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A developmentally controlled change in the post-translational modifications on the lysosomal α-mannosidase of the cellular slime mould *Dictyostelium discoideum*

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During the development of the cellular slime mould *Dictyostelium discoideum*, a second form of a number of lysosomal enzymes begins to accumulate. The second ('late') form of these enzymes differs from the pre-existing ('early') form in post-translational modification. Pulse–chase experiments using [^{35}S]methionine show that the late form of α-mannosidase-1 is made by synthesis *de novo* starting 8 h after the onset of development. These experiments show there is no interconversion between early and late forms *in vivo*. A one-dimensional peptide map indicated that the early and late forms of α-mannosidase have similar amino acid sequences. The two forms have a similar half-life *in vivo* when measured during the same period of development. Double-labelling studies were performed with $^{35}SO_4$ and [^{3}H]leucine or $^{32}PO_4$ and [^{3}H]leucine, and these studies indicated that the oligosaccharides present on the early form of α-mannosidase contained more sulphate and phosphate than did those on the late form. The early enzyme had a 10-fold higher $^{35}S/^{3}H$ ratio and a 4-fold higher $^{32}P/^{3}H$ ratio. Endocytosis experiments using early and late α-mannoside showed that the early form was efficiently taken up by human fibroblasts, whereas the late form was poorly endocytosed. This suggests that the late form lacks the mannose 6-phosphate residue required for efficient uptake.

INTRODUCTION

Dictyostelium discoideum is a cellular slime mould that lives vegetatively as a single-cell amoeba, ingesting bacteria as its natural food source. Under conditions of starvation the amoebae aggregate to form a group of approx. 10^5 cells. The aggregate then undergoes a series of morphological and biochemical changes resulting in the formation of a fruiting body consisting of a stalk and a sorocarp containing spores. This developmental phase of *Dictyostelium* is completed in 24–28 h and provides an ideal system to study developmental phenomena (Loomis, 1975).

It has been shown that there is transcriptional and translational control during the developmental phase. Blumberg & Lodish (1980*a*,*b*, 1981) have reported the production of approx. 2500 new gene products during differentiation. Many lysosomal enzymes are known to be controlled by the developmental programme (Loomis, 1975). Lysosomal α-mannosidase (EC 3.2.1.24) has been studied in some detail. It is synthesized as a 140 kDa precursor molecule. This precursor is then modified and proteolytically processed into two subunits of 60 kDa and 58 kDa (Pannell *et al.*, 1982; Mierendorf *et al.*, 1983). The holoenzyme exists as a tetramer consisting of two 60 kDa and two 58 kDa subunits. Not only does the level of α-mannosidase change during development, but the post-translational modifications present on the molecules also change. Although both the 'early' and 'late' forms of α-mannosidase have extremely low pI values, it has been shown that the two forms differ in charge (Moore *et al.*, 1985; Knecht *et al.*, 1985). The low pI value of α-mannosidase is due to the extensive phosphorylation and sulphation of high-mannose oligosaccharides (Freeze & Miller, 1980; Freeze *et al.*, 1983). Free *et al.* (1978) have shown that in addition to phosphorylated oligosaccharides, α-mannosidase contains phosphoserine and phosphothreonine. The phosphate moieties on the carbohydrates are present as an unusual methyl-phosphomannose residue in which a methyl group is an ester linkage to the phosphate (Gabel *et al.*, 1984).

Here we present evidence that the late form of α-mannosidase is made by synthesis *de novo* and that the degradation rates of the early and late enzyme are similar. We show that the lower net negative charge of the late form of α-mannosidase as compared with the early form is, at least in part, due to a decrease in the number of sulphate and phosphate residues present in the oligosaccharides.

MATERIALS AND METHODS

Materials

Proteinases, Fast Garnet GBC salt, *p*-nitrophenyl α-D-mannopyranoside and concanavalin A were purchased from Sigma (St. Louis, MO, U.S.A.). Naphthyl α-D-mannoside was a product of Koch–Light (Haverhill, Suffolk, U.K.). Materials for polyacrylamide-gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Fluorography materials were purchased from National Diagnostics (Somerville, NJ,

Abbreviation used: HBSS, Hanks balanced salt solution.
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U.S.A.). Radioactively labelled methionine was obtained from New England Nuclear (Boston, MA, U.S.A.). Radioactively labelled sulphur, phosphate and leucine were obtained from ICN Radiochemicals (Irvine, CA, U.S.A.).

Organism and development

Strain AX3 of *Dictyostelium discoideum* was grown in axenic cultures in HL-5 medium [1% (w/v) 'proteinase peptone'/0.5% (w/v) yeast extract/1% (w/v) glucose/ 2.5 mM-Na_2HPO_4/2.5 mM-KH_2PO_4, pH 6.5]. To initiate development, cells were harvested by centrifugation, washed with PDF buffer [20 mM-KCl/5.3 mM-$MgCl_2$/ 9.2 mM-KH_2PO_4/0.1% streptomycin sulphate, pH 6.4) and 1×10^8 cells were placed on nitrocellulose filters supported by two absorbent pads (Sussman, 1966). Cells were incubated under constant illumination at 21 °C.

