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TURNOVER OF PROTEIN COMPONENTS OF THE PLASMA MEMBRANE OF *SACCHAROMYCES CEREVISIAE*

ENRIQUE HERRERO, JAVIER PASTOR and RAFAEL SENTANDREU *

Departamento de Microbiologia, Facultad de Farmacia, Universidad de Valencia, Avda Blasco Ibáñez 13, Valencia 10 (Spain)

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The peptide composition of plasma membrane in *Saccharomyces cerevisiae* cells growing at different temperatures between 18 and 38°C was studied using SDS-polyacrylamide gel electrophoresis. Stability of the proteins, both qualitative and quantitative, was observed at the tested temperatures. Treatment for 2 h with cycloheximide decreased by about 50% the amount of a 80 kDa membrane peptide at 18, 23, 28 and 33°C, with no other apparent effects. At 38°C the 80 kDa peptide was not affected by the presence of the drug. Addition of tunicamycin to cultures at concentrations partially inhibitory to growth caused a large accumulation of the 80 kDa peptide in the plasma membrane, which cycloheximide did not modify. Pulse-chase experiments indicated a low rate of turnover of total plasma membranes in cells growing at 18 and 28°C. In contrast, at 38°C about 50% of the radioactivity in plasma membranes disappeared after a 2 h chase. The 80 kDa protein band was the only one with significant differential decay.

Introduction

The plasma membrane of cells is a highly organized organelle specifically designed to fulfil essential functions, many of them being involved either in the communication of the cell with the environment or in the formation of the cell wall. Internal membranes are used in the transport of molecules to the outside of the cell and some regions of the plasma membranes are probably internalized. Thus, membrane turnover (see Ref. 1 for a review) implies not only degradation of structural components but also reutilization of significant parts after their internalization. The *Saccharomyces cerevisiae* plasmalemma is a good model for the study of membrane turnover, since yeast cells in exponential growth are actively involved in the secretion of molecules to the cell wall and to the external medium. Also, the secretion process seems to occur in a way similar to that in higher cells [2–4] and final steps involve the fusion of internal vesicles with the plasmalemma, as demonstrated by the study of mutants altered in their secretory pathways [5–7]. Whether or not some regions of this plasmalemma are again internalized remains to be elucidated.

The structure and asymmetrical location of some proteins in plasma membranes of *S. cerevisiae* have recently been reported [8]. Also, several enzyme activities associated with the cell plasma membrane have been detected: an oligomycin-resistant Mg^{2+}-ATPase activity probably involved in active transport [9,10], a permease system involved in general amino acid transport [11,12] and a chitin synthetase activity [13] among others. However, the technical problems arising from the methods employed in plasma membrane isolation

* To whom correspondence should be addressed.

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

have not been entirely solved. Our group has adapted [14] a method initially developed by Scarborough [15] for plasma membrane isolation from a slime mutant of *Neurospora crassa*. Concanavalin A cross-links the glycoprotein molecules present in plasma membranes and prevents the vesicularization during protoplast lysis and contamination by other membrane fractions. This method leads to the preparation of sufficient quantities of plasma membrane and permits kinetic studies on the turnover of its protein components.

In this context, we have studied the peptide composition of the plasma membrane of cells growing at different temperatures and the effect of temperature and some inhibitors on their stability.

Materials and Methods

Strain and growth conditions. S. cerevisiae X-2180-1A (*a, SUC2, mal, gal*$_2$*, CUP1*) was grown in YNB medium without amino acids (Difco), supplemented with 4% (w/v) glucose as carbon source. They were initially inoculated at about 5 μg (dry weight) cells/ml culture and incubated in a rotatory shaker for 14–20 h, depending on the temperature. Growth rate was followed by taking samples periodically and measuring the absorbance at 600 nm. Cells for membrane purification were taken at the early exponential phase of growth, when the absorbance of cultures was 0.2–0.3 (about 500 μg dry wt. cells/ml culture).

Doubling times during exponential growth at the temperatures of incubation were: 190 min (18°C), 164 min (23°C), 130 min (28°C), 114 min (33°C) and 110 min (38°C).

