ABNORMALITIES IN MITOCHONDRIAL