α-Mannosidase assay and polyacrylamide-gel electrophoresis

α-Mannosidase activity was assayed as described previously using *p*-nitrophenyl α-D-mannopyranoside as the substrate (Loomis, 1970). One unit of activity was defined as the hydrolysis of 1 μmol of substrate/min at 35 °C. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Cellular extracts were prepared from cell suspensions by adding Triton X-100 to a final concentration of 0.35%. When immunoprecipitated α-mannosidase in non-denaturing gels was analysed, the enzyme was released from the IgG by resuspending the immunoprecipitates in 50 μl of 200 μg of proteinase (*Streptomyces griseus*, type VI, in 10 mM-NaCl/10 mM-Tris/HCl, pH 7.4)/ml and incubating for 30 min at 37 °C. The sample was then spun in a Fischer model 235B Microfuge for 5 min. Greater than 95% of the initial α-mannosidase activity was found in the supernatant and could be analysed by staining non-denaturing polyacrylamide gels with naphthyl α-D-mannoside and Fast Garnet GBC salt as previously described by Knecht *et al.* (1985). SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970). Fluorography was performed by submerging the gel in Autofluor (National Diagnostics), drying the gel on a Bio-Rad model 334 slab-gel dryer and exposing the gel with Kodak XAR-5 film at −70 °C. Radioactive bands were excised from the gel, solubilized, and counted for radioactivity in a Beckman LS7000 liquid-scintillation counter (Mahin & Lofberg, 1966).

Immunoprecipitation

Monoclonal antibody 2H9, an antibody directed against *Dictyostelium* α-mannosidase, was kindly given by Dr. R. C. Mierendorf and Dr. R. L. Dimond (Mierendorf *et al.*, 1983). The 2H9 antibody is able to recognize both the precursor and mature forms of α-mannosidase. α-Mannosidase was immunoprecipitated as previously described (Knecht & Dimond, 1981) with minor modifications. The buffer used for immunoprecipitation was 10 mM-Tris/HCl/10 mM-NaCl/0.01% bovine serum albumin/2 mM-methionine, pH 7.4 (referred to hereafter as 'TNB' buffer). Briefly, 50 μl of a 10% suspension of *Staphylococcus aureus* cells (Pansorbin; Calbiochem–Behring) in TNB buffer was added to all extracts and incubated for 30 min on ice. The bacteria were removed by centrifugation and the extracts transferred to new tubes. Antibody was added to the extracts and the tubes were incubated for 15 min at room temperature. The tubes were then placed on ice and, after a 30 min incubation, Pansorbin (in TNB buffer) was added. After Pansorbin addition, the tubes remained on ice for another 15 min. Immunoprecipitation complexes were collected through a 30% (w/v) sucrose (in TNB buffer) cushion by layering the sucrose under the sample and centrifuging for 4 min in a Microfuge (Fisher model 235B). The immunoprecipitated pellets were then washed at least five times with TNB buffer. These operations were carried out at 4 °C. The immunoprecipitation from labelled cell extracts was performed as described above, except that the buffer used was 50 mM-Tris/HCl/ 150 mM-NaCl/5 mM-EDTA/0.5% Nonidet P40/2 mM-methionine/0.02% NaN_3, pH 7.6.

Labelling of cells

Cells were labelled by placing nitrocellulose filters with 1×10^8 cells at various stages of development into Petri dishes spotted with 30–50 μl of PDF buffer containing the indicated amount of [^{35}S]methionine (New England Nuclear; sp. radioactivity 1000 mCi/mmol). The cells were allowed to incorporate the labelled amino acid for 60 min. Cells were harvested either immediately after labelling or after a chase. For pulse–chase experiments the filters were placed back on absorbent pads containing 10 mM-unlabelled methionine in PDF buffer after the pulse-labelling. Cells were harvested by scraping them off filters and placing them in 0.5 ml of ice-cold PDF. Cells were collected by centrifugation in a Microfuge and washed twice with ice-cold PDF. Cells were then resuspended in 0.2 ml of PDF, lysed by the addition of Triton X-100, and frozen at −20 °C. Trichloroacetic acid precipitation was performed by adding a given amount of sample to 1 ml of bovine serum albumin (1 mg/ml), adding 1 ml of ice-cold 20% trichloroacetic acid and incubating for 15 min on ice. Trichloroacetic acid-insoluble material was collected on a Whatman GF-B glass-fibre filter. The filter was washed extensively with cold 10% trichloroacetic acid and dried. The filters were counted for radioactivity after the addition of 10 ml of Liquiscint (National Diagnostics).