Purification of plasma membranes. Basically, the method described by Santos et al. [14] was employed, with only minor changes. Prior to protoplast preparation, about 100 mg cells (dry wt.) were treated with 15 ml of 5 mM DL-dithiothreitol in 100 mM Tris-HCl (pH 8) plus 5 mM EDTA for 30 min at 28°C. The cells were washed twice and resuspended in 30 ml of 1 M sorbitol containing 30 mg of Zymolyase 5000. Incubation was at 28°C and formation of protoplasts was followed by phase-contrast microscopy. Under the above-described conditions, 100% protoplasts were obtained after about 20 min. Isolation of plasma membranes was carried out as previously described [14].

Analysis of membrane peptides by SDS-polyacrylamide gel electrophoresis. The peptide composition of membranes was analyzed by SDS-polyacrylamide gel electrophoresis using slab gels. Preparation of the gels and buffer system were as described by Laemmli [16]. The concentration of the stacking gel was 6% (w/v) acrylamide, while that of the separating gel was 10% (w/v) acrylamide. Bisacrylamide was always 1:55 with respect to acrylamide.

About 100 μg membrane protein were run in each slot. Electrophoresis was carried out at a constant current of 15 mA through the stacking gel and 25 mA through the separating gel. Gels were stained overnight in a solution of 0.05% (w/v) Coomassie blue in 15% (v/v) isopropanol plus 10% (v/v) acetic acid. Destaining was carried out by three successive washings (3 h each) in 15% (v/v) isopropanol plus 10% (v/v) acetic acid. Stained gels were scanned using a Vitatron TLD100 scanner at a wavelength of 540 nm, and the relative areas under the peaks measured.

In all the electrophoresis experiments, the following standard proteins were run in parallel: α-lactalbumin (14000), soybean trypsin inhibitor (20100), carbonic anhydrase (30000), ovalbumin (43000), bovine serum albumin (67000) and phosphorylase *b* (94000).

Nomenclature of peptide from the gels. Peptides are named according to their apparent mobility in the gels, by giving them a number corresponding to the size in kilodaltons (i.e., a peptide with an apparent size of 40 kDa would be termed as a 40 kDa peptide).

Determination of the rate of turnover of bulk and individual proteins in labelled plasma membranes. Exponentially growing cells were labelled for 15 or 20 min with 0.4 μCi/ml of [U-^{14}C]protein hydrolysate (57 mCi/matom). Incorporation of the radioactive precursors was stopped by diluting with 3 vol. of YNB/glucose medium plus 1% (w/v) casein hydrolysate. Samples (10 ml) were taken periodically and supplemented with 5 ml of cells previously incubated for 4 h with DL-[G-^{3}H]-threonine (5 μCi/ml, 316 mCi/mmol). This addition was used as an internal standard. 10 absorbance units of unlabelled cells were also added to each sample. Membranes were obtained as indicated above and finally resuspended in 10 mM

Tris-HCl buffer (pH 7.4) at a concentration of 2–5 mg protein/ml.

Radioactivity in whole plasma membranes (10–20 μl suspension) was measured by adding 2 ml of the scintillation fluid (4 g PPO and 100 mg POPOP/l toluene plus 500 ml Triton X-100) in a Beckman scintillation spectrophotometer model 7500.

To determine the ratio $^{14}C/^{3}H$ in individual peptide bands, the peptides were separated by SDS-polyacrylamide gel electrophoresis and, after gel staining, the bands were cut with a razor blade and digested overnight with 1% (v/v) NH_4OH in 30% (v/v) H_2O_2 at 40°C. Radioactivity was measured as described above.

Continuous labelling of plasma membrane proteins. Exponentially growing cells were labelled for 4 h with 0.2 μCi/ml [U-^{14}C]protein hydrolysate (57 mCi/matom). After this period membranes were purified as described and individual peptide bands were separated by SDS-polyacrylamide gel electrophoresis.

Treatment with antibiotics. Cycloheximide was used at a final concentration of 100 μg/ml from a stock solution at 10 mg/ml in distilled water. After addition of the antibiotic, protein synthesis was halted almost immediately. Treatment was continued for 2 h before the samples were taken.

Tunicamycin (a gift from J. Tkacz, Rutgers University) was employed at 10 μg/ml in the growth medium. It was prepared immediately before use from a stock solution of the antibiotic at 2.5 mg/ml in 0.3 M NaOH.

