop's Stortford, Herts., U.K. α -D-[U- 14 C]glucose 1-phosphate (4.02 mCi/mmol), cyclic [8- 3 H]AMP (30 Ci/mmol), UDP-D-[U- 14 C]galactose (347 mCi/mmol), 45 CaCl₂ (11.49 mCi/mg of Ca), 32 P (carrier-free as phosphate, 10 mCi/ml) and Na 125 I (13–17 mCi/mmol) were all from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were purchased from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Cell culture

Dictyostelium discoideum strain NC4 was used

throughout the present study and grown in association with *Escherichia coli* B/r either on nutrient-agar plates as described by Sussman (1966) or in liquid culture as described by Gerisch (1960). Synchronous development was induced by separating vegetative amoebae from bacteria by differential centrifugation (Sussman, 1966) and spreading 0.5 ml of a suspension of cells (10⁸/ml) on the surface of a Millipore filter (AAWP 04700) supported by an absorbent pad saturated (1.5 ml) with a solution of buffered salts [lower-pad solution (LPS): 20 mM-sodium phosphate buffer, pH 7.5, containing KCl (1.5 g/litre) and MgCl₂·6H₂O (1.07 g/litre)]. For development in α -chymotrypsin, the proteinase was dissolved in LPS at 3 mg/ml. Growth and development were in a humid atmosphere at 22°C. At appropriate times during development, cells were washed from Millipore filters with 20 mM-sodium phosphate, pH 6.0, pelleted by centrifugation (300 g for 3 min) and used immediately or stored at –20°C at a concentration of 10⁸ cells/ml in the appropriate buffer for each enzyme determination.

Cell disruption

Amoebae were disrupted in suspension (10⁸/ml) using a 150 W MSE ultrasonic disintegrator fitted with an exponential probe. Cells at any stage before culmination required 1 min at setting 6 for more than 95% breakage and culminates required 5 min treatment. Spores were treated for 15 min, but breakage was never more than 50%. During sonication, samples were cooled by immersion of the vessel containing the cells in an ice/water mixture.

Enzyme assays

β -N-Acetylglucosaminidase, α -D-mannosidase and β -D-glucosidase were assayed as described by Kilpatrick & Stirling (1976). Suitable dilutions of enzyme (20 μ l) were incubated with 100 μ l of the appropriate substrate (0.38 mM-4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside, 1 mM-4-methylumbelliferyl α -D-mannopyranoside or 1 mM-4-methylumbelliferyl β -D-glucopyranoside in 0.15 M-sodium citrate/sodium phosphate buffer, pH 4.5) at 37°C for up to 30 min. The reaction was stopped by the addition of 1 ml of 0.5 M-sodium glycinate buffer, pH 10.4. The 4-methylumbelliferone released was measured with a Locarte fluorimeter (activating at 330 nm and measuring at 440 nm), standardized with a solution of quinine sulphate (4 μ g/ml in 0.05 M-H₂SO₄). Fluorescence values were converted into concentrations by using a calibration curve of 4-methylumbelliferone (up to 20 nmol/ml) in the reaction-stopping buffer.

UDP-glucose pyrophosphorylase was assayed both in the direction of glucose 1-phosphate formation (Franke & Sussman, 1971) and in the direction of UDP-glucose formation (Hames, 1976). Assay

mixtures for measurement of activity in the direction of glucose 1-phosphate formation contained, as final concentrations in 0.5 ml: 1 mM-UDP-glucose, 1.6 mM-NADP⁺, 5 mM-MgCl₂, 1 mM-EDTA, 0.05 mM-glucose 1,6-bisphosphate, 0.075 unit of glucose 6-phosphate dehydrogenase, 0.0175 unit of phosphoglucosyltransferase, 0.15 unit of 6-phosphogluconate dehydrogenase, suitably diluted enzyme (about 5 μ l of a sonicated solution of 10⁸ cells/ml) and 2 mM-sodium pyrophosphate (added to start the reaction) all in 0.08 M-Tricine/NaOH buffer, pH 7.6. The initial rate of change in absorbance at 340 nm at 37°C was recorded. Activity in the direction of UDP-glucose formation was determined by quantification of radiolabelled product. Reaction mixtures contained, as a final concentration in 0.1 ml of 0.8 M-Tricine/NaOH, pH 7.4, 5 mM-UTP, 5 mM- α -D-[U-¹⁴C]glucose 1-phosphate and 10 mM-MgCl₂. The reaction was started with the addition of 0.15 ml of enzyme in the Tricine buffer (from cells at 2 $\times$ 10⁶/ml) and incubated at 37°C for 30 min. To stop the reaction, the mixture was heated at 100°C for exactly 2 min and cooled on ice. Excess [¹⁴C]-glucose 1-phosphate was destroyed by incubation of the reaction mixture with 0.5 unit of alkaline phosphatase (phosphodiesterase-free) at 45°C for 30 min. A 50 μ l sample of the reaction mixture was spotted on to a moist Whatman DE81 filter paper (2.3 cm diameter) which after 2 min was washed with 5 ml of water and 5 ml of 96% (v/v) ethanol under gentle suction. The filter papers were dried and counted for radioactivity.