When double labelling with $^{35}SO_4$ and [^{3}H]leucine, nitrocellulose filters with 10^8 cells were placed on to 30–50 μl of PDF (without the streptomycin sulphate) containing the indicated amount of $^{35}SO_4$ (800 Ci/mmol) and [^{3}H]leucine (40–60 Ci/mmol). After the cells had been labelled for 1 h, the chase was initiated by placing the filters on pads soaked in PDF buffer supplemented with 10 mM-leucine and 10 mM-$MgSO_4$. At various times cells were harvested and the α-mannosidase was immunoprecipitated and subjected to native or SDS/ polyacrylamide-gel electrophoresis. When double labelling with $^{32}PO_4$ and [^{3}H]leucine the filters were placed on to 50 μl of 10 mM-Mes/PDF (Mes was substituted for the potassium phosphate normally found in PDF) containing the indicated amount of radioactivity. The cells were labelled for 1 h and the chase was initiated by placing the filters on pads soaked in Mes/PDF containing 10 mM-leucine and 10 mM-KH_2PO_4. In labelling experiments where cells were labelled at 4 h and at 14 h of development we found that the amounts of [^{3}H]leucine, [^{35}S]methionine, and $^{32}PO_4$ incorporated into trichloroacetic acid-precipitable material per mg of protein were similar (within 20%) for the two labelling periods. As

previously reported by Ivatt *et al.* (1984), we found that the incorporation of $^{35}SO_4$ into trichloroacetic acid-precipitable material decreased during development.

Proteolytic digestions

Gel slices from the SDS/polyacrylamide gels containing the α-mannosidase subunits were equilibrated in 0.125 mM-Tris/HCl (pH 6.8)/0.1% SDS/1 mM-EDTA, loaded in the wells of a second gel and digestion and electrophoresis were performed as described by Cleveland *et al.* (1977).

Sepharose 4B–concanavalin A column chromatography of glycopeptides

α-Mannosidase labelled with [^{3}H]leucine and $^{35}SO_4$ was immunoprecipitated and subjected to SDS/polyacrylamide-gel electrophoresis. The subunits of each form were excised from the gel and electroeluted. The electroeluted samples were dialysed against water and freeze-dried. A 0.5 ml portion of Pronase buffer [100 mM-Tris/HCl (pH 8.0)/10 mM-$CaCl_2$] containing 2 mg of Pronase (*Streptomyces griseus* proteinase, type VI) was added to each sample. Whereas the native holoenzyme is quite resistant to proteinase (Moore *et al.*, 1985) the denatured subunits are easily digestible. Digestion took place at 37 °C for 48 h, after which an additional 2 mg of Pronase was added and the incubation was continued for 20 h. The proteinase preparation was devoid of sulphatase activity and contained a minimum of phosphatase activity as determined by assays with *p*-nitrophenol substrates. The samples were boiled for 5 min and centrifuged in the Microfuge for 5 min to remove insoluble material. Before applying the samples to the column they were first diluted with stock solutions of NaCl and $MgCl_2$ to give the same concentrations of ions as in the concanavalin A column buffer. Samples were applied to a 1 cm Sepharose 4B–concanavalin A column that had previously been equilibrated with column buffer [100 mM-Tris/HCl (pH 8.0)/10 mM-$CaCl_2$/0.5 M-NaCl/1.25 mM-$MgCl_2$]. Column chromatography was carried out at room temperature. After sample application, the column was washed extensively with buffer, and 200 μl fractions were collected. Bound material was specifically eluted with 0.3 M-α-methyl D-mannoside in buffer.

Endocytosis of *Dictyostelium* α-mannosidase

Cultures of human skin fibroblasts from a patient with mannosidosis (GM-654; Genetic Mutant Cell Repository, Camden, NJ, U.S.A.) were grown to confluency in 25 cm^2 plastic flasks (Corning Glass Co., Corning, NY, U.S.A.) in Ham's F10 medium/15% (v/v) fetal-calf serum/kanamycin (20 μg/ml) (Grand Island Biologicals, Grand Island, NY, U.S.A.). Uptake experiments were performed by aspirating the medium and adding 1200 units of partially purified early or late α-mannosidase in 2.5 ml of Ham's F10 (serum-free) medium+0.25% human serum albumin, pH 6.5. The two forms of α-mannosidase were partialy purified as described previously (Moore *et al.*, 1985). Cell cultures were then incubated for 4 h at 37 °C, the culture fluid was aspirated and the cells washed with HBSS (Grand Island Biologicals). Cells were harvested by treatment with 0.25% trypsin in HBSS for 10 min, washed twice with HBSS, suspended in 75 μl of 50 mM-citrate/phosphate buffer, pH 4.5, and sonicated for three 15 s bursts while on ice. The fluorogenic substrate 4-methylumbelliferyl α-D-mannopyranoside was used for the assay of α-mannosidase. The assay was performed with 2.7 mM-substrate in 50 mM-citrate/phosphate buffer, pH 4.5. For the uptake experiments, one enzyme unit was defined as the activity liberating 1 μmol of 4-methylumbelliferone/h at 37 °C. Protein concentrations were measured by Coomassie Blue dye binding using bovine serum albumin as a standard (Bradford, 1976).

