A bacterial factor induces changes in cysteine proteinase forms in the cellular slime mould *Dictyostelium discoideum*

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The electrophoretic pattern of cysteine proteinases in axenically grown myxamoebae of *Dictyostelium discoideum* can be altered by the addition of either Gram-negative (*Klebsiella aerogenes*, *Escherichia coli*) or Gram-positive (*Micrococcus lysodeikticus*, *Bacillus subtilis*) bacteria to the culture. No changes occurred, however, if either yeast or latex beads were used in place of bacteria. The changes involved the simultaneous loss of proteinases characteristic of the axenic cells (the A-forms) and the acquisition of those found in cells which have been grown on bacteria (the B-forms). Using *K. aerogenes* the conversion was complete within 4 h. Extracellular proteinase activity was unaffected during this period. After the *D. discoideum* cells had been lysed, no equivalent change in proteinase band pattern could be produced either by prolonged incubation of cell extracts or by treatment with proteinases. An identical conversion could be induced in cultures of myxamoebae by a factor, cysteine proteinase converting factor (CPCF), present in the 15000 g supernatant of a sonicated suspension of *K. aerogenes*. CPCF was macromolecular, as demonstrated by both ultrafiltration and gel filtration, acid-precipitable, but was soluble in ethanol or alkali. Its activity was unaffected by treatment with trypsin. The results suggested that CPCF might be a component of the bacterial cell wall, and since its activity was affected by lysozyme treatment, peptidoglycan is implicated. The results can be interpreted in terms of a novel nutrient-dependent post-translational change which affected most of the cysteine proteinases present in *D. discoideum* myxamoebae.

INTRODUCTION

The study of the regulation of microbial enzyme activities in response to changes in nutrients has been invaluable in providing insights into the molecular basis of biological control mechanisms. Although the cellular slime mould *Dictyostelium discoideum* has been widely used as a model for studying the control mechanisms involved in development (see Loomis, 1982), there has been less study of the factors which regulate enzyme activity during the growth phase. Nutrient-dependent differences are well documented, however, and can influence events during the developmental phase of the life cycle.

D. discoideum may be grown on two types of nutrient. All strains can be cultured in association with bacteria, usually *Klebsiella aerogenes* or *Escherichia coli*, while certain laboratory-isolated strains (so-called axenic strains) are also capable of growth in semi-defined medium consisting of yeast extract and peptone to which glucose is normally added. Ashworth and co-workers (Watts & Ashworth, 1970; Wiener & Ashworth, 1970; Ashworth & Quance, 1972) reported differences in the specific activity of a number of acid hydrolases between bacterially grown and axenically grown cells. Axenic cells have much higher levels of enzymes such as β -N-acetylglucosaminidase and α -mannosidase. In bacterially grown cells, high levels of activity are only achieved during the early phase of development when the cells are starved, and the developmental changes in these hydrolase activities depend on the manner in which the cells are grown (Quance & Ashworth, 1972). Axenic growth of

cellular slime moulds does not invariably lead to higher levels of activity of hydrolytic enzymes. In *Polysphondylium pallidum*, the level of cysteine proteinase activity is highest in cells grown in association with *E. coli* (North *et al.*, 1984). Since cysteine proteinase activity decreases during development, this situation is in fact analogous to that for the other hydrolases, that is, axenic growth gives rise to enzyme levels equivalent to those encountered in bacterially grown cells only during development. Thus the difference between the two types of cell has been explained in terms of the slower growing axenic cells undergoing prematurely certain events which are normally integral parts of the developmental programme. Results from two studies have, however, implied the involvement of bacterial factors in enzyme regulation in cellular slime moulds. These have shown the repression of β -N-acetylglucosaminidase in *D. discoideum* by lipopolysaccharide (LPS) (Longmore & Watts, 1980) and the induction of cysteine proteinase activity in *P. pallidum* by *E. coli* cells (North *et al.*, 1984).

In the accompanying paper (North *et al.*, 1988) we reported a nutrient-dependent effect in which it is the bacterially grown myxamoebae, but not axenic cells, that show a close resemblance to developmental cells. In *D. discoideum*, unlike *P. pallidum*, the levels of cysteine proteinase activity in axenic and bacterially fed cells are similar. There are, however, two electrophoretically distinct enzyme patterns whose appearance is dependent on how the cells are grown. The A-pattern is characteristic of axenically grown cells, the B-pattern of bacterially grown cells. During the early stage of development, the cells have a pattern which is very similar to the B-pattern.

