Linked Pools of Processed $\alpha$-Mannosidase in Dictyostelium discoideum*

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We are studying the fate of $\alpha$-mannosidase, a lysosomal enzyme, in Dictyostelium discoideum. $\alpha$-Mannosidase is synthesized as a 150,000-dalton precursor which becomes proteolytically cleaved to mature (56,000–62,000 dalton) forms. When cells are shifted into starvation buffer (5 mM phosphate, pH 6.5), the enzyme is secreted. We compared the kinetics of secretion of newly processed $\alpha$-mannosidase with that of bulk forms. After 2 h in phosphate buffer less than 20% of the newly processed forms but as much as 50% of the bulk enzyme activity was secreted. During the course of the experiments, the total $\alpha$-mannosidase activity remains constant, cells remain viable, and there is no evidence of degradation of enzyme. Furthermore, a 4-h chase period prior to starvation is required before the newly processed forms are secreted to the same extent as the bulk forms. On the basis of these results we propose that the enzyme is present in at least two pools and that it is transferred from a newly processed to an efficiently secreted pool.

Cells secrete either uncleaved precursor or mature forms of several lysosomal enzymes. Most precursor molecules enter functional lysosomes, but a minor fraction has been shown to be transported directly to the media by fibroblasts (1, 2), Chinese hamster ovary cells (3, 4), and porcine kidney cells (5, 6). The extent of secretion of precursor forms is much greater in cells which are genetically defective in elements of the phosphomannosyl recognition system (2, 4) or in cells which have been poisoned with amines (1, 7).

In normal cells most precursor molecules enter functional lysosomes where they are soon converted by proteases to mature products. For example, pulse-labeled $\alpha$-iduronidase precursors were found in an endoplasmic reticulum fraction of human fibroblasts and could be chased into a lysosomal fraction in which they were cleaved (8). In vitro experiments of Frisch and Neufeld (9) lend support to these ideas.

The contents of functional lysosomes are secreted under certain conditions. For example, kidney cells have been shown to exocytose lysosomal enzymes in a coordinated manner (10), particularly in response to hormonal stimulation (11). Macrophages and polymorphonuclear leukocytes secrete enzymes after stimulation by zymosan granules (12), and mast cells secrete histamine and acid hydrolases in response to a variety of secretagogues (13). Although it often has gone underrecognized, it is reasonable to assume that these secreted hydrolases are mature cleaved forms. We have reported the secretion of both unprocessed precursors and mature forms of $\alpha$-mannosidase by D. discoideum (14). Analysis of the latter is the focus of this paper.

D. discoideum synthesizes acid hydrolases which either accumulate intracellularly or in the medium. Measurements of changes in net enzyme activity levels indicated that the extent and rate of secretion of these enzymes is controlled by genetic (15), developmental (16), and nutritional factors (17). For example, $\alpha$-mannosidase, $\beta$-hexosaminidase, and $\beta$-glucosidase are extensively secreted when cells are shifted from growth medium to starvation buffer. We previously reported on the biosynthesis and processing of $\alpha$-mannosidase from this organism (14, 18). Many of these findings have since been confirmed by Mierendorf et al. (19). We purified the enzyme, and prepared a specific antibody against it. The antibody was utilized in pulse-chase studies which indicated that 150,000- and 80,000-dalton precursors are processed to a group of mature forms (56,000–62,000 daltons). We noticed that, under conditions which would be expected to produce marked secretion of $\alpha$-mannosidase, little of the newly processed forms were secreted.

Here we document the finding that newly processed $\alpha$-mannosidase polypeptides are secreted to a much lesser extent than the bulk enzyme activity, when growing cells are subjected to starvation. We present further evidence that the radioiodinated polypeptides are indeed derived from $\alpha$-mannosidase, and we show results which establish a kinetic relationship between newly processed and efficiently secreted forms. The possibility that mature $\alpha$-mannosidase exists in two linked pools and the relation of the results to an understanding of the generation of lysosome heterogeneity are discussed.