Threonine deaminase was assayed as described by Pong & Loomis (1973). Assay mixtures contained 25 μ l of 0.25 M-L-threonine, 50 μ l of cell extract (10⁸ cells/ml) containing 20 μ g of pyridoxal phosphate/ml (added before freezing the cell suspension), 12.5 μ l of 0.1 M-Tris/HCl, pH 8.5, and 37.5 μ l of 20 μ g/ml solution of fresh pyridoxal phosphate in water. After incubation at 25°C for 1 h, the product was allowed to react with 2,4-dinitrophenylhydrazine (0.9 ml of a 0.34 mg/ml solution in 1.2 M-HCl) for 20 min at 25°C, followed by the addition of 2.5 M-NaOH (1.7 ml) and measurement of the A_{520} after further incubation for 10 min. A standard curve of α -oxobutyrate (0–2 μ mol/ml), obtained by the same procedure, was used.

UDP-galactose 4-epimerase was assayed by the method of Telser & Sussman (1971). Assay mixtures of 250 μ l containing (final concns.) 0.8 mM-UDP-galactose, 4 mM-NAD⁺, 0.08 M-Bicine/NaOH buffer, pH 8.5, 0.004 unit (in 10 μ l) of UDP-glucose dehydrogenase and 50 μ l of suitably diluted slime-mould sonicated material in the Bicine buffer were incubated at 37°C in a quartz cuvette. The production of NADH was monitored by the increase in A_{340} .

Glycogen phosphorylase was assayed in the

direction of glucose 1-phosphate formation (Firtel & Bonner, 1972). Assay mixtures contained (as final concentrations in 0.5 ml): 50 mM-imidazole/HCl, pH 7.1, 0.2% (w/v) oyster glycogen, 20 mM-potassium phosphate, pH 7.1, 5 mM-MgCl₂, 1 mM-dithiothreitol, 0.5 mM-NADP, 10 μ g of phosphoglucosyltransferase, 0.2 unit of glucose 6-phosphate dehydrogenase and 10 μ l of slime-mould sonicated material (in the potassium phosphate buffer). The initial rate of change in A_{340} was recorded during incubation at 37°C.

UDP-galactose polysaccharide transferase was assayed as described by Sussman & Osborn (1964). Assay mixtures contained (as a final concentration in 50 μ l): 54 mM-dimethylglutarate buffer, pH 7.5, 14 mM-KCl, 1.8 mM-MgCl₂, 50 nM-UDP-[U-¹⁴C]-galactose, non-radioactive D-galactose to give a final substrate concentration of 100 μ M, 10 μ l of mucopolysaccharide acceptor [prepared as described by Sussman & Osborn (1964)] and 10 μ l of slime-mould sonicated material. Incubation was at 37°C for 1 h and the reaction was stopped by the addition of 50 μ l of 0.1 M-HCl. The mixture was heated in a boiling-water bath for 15 min and spotted on to Whatman no. 1 filter-paper circles (2.4 cm diam.), dried and dropped into 95% ethanol at –20°C. The filter papers were washed in cold ethanol three times, dried and counted for radioactivity.

Tyrosine aminotransferase was assayed as described by Pong & Loomis (1971). Assay mixtures contained 0.7 ml of 7 mM-L-tyrosine in 150 mM-Tris/HCl, pH 8.5, 50 μ l of 2 mM-pyridoxal phosphate, 50 μ l of sonicated cell extract (from 10⁸ cells/ml, diluted 1:1 with the Tris buffer) and 75 μ l of 0.15 M- α -oxoglutarate (added to start the reaction). After 15 min incubation at 32°C, the reaction was stopped with 0.1 ml of 10 M-NaOH, left at 25°C for 30 min, clarified by centrifugation and the absorbance read at 331 nm. A molar absorption coefficient of the product of 2.49×10^4 litre $\cdot$ mol^{–1} $\cdot$ cm^{–1} (Diamondstone, 1966) was used for calculation of enzyme activities.