Abbreviations used: CPCF, cysteine proteinase converting factor; DTT, dithiothreitol; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane; LPS, lipopolysaccharide; PAGE, polyacrylamide-gel electrophoresis.

The proteinases of axenic cells change immediately upon starvation. In order to investigate the nature of the regulatory factors responsible for these differences in pattern the effect of bacteria on the proteinases of axenic cells has been examined. In this paper we demonstrate that a change from the A- to the B-pattern can be readily induced by adding whole bacteria to axenic cells and that a cell-free preparation which induces this cysteine proteinase conversion can be obtained from *K. aerogenes*. A preliminary account of this work has been presented previously (North, 1987).

MATERIALS AND METHODS

Materials

Gelatin (porcine type 1), latex beads (0.46 μ m), trypsin (type IX, EC 3.4.21.4), chymotrypsin (type II, EC 3.4.21.1), papain (type IV, EC 3.4.22.2), lysozyme (grade 1, mucopeptide *N*-acetylmuramoylhydrolase, EC 3.2.1.17), LPS (*E. coli* and *Salmonella typhimurium*), freeze-dried *Bacillus subtilis* (A.T.C.C. 6633) and freeze-dried *Micrococcus lysodeikticus* (A.T.C.C. 4698) were supplied by Sigma Chemical Co. (Poole, England). Antipain, leupeptin, pepstatin, chymostatin, phosphoramidon and E64 were all products of the Peptide Institute Inc. (Osaka, Japan) and were obtained through Scientific Marketing Associates (London, England).

Organisms and growth conditions

Unless otherwise indicated *D. discoideum* strain AX2 (A.T.C.C. 24397) was grown at 22 °C in axenic HL5 medium containing glucose (Watts & Ashworth, 1970). The cells were harvested during the exponential phase by centrifugation (500 *g*, 5 min). To obtain bacterially grown cells myxamoebae were inoculated into suspensions of autoclaved *K. aerogenes* and grown as described in the accompanying paper (North *et al.*, 1988). Overnight cultures (1 l) of *K. aerogenes* were grown at 37 °C in nutrient broth (Oxoid).

Preparation and testing of samples of *K. aerogenes*

The bacteria were harvested by centrifugation (7000 *g*, 10 min in a Sorvall model RC-5B), washed once with 20 mM-sodium phosphate buffer, pH 6.0, and resuspended in the same buffer to give an A_{650} of 2.5. The suspension was added to axenic cultures of myxamoebae in a ratio of 1:10 (v/v). The bacteria were heat-treated by incubating the suspension at 70 °C for 5 min. For the preparation of cysteine proteinase converting factor (CPCF), the suspension was sonicated for 2 min at an amplitude of 9 μ m and then centrifuged at 15000 *g* for 10 min. The supernatant represented a crude preparation of CPCF which was normally added to the *D. discoideum* cultures in a ratio of 1:10 (v/v). Testing was carried out using 10 ml of axenic cultures in 50 ml Erlenmeyer flasks. After 4 h at 22 °C, the cells were harvested by centrifugation, washed once with water and the cell pellet stored frozen until required. For time courses, 100 ml cultures in 500 ml flasks were used, 10 ml samples were withdrawn periodically and cell pellets obtained and frozen. Whenever particulate materials (bacteria, yeast, latex beads) were tested in this system they were added as a suspension having an A_{650} of 2.5, with 1 ml added to a 10 ml culture.

Ultrafiltration of CPCF was carried out at 4 °C under N_2 in an Amicon ultrafiltration unit fitted with a PM10,

Fig. 1. Change in proteinase band pattern induced by the addition of bacteria to axenic cultures

Axenic cultures were treated with 20 mM-phosphate buffer, pH 6.0 (lane 1); resuspended in fresh medium with no addition made (lane 2); treated with *K. aerogenes* (lanes 3–5) or treated with *E. coli* (lanes 6–8). The bacteria were either heat-treated (lanes 3 and 6), untreated (lanes 4 and 7) or sonicated (lanes 5 and 8). Myxamoebae were harvested 8 h after treatment was initiated. Extracts were prepared and analysed by gelatin/PAGE as described under Materials and methods. Unless otherwise indicated in this and subsequent Figures, only the segment of the gel containing the proteinase bands is shown.