MATERIALS AND METHODS

D. discoideum (strain Ax2) was generously donated by Dr. Claudette Klein (St. Louis University School of Medicine) and was maintained in log phase by daily dilution with HL-5 medium (20). The purification of $\alpha$-mannosidase and preparation of antibody directed against this enzyme were performed as previously described (14). D. discoideum mutants were gifts from Dr. Randall Dimond (University of Wisconsin-Madison). [35S]Methionine was purchased from American Corp. Pansorbin, a commercial preparation of formaldehyde-treated Staphylococcus aureus was purchased from Calbiochem. Protocol (a tissue and gel solubilizer) was purchased from New England Nuclear.

Secretion of $\alpha$-Mannosidase Forms during Starvation—Cells (2–6 $\times$ 10^6 cells/ml) were centrifuged at 200 $\times$ g for 8 min. The medium was aspirated and cells were resuspended to 7.5 $\times$ 10^6 cells/ml in HL-5 (growth medium). Resuspended cells were incubated in the presence or absence of [35S]methionine under conditions described in the figure legends. At the end of this incubation, medium was aspirated and
Linked Pools of α-Mannosidase

A

\[ \alpha \text{Man}_p \]

\[ \alpha \text{Man}_m \]

hrs 0 1 2 3 4

B

SECRETION (%) vs. STARVATION TIME (HRS)

C

STARVATION TIME (HRS)

D

SECRETION (%) vs. STARVATION TIME (HRS)

FIG. 1. Processing and secretion of newly synthesized α-mannosidase during starvation. Cells (7.5 × 10^6 cells/ml) were incubated 3.5 h in 5 ml of HL-5 medium. [\(^{35}\)S]Methionine (600 μCi) was added and the incubation was continued for 1.5 h. The suspension was made 50 mM in methionine and centrifuged. Cells were resuspended to their original volume in starvation buffer. Aliquots (1 ml) were removed at the indicated times, cells and medium were separated, and samples were assayed for enzyme activities. Subsequent treatment of cells and medium, which included immunoprecipitation, SDS-polyacrylamide gel electrophoresis and autoradiography, is described under “Materials and Methods.” A is a reproduction of the exposed autoradiograph. The gel concentration was 7.5%. Film exposure time was 10 days. C and M refer to cells and medium, respectively. α-Man\(_p\) is the 150,000-dalton precursor form of α-mannosidase. α-Man\(_m\) is the group of mature forms (56,000-62,000 daltons). B depicts the relationship between starvation time and secretion of α-Man\(_m\) bulk α-mannosidase forms, and acid phosphatase. The direct counting method was used to quantify the distribution of labeled α-mannosidase forms: ×, α-mannosidase activity; ○, α-Man\(_m\); □, acid phosphatase activity.

FIG. 2. Comparison of extents of secretion of newly labeled and bulk α-mannosidase. Cells (7.5 × 10^6 cells/ml) were incubated 2 h in 5 ml of HL-5 medium. The suspension was centrifuged and cells were resuspended to their original volume in a defined medium (22) minus methionine. [\(^{35}\)S]Methionine (850 μCi) was added and the incubation was continued for 20 min. The suspension was centrifuged, cells were resuspended in 5 ml of HL-5 medium and incubated 1 h to allow for precursor processing. Cells were centrifuged and resuspended in 5 ml of starvation buffer. One-ml aliquots were removed at the indicated times. Gel patterns and secretion percentages were obtained as described in the legend to Fig. 1. A is a reproduction of the exposed autoradiograph. The gel concentration was 7.5%. Film exposure time was 8 days. B depicts the extent of secretion of newly synthesized and bulk α-mannosidase from starved cells. Densitometric tracings were used to quantitate the distribution of labeled (Y- mannosidase: ×, α-mannosidase activity; ○, labeled α-mannosidase activity. which did not contain samples was subtracted. The data were reported as per cent secretion:

\[
\text{% secretion} = \frac{\text{Media}}{\text{Media and cells}} \times 100
\]

In some figures these results are compared to the per cent secretion of enzyme activity. For these measurements, aliquots of lysed cells or medium were removed before immunoprecipitation. α-Mannosidase was assayed as previously described (14). Acid phosphatase was assayed using 4-methylumbelliferyl phosphate made 1 mM in 50 mM sodium acetate buffer, pH 4.5 (21). Per cent secretion was calculated as described for α-Man\(_m\).

