STUDIES ON MEMBRANE PROTEIN TURNOVER IN GROWING YEAST CELLS

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1. Introduction

The metabolic stability of soluble proteins in exponentially growing yeast has been reported [1]. The absence of a demonstrable protein turnover strongly differentiates the yeast cells from the mammalian systems where extensive degradation of soluble or membrane proteins has been found [2].

In this report, the degradation rates of yeast membrane proteins have been compared. The turnover process is often described as a regulatory mechanism used by the cells in order to adjust their transport systems to their needs [3,4]. The experimental indications in favour of this kind of regulation result essentially from uptake measurements in cells incubated with cycloheximide. The decrease of transport activity observed is often explained by the degradation of a rapidly turning over component of the transport system, but it can also result from the intracellular accumulation of free metabolites which inhibit the permease [5]. These two possibilities are not mutually exclusive and it is possible that both may be involved in the regulation of cellular transport systems. In order to demonstrate the existence in yeast cells of membrane proteins turning over rapidly, cellular proteins were labelled with radioactive amino acid and the decay of the radioactivity associated with the membrane polypeptides (separated by polyacrylamide–SDS gel electrophoresis) was measured after different lengths of chase. Our data indicate that there is no detectable loss of radioactivity from major proteins during 3 h. It may be concluded that in growing yeast cells the precursor amino acids are incorporated into essentially stable membraneous end products. The same stability was observed for cytoplasmic proteins.

2. Materials and methods

2.1. *Growth of cells*

Saccharomyces cerevisiae strain FL 100 (wild-type haploid, mating type) was grown in liquid minimal medium (yeast nitrogen base Difco without amino acids) supplemented by 4% glucose. Cultures were incubated at 28°C on a reciprocal shaker with a generation time of 110 min.

2.2. *Preparation of membrane proteins*

The membrane preparation procedure followed the general method [6] with slight modifications.

Each cell pellet was resuspended in equal vol. 0.5 M sorbitol; glass beads were added and the cells broken by 5 min agitation with a Chemap Vibromixer. The homogenized samples were then sedimented for 10 min at 10 000 × *g*. The supernatants were removed and then centrifuged for 60 min at 150 000 × *g* in final 39% (w/w) sucrose. The pellets from this centrifugation were resuspended in 55% (w/w) sucrose and centrifuged 60 min at 150 000 × *g*. The resulting precipitates were washed with 0.5 M sorbitol and again centrifuged for 20 min at 150 000 × *g*. The pellets obtained constitute the crude plasmalemma, as defined [7]. Each of them was mixed with 1 vol. 10% trichloroacetic acid. The precipitates were collected by centrifugation and washed 3 times with 1 ml ether to remove traces of trichloroacetic acid. The membrane pellets were resuspended in 50 μl buffer: 90 mM Tris-borate; 2.5 mM Na_2 EDTA; 20% (w/v) sodium lauryl-sulfate (SDS); 5% (v/v) β-mercaptoethanol; 10% (w/v) sucrose; 0.05% (w/v) bromophenol blue, pH 8.3. Just before electrophoresis the samples were placed in a

boiling water bath for 3 min. The final protein concentration was 3–5 mg/ml.

2.3. *Polyacrylamide gel electrophoresis*

Polyacrylamide gels (11 X 13 X 0.15 cm) were prepared as in [8]. The running gels of 10% acrylamide (prepared from a stock solution 30 g acrylamide, 0.8 g *N,N'*-bis-methyleneacrylamide) contained 0.375 M Tris–HCl (pH 8.8) and 0.1% SDS, and were polymerized chemically by the addition of tetramethylethylenediamine and ammonium persulfate. The stacking gels (1 cm) contained 5% acrylamide, 0.125 M Tris–HCl (pH 6.8) and 0.1% SDS. Polymerization was performed in the same way as for the running gels. The electrode buffer was 0.040 M Tris–glycine (pH 8.3) with 0.1% SDS.

After incubation of the samples (15–20 μl) a current 12.5 mA/gel was applied for 4 h at room temperature; 50 V at the beginning and 130 V at the end of the run. Immediately after electrophoresis, the gels were immersed into staining solution consisting of 20% (v/v) isopropanol, 7.5% (v/v) acetic acid, 0.5% (w/v) Coomassie blue R250 in distilled water and agitated gently during 90 min. Destaining was achieved by repeated washing in 15% methanol–7.5% acetic acid. The gels were then dried onto a piece of filter paper under vacuum. For molecular weight determinations the following proteins were employed as standards: ββ' and α subunits of the *E. coli* polymerase, bovine serum albumin and trypsin inhibitor.

2.4. *Radioactivity determination*

Gels were cut into 2 mm slices. Each slice was incubated overnight at 37°C in 1 ml 30% H_2O_2 containing 5% (v/v) NH_4OH and their radioactive content was determined after addition of 10 ml Triton (33%) – toluene – PPO ($5^0/_{00}$) scintillation fluid, in an Intertechnique LS 30 spectrometer. Channels were so adjusted that the extent of overlap of ^{14}C into ^{3}H channel was restricted to about 8% and that no counts from ^{3}H were detected in the ^{14}C channel. Samples of hydrolysed gel containing only one of the isotopes served as standards to evaluate the cross contaminations in each channel. The ratios of $^{14}C/^{3}H$ counts were then estimated by using the appropriate corrections.