PM30 or XM50 membrane with pressure applied at 276 kPa. For gel filtration, a 1 ml sample of CPCF, concentrated 10-fold by ultrafiltration using an XM50 filter, was applied to a column of Sephacryl S-300 (290 mm $\times$ 10 mm) and 1 ml samples collected. The material was eluted in 20 mM-sodium phosphate buffer, pH 6.0. The void volume was determined from the elution of Blue Dextran. Partial purification of CPCF was achieved by treating 1 vol. of crude CPCF with 80 % (v/v) ethanol, the precipitate was then removed by centrifugation (7000 *g*, 10 min, Sorvall RC5B) and the supernatant dialysed overnight against 10 % (w/v) trichloroacetic acid. The resultant precipitate was collected by centrifugation (7000 *g*, 10 min, Sorvall RC5B), dissolved in 0.2 vol. of 1 M-KOH and dialysed overnight against 20 mM-sodium phosphate buffer, pH 6.0.

Proteinase analysis

Proteinases were analysed by gelatin/polyacrylamide-gel electrophoresis (PAGE) exactly as described in the accompanying paper (North *et al.*, 1988). Unless otherwise indicated, proteinase bands were developed by incubating gels in 0.1 M-sodium acetate buffer, pH 4.0, in the presence of 1 mM-dithiothreitol (DTT). The same system of nomenclature has been used, and bands shown in Figures are labelled accordingly.

RESULTS

The conversion of cysteine proteinase forms induced by bacteria

When myxamoebae of *D. discoideum* AX2 are grown in axenic medium containing glucose the major proteinase forms detected using gelatin/PAGE have apparent M_r s of 38000, 42000, 45000, 51000 and 54000. These comprise the A-pattern. Bacterially grown myxamoebae have a distinct B-pattern consisting of

Fig. 2. Lack of effect on the proteinase pattern of transferring bacterially grown myxamoebae to axenic medium

Cells grown in association with *K. aerogenes* (lanes 1–3) or *E. coli* (lanes 4–6) were washed free of bacteria and resuspended in fresh HL5 medium. Samples were taken at 0 h (lanes 1 and 4), 3 h (lanes 2 and 5) and 6 h (lanes 3 and 6) after resuspension and analysed by gelatin/PAGE.

Fig. 3. Effect of incubation on the proteinase patterns in myxamoebal extracts

Extracts were prepared from untreated axenic cells (lanes 1–4) or axenic cells treated with *K. aerogenes* (lanes 9–12) or a mixture containing equal volumes of the two extracts (lanes 5–8). The original extracts and the mixture were incubated for 6 h at -20°C (lanes 1, 5 and 9), 4°C (lanes 2, 6 and 10) or 50°C (lanes 3, 7 and 11) and at 50°C with an equal volume of electrophoresis sample buffer (lanes 4, 8 and 12) before analysis by gelatin/PAGE.

38000, 43000 and 48000- M_r forms (North *et al.*, 1988). When a suspension of either *K. aerogenes* or *E. coli* was added to axenic cells a conversion from the A-pattern to the B-pattern occurred (Fig. 1). The presence of bacteria resulted in the loss of those cysteine forms characteristic of the A-pattern and the acquisition of enzymes with mobilities identical with those of the B-pattern. The most distinct and characteristic feature of the change was the loss of ddCP51 and the appearance of a 48000- M_r proteinase which appeared identical with ddCP48. The same effect was achieved using two species of Gram-positive bacteria, *M. lysodeikticus* and *B. subtilis*, but there was no effect with either yeast or latex beads (results not shown). The changes induced by *K. aerogenes*

Fig. 4. Lack of effect of exogenous proteinases on the proteinase band pattern

Extracts were prepared from untreated axenic cells (lanes 1–5) or axenic cells treated with *K. aerogenes* (lanes 6–10). Extracts were either stored frozen (lanes 1 and 6) or incubated for 1 h at 25°C with no addition made (lanes 2 and 7) or with added trypsin (lanes 3 and 8), chymotrypsin (lanes 4 and 9) or papain (lanes 5 and 10) before analysis by gelatin/PAGE. The proteinases were added at a concentration of 35 $\mu\text{g}/\text{ml}$.

were initiated within 2 h (see North, 1987) and complete within approx. 4 h. The most reproducible and rapid change in pattern was achieved if the bacteria were heated before addition to the *D. discoideum* culture. If bacterially grown myxamoebae were washed free of bacteria and transferred to HL5 medium, the reverse B- to A-pattern conversion did not occur within 6 h (Fig. 2).