RESULTS

Late-form α-mannosidase arises *de novo*

It has been reported that the late form of α-mannosidase begins to appear at 8 h of development (Moore *et al.*, 1985; Knecht *et al.*, 1985). As shown in Fig. 1, lane 1, cells labelled at 4 h of development synthesize early-form enzyme. The faint band of radioactive material above the major band seen in Fig. 1, lane 1, was not routinely seen in other experiments and may represent α-mannosidase that was not completely freed from the IgG. If the late form arises by an intracellular conversion from early-form enzyme, we would expect that, during a chase period, some of the enzyme labelled at 4 h would be converted into the late form. There was no detectable conversion of labelled early-form enzyme into the late form during subsequent development (Fig. 1, lanes 2 and 3). Labelling after 8 h of development provided further evidence that late enzyme is synthesized *de novo*. As shown in Fig. 1, lane 4, all of the α-mannosidase immunoprecipitated from cells labelled at 14 h of development was in the late form, and there is no evidence of an early-form precursor. In

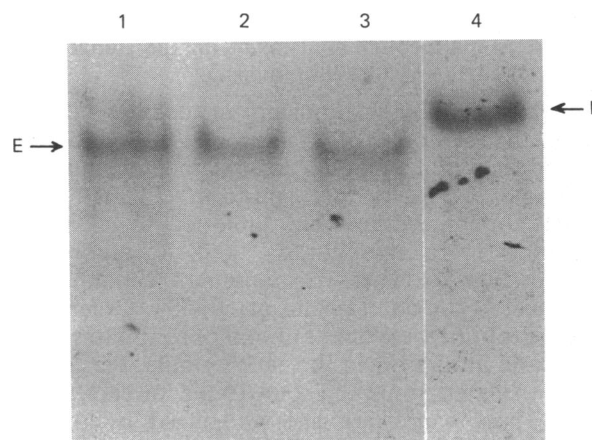

Fig. 1. Native polyacrylamide-gel electrophoresis of immunoprecipitated α-mannosidase

Cells were labelled for 1 h with 300 μCi of [^{35}S]methionine at 4 h or 14 h of development and then allowed to continue development in the presence of unlabelled methionine for the indicated time. The cells were harvested and the α-mannosidase was immunoprecipitated and subjected to electrophoresis in a non-denaturing ('native') gel. Fluorography was performed and the film was exposed for 30 days. Arrows indicate positions of early (E) and late (L) enzyme. Lanes 1, 2, and 3 contain enzyme from cells labelled at 4 h, whereas lane 4 contains enzyme from cells labelled at 14 h. Lane 1, 0 h chase; lane 2, 5 h chase; lane 3, 10 h chase; lane 4, 0 h chase.

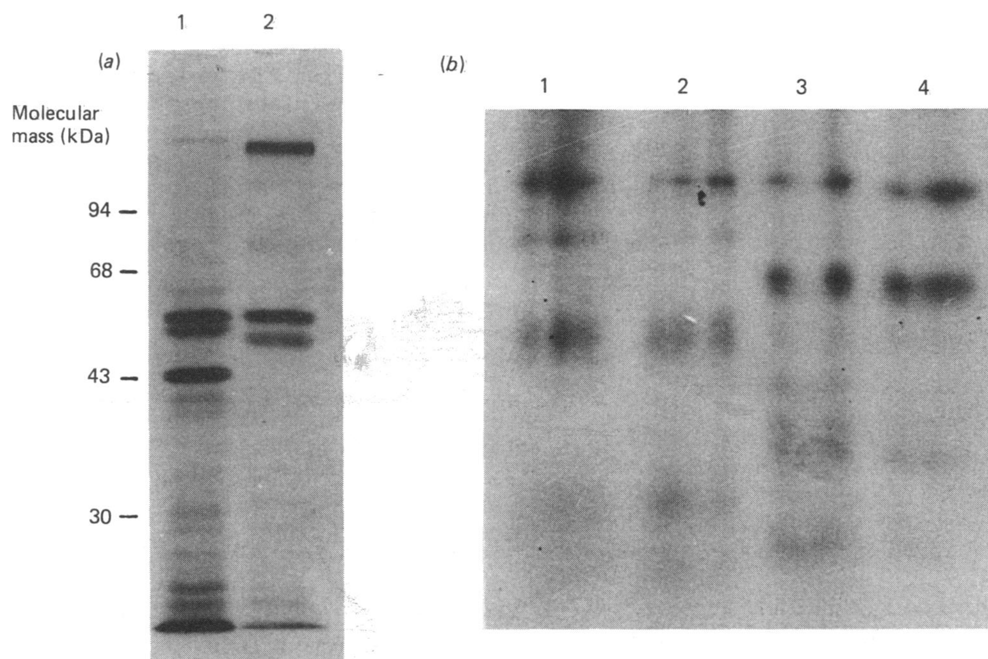

Fig. 2. One-dimensional peptide analysis of early and late α-mannosidase

Cells were labelled for 1 h with 500 μCi of [^{35}S]methionine at 4 h and at 14 h of development and then allowed to continue development for an additional 1 h in the presence of unlabelled methionine. α-Mannosidase was immunoprecipitated and subjected to SDS/polyacrylamide-gel electrophoresis. Proteolytic digestions were carried out by using 2 μg of chymotrypsin. (*a*) Gel from which early and late subunits were excised and used for proteolytic digestions; lane 1, early enzyme; lane 2, late enzyme. (*b*) Fluorograph of the products of digestion of individual subunits of enzymes: lane 1, early 60 kDa; lane 2, late 60 kDa; lane 3, early 58 kDa; lane 4, late 56 kDa.

experiments where cells were labelled at 14 h and development was allowed to continue, it was clear that the late enzyme was not converted into the early form.