Alkaline phosphatase was assayed by the method of Loomis (1969). Sonicated extract (50 μ l, suitably diluted) was mixed with 50 μ l of substrate [20 mM-*p*-nitrophenyl phosphate (disodium salt) in 40 mM-Tris/HCl, pH 8.5, containing 20 mM-MgCl₂] and incubated at 37°C for up to 1 h. The reaction was stopped with 900 μ l of 1 M-Na₂CO₃ and the A_{405} measured. Concentrations were determined against a standard curve of *p*-nitrophenol (0–60 nmol/ml) in 1 M-Na₂CO₃.

Erythrocyte-agglutination assay for discoidin

The method of Rosen *et al.* (1975) was used. Fresh human blood (group O; rhesus-positive; 0.3 ml approx.) was taken from a finger prick into 3% (w/v) sodium citrate. The red cells were washed

three times in 75 mM-sodium/potassium phosphate buffer, pH 7.2, containing 75 mM-NaCl (phosphate buffered saline) and made up as a 3% (v/v) suspension in this buffer. Assays were carried out in Cooke microtitre V plates. Samples containing discoidin (25 μ l) were diluted with phosphate-buffered saline (25 μ l) and subjected to a series of 2-fold dilutions in phosphate-buffered saline (25 μ l/well). An equal volume of 0.9% (w/v) NaCl was added to each well, followed by 25 μ l of the erythrocyte suspension. The plates were given a sharp tap to mix the contents of the wells, covered, and the patterns allowed to develop for 1 h at room temperature. Agglutination was positive if the red cells were prevented from forming a distinct spot in the bottom of the well. The end point was defined as the lowest dilution at which detectable agglutination took place, and agglutination activity units are the reciprocal of this value.

Preparation of 125 I-labelled α -chymotrypsin

125 I-labelled α -chymotrypsin was prepared by the method of Greenwood *et al.* (1963).

Estimation of membrane cyclic AMP-binding sites

Normally developing cells at the 10 h stage of development were washed from Millipore filters with 50 mM-sodium phosphate buffer, pH 7.5, washed three times in the same buffer and resuspended at 2×10^7 cells/ml in two equal portions. An equal volume of α -chymotrypsin (Pronase or trypsin) (1 mg/ml) in the same pH 7.5 buffer was added to one portion and proteinase-free buffer to the other. After incubation at room temperature for 15 min with gentle agitation, cells were removed from the enzyme by centrifugation (300 *g* for 3 min), washed twice in cold (0–2°C) 50 mM-sodium phosphate buffer, pH 6.0, containing 2 mM-dithiothreitol and finally suspended at 2×10^7 cells/ml in the dithiothreitol-containing buffer. Cells developing in α -chymotrypsin were harvested from Millipore filters and washed three times in 50 mM-sodium phosphate buffer, pH 6.0, containing 2 mM-dithiothreitol. These cells were also finally resuspended at 2×10^7 cells/ml.

Cyclic AMP binding was assayed as described by Green & Newell (1975). Cell suspension, prepared as described above (1 ml), was mixed with 25 μ l of cyclic [8- 3 H]AMP to give a final concentration range of the nucleotide of 0.04–300 nM and incubated at 0–2°C for 2 min. Bound cyclic [8- 3 H]AMP associated with cells was separated from free label by rapid filtration (under 10 s) with gentle suction through previously wetted Millipore filters (HAWP 02412; 24 mm diameter; 0.45 μ m pore size). Millipore filters were dissolved in 1 ml of 2-methoxy-methanol, scintillation cocktail {1% butyl PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole] in toluene/Triton X-100 (2:1, v/v)} was

added and the radioactivity counted. The volume of fluid remaining in the filter was determined by using 25 μ l of [32 P] P_i suitably diluted to contain about 100 000 c.p.m./25 μ l, in place of the cyclic [8- 3 H]-AMP as suggested by Mullens & Newell (1978). Phosphodiesterase activity was not detected in the α -chymotrypsin preparation.