The new proteinase forms which appeared in axenically grown myxamoebae in response to bacteria had properties identical to those of the B-form enzymes of myxamoebae grown throughout on bacteria (North *et al.*, 1988). Thus they were most active in the acid pH range, but displayed a broad pH-dependence, their activity was enhanced by DTT and they were inactivated by some, although not all cysteine proteinase inhibitors. *trans*-Epoxy succinyl-L-leucylamido-(4-guanidino)-butane (E64) was the most effective of the inhibitors tested, but antipain and chymostatin had considerably less effect.

To determine whether equivalent changes in proteinase forms could be induced after cells had been lysed with Triton X-100, extracts prepared from axenically grown cells were subjected to a number of treatments. Prolonged dialysis against 20 mM-sodium phosphate buffer, pH 6.0, had no effect on the band pattern. Incubation of cell extracts at -20°C , 4°C or 50°C for up to 6 h failed to produce a qualitative change in the band pattern, although the activity of many of the proteinases did decrease at the higher temperatures (Fig. 3). When extracts were incubated at room temperature with electrophoresis sample buffer, which contained SDS and 2-mercaptoethanol, the band pattern was not affected (results not shown), but at 50°C the treatment completely abolished the activity of all forms. When extracts prepared from cells exposed to bacteria were similarly treated, again there were no changes in the band pattern, although activity was reduced after incubation at 50°C . ddCP38B was unique in that it retained some activity after incubation at 50°C with sample buffer, a property which distinguished it from the less stable ddCP38A. Electrophoresis of mixtures of extracts prepared from untreated and bacterially treated axenic cells, gave exactly

those patterns expected from a combination of the proteinases present in the individual extracts; this is seen most clearly in lane 7 (Fig. 3). This result eliminated the possibility that either extract contained factors which could bring about the direct conversion of the proteinases *in vitro*.

Fig. 5. Lack of effect of proteinase inhibitors on proteinase conversion *in vivo*

With the exception of the cells analysed in lane 1, axenic cells were treated with crude CPCF. Other additions (all at a concentration of 20 $\mu\text{g/ml}$) were made as indicated. Proteinase patterns were determined after 4 h. Lane 1, no CPCF treatment; lane 2, phosphoramidon; lane 3, pepstatin; lane 4, chymostatin; lane 5, leupeptin; lane 6, antipain; lanes 7 and 8, no inhibitor, CPCF only.

The loss of the A-form proteinases coincided exactly with the acquisition of the B-form proteinases, an observation consistent with the proteinase pattern changes occurring at a post-translational level. The lower apparent M_r of the B-form proteinases suggested that the bacteria might have induced proteolytic processing of the pre-existing A-form enzymes. To test the susceptibility of the enzyme forms to general proteolysis, papain, trypsin or chymotrypsin were added to extracts of treated and untreated cells. There was no effect on the mobility of the proteinases (Fig. 4). Indeed, the proteinase forms proved to be remarkably stable, all of the A-form as well as the B-form enzymes displaying resistance to non-specific proteolytic attack. Some potentiation of the B-form enzyme activity was noted, but the reasons for this are not known.

As a further approach to investigating whether proteolysis might be involved, the effect of proteinase inhibitors on the proteinase conversion in whole cells was tested. In these experiments CPCF, the cell-free factor obtained from *K. aerogenes* (see below), was used to induce the proteinase changes. The presence of inhibitors of aspartic (pepstatin), serine (leupeptin, chymostatin), cysteine (leupeptin, chymostatin, antipain) and metallo- (phosphoramidon) proteinases had no effect on the switch in proteinase pattern induced by CPCF (Fig. 5). B-forms were apparent throughout. When the proteinase inhibitors were added without CPCF only the A-form proteinases were present (results

Fig. 6. Change in proteinase pattern induced by CPCF

(a) Crude CPCF was added to an axenic culture at a concentration of 0.12 units/ml and samples were taken periodically for analysis by gelatin/PAGE. Lanes 1–8, cell extracts; lanes 9–12, cell-free medium. The time given is that after addition of CPCF. These samples were analysed using a Bio-Rad mini Protean system. (b) An axenic culture was harvested and resuspended in fresh medium and the culture split into two portions. To one (+) CPCF was added at a concentration of 0.27 units/ml. Samples of the cell-free medium were collected periodically for analysis by gelatin/PAGE. In cell extracts of the (+) culture B-form enzymes were present after 2 h.