One-dimensional peptide analysis of the individual subunits of early and late α-mannoside

To examine the relationship in peptide sequence between the two forms of α-mannosidase, chymotrypsin was used to obtain a one-dimensional peptide map (Fig. 2). Samples of early- and late-form [^{35}S]methionine-labelled α-mannosidase were subjected to SDS/polyacrylamide-gel electrophoresis and a fluorograph prepared (Fig. 2*a*). The two subunits of α-mannosidase are seen at 56 or 58 kDa and at 60 kDa. Other bands which we routinely see in our immunoprecipitates are the major band at 43 kDa in the early sample, the high-M_r band in the late sample and some of the lower-M_r minor bands. These bands are not recognized by the 2H9 antibody in Western blots. We have not been able to assign proteins to these bands. The α-mannosidase subunits were excised from the gel, peptide fragments were generated by proteinase treatment, and the fragments separated by SDS/polyacrylamide-gel electrophoresis as described by Cleveland *et al.* (1977). As shown in Fig. 2(*b*) (a fluorograph of the [^{35}S]methionine-labelled fragments), the peptide fragments generated from the large subunit (60 kDa) of early and late enzyme are indistinguishable. This is also true for the smaller (58 kDa and 56 kDa) subunits of the enzymes, except that some of the fragments from the late subunit are slightly smaller than those generated from the early subunit. Since the pattern of the peptides is indistinguishable, this small difference in M_r is likely to be due to differences in the oligosaccharides present on the enzyme.

Late enzyme differs from early enzyme in the level of sulphation and phosphorylation

It has been reported that early α-mannosidase is highly sulphated and phosphorylated (Freeze & Miller, 1980; Freeze *et al.*, 1980, 1983; Knecht *et al.*, 1984; Cladaras & Kaplan, 1984; Ivatt *et al.*, 1984). An experiment in which cells were labelled with $^{35}SO_4$ and [^{3}H]leucine and the α-mannosidase immunoprecipitated is shown in Fig. 3. There was substantial incorporation of $^{35}SO_4$ into the early-form, but very little into the late-form, enzyme. The ratio of ^{35}S incorporated to ^{3}H incorporated was 10-fold higher in the early form than in the late form. The radioactivity shown in Fig. 3 co-migrated with immunoprecipitated early- and late-form α-mannosidase that was stained for activity in adjacent lanes.

A similar double-labelling experiment was performed using $^{32}PO_4$ and [^{3}H]leucine, and the labelled immunoprecipitated α-mannosidase was analysed by SDS/polyacrylamide-gel electrophoresis. Gel slices containing both radioactively labelled subunits of α-mannosidase were excised, solubilized and counted for radioactivity in a liquid-scintillation counter. A 4-fold decrease in the incorporation of $^{32}PO_4$ into late-form enzyme was found. The ^{32}P/^{3}H ratio in the early form was 1.32, whereas that for late α-mannosidase has a ratio of 0.32.

Column chromatography of α-mannosidase-derived glycopeptides on Sepharose 4B–concanavalin A

A column of Sepharose 4B–concanavalin A was used to investigate whether there was a difference in the

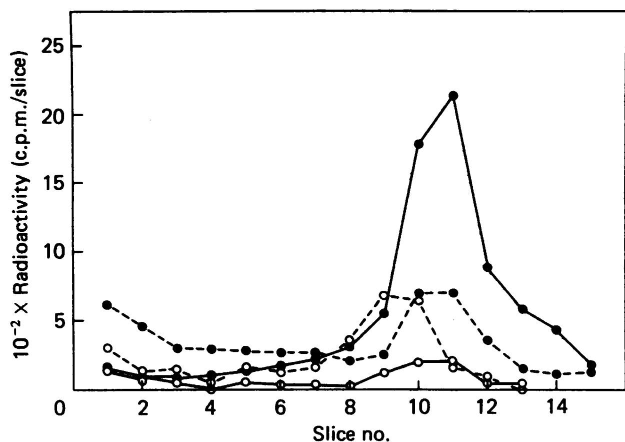

Fig. 3. Late enzyme differs from early enzyme in the level of sulphation

Cells were labelled for 1 h starting at 4 h (early) or 14 h (late) with 2 mCi of $^{35}SO_4$ and 2 mCi of [^{3}H]leucine and then allowed to continue development for an additional 30 min in the presence of unlabelled leucine and $MgSO_4$. Immunoprecipitated α-mannosidase from the two samples were subjected to non-denaturing polyacrylamide-gel electrophoresis and the lanes were sliced into 2 mm sections; these were solubilized and counted for ^{35}S and ^{3}H radioactivity. ●——●, ^{35}S, early; ●----●, ^{3}H, early; ○——○, ^{35}S, Late; ○----○, ^{3}H, late.