Results

Effect of α -chymotrypsin on marker enzymes during early development

The developmentally regulated marker enzymes β -*N*-acetylglucosaminidase, α -mannosidase, threonine deaminase and tyrosine aminotransferase, which normally increase in specific activity during interphase and the early stages of aggregation, were also found to increase in *D. discoideum* cells developed in the presence of α -chymotrypsin (Fig. 1). The rates of appearance of these enzymes were unaffected by development in α -chymotrypsin, but β -*N*-acetylglucosaminidase had a higher specific activity throughout. Production of β -glucosidase I normally ceases early in development and the increased activity during late development is due to β -glucosidase II (Fig. 1). Cells developing in α -chymotrypsin showed a similar decrease in the specific activity of β -glucosidase I, although the specific activity remained higher than in the untreated cells and increased between 16 and 22 h. Examination of this β -glucosidase activity by electrophoresis on Cellogel (results not shown) revealed that the enzyme co-migrated with β -glucosidase I and not the late-developmental form, β -glucosidase II.

The developmentally regulated carbohydrate-binding protein, discoidin, normally appears during interphase and reaches maximal levels after aggregation is complete (Rosen *et al.*, 1973). Development in α -chymotrypsin advanced the expression of discoidin by about 2 h (Fig. 1), and its activity in extracts of developmentally blocked cells could be measured by haemagglutination after 20 h of starvation. In contrast, discoidin haemagglutination activity falls to undetectable levels during culmination of normal cells. The haemagglutination activity of discoidin was unaffected by assay in the presence of α -chymotrypsin (0.5 mg/ml).

Effect of α -chymotrypsin on enzymes appearing after aggregation

UDP-glucose pyrophosphorylase, glycogen phosphorylase, UDP-galactose 4-epimerase, UDP-galactose polysaccharide transferase and alkaline phosphatase were assayed as markers of post-aggregative development. Production of all these enzymes was inhibited in cells exposed to α -chymotrypsin during development (Fig. 2). In the presence of α -chymotrypsin, the specific activity of glycogen phosphorylase was 40% of that in untreated control

Fig. 1. Effect of α -chymotrypsin on marker enzymes during early development

D. discoideum cells were allowed to develop on filters in the presence (●) or absence (O) of α -chymotrypsin in the lower pad solution. Cells were harvested at intervals and assayed for (a) α -mannosidase, (b) β -N-acetylglucosaminidase, (c) threonine deaminase, (d) tyrosine aminotransferase, (e) β -glucosidase and (f) the carbohydrate-binding protein, discoidin, by the methods described in the text.

Fig. 2. Effect of α -chymotrypsin on enzymes appearing after aggregation

D. discoideum cells were allowed to develop on filters in the presence (●) or absence (O) of α -chymotrypsin in the lower pad solution. Cells were harvested at intervals and assayed for (a) glycogen phosphorylase, (b) UDP-glucose pyrophosphorylase, (c) UDP-galactose 4-epimerase, (d) UDP-galactose polysaccharide transferase and (e) alkaline phosphatase by the methods described in the text.

cells. Similarly, 17% of UDP-glucose pyrophosphorylase, 32% of UDP-galactose polysaccharide transferase and 9% of UDP-galactose 4 epimerase were produced compared with the maximum specific activities in the untreated controls.

UDP-glucose pyrophosphorylase activity during recovery from α -chymotrypsin treatment

Amoebae blocked in development by α -chymotrypsin can complete development normally after removal of the proteinase (Kilpatrick *et al.*, 1980). This recovery was monitored by measuring the specific activity of UDP-glucose pyrophosphorylase. We chose this enzyme because it normally

begins to accumulate during late aggregation and is the first of the developmentally regulated enzymes to be substantially affected by development in α -chymotrypsin.

Amoebae were allowed to develop in α -chymotrypsin for 12 or 24 h on filters and then transferred to fresh support pads containing 20 mM-sodium phosphate buffer, pH 7.5. Samples were taken at intervals for UDP-glucose pyrophosphorylase assay until fruiting bodies had formed. This occurred within 8–10 h in the case of transfer after exposure to α -chymotrypsin for 12 h and 6–8 h after exposure for 24 h. The results are shown in Fig. 3. The specific activity increased 3-fold in cells treated for 12 h with

Fig. 3. UDP-glucose pyrophosphorylase activity during recovery from α -chymotrypsin treatment

D. discoideum cells that had been allowed to develop on filters in the presence of α -chymotrypsin for (a) 12 h or (b) 24 h were transferred to fresh support pads containing either lower-pad solution (○) or α -chymotrypsin (3 mg/ml) in lower-pad solution (●). Cells were harvested at intervals and assayed for UDP-glucose pyrophosphorylase by the method of Hames (1976).