Determination of protein concentration in membranes. The method of Lowry et al. [33] was employed, with a slight modification: sodium deoxycholate at 0.3% (w/v) was included in the treatment in order to solubilize membrane proteins.

Chemicals. Glucose, DL-dithiothreitol, Coomassie blue, PPO, POPOP, Triton X-100 and cycloheximide were purchased from Sigma Chemical Co. Toulene was obtained from Merck. Acrylamide and bisacrylamide were purchased from Bio-Rad. Zymolyase 5000 was acquired from Kirin Breweries, Gumma, Japan. Protein standards were from Pharmacia. Radioactive materials were purchased from The Radiochemical Centre, Amersham, U.K.

Results

Peptide composition of S. cerevisiae plasma membrane at different growth temperatures

Previous studies [8,14] have led to the proposal of the peptide composition of *S. cerevisiae* plasma membrane growing at 28°C. We have extended this work to determine the influence of growth temperature (18, 23, 28, 33 and 38°C) on the plasma membrane composition.

Fig. 1 shows the peptide bands and the corresponding densitometric profile. Between 30 and 40 different bands were easily distinguishable in the gels. The intensities of the bands were quantified by measuring the areas under each peak and values were made relative to that of a 46 kDa peptide, of which the intensity remained constant with respect to the sum of the bands.

A 23.5 kDa band appeared in the gels, with an

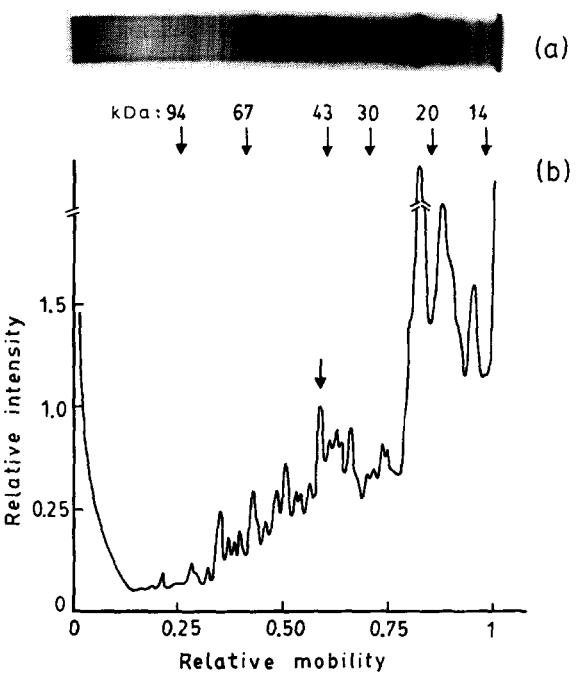

Fig. 1. *S. cerevisiae* plasma membrane peptide bands. Cells were grown at 28°C and peptides separated in 10% acrylamide gels. (a) Photograph of a gel. (b) Densitometric profile (at 540 nm wavelength) of the gel. The positions of the standards run in parallel are also shown. The arrow of the gel profile corresponds to the 46 kDa peptide band.

intensity of about 4-fold that of the reference band. Since the size of this peptide is about the same as that of concanavalin A monomers [17], the possibility that it might be the lectin left behind during the process of membrane purification was considered [14]. In fact, about 10% of [^{3}H]concanavalin A remained associated to the plasma membrane after the isolation process, because it was not removed by α-methylmannoside (unpublished results). Therefore, to check the above possibility, cells growing at 28°C were labelled for almost two generations with [^{14}C]protein hydrolysate. Membranes were purified, the peptide components separated by gel electrophoresis and the radioactivity in sections of the gel measured (see Materials and Methods). The relationship between the densitometric profile and the radioactivity in the stained gel is shown in Fig. 2. A significant amount of radioactivity appeared in the 23.5 kDa region, indicating that although part of the band may correspond to concanavalin A, a protein(s) of this size is actually present in the yeast membrane.

The analysis of plasma membrane densitometric and radioactive profiles from cells growing at 18, 23, 28, 33 and 38°C showed no apparent differences. Therefore, it seems that the temperature of growth does not significantly influence the peptide composition of *S. cerevisiae* plasma membranes.