number of sulphate and phosphate residues on the oligosaccharides of the two enzyme forms. Fig. 4 shows an experiment in which cells were labelled with $^{35}SO_4$ and [^{3}H]leucine. The α-mannosidase was immunoprecipitated, purified by electrophoresis in SDS/polyacrylamide-gels, and digested with proteinase. The glycopeptides of early and late enzymes were loaded on to the concanavalin A column, and bound and unbound material was eluted and counted for ^{35}S and ^{3}H radioactivity. As shown in Fig. 4(*a*), 42% of the $^{35}SO_4$ from early α-mannosidase bound to the column and was specifically eluted with α-methyl-D-mannoside, indicating that this $^{35}SO_4$ was incorporated into oligosaccharide. Since all the ^{3}H was eluted as unbound material, the enzyme was completely digested by the proteinase. All of the ^{35}S incorporated into the late forms was eluted from the column as unbound material (Fig. 4*b*).

Endocytosis of early and late α-mannosidase

Several *Dictyostelium* lysosomal enzymes from axenically grown cells (early-form enzymes) are efficiently endocytosed via the mannose 6-phosphate-receptor system (Freeze *et al.*, 1980, 1983). Preparations of early-form and late-form α-mannosidase were prepared as described by Moore *et al.* (1985) and used to determine the extent of endocytosis by a human α-mannosidosis fibroblast cell line. As shown in Table 1, early α-mannosidase was efficiently taken up by the fibroblasts, and this endocytosis was inhibited by the addition of mannose 6-phosphate during the uptake period. The late form of α-mannosidase was endocytosed poorly, suggesting that late enzyme either lacks the mannose 6-phosphate residue or this residue is blocked and unable to participate in receptor binding.

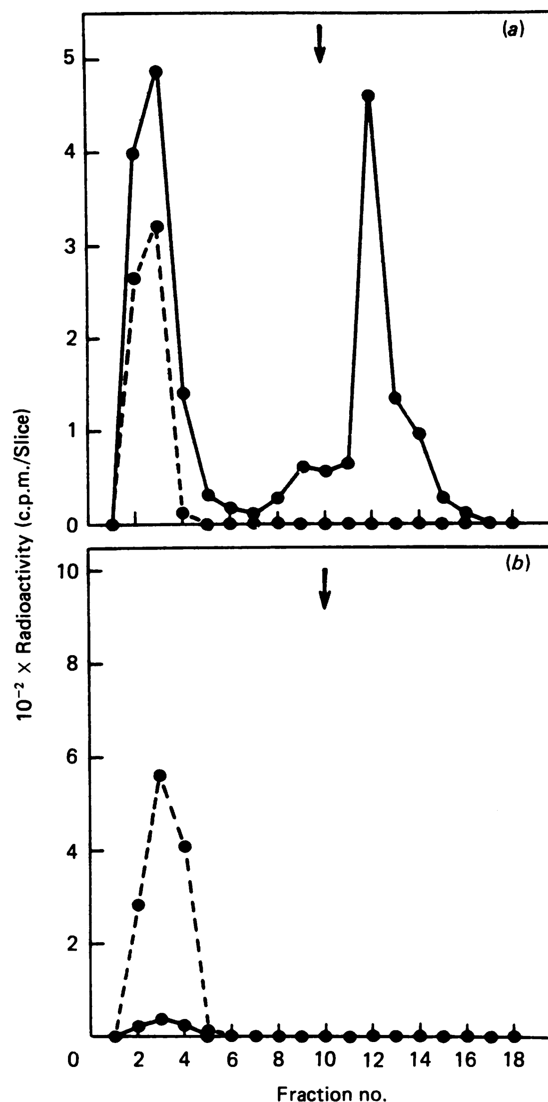

Fig. 4. Concanavalin A column chromatography of glycopeptides from early and late enzyme labelled with $^{35}SO_4$ and [^{3}H]leucine

Cells were labelled for 1 h at 4 h (*a*) and 14 h (*b*) of development with 7.5 mCi of $^{35}SO_4$ and 2 mCi of [^{3}H]leucine and then allowed to continue development for an additional 30 min in the presence of unlabelled leucine and $MgSO_4$. Glycopeptides were prepared and subjected to chromatography on a Sepharose 4B–concanavalin A column. The bound material was eluted with 0.3 M-α-methyl D-mannoside. The arrow indicates start of elution with α-methyl D-mannoside. ●----●, ^{3}H; ●——●, ^{35}S.

Degradation rates for early and late α-mannosidase are similar

To assess how the changing of modification affects the fate of α-mannosidase, a number of experiments were performed in order to compare the degradation and secretion rates of the two α-mannosidase forms. Cells were labelled with [^{35}S]methionine for 1 h at 4 and 14 h of development. After the labelling, the cells were allowed to continue development in the presence of unlabelled methionine and collected between 17 and 27 h of development. In this way, the degradation and

Table 1. Endocytosis of early and late α-mannosidase

The endocytosis experiment was performed as described in the Materials and methods section. Results are means±S.E.M. Abbreviations: N.D., not determined; Man6*P*, mannose 6-phosphate.