α -chymotrypsin and 9-fold in cells treated for 24 h. During normal development the increase in specific activity is about 8-fold (Fig. 2). It should be noted that in these recovery experiments the method of Hames (1976) was used to assay UDP-glucose pyrophosphorylase enzyme activity, whereas that of Franke & Sussman (1971) was used in the experiments shown in Fig. 2.

Permeability of α -chymotrypsin-treated cells to $^{45}\text{Ca}^{2+}$

The effect of α -chymotrypsin on the integrity of the plasma membranes of treated cells was investigated by monitoring efflux and uptake of $^{45}\text{Ca}^{2+}$. An intracellular pool of $^{45}\text{Ca}^{2+}$ was established by incubating 5×10^7 freshly harvested vegetative cells on a Millipore filter in the presence of $5 \mu\text{Ci}$ of $^{45}\text{Ca}^{2+}$. Normal development was allowed to proceed for 8 h and then the cells harvested and treated with 0.5 mg of α -chymotrypsin/ml in 20 mM-sodium phosphate buffer, pH 7.5, for 15 min at room temperature. Cells treated in this way released 1.2% of the total cellular $^{45}\text{Ca}^{2+}$, whereas 0.9% was released in cells incubated in buffer alone.

Influx of Ca^{2+} was measured by adding $5 \mu\text{Ci}$ of $^{45}\text{Ca}^{2+}$ to 5×10^7 amoebae that had been allowed to develop in the presence of α -chymotrypsin for 8 h. After 15 min at 22°C , amoebae were washed free of unincorporated $^{45}\text{Ca}^{2+}$ and the cells counted for radioactivity. Cells that had developed in α -chymotrypsin incorporated 3.5×10^4 c.p.m., whereas the same number of normally developing cells incorporated 4.4×10^4 c.p.m. under the same conditions.

Uptake of ^{125}I -labelled α -chymotrypsin by developing amoebae

The extent of uptake of α -chymotrypsin by developing cells was investigated by adding ^{125}I -labelled α -chymotrypsin ($0.07 \mu\text{Ci}$) to lower-pad solution. After 24 h the cells were harvested and washed thoroughly with 20 mM-sodium phosphate buffer, pH 6.0, and counted for radioactivity. Less than 0.5% of the ^{125}I -labelled α -chymotrypsin was associated with the cells.

Estimation of the number and type of cell-surface cyclic AMP-binding sites

Binding of cyclic $[8\text{-}^3\text{H}]\text{AMP}$ to cells at various concentrations of the nucleotide was determined and the results analysed by the method of Scatchard (1949). In addition to measuring binding of cyclic $[8\text{-}^3\text{H}]\text{AMP}$ to normal cells and cells allowed to develop in α -chymotrypsin, cells allowed to develop normally for 10 h were treated for 15 min with α -chymotrypsin immediately before the binding studies. The results are shown in Fig. 4. Short exposure (15 min) of normally developing amoebae to α -chymotrypsin destroyed the low-affinity component of the cyclic AMP-binding sites. In the experiment shown, the number of sites was decreased from 1.34×10^5 to 0.37×10^5 per cell, with an apparent decrease in the equilibrium dissociation constant (K_a) from 198 nM to 125 nM. Cells allowed to develop in α -chymotrypsin also displayed a lower number of low-affinity binding sites (7.1×10^4 per cell) with a K_a of 125 nM. Results from three experiments were in the range of $(4.5\text{--}9) \times 10^4$.

Table 1. Comparison of the effects of α -chymotrypsin, Pronase and trypsin on low- and high-affinity binding of cyclic $[8\text{-}^3\text{H}]\text{AMP}$ to *D. discoideum* cells

Cells that had developed normally for 10h were harvested and duplicate samples treated with proteinase for 15 min, washed and used for analysis of cyclic $[8\text{-}^3\text{H}]\text{AMP}$ binding. Cells treated with proteinases were compared with normal 10h cells and with cells allowed to develop in α -chymotrypsin for the same period. Values are the individual results of duplicate experiments.