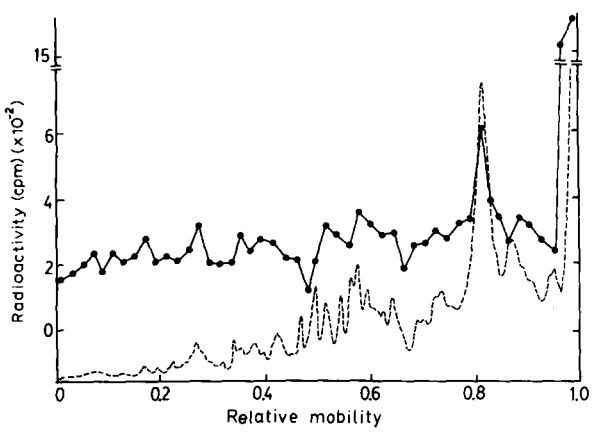

Fig. 2. Relationship between densitometric and radioactive profile of *S. cerevisiae* plasma membrane peptide bands. Cells were grown at 28°C for 4 h in the presence of 0.2 μCi/ml [U-^{14}C]protein hydrolysate. Peptides were separated in 10% acrylamide gels and the radioactivity (●) measured as indicated in Materials and Methods. (— — —) Densitometric profile of the stained gel.

Effect of cycloheximide on peptide composition

Since cycloheximide inhibits protein synthesis, the formation and accumulation of newly synthesized membrane peptides may be halted after the addition of the drug. Thus, it was thought that analysis of plasma membrane peptide bands before and after treatment of yeast cells with cycloheximide for 2 h might give an indication of the turnover of individual bands. This was done with cells grown in the range between 18 and 38°C and the only apparent difference detected was in the 80 kDa peptide band. The amount of this band decreased by 40–60% in the presence of the antibiotic at 18, 23, 28 and 33°C. This decrease did not take place at 38°C (Fig. 3).

The small differences found may only be apparent because cycloheximide might inhibit the synthesis of a protease(s) or a protease activator(s) needed in normal protein turnover, as has been reported recently in the case of mouse adipocytes [18].

Effect of tunicamycin on peptide composition of plasma membranes

Many proteins in the range of 100–200 kDa of the yeast plasma membrane appear to be glycosylated (Ref. 8; and unpublished results), because

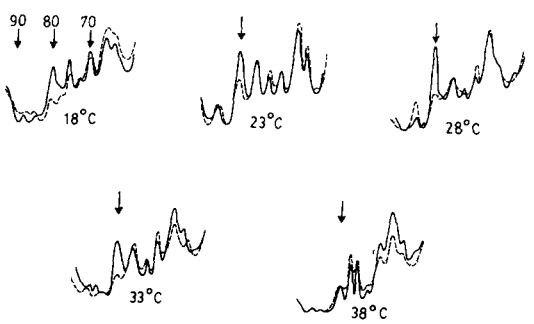

Fig. 3. Effect of cycloheximide on peptide composition of plasma membranes. The figure shows the densitometric profiles of the regions of the gels where peptides between 70 and 90 kDa band, from cultures grown at the indicated temperatures. (———) Untreated cells; (— — —) cells treated for 2 h with cycloheximide (100 μg/ml). The arrow indicates the position of the 80 kDa peptide band. The values 70, 80 and 90 (top left correspond to values in kDa.

they are stained by the periodic acid-Schiff method [19]. Since tunicamycin is an inhibitor of the formation of *N*-glycosidic linkages in glycoproteins [20–22], the effect of the antibiotic on the peptide pattern of *S. cerevisiae* plasma membranes was studied. Cells grown at 28°C in the presence of 10 μg/ml tunicamycin showed a growth inhibition of about 40% (checked by measuring the absorbance and total cell number). Plasma membrane peptides analyzed after 2 and 4 h treatment were compared with the controls. The only significant difference found was a large accumulation of the 80 kDa peptide (Fig. 4).

As cycloheximide decreases the amount of the 80 kDa peptide band found in the plasma membrane, we tested the effect of cycloheximide on cells in which *N*-glycosylation had previously been inhibited with tunicamycin. Cells incubated for 2 h with tunicamycin were supplemented with cycloheximide and incubation was extended for 2 h more. After that time, the amount of the 80 kDa peptide band did not decrease; on the contrary, a greater accumulation took place (Fig. 4d).

