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A COMPARISON OF INSOLUBLE MITOCHONDRIAL MEMBRANE PROTEINS FROM GLUCOSE REPRESSED AND DEREPRESSED *SACCHAROMYCES CARLSBERGENSIS*

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SUMMARY

Yeast cells (*Saccharomyces carlsbergensis*) were grown under conditions of glucose repression in a medium containing ^{14}C-labeled amino acids and also under derepressing conditions in a medium containing ^{3}H-labeled amino acids. The mitochondria were isolated, mixed and the mitochondrial membrane proteins fractionated. The distribution of ^{3}H- and ^{14}C-labeled amino acids indicated that there were no qualitative differences in membrane proteins. However, the amount of one component was greatly increased relative to all others upon derepression. The distribution of label during protein fractionation suggests that membrane solubilization with cholate and deoxycholate proceeds by solubilization of discrete membrane units containing all of the membrane proteins.

INTRODUCTION

It has been demonstrated that both morphological as well as enzymatic changes occur in mitochondria accompanying the transition from the glucose repressed to the derepressed (aerobically adapted) state in Saccharomyces[1,2].

There have been no reports describing the changes that may occur in the insoluble mitochondrial membrane protein fraction as a result of derepression. It has been reported that a cytoplasmic petite mutant of yeast lacks one specific component of the insoluble mitochondrial membrane protein fraction[3]. This was determined primarily from the absence of a zone on acrylamide gels and was further investigated using immunological techniques. Unfortunately the methods employed did not allow investigation of quantitative differences among membrane proteins from the petite and the wild-type cells. However, in addition, it has been suggested that the component reported missing from the petite was lost during mitochondrial preparation and does not truly reflect a protein absent from petite mitochondria[4]. Moreover, the lack of appropriate controls makes it impossible to determine whether the gel pattern observed was characteristic of a primary genetic effect of petite mutation or a secondary physiological effect characteristic of anaerobic growth. In this communication

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an initial approach to the resolution of this problem is presented. A comparison is made between the distribution of components in the insoluble mitochondrial membrane protein fractions from glucose repressed and derepressed cells of *Saccharomyces carlsbergensis*. Proteins of the repressed and derepressed cells were labeled with ^{14}C- and ^{3}H-labeled amino acids respectively. This procedure facilitates a determination of quantitative as well as qualitative differences between the two different states and overcomes the difficulties previously encountered in determining differences either by mass or by staining density on gels.

METHODS AND MATERIALS

Isolation of mitochondria from aerobic yeast

Yeast cells (*S. carlsbergensis*) were grown on 1 % glucose, 1 % peptone and 1 % yeast extract. Generally labeled amino acids (^{3}H, New England Nuclear) were added to the growth medium at a level of 100 μC/l at the time of inoculation. The cultures were grown to early stationary phase (3.5 generations) at room temperature on a rotary shaker. The cells were harvested by centrifugation and resuspended in a 0.25 M sucrose, 0.01 M Tris buffer (pH 7.4), 0.001 M EDTA, 1 % casein hydrolysate solution in a ratio of 1 vol. of buffer to 4 parts of wet yeast by wt. 15-ml aliquots of suspended cells were mixed with 20 ml glass beads (0.45–0.50 mm) and homogenized for 45 sec in a CO_2-cooled Braun homogenizer. The glass beads were washed 3 times with buffer and the total cell suspension was centrifuged at 1000 $\times$ g for 10 min to remove the heavy debris of cell walls, nuclei and unbroken cells. The supernatant fraction from this centrifugation was recentrifuged at 7700 $\times$ g for 20 min to sediment the mitochondria. The mitochondrial pellet was resuspended in the homogenizing buffer and centrifuged at 1000 $\times$ g for 10 min. The supernatant fraction was centrifuged at 27000 $\times$ g for 20 min to obtain the final mitochondrial pellet. The mitochondria were then suspended in undiluted Renografin solution (obtained from E. R. Squibb, N.Y.), using approx. 1 vol. of mitochondrial pellet to 1.5 vol. of Renografin. 4.4 ml of this suspension were placed into the bottom of each of three Spinco SW-25 cellulose nitrate tubes. Linear gradients of Renografin in 0.01 M Tris–0.001 M EDTA buffer (pH 7.4) ranging from 1.1 to 1.2 g/ml, were layered over the suspension. The mitochondria were floatated by centrifugation for 6 h at 25000 rev./min in a Spinco Model L ultracentrifuge. The red-brown mitochondrial band was collected using a side collecting No. 13 hypodermic needle. The pooled mitochondria were diluted with the pH-7.4 buffer about 1.5 times and centrifuged 30 min at 30000 rev./min in a Spinco No. 30 rotor in the Model L centrifuge. The pellets were suspended in a solution of 1 part 0.9 % KCl and 2 parts pH-7.4 buffer at a concentration of 10–20 mg/ml and the suspensions were frozen.