RESULTS

Secretion of Newly Processed and Bulk Forms of α-Mannosidase—Newly processed α-mannosidase is secreted to a much lesser extent than bulk forms of the enzyme when D. discoideum are starved. Evidence for this is shown in Fig. 1. Here, cells were labeled with [\(^{35}\)S]methionine under growth condi-

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; α-Man\(_m\), mature α-mannosidase.
mannosidase was secreted. We also considered the possibility that differential degradation of intracellular and extracellular newly processed forms could lead to this result. This has been eliminated since the total amounts of both α-mannosidase activity and newly processed α-mannosidase remain constant during the experiment. Furthermore, in experiments not shown, labeled α-mannosidase in secretions was found to be undegraded for as long as 24 h.

Confirmation That the Labeled Polypeptides Are Derived from α-Mannosidase—We had previously shown that the labeled polypeptides were immunoprecipitated with an antisera specific for α-mannosidase and that they co-migrated on SDS gels with purified α-mannosidase (14). Still it was formally possible that the labeled bands might not be the enzyme. This could explain the discrepancies in secretion of the bands and the enzyme activity. Two results yield strong evidence against this possibility. As shown in Fig. 3, two α-mannosidase-negative mutants of D. discoideum show no detectable incorporation of [35S]methionine into newly processed bands even though mutant cells incorporated [35S]me-

FIG. 3. α-Mannosidase-negative mutants do not synthesize detectable α-Man\(_\text{p}\). Wild type (A x 2) or mutant (M-1 or M-4) cells lacking α-mannosidase activity (23) were incubated at a density of 7.5 × 10^6 cells/ml for 4 h in HL-5; 170 μCi of [35S]methionine were added for each milliliter of suspension and the incubation continued for 4 h. The suspension was made 10 mM in methionine and 1 ml aliquots were lysed as described under “Materials and Methods.” Subsequent treatment of lysed suspensions which included immunoprecipitation SDS-polyacrylamide gel electrophoresis, and autoradiography is described under “Materials and Methods.” Film exposure time was 7 days and the gel concentration was 7.5%; lanes a and d, wild type; lane b, M-1; lane c, M-4.

Quantitation of the fraction of newly processed α-mannosidase secreted in this experiment was complicated by the transfer of radioactivity from intracellular precursor to product during the starvation period. This might lead to miscalculation of the extent of secretion. To avoid this complication, kinetic experiments were modified as described in Fig. 2. Cells were briefly labeled with [35S]methionine in a defined growth medium lacking methionine to obtain sufficient incorporation of label. The label was then chased with unlabeled methionine in growth medium for 1 h to allow complete processing of precursor previous to starvation. The remainder of the analysis was performed as described above. As shown in Fig. 2, by comparison to bulk enzyme activity, little newly processed α-

This experiment is similar to, but not identical with Fig. 4B in Ref. 14. The latter employed a 20-min pulse and was designed to detect secretion of precursor. Precursor is barely detectable in the 2-h chase time of the experiment presented here in Fig. 1. The 90-min pulse period employed was designed to increase the sensitivity of detection of secretion of mature forms. This condition allows for considerable processing of precursor and build-up of mature forms before the chase. As a consequence relatively less precursor is seen.
Fig. 5. Transit of newly processed α-mannosidase to an efficiently secreted pool. Cells (7.5 x 10^6 cells/ml) were incubated 2 h in 5 ml of HL-5 medium. [35S]Methionine (850 μCi) was added and the incubation continued for 1 h. The suspension was made 10 mM in methionine and at 0, 1, 2, 3, and 4 h, 1-ml aliquots were removed. These aliquots were centrifuged and cells were resuspended in 1 ml of starvation medium. After 2 h, cells and medium were separated, assayed for α-mannosidase activity, and processed as described under "Materials and Methods." The gel concentration was 8.5%. Film exposure time was 14 days. Cells which were continuously labeled with [35S]methionine as described in Fig. 4 were included as a control. Bars in the graph represent the per cent secretion of α-Man, as quantitated by densitometry. The line indicates the per cent secretion of α-mannosidase activity.