Turnover of plasma membrane proteins at different temperatures

The turnover of bulk plasma membrane proteins at 18, 28 and 38°C was determined by pulse-chase experiments, as described in Materials and Methods. Proteins were labelled for about one-tenth of the generation time with a mixture of ^{14}C-labelled amino acids (20 min at 18°C or 15 min at 28 and 38°C). After this period, a large excess of unlabelled amino acids was added to the culture and incubation continued during the next 6 h.

As shown in Fig. 5, plasma membrane protein bands remained stable at 18 and 28°C during the 6 h chase. By contrast, at 38°C, about 50% of the radioactivity incorporated during the pulse disappeared during the next 2 h after which the radioactivity also remained constant.

The turnover of individual peptide bands was measured by determining the ratio $^{14}C/^{3}H$ from SDS-polyacrylamide gels. Table I shows the results obtained on three plasma membrane peptides. The 80 kDa peptide band showed a significant turnover at 18 and 28°C; in fact, this seems also to be the case at 38°C when $^{14}C/^{3}H$ ratios are expressed relative to total cell protein but not when they are expressed relative to plasma membrane protein (since all plasma membrane proteins are affected by turnover; Fig. 5). These results are in agreement with those obtained with cycloheximide (Fig. 3). The stability of two other membrane peptides (46

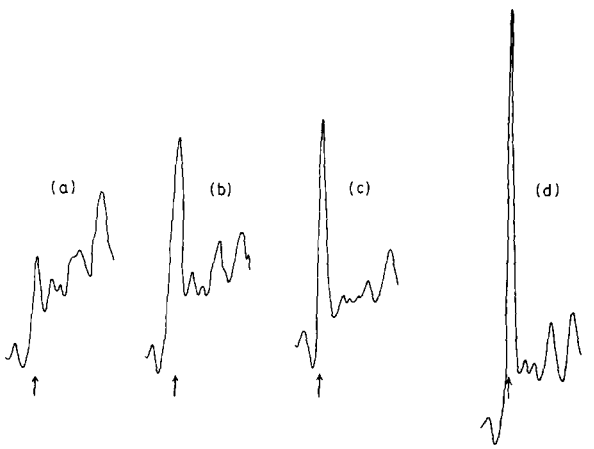

Fig. 4. Effect of tunicamycin on peptide composition of plasma membranes. Cells were grown at 28°C under the following conditions: (a) untreated cells; (b) cells treated for 2 h with tunicamycin (10 μg/ml); (c) cells treated for 4 h with tunicamycin; and (d) cells treated for 4 h with tunicamycin, cycloheximide also being added during the last 2 h. The arrow indicates the position of the 80 kDa peptide.

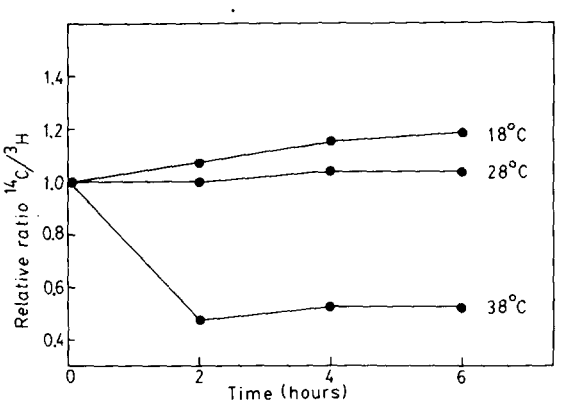

Fig. 5. Relative ratio $^{14}C/^{3}H$ in total plasma membrane protein (relative to the ratio in total cell protein) during pulse-chase experiments. A pulse of [U-^{14}C]protein hydrolysate was given to *S. cerevisiae* cells growing at the indicated temperatures and incorporation of ^{14}C label was stopped at time 0. Addition of ^{3}H-labelled internal standard and normalization of $^{14}C/^{3}H$ ratios were as described in the text and Table I.

TABLE I

TURNOVER OF INDIVIDUAL PLASMA MEMBRANE PEPTIDE BANDS FROM *S. CEREVISIAE* GROWING AT DIFFERENT TEMPERATURES

All values correspond to the ratio $^{14}C/^{3}H$ 4 h after the pulse and are made relative to the ratio $^{14}C/^{3}H$ at time 0, which is given the unit value. (A) Ratio $^{14}C/^{3}H$ in each peptide band, relative to the ratio in total cell protein. (B) Ratio $^{14}C/^{3}H$ in each peptide band, relative to the ratio in total plasma membrane protein.