Isolation of mitochondria from repressed yeast

For the repressed culture of yeast, 10 % glucose, 1 % peptone and 1 % yeast extract was used as the medium. 50 μC of uniformly ^{14}C-labeled amino acids (Amhersham/Searle) were added to each liter of media at the time of inoculation. The cultures were grown at room temperature on a rotary shaker to early stationary phase and the cells harvested and broken as described for the aerobic yeast. After the cells were broken, the suspension was centrifuged at 1000 $\times$ g for 10 min and the supernatant

fraction removed and centrifuged again at 1000 × *g* for 10 min. The supernatant fraction from this centrifugation was then centrifuged for 45 min at 30000 rev./min in a No. 30 rotor in the Model L centrifuge and the pellets obtained were resuspended in undiluted Renografin (1 g pellet per ml Renografin) and banded by density gradient centrifugation as above. The mitochondrial bands were collected, repelleted, suspended and frozen as described for the aerobic mitochondria.

Isolation of membrane proteins

The tritium-labeled "derepressed" and ^{14}C-labeled "repressed" mitochondrial suspensions were thawed and combined. These were then centrifuged at 40000 rev./min in the Spinco No. 40 rotor for 40 min. The resulting pellet was resuspended and washed twice with a 0.6 % KCl solution (2 parts 0.9 % KCl *plus* 1 part Tris buffer (pH 7.4)) using 1 ml of this solution for every 10–20 mg of protein. After the last wash, the residue was suspended in pH-7.4 buffer and the protein determined using the procedure of LOWRY *et al.*[5]. A 10 % sodium deoxycholate, 5 % sodium cholate solution in water was added to the suspension to yield a final concentration of 2 mg of deoxycholate and 1 mg of cholate per mg of protein. This was stirred for 1 h at room temperature and then centrifuged at 48000 × *g* for 20 min. The supernatant fraction was removed from the pellet, and both kept for further treatment. The supernatant fraction is referred to as the deoxycholate supernatant and the pellet as the deoxycholate pellet.

The deoxycholate supernatant was brought to 15 % saturation by addition of saturated (at room temperature) $(NH_4)_2SO_4$ and stirred at 4° for 1 h. The turbid suspension was centrifuged at 48000 × *g* for 20 min. The pellet from this centrifugation was homogenized with a small volume of buffer and extracted 3 times with 25 vol. of a mixture of 95 % ethanol and diethyl ether (3:1, v/v). The extracted precipitate was washed 3 times with 0.01 M Tris buffer (pH 8.5) and finally resuspended in the pH-8.5 buffer at a final concentration of 20 mg/ml. The deoxycholate pellet fraction was treated in an analogous manner, however, without the $(NH_4)_2SO_4$ fractionation step.

Solubilization of the membrane protein

The protein concentration of the above suspension was adjusted to between 5 and 10 mg/ml. This suspension was made 1 % in sodium dodecyl sulfate and after N_2 had been blown over the surface of the rapidly stirred solution for 10 min to remove O_2, a 100-fold excess (assuming one sulfhydryl per 25000 mol. wt.) of dithiothreitol was added. The reaction vessel was sealed and the reduction allowed to proceed for approx. 4 h at room temperature. At the end of this time a 10-fold (relative to the dithiothreitol) excess of recrystallized sodium iodoacetate in 1 M Tris–HCl buffer (pH 8) was added to the reduced protein solution and allowed to react in the dark for 10 min. The resulting solution was then dialyzed 18 h using three changes of Tris buffer (pH 8.5) in 500-fold volume excess.