	Activity (units/mg)*	
Enzyme	No additions	+Man6*P* (2 mM)
Early	13.2±0.89	0
Late	1.78±0.12	N.D.

* The activity represents specific activity after subtraction of activity due to endogenous enzyme.

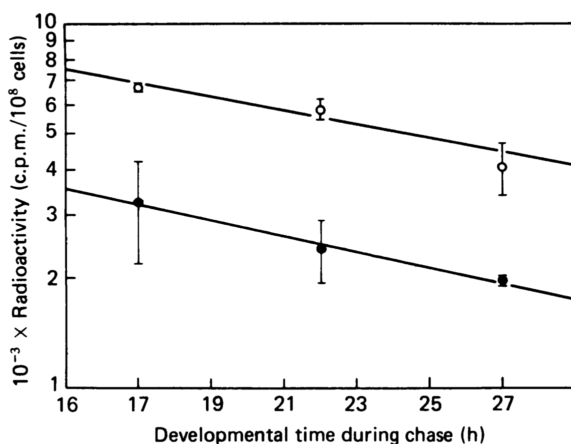

Fig. 5. Early and late α-mannosidase degradation rates

Cells were labelled with 300 μCi of [^{35}S]methionine at 4 h (●——●) and at 14 h (○——○) of development and then development was allowed to continue in the presence of unlabelled methionine until the indicated times. Cells were collected and the α-mannosidase was immunoprecipitated and subjected to polyacrylamide-gel electrophoresis under non-denaturing conditions. The enzyme bands were excised from the gel and the radioactivity associated with these bands was counted as described in the Materials and methods section. Each point on the graph represents the data from two independent experiments (each experiment having two or three samples for each time point) and the line was plotted by using linear regression.

secretion rate of the two forms of enzyme could be measured and compared during the same developmental time. As shown in Fig. 5, both forms of α-mannosidase had similar degradation rates. The early form had a half-life of 13.5 h, which was slightly shorter than the 15 h half-life of the late form.

The half-life of the individual subunits of early and late enzyme were determined by immunoprecipitation of labelled enzyme from a pulse–chase experiment and subjecting the immunoprecipitate to SDS/polyacrylamide-gel electrophoresis. The half-life of the 60 kDa subunit was found to be 12 h for the early-form enzyme and 13.2 h for the late form. These half-lives are in reasonable agreement with the half-lives of the holoenzymes. The lower-M_r subunits of the early and late enzyme were found to have half-lives of 8.2 h and 6.7 h respectively. In these experiments we noticed that a radioactively labelled 37 kDa protein appeared during the chase period for both early-labelled and late-labelled samples. This 37 kDa protein is recognized by the 2H9 antibody on Western blots. The 37 kDa α-mannosidase fragment has been previously noted by Livi *et al.* (1985).

When conducting these pulse–chase experiments, we also determined the loss of enzyme from the cell via secretion. It was found that less than 10% of the labelled enzyme was secreted during the course of the experiment (17–27 h of development). Of the enzyme that was secreted, the early form predominated.

DISCUSSION

Lysosomal enzymes from a number of different cell types, including *Dictyostelium discoideum*, are known to be highly modified. During the development of *Dictyostelium*, a unique situation arises wherein the cell begins to accumulate a second form of several lysosomal enzymes. This phenomenon has been noted for α-mannosidase, acid phosphatase, *N*-acetylglucosaminidase and β-galactosidase-2 (Oohata, 1983; Loomis & Kuspa, 1984; Knecht *et al.*, 1985; Moore *et al.*, 1985). The second form (late) of these enzymes appears at 8 h of development. We present evidence that the late form of α-mannosidase is made by synthesis *de novo*. Our data would suggest that the system used to modify lysosomal enzymes changes as part of the developmental programme. No evidence could be found for an intralysosomal conversion of the enzyme form. As a result of this changing of the modification system, the cell contains two discrete populations of each of these lysosomal enzymes that differ in their post-translational modification. This finding has prompted us to ask how the two forms differ and how post-translational modification might affect the fate of an enzyme.

As previously reported, the two forms of α-mannosidase, as well as other lysosomal enzymes, differ in their pI (Knecht *et al.*, 1985; Moore *et al.*, 1985). The late enzyme has been shown to be less negatively charged than the early form. In order to discover the possible reasons for this difference, cells were labelled with [^{3}H]leucine and $^{35}SO_4$, and the incorporation of labelled material into α-mannosidase was investigated. It was found that the ^{35}S/^{3}H ratio was 10-fold higher for the early form than for the late form. Concanavalin A column chromatography of glycopeptides derived from the early form showed that at least 40% of the $^{35}SO_4$ incorporated into the molecule was associated with oligosaccharides. All of the sulphate associated with the late enzyme was eluted from the concanavalin A column as unbound material. These results suggest that the late-form enzyme contains little, if any, sulphate associated with its oligosaccharides.