Fig. 7. Effect of ultrafiltration and other treatments on CPCF

Axenic cultures were treated with the following for 4 h and then analysed by gelatin/PAGE. Lane 1, buffer only; lane 2, suspension of untreated *K. aerogenes*; lane 3, 15000 g supernatant of sonicated *K. aerogenes* (i.e. crude CPCF, final concentration after addition to culture of 0.71 units/ml); lane 4, crude CPCF dialysed against 20 mM-sodium phosphate buffer, pH 6.0, overnight; lane 5, crude CPCF heated at 100 °C for 5 min; lane 6, crude CPCF incubated with trypsin (1 mg/ml) for 2 h and then heated at 100 °C for 5 min; lane 7, crude CPCF filtered through a PM10 membrane; lane 8, material retained by PM10 membrane; lane 9, crude CPCF filtered through PM30 membrane; lane 10, material retained by PM30 membrane; lane 11, crude CPCF filtered through XM50 membrane; and lane 12, material retained by XM50 membrane.

Fig. 8. Effect of acid, alkali and ethanol on CPCF

Samples (5 ml) of crude CPCF were treated with 1 M-HCl, 10% (w/v) trichloroacetic acid, 1 M-KOH or 80% (v/v) ethanol. Precipitates obtained with acid and ethanol treatment were collected by centrifugation and the pellets resuspended in 5 ml of 20 mM-sodium phosphate buffer, pH 6.0. Both resuspended pellets and supernatants were dialysed against buffer overnight and then the CPCF-activity tested in the standard system. Additions were made to the cultures as follows: lane 1, buffer only; lane 2, whole *K. aerogenes*; lane 3, crude CPCF; lane 4, HCl precipitate; lane 5, HCl supernatant; lane 6, trichloroacetic acid precipitate; lane 7, trichloroacetic acid supernatant; lane 8, dialysed KOH-treated CPCF; lane 9, ethanol precipitate and lane 10, ethanol supernatant.

not shown). It is noteworthy that incubation of the cells for 4 h with antipain, chymostatin or leupeptin, all of which inhibit the cysteine proteinases *in vitro*, did not result in the loss of any of the proteinase bands. Since the inhibitors would have been present in the cell pellets during extract preparation this supports the view that none of the proteinase forms resulted from autodigestion. E64, a specific cysteine proteinase inhibitor, had an irreversible effect on enzyme activity, and the proteinase bands were diminished when extracts were prepared from cultures incubated for 4 h with 10 µg of E64/ml and absent when the E64 concentration was 100 µg/ml (results not shown).

Isolation of a factor from *K. aerogenes* which induces proteinase conversion: the CPCF

When preparations of *K. aerogenes* were sonicated and centrifuged, the 15000 g supernatant was as active in inducing proteinase changes as the original suspension (Fig. 6). Activity was also retained in the pelleted material. The factor responsible for the supernatant activity has been named CPCF. Although it is not yet known whether the molecule(s) responsible for activity absorb(s) u.v. light, for convenience crude preparations were quantified in terms of their A_{280} ; a preparation with an A_{280} of 1.0 was defined as containing 1 unit of CPCF/ml. The A- and B-pattern conversion could be induced in axenic cultures within 4 h by the addition of CPCF at a concentration of 0.01 unit/ml or greater. The time course (Fig. 6) was almost identical with that achieved with bacterial suspensions (see North, 1987). During the period when the intracellular enzyme forms were changing, the extracellular proteinase forms remained unchanged. ddCP42 was the major enzyme in the medium. Confirmation that there was no significant change in the nature of the proteinase released was obtained by adding CPCF to myxamoebae which had been washed and resuspended in fresh medium. Any extracellular proteinase detected would have been released only during

the period of CPCF treatment, but the pattern was the same as that for the medium of similar cultures to which CPCF was not added (Fig. 6b).

CPCF mediated an effect only when added to whole cells. If extracts of axenically grown myxamoebae were mixed with CPCF, or if frozen cell pellets were thawed in 0.1% (v/v) Triton X-100 containing CPCF, the proteinase band pattern was unaffected.