Fractionation of the solubilized protein on gels

The solubilized protein was fractionated on 8 % acrylamide gels using the discontinuous method of ORNSTEIN AND DAVIS[6] modified to contain 0.03 % dodecyl sulfate in the upper buffer. The migrating dye fronts were marked by insertion of a

small piece of copper wire and the gels stained in a 0.5 % amido schwartz solution in 40 % ethanol, 15 % acetic acid. Gels were destained after 30 min in the dye with 10 % acetic acid to remove most of the dye and then transferred to a solution of 50 % ethanol, 5 % acetic acid for color development of the protein bands. The stained protein bands were cut out and placed into scintillation counting vials and heated at 80–100° until they were slightly opaque. Then, 0.2 ml of 30 % H_2O_2 was added to each vial containing approx. ten gel slices (of the 5-mm diameter gel size), and the vials were sealed and placed into water bath at approx. 80° until the gels were completely solubilized. After cooling, 1 ml of NCS solubilizing fluid was added to each vial and then 10 ml of toluene scintillation fluid. ^{14}C and ^{3}H levels were counted simultaneously in a Packard Tri-Carb liquid-scintillation counter.

RESULTS AND DISCUSSION

A photograph of the gel patterns derived from the deoxycholate supernatant and deoxycholate pellet is presented in Fig. 1. The bands are designated by numbers for subsequent discussion. The respective distributions of ^{3}H and ^{14}C in the solubilized gel bands are presented in Tables I and II for the deoxycholate supernatant and deoxycholate pellet, respectively.

The total absence of any component in either the repressed or derepressed state would be indicated by a ratio of $^{3}H/^{14}C$ of either infinity or zero for the respective states. However, as is clearly demonstrated by the data in Tables I and II a significant amount of each component is found in both states, indicating that there is no such qualitative difference detectable between the proteins of the two states. Quantitatively, however, gel component 4 shows a very high increase in its concentration in the derepressed membrane fraction relative to the other components. This is best

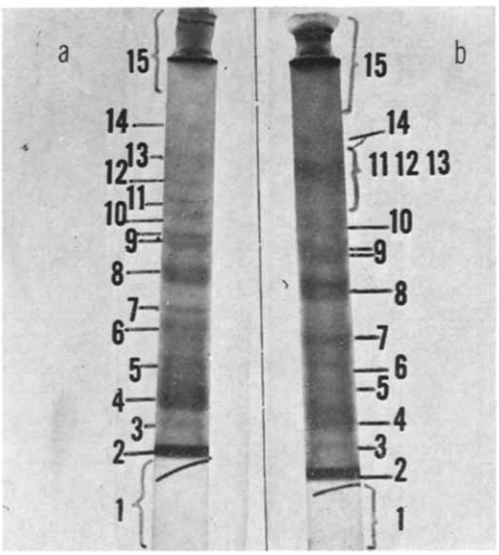

Fig. 1. Gel-electrophoresis patterns of deoxycholate-soluble (a) and deoxycholate-insoluble (b) mitochondrial membrane proteins.

TABLE I

DISTRIBUTION OF RADIOACTIVE AMINO ACIDS IN THE SODIUM DEOXYCHOLATE-SOLUBLE MEMBRANE PROTEINS OF YEAST MITOCHONDRIA

*Band No.:**	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
$^{3}H/^{14}C$**	4019/4940	630/730	3515/1416	1267/1084	1110/1564	402/538	1309/1459	821/1097	241/431	295/617	192/519	335/1081	326/1273	442/1689
Ratio	0.81	0.86	2.48	1.17	0.71	0.75	0.90	0.75	0.56	0.48	0.37	0.31	0.26	0.26
^{3}H component of total ^{3}H fraction (%)	27	4	24	8	7	3	9	6	2	2	1	2	2	3
^{14}C component of total ^{14}C fraction (%)	27	4	8	6	8	3	8	6	2	3	3	6	7	9

* Band 1 = blank used for background determination.
** Values are 10-min counts corrected for background and ^{14}C overlap into ^{3}H.