Peptide (kDa)	Growth temperature					
	18°C		28°C		38°C	
	A	B	A	B	A	B
80	0.82	0.71	0.67	0.58	0.74	1.38
66	0.95	0.83	1.17	1.02	0.71	1.32
46	1.12	0.98	1.07	0.93	0.66	1.23

and 66 kDa) at the tested temperatures is also shown in Table I. The same behaviour was observed for two other plasma membrane peptide bands.

Discussion

Analysis of the peptide bands obtained by one-dimensional SDS-polyacrylamide gel electrophoresis of *S. cerevisiae* plasma membranes showed that they were affected neither qualitatively nor quantitatively by the growth temperature (between 18 and 38°C). McAlister and Finkelstein [23] have recently reported that after transfer of *S. cerevisiae* from 23 to 36°C a transitory change in the rate of synthesis of some cell proteins takes place. This change disappears after 90 min, because the rate of protein synthesis returns to the previous level. This is in agreement with our results using steady-state growth conditions.

The turnover of the total protein and many individual peptide bands of the plasmalemma was not significant at physiological temperatures. Following pulse-chase and cycloheximide experiments, the protein bands were found to be stable, at least for a period of two to three generations. For higher cells, Reed et al. [18] have described that cycloheximide specifically inhibits the synthesis of a protease involved in the degradation of membrane peptides. Therefore, addition of the antibiotic to growing cultures leads to abnormally low turnover rates. This interpretation does not hold for *S. cerevisiae*, since similar turnover rates were found both by pulse-chase experiments and by growing cells in the presence of cycloheximide.

The protein stability of yeast plasmalemma in cells growing at 18 and 28°C contrasts with the situation in higher cells. Mammalian cells in tissue cultures show significant turnover rates in their plasma membranes [1,24–27], a phenomenon that mainly affects low molecular weight peptides [28,29].

Results from cells grown at 38°C contrast with those obtained from cells grown at lower temperatures. About half of the radioactivity incorporated into the plasmalemma proteins disappeared during the next 2 h. This may be explained by an uncoupling between synthesis of proteins, incorporation into the plasma membrane and cell surface extension. This temperature is not a physiological one for *S. cerevisiae* and though cells can initially grow at a higher rate at this temperature than at 28°C, an uncoupling between macromolecular synthesis and cell growth may take place that would result in a smaller yield of yeast mass. The excess of membrane material would be rapidly degraded, with no preferential specificity for individual peptides (Table I).

Among the individual peptide bands of the plasma membrane, only the 80 kDa peptide exhibited a significant turnover rate in cells growing at 28°C. The fact that tunicamycin induced its accumulation (Fig. 4) might be explained in two different ways. First, the 80 kDa peptide is processed at the membrane level to give two or more smaller peptides; if processing requires previous glycosylation of the protein molecules (either at the plasmalemma or at an intracellular level), the addition of tunicamycin would cause its accumulation in the form of the unprocessed molecules. Second, the 80 kDa molecules are transitorily located at the plasmalemma on their way to an extracellular location. If this process requires previous glycosylation, the addition of tunicamycin would also cause their accumulation at the plasma membrane. Both hypotheses point to glycosylation taking place at the level of the plasma membrane.

Although *N*-glycosylation seems to occur mainly at an intracellular level [30,31], the possibility that mannosyltransferase activity exists in the yeast plasmalemma cannot be ruled out. In fact, Santos and Sentandreu [32] have suggested that the addition of at least part of the sugar molecules *O*-glycosidically linked to peptide chains occurs at this level. However, to date, we have no conclusive results on the possible mechanism affecting the 80 kDa membrane peptide.

Although none of the gel bands have been characterized from a physiological point of view, we have observed (unpublished results) that addition to the growth medium of sugars different from glucose affects peptide composition of the plasma membrane. This opens up the possibility of studying the kinetics of dilution of these peptides when cells are shifted from one carbon source to another as a means of analyzing a particular type of protein turnover.

Acknowledgements

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