Linked Pools of α-Mannosidase

![Diagram of linked pools of α-Mannosidase](https://example.com/diagram.png)

Discussion

One way to explain these results is to assume that α-mannosidase resides in a presecretory pool before it enters a pool of enzyme which can be efficiently secreted when D. discoideum is starved. This idea is in consonance with the differential extents of secretion of newly processed and bulk α-mannosidase (Figs. 1 and 2). Trivial interpretations such as miscalculations of secretion rates due to replenishment of intracellular newly processed enzyme by precursor, or differential degradation of intracellular and extracellular enzyme, have been eliminated by the results of control experiments. The possibility that the labeled polypeptides are not derived from α-mannosidase is also unlikely since they were immunoprecipitated by antibody specific for α-mannosidase and have the same mobility as the purified enzyme on SDS gels (14), are absent in extracts from α-mannosidase-negative mutants (Fig. 3), and are co-secreted with α-mannosidase when steady state pools are labeled (Fig. 4). The idea of separate linked pools is further supported by pulse-chase experiments (Fig. 5) which indicate the transfer of the enzyme from the presecretory to the efficiently secreted pool.

The physical nature of these pools is unclear. If the fate of acid hydrolases in D. discoideum is analogous to that in higher organisms (8, 9), it is converted from proenzyme to mature enzyme in functional lysosomes. Since the enzyme in the presecretory pool has been proteolytically processed, it seems reasonable to assume that this pool is in a population of functional lysosomes. However, it is also possible that cleavage of acid hydrolase precursors in D. discoideum may occur in prelysosomal organelles (Golgi, Gerl, transport vesicles) in a manner similar to proinsulin (24) or prolactin (25). If cleavage takes place in functional lysosomes, it is possible that the presecretory pool is in a different population of lysosomes than the efficiently secreted pool. This view was suggested as one of the explanations of the bimodal distribution of administered asialocereuloplasmin in rat liver (26). If it correct it would extend to lysosomes the well documented pool separation already demonstrated for the secretory vesicle-condensing vacuole system by electron microscopy (27).

An alternate explanation of our results is that young cleaved enzyme is chemically different from old cleaved enzyme. The chemical difference may allow young enzyme to be sequenced within a lysosome in a manner which supports its retention by the cell. Indeed, differential sequestration of β-glucuronidase forms based on their ability to interact with the phosphomannosyl receptor has been reported (28, 29) in higher organisms. This particular recognition system is an unlikely candidate for such differential sequestration in D. discoideum because essentially all α-mannosidase molecules secreted during starvation bear the phosphomannosyl recognition marker (30). Also, numerous attempts to detect the receptor in this organism have been unsuccessful. However, the general mechanism is a reasonable one especially since indirect evidence for the involvement of other recognition systems in lysosome biogenesis has been reported (31–33).

Experiments are underway to determine whether the existence of the two pools is the result of heterogeneity of lysosomal vesicles or of α-mannosidase structure. These studies may highlight mechanisms of sorting which occur after proteins leave the Golgi region. It should be stressed that the secretion of acid hydrolases induced by starvation may be different in mechanism from secretion during growth. Experiments are planned to determine if the pools of mature enzyme described here also are involved in other secretion modes. The reported findings may alert others studying lysosome biogenesis to the possibility of linked pools of acid hydrolases in higher organisms.

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