TABLE II

DISTRIBUTION OF RADIOACTIVE AMINO ACIDS IN THE SODIUM DEOXYCHOLATE-INSOLUBLE MEMBRANE PROTEINS OF YEAST MITOCHONDRIA

*Band No.:**	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11,12,13*	*14*	*15*
$^{3}H/^{14}C$**	2736/7345	485/998	2148/1817	691/1226	504/1457	647/1972	1017/2631	617/1908	422/1533	589/2499	526/2662	815/4294
Ratio	0.37	0.49	1.18	0.56	0.35	0.33	0.39	0.32	0.28	0.24	0.20	0.19
^{3}H component of total ^{3}H fraction (%)	24	4	19	6	5	6	9	6	4	5	5	6
^{14}C component of total ^{14}C fraction (%)	24	3	6	4	5	7	9	6	5	8	9	14

* Band 1 = blank used for background determination.
** Values are 10-min counts corrected for background and ^{14}C overlap into ^{3}H.

illustrated in Fig. 2 by the abrupt increase in $^{3}H/^{14}C$ ratio for this component. As a similar distribution of membrane proteins of the repressed and derepressed states should be reflected in a constant ratio of $^{3}H/^{14}C$ throughout the component in the gel, it is apparent that the distribution of proteins in the insoluble mitochondrial membrane fractions differs markedly for the repressed and derepressed states only in the concentration of this single component.

It may be noted that the ratio of $^{3}H/^{14}C$ is lower for the deoxycholate pellet than for the deoxycholate supernatant. This is consistent with previous observations in this laboratory that repressed yeast yield higher proportions of deoxycholate-insoluble material (approx. 60 %) than derepressed cells (approx. 10 %)[7]. In lieu of other evidence it could be suggested that this is a reflection of gross differences in the protein composition of these membranes. However, the data show that there is a definite and constant relationship in the distributions of the proteins from these two fractions, *i.e.* the overall gel patterns of the two preparations are nearly identical. A doubling of the ratios observed for each component in the deoxycholate pellet presents strikingly similar ratios for the comparable components in the deoxycholate supernatant. While the particular factor of 2 may be fortuitous, the overall implications of the constant doubling ratio should remain valid regardless of the particular factor used. In addition the similar distributions of proteins in the two fractions suggest that all the components from the repressed and the derepressed yeast are being acted upon by deoxycholate as a group of proteins rather than as separate components. It might therefore be concluded from this observation that the individual components of each fraction are actually being isolated as "complexes" which are only resolved into separate components by the treatment after the deoxycholate step

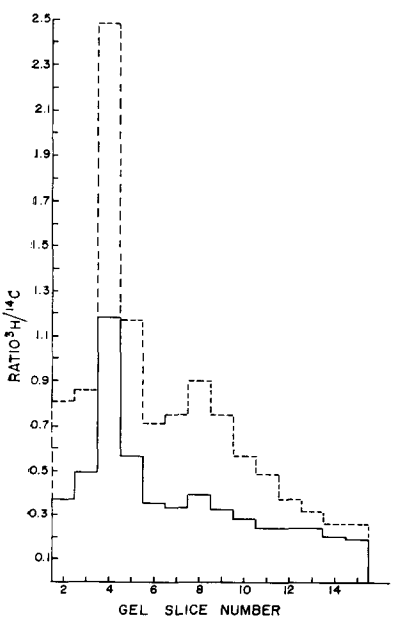

Fig. 2. Distribution of $^{3}H/^{14}C$ ratios in membrane protein fractions. Upper curve, data from deoxycholate-soluble protein (Table I) and lower curve, data from deoxycholate-insoluble protein (Table II).

of the isolation procedures. It appears therefore that the characteristics peculiar to each of the complexes that facilitate their differential solubilization and resolution from one another must result from structural differences within the protein complexes or from constituents other than the proteins themselves. Such differences could result from association with lipid, carbohydrate, *etc.*

These studies have demonstrated that there are quantitative but no apparent qualitative differences between the insoluble mitochondrial membrane proteins from glucose repressed and derepressed yeast and that there is a similar (if not identical) distribution of protein components in both the deoxycholate pellet and deoxycholate supernatant fractions. Therefore, there appear to be definite complexes or units of membrane with common protein components in the deoxycholate pellet and deoxycholate supernatant fractions with solubility determined by some other membrane constituent or state of membrane structure. It cannot be ruled out that these may even represent separate classes of mitochondria. In addition this suggests that these complexes may be a reflection of a basic structural unit of the mitochondrial membrane.

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