2.5. *Chemicals*

[^{14}C]Leucine, spec. act. 288 mCi/mmol, and [^{3}H]leucine, spec. act. 750 mCi/mmol, were obtained from CEA, France. Acrylamide (purum) was purchased from Fluka AG and *N,N'*-bis-methyleneacrylamide from Eastman Kodak. All other reagents were of analytical grade.

3. Results and discussion

To measure the turnover of membrane polypeptides in *Saccharomyces cerevisiae*, the kinetics of incorporated [^{14}C]leucine disappearance from proteins were followed. The ^{14}C radioactivity was normalized to an independent long-time ^{3}H-labelling and the specific activities of membrane proteins were expressed by the ratio $^{14}C/^{3}H$.

To label cellular proteins, 50 μCi [^{14}C]leucine (288 mCi/mmol) were added to 200 ml exponentially growing culture. Figure 1 illustrates the kinetics of exogenous [^{14}C]leucine incorporation into trichloroacetic acid-precipitable material. The rate of incorporation remained constant over 10 min. The radio-

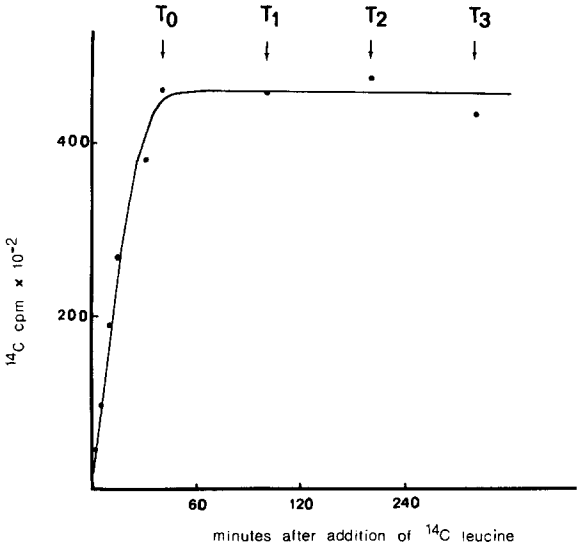

Fig.1. Incorporation of [^{14}C]leucine into total protein. At time zero [^{14}C]leucine (0.25 μCi/ml; 0.1 μg/ml) was added to the culture. Samples (0.1 ml) were removed at indicated times and mixed with an equal vol. cold 10% trichloroacetic acid, then filtered, dried and counted.

activity was completely incorporated into the proteins 40 min after [^{14}C]leucine addition. For this reason, this time was chosen as time zero. Membrane fractions were prepared at time T_0 and at 1 h intervals during the next 3 h at times T_1, T_2, T_3. For each preparation, 40 ml sample was removed from the culture and treated rapidly with 10^{-3} M sodium azide to prevent further cellular metabolic activity. The cells were collected by centrifugation and the washed pellet was mixed with a pellet of cells labelled with [^{3}H]leucine (by growth in a minimal medium supplemented for 2 h with [^{3}H]leucine at 0.3 μCi/ml (20 μg/ml) and with isoleucine and valine (each at 20 μg/ml)). The membrane fractions were isolated from each cellular pellet as in section 2.

Figure 2 shows the radioautogram of a gel where proteins of the different membrane preparations were run simultaneously. It can be noted that the membrane proteins in yeast cells exhibit a wide range of molecular weights. About 30 discrete bands of varying intensity are visible in each pattern. The most characteristic band corresponds to mol. wt 55 000.