Properties of CPCF

The activity of CPCF was retained completely by Amicon PM10, PM30 and PM50 ultrafiltration membranes (Fig. 7) which suggested an M_r in excess of 50000. CPCF activity did pass through a 0.20 µm filter and so could not have been due to whole bacteria remaining in the supernatant. The macromolecular nature of CPCF was confirmed, however, by gel filtration. When samples of concentrated CPCF were applied to a column of Sephacryl S-300 the activity was eluted with the void volume.

The active factor of crude preparations was not affected by treatment with trypsin nor by incubation at 100 °C (Fig. 7). It was precipitated by HCl and by trichloroacetic acid, and the activity could be recovered by dissolving the precipitates in 20 mM-sodium phosphate buffer, pH 6.0, and dialysing against the same buffer. It was not, however, precipitated by 80% (v/v) ethanol and 1 M-KOH had no effect on the activity (Fig. 8). A partially purified preparation could be obtained from the ethanol-soluble supernatant of crude CPCF by dialysing the latter against trichloroacetic acid, collecting the precipitate and dissolving the latter in KOH. Dialysis against buffer gave a solution with high CPCF activity.

CPCF as a component of the bacterial cell wall

The results described above indicated that CPCF must be a large molecule or complex. However, its properties were not all consistent with it being either protein or

Fig. 9. Effect of lysozyme treatment on the activity of CPCF

Samples of CPCF, purified as described under Materials and Methods, were treated with lysozyme (1 mg/ml) in 0.1 M-Tris/HCl, pH 8.0, for 2 h. Samples (1 ml) were either frozen immediately or heated at 100 °C for 5 min and then frozen. Control samples of CPCF were incubated without lysozyme. All the samples were subsequently tested on axenic cells in the standard test system. (a) Buffer only, no CPCF; (b) untreated CPCF; (c) heat-treated CPCF; (d) lysozyme-treated CPCF; (e) lysozyme- and heat-treated CPCF. The Figure shows the inverse densitometric scans at 550 nm of the lanes loaded with the samples indicated. Apparent $10^{-3} \times M_r$ indicated on (a) and (b).

nucleic acid. The possibility that it might be a component of the bacterial cell wall was considered. Since both Gram-negative and Gram-positive species were capable of inducing the proteinase switch, it was unlikely that CPCF was a component specific to one group of bacteria. Nevertheless, in view of the reported effects of LPS on the β -N-acetylglucosaminidase activity of *D. discoideum* (Longmore & Watts, 1980), this Gram-negative specific factor was tested for CPCF-like activity. When added to axenic cells at a concentration of 0.5 mg/ml neither *E. coli* nor *S. typhimurium* LPS altered the A-pattern within the 4 h time period in which CPCF was effective. CPCF had no effect on the level of β -N-acetylglucosaminidase activity within 4 h of addition (results not shown), although it should be noted that Longmore & Watts (1980) did use considerably longer time periods in their study of the effect of LPS on the latter enzyme.

Peptidoglycan is the one cell wall component that is common to Gram-negative and Gram-positive bacteria, and experiments were carried out to test the possibility that it was involved in CPCF activity. Samples of CPCF were treated with lysozyme, an enzyme which specifically hydrolyses glycosidic bonds in peptidoglycan, and then tested for their effect on the proteinase pattern of axenic cells. Lysozyme treatment abolished the converting activity provided the samples were heated before being

tested (Fig. 9). The Figure shows the effect on a purified preparation of CPCF, but identical results were obtained when crude preparations of CPCF or suspensions of *M. lysodeikticus* were similarly treated (results not shown). In all cases, the activity was abolished by a combination of lysozyme and boiling. Thus CPCF activity was dependent on a molecule which was susceptible to attack by lysozyme which suggests strongly that it must have been peptidoglycan.