Treatment	[Cyclic $[8\text{-}^3\text{H}]\text{AMP}$] (mM) ...	No. of molecules bound per cell	
		1	150
None		590, 702	22 000, 23 200
α -Chymotrypsin during development		481, 555	10 600, 14 900
α -Chymotrypsin		508, 554	7100, 11 100
Pronase		758, 994	4700, 13 000
Trypsin		592, 692	25 700, 17 300

Fig. 4. Scatchard-plot analysis of cyclic $[8\text{-}^3\text{H}]\text{AMP}$ binding to *D. discoideum* cells

Scatchard plots of cyclic $[8\text{-}^3\text{H}]\text{AMP}$ binding to (a) amoebae harvested after 10h of normal development, (b) amoebae harvested after 10h of normal development and treated with α -chymotrypsin for 15 min ($\blacktriangle$) and amoebae harvested after 10h of development in α -chymotrypsin ($\bullet$). N is the number of molecules bound per cell.

binding sites. Accurate determinations of the numbers of high-affinity sites per cell were not possible, but were in the order of 3000 sites per cell, with a K_a of 0.6 nM.

Effects of treating cells with Pronase and trypsin on cyclic AMP binding were compared with those of α -chymotrypsin. The results summarized in Table 1 show that at 1 mM-cyclic $[8\text{-}^3\text{H}]\text{AMP}$ (a concentration at which predominantly high-affinity binding sites are occupied), binding is unaffected by exposure to any of the enzymes. Low-affinity cyclic AMP binding (determined at 150 mM-cyclic $[8\text{-}^3\text{H}]\text{AMP}$) was decreased to the same extent by α -chymotrypsin and Pronase treatment, but trypsin treatment had no effect.

Discussion

Analysis of developmentally regulated enzymes has shown clearly that α -chymotrypsin affects a process necessary for the expression of post-aggregative enzymes. Aggregation was unaffected by α -chymotrypsin, and developmentally regulated enzymes that begin to increase in specific activity before aggregation had patterns of accumulation which closely paralleled those of normal development.

Although development in α -chymotrypsin inhibited the production of all the post-aggregative enzymes we measured, those which normally appear immediately after aggregation, glycogen phosphorylase, UDP-glucose pyrophosphorylase and UDP-galactose polysaccharide transferase, accumulated to a greater extent than the culmination-specific enzymes UDP-galactose 4-epimerase, β -glucosidase II and alkaline phosphatase. There is a leakiness of the 'early' blocking effect of α -chymotrypsin and we have previously observed that about 1% of the expected number of fruiting bodies are formed. Spore maturation appears to be more sensitive to α -chymotrypsin (Kilpatrick *et al.*, 1980; Peacey & Gross, 1981), and this may be

low-affinity binding sites per cell. Neither type of treatment with α -chymotrypsin had any effect on the number or affinity of the high-affinity cyclic AMP-

reflected in the greater effect that the proteinase has on culmination-specific enzymes. Treatment of developing slime-mould cells with concentrations of Pronase greater than $250\text{ }\mu\text{g/ml}$ also decreases the accumulation of the prespore-cell-specific enzyme UDP-galactose polysaccharide transferase, but does not appear to affect the accumulation of glycogen phosphorylase (Peacey & Gross, 1981). Whether this is due to specific actions of Pronase and α -chymotrypsin, to the inclusion of 5 mM-cyclic AMP in the agar or to differences in the responses of strains NC-4 and HM18 to the proteinases is not known. However, there appears to be a lower specific activity of glycogen phosphorylase in untreated HM18 cells than we expected in NC-4 (Firtel & Bonner, 1972), and were able to measure in the results shown in Fig. 2.

Development in the presence of α -chymotrypsin produces a phenocopy of a mutant that aggregates normally but fails to develop past aggregation, which was described by Loomis *et al.* (1976). This temperature-sensitive morphological mutant, DTS6, has the same patterns of enzyme accumulation at its non-permissive temperature as wild-type cells developing in the presence of α -chymotrypsin. Further study of the lesion in DTS 6 has not, however, been reported.

Morphologically normal fruiting bodies were formed when α -chymotrypsin-blocked cells were transferred to fresh support pads containing buffer alone, and this recovery was accompanied by a rise in the specific activity of intracellular UDP-glucose pyrophosphorylase. The increase in specific activity was greater in cells exposed to the proteinase for 24 h before transfer than in those exposed for only 12 h. In part this might be due to differences in the amounts of residual proteinase transferred with the cells on Millipore filters. The activity of α -chymotrypsin decreases with time when incubated with slime-mould cells (Kilpatrick *et al.*, 1980), and thus higher activities would be expected at 12 h than at 24 h. Inactivation of α -chymotrypsin is probably not due to cellular uptake of the proteinase, since less than 1% of ^{125}I -labelled α -chymotrypsin was taken up by the cells in 24 h, but this experiment does not exclude the possibility that labelled enzyme was taken up, degraded and ^{125}I excreted.