Inasmuch as most of the sulphate is known to be incorporated into glycoproteins (Ivatt *et al.*, 1984), it might seem surprising that some 58% of the $^{35}SO_4$ in the early-form α-mannosidase glycopeptide preparation was eluted from the concanavalin A column as unbound material. It is noteworthy that Freeze & Miller (1980) found that 40% of [^{3}H]mannose-labelled glycopeptides of early-form α-mannosidase did not bind to a

concanavalin A column. Freeze & Wolgast (1986) have shown that some sulphated dictyostelial oligosaccharides do not bind to concanavalin A. Thus it is likely that some of the unbound ^{35}S was associated with oligosaccharides that did not bind to the column. Since all glycopeptides from *Dictyostelium* studied thus far contain only high-mannose oligosaccharides, it is unlikely that the $^{35}SO_4$ is incorporated into complex or hybrid-type oligosaccharides. A second possibility is that the $^{35}SO_4$ might be bound to tyrosine, the only amino acid known to be sulphated (Wold, 1981). Another possible explanation is that some of the $^{35}SO_4$ was removed by sulphatases during the proteolysis generating the glycopeptides. However, attempts to release the SO_4 and PO_4 by treatments with phosphatases and sulphatases have been unsuccessful, and assay of the proteinase preparation for sulphatase using the *p*-nitrophenol substrate showed the preparation to be devoid of sulphatase activity. Further testing of the unbound-material fraction will be necessary to identify the source of the $^{35}SO_4$.

In double-labelling experiments using [^{3}H]leucine and $^{32}PO_4$ the early-form α-mannosidase was found to have a 4-fold higher ratio of $^{32}PO_4$ to [^{3}H]leucine than the late form. Early-form α-mannosidase is endocytosed by human fibroblasts via the mannose 6-phosphate-receptor system (Freeze *et al.*, 1980, 1983). Gabel *et al.* (1984) have shown that the mannose 6-phosphate is covered by a methyl group, creating a methylphosphomannose residue. This methyl group affects, but does not abolish, uptake by the mannose 6-phosphate receptor (Freeze, 1985). Unlike the early form, the late enzyme was not endocytosed by the human fibroblast cell line. This lack of endocytosis suggests that the late enzyme does not contain substantial amounts of the mannose 6-phosphate residue. The reduced, but measurable, level of $^{32}PO_4$ incorporation into the late enzyme could be due to the previously reported existence of phosphorylated amino acids (Free *et al.*, 1978). Further experimentation will be needed to determine the nature of the sulphate and phosphate associated with the late-form enzyme. Our data indicate that the difference in the charge of the molecules is, at least in part, due to a difference in the amount of sulphate and phosphate incorporated into these molecules.

Both forms of α-mannosidase are the products of a single structural gene (Free *et al.*, 1976). We considered the possibility that some differences in amino acid sequence could be generated by differences in processing of either the mRNA or the polypeptide. Thus a one-dimensional peptide map was generated. SDS/polyacrylamide-gel electrophoresis of the fragments generated by a chymotryptic digest of α-mannosidase subunits showed no difference in the pattern of peptides generated from early-form and late-form enzymes (Fig. 2*b*). The small differences which we did see could be easily explained on the basis of differences in oligosaccharides associated with the peptides.

Post-translational modifications are known to affect the rate at which some proteins are degraded. Mutations in post-translational modification affect the fate *in vivo* of some *Dictyostelium* lysosomal enzymes (Free & Schimke, 1978). Thus the rate of degradation *in vivo* for the two forms of α-mannosidase was measured. Our data showed a 13.5 h and 15 h half-life for the early- and late-form holoenzymes respectively. The small difference in degradation rates is unlikely to be of physiological importance during the 24–28 h of *Dictyostelium* development.

Rates of degradation *in vivo* for the individual α-mannosidase subunits were also determined. The large subunit was found to have a half-life of 12 h (early form) and 13.2 h (late form). This is in good agreement with the holoenzyme data. The smaller subunit was found to have a half-life of 8.2 h (early form) and 6.7 h (late form). We hypothesize that the smaller subunit is 'clipped' to generate the 37 kDa α-mannosidase fragment previously noticed by Livi *et al.* (1985) and which we observed appearing in our immunoprecipitates from samples during the chase period of pulse–chase experiments (results not shown). The clipped fragments from the smaller subunit would be retained in the holoenzyme. This would explain the apparent discrepancy between the half-lives for the smaller subunit and for the large subunit and holoenzyme.

We have shown that, as part of its developmental programme, *Dictyostelium* alters the form of oligosaccharide-associated modification that newly synthesized α-mannosidase receives. This change in the modification on newly synthesized enzyme may be due to a loss of the endoplasmic-reticulum and Golgi-apparatus transferases that add sulphate and phosphate to the oligosaccharide, or it could be due to a change in the processing steps preceding sulphate and phosphate addition such that the enzyme-associated oligosaccharides are no longer substrates for these transferases. The physiological consequences of changing the oligosaccharide-associated modifications on newly synthesized enzymes are unclear.

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