DISCUSSION

The phenomenon described here represents a novel aspect of control in *D. discoideum*. Cysteine proteinase activity is present in myxamoebae whatever the food source, but the nutrients have a pronounced effect on the nature of the physical forms of the enzymes which are present. Much of the evidence obtained to date is consistent with the view that, when bacteria or a bacterial factor are added the pre-existing A-form proteinases of axenic cells are converted into the B-forms normally found in bacterially grown cells, and that the changes are dependent on some alteration to the machinery involved in post-translational modification. An alternative mechanism, for which there is no experimental support, would require that bacteria induced synthesis *de novo* of B-form enzymes and this was tightly coupled to the loss of A-form enzymes. The loss would have to involve proteinase inactivation rather than release from the cells, since the appearance of A-form enzymes in the medium was not stimulated by CPCF (Fig. 6). Changes in the physical form of other hydrolytic enzymes of *D. discoideum* have been observed, but these occur during the developmental phase and not in response to an environmental factor such as a nutrient. The most notable involves a change in post-translational modification which affects a number of different hydrolytic enzymes (Bennett & Dimond, 1986; Livi *et al.*, 1987; Moore *et al.*, 1987). In this case, however, there is no conversion of pre-existing enzymes and the substrate for the new type of modification is restricted to newly synthesized proteins (Livi *et al.*, 1987).

The factor responsible for the cysteine proteinase changes was present in all four species of bacteria tested. The characterization of the cell-free factor CPCF, derived from *K. aerogenes*, clearly established that it was macromolecular, and the effect of lysozyme provided strong evidence in support of the view that its activity was dependent on peptidoglycan, a material found exclusively in bacteria. The involvement of a common bacterial component suggests that the proteinase changes may form part of a general response by the myxamoebae to the presence of bacteria. It will now be of interest to examine the relationship between the proteinase conversion and processes involved in feeding such as phagocytosis and digestion of bacteria, and to establish whether the mechanism of action of CPCF requires its internalization by endocytosis and if a surface receptor is required. This may provide clues as to how the initial detection of the inducer is coupled to the process(es) responsible for changing the enzyme forms.

A bacterial factor has previously been shown to be involved in the regulation of another hydrolase of *D. discoideum*. Lower β -N-acetylglucosaminidase levels in cells grown on Gram-negative bacteria than in axenic cells were accounted for by the presence of LPS, which

acts as a repressor of the enzyme (Longmore & Watts, 1980). The developmental increase in β -N-acetylglucosaminidase activity of bacterially grown cells can thus be explained by the withdrawal of repressor. In contrast, the formation of the B-form cysteine proteinases was dependent on the presence of a bacterial factor, and this raises two questions regarding the developmental changes in cysteine proteinase patterns in *D. discoideum* (North *et al.*, 1988). First, why does starvation of bacterially grown cells, and therefore withdrawal of inducer, not result in a change to the A-pattern? This could relate to the observation that a B-pattern to A-pattern switch does not readily occur when bacterially grown cells are transferred to axenic medium (Fig. 2), indicating a greater stability of the B-form enzymes *in vivo*. Cysteine proteinase activity decreases during development (North, 1985), which suggests that starvation prevents further synthesis of vegetative cysteine proteinases. The pre-existing B-form enzymes would thus remain as the only cysteine proteinases in bacterially grown cells during early development. The second question concerns why, in spite of the absence of a bacterially derived inducer, starvation of axenically grown cells results in a switch to a pattern which is similar to the B-pattern. It is possible that growth in axenic medium blocks the A- and B-pattern conversion but that the blockage can be overridden by bacteria. Starvation would result in withdrawal of repressor, thus allowing B-form enzymes to appear. Axenic growth is unnatural (wild-type strains can only feed on micro-organisms), and so the A-pattern could represent a phenomenon which is never seen in the wild. However, a suggestion that this may not be entirely so has come from experiments in which *D. discoideum* was incubated with buffered suspensions of baker's yeast which contained no other nutrients. Although growth was poor, some myxamoebae were recovered, and these had a proteinase pattern almost identical with that found in cells grown in axenic medium lacking glucose, that is an A-pattern (North, M. J., unpublished results). The latter may thus be characteristic of cells which are feeding on non-bacterial food sources.

The availability of a system in which physical forms of

enzymes can be so easily altered offers a number of unique advantages. First, it will be possible to investigate the cellular components responsible for generating differences in enzyme forms by comparing axenic cells before and after CPCF treatment. To this end, work is underway to raise antibodies specific to individual cysteine proteinase forms so that the relationships between the different enzymes can be probed directly by immunological techniques and the processes involved in their formation in the cells established. Secondly, it is already known that the A-form and B-form enzymes display differences in their properties (pH-dependence, inhibitor sensitivity) (North *et al.*, 1988). Using this system it should be possible to explore in detail the effect that variations in the structure of cysteine proteinases have on their specificity and on their function *in vivo*.

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