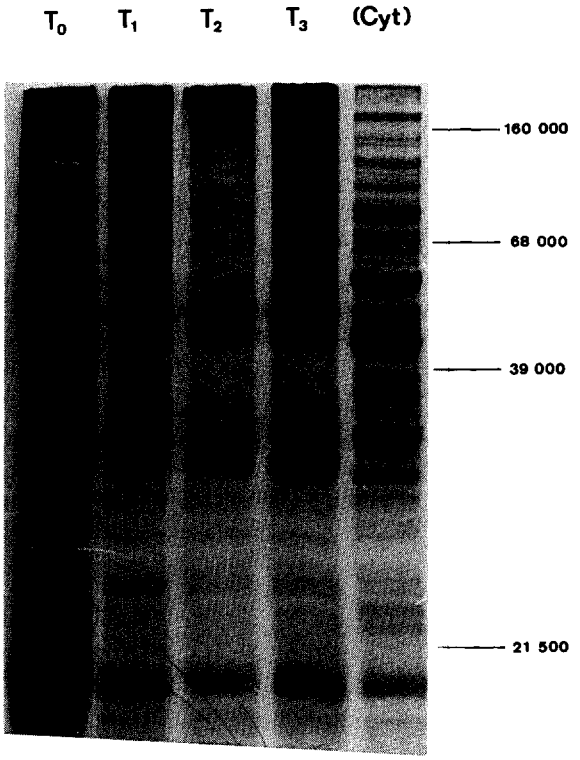

Figure 2 shows also clearly that the pattern of membrane proteins differs from the pattern of cytoplasmic proteins. For each time point identical patterns with the same number of protein bands are obtained. As in these conditions of autoradiography the film is only sensitive to ^{14}C counts, this suggests strongly that the process of turnover is not important. To quantify better the latter, the proteins present in the fractions prepared at times T_0 and T_3 were analyzed for their specific radioactivities. In fig.3 the standardized ratios were plotted versus molecular weight. It appears from fig.3 that the patterns of labelled proteins are very similar whether prepared at time T_0 or at time T_3. The double ratio of specific activities remains relatively constant along the gel, indicating that there is no differential loss of radioactivity in any of the major proteins. Therefore it may be concluded that in rapidly growing yeast cells major membrane proteins do not turn over but are conserved during 3 h at least. Proteins that turned over rapidly might be expected to show a progressive decay of ^{14}C counts that would be detectable as a variation of the ^{14}C/^{3}H ratio. Indeed, one important reservation must be formulated. Since each gel slice contains several different polypeptides, the existence of minor proteins turning over rapidly cannot be excluded.

In spite of this remark, yeast membrane proteins from exponentially growing cells can be regarded as essentially stable products. In mammalian cells, it was found that plasma membranes are in a dynamic state and that there is a marked heterogeneity in turnover rates of individual protein species as separated by polyacrylamide gel electrophoresis [9].

Fig.2. Radioautogram of the electrophoresis pattern of membrane fractions isolated at various times after incorporation of [^{14}C]leucine into proteins. A culture was grown on minimal medium in the presence of [^{14}C]leucine (0.25 μCi/ml; 0.1 μg/ml). At times T_0, T_1, T_2 and T_3 (see fig.1.) membrane fractions were isolated after previous addition to each ^{14}C-labelled sample of an aliquot of ^{3}H-labelled culture (0.3 μCi/ml; 20 μg/ml; 2 h labelling). Solubilization and electrophoretic separation of membrane proteins are described in section 2. Approx. 3×10^4 cpm ^{14}C and 2×10^4 cpm ^{3}H were applied in each sample. The film (Kodirex RP) sensitive to ^{14}C counts only was exposed to the gel for a period of 8 days. Band Cyt: electrophoretic pattern of cytoplasmic proteins (39% saccharose supernatant).

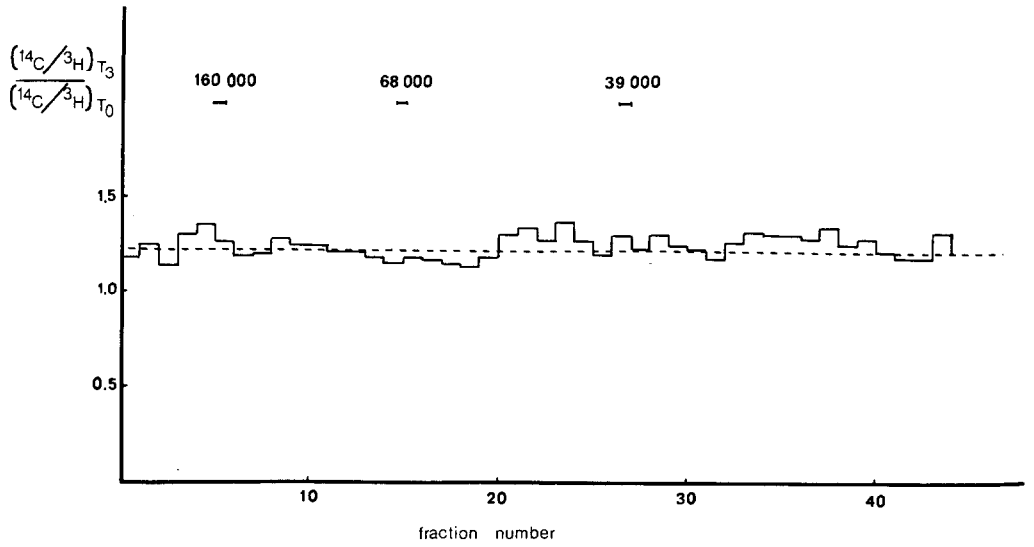

Fig.3. Evolution of the radioactivity present in membrane proteins. $^{14}C/^{3}H$ ratios were calculated for each gel slice resulting from the electrophoresis of T_0 and T_3 preparations (see fig.2.). Each slice contained between 300 and 1000 cpm of ^{14}C and between 150 and 800 cpm of ^{3}H. The double ratios of specific radioactivities measured at both times are plotted versus molecular weight of proteins; (– – – – –) average ratio.

These turnover rates are not very high (1%/h) but they are nearly the same as the growth rate. In such a case the process of turnover can exert a physiological role at the cellular level. In yeast, such a turnover rate would hardly be detected from our experiment which covers period of 3 h. It is interesting to remark that for a prokaryote like *Escherichia coli* growing in conditions similar to yeast, no significant protein turnover has been detected for total cellular proteins [10].

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