Interestingly, the rate of morphological development was not related to the specific activity of UDP-glucose pyrophosphorylase in the amoebae. In both 12 h- and 24 h-blocked cells there was a 4 h lag on transfer of the cells to buffer before the specific activity of UDP-glucose pyrophosphorylase increased, but we observed no macroscopic changes in the behaviour of cells during this period. Thereafter completion of development was extremely rapid in both groups of cells, taking place in only 4 h, although there was a 3-fold difference between them

in the peak specific activity of UDP-glucose pyrophosphorylase.

Previously we have shown that EDTA-resistant cohesion is unimpaired in cells treated with α -chymotrypsin (Schmidt *et al.*, 1978) and thus it would appear that cohesive cell contact is not the only requirement for the expression of post-aggregative enzymes. In view of recent reports linking cyclic AMP signalling with the expression of post-aggregative enzymes (Town & Gross, 1978) and specific mRNA molecules (Landfear & Lodish, 1980), we investigated the possibility that cell-surface cyclic AMP binding is affected by α -chymotrypsin. Green & Newell (1975) demonstrated that cyclic AMP bound to the membranes of aggregating amoebae with two distinct binding affinities. Cells allowed to develop in α -chymotrypsin or even treated with the enzyme for a short time have markedly altered abilities to bind cyclic AMP with low affinity, but the high-affinity binding is unaffected. The differences in cyclic AMP binding observed after these two types of treatment with α -chymotrypsin (Fig. 4) were reproducible and may be due to the lower effective concentration of α -chymotrypsin in contact with the cells after 10 h incubation, even though the total proteinase activity was only decreased to 50% (Kilpatrick *et al.*, 1980). Juliani & Klein (1977) found that the number of cell-surface cyclic AMP-binding sites was dependent on the Ca^{2+} concentration. One way in which α -chymotrypsin could affect the Ca^{2+} concentration is by causing increased uptake or decreased release from the cells, but we consider that this is unlikely because $^{45}\text{Ca}^{2+}$ uptake and release by cells was not appreciably affected by treatment with the enzyme.

A mutant strain, NP 144 (Newell, 1977), is capable of only poor aggregation, but once aggregated proceeds normally through development. This strain displays normal low-affinity but few high-affinity binding sites. Thus it appears that the high-affinity cyclic AMP binding might be required for aggregation, and it is noteworthy in the present context that neither high-affinity binding nor aggregation is affected by α -chymotrypsin. It seems possible that low-affinity cyclic AMP binding may be required for post-aggregative development, and it may be significant that this component is destroyed by treatment with α -chymotrypsin. Pronase affects low-affinity cyclic AMP binding in a similar manner to α -chymotrypsin, whereas trypsin has no effect. It is notable that Pronase has an inhibitory effect on development similar to that caused by α -chymotrypsin, whereas trypsin has only weak inhibitory activity, despite its generalized effects on membrane proteins (Kilpatrick *et al.*, 1980).

In recent studies with 8-azido cyclic [^{32}P]AMP, only one specific cell-surface binding protein has been detected (Juliani & Klein, 1981), and it seems

likely that the high- and low-affinity cyclic AMP binding can be explained by negative co-operativity (Mullens & Newell, 1978). Nevertheless, the possibility remains that the two distinct binding-affinity components of one site might mediate separate cellular responses (Mullens & Newell, 1978). As yet there is no information available on the normal turnover rate of membrane cyclic AMP-binding sites and therefore it is not known how quickly the affected sites can be replaced. It may be that the decrease in the number of sites seen on development of cells in α -chymotrypsin along with an alteration in affinity for cyclic AMP is enough to prevent further development, but we cannot discount the possibility that there are other α -chymotrypsin-sensitive proteins acting with, or independently of, the cyclic AMP-binding sites. Indeed, the differentiation-inducing effects of 6-aminoheptyl cyclic AMP indicate a 'receptor-independent' mechanism for the effects of cyclic AMP on post-aggregative development (Juliani *et al.*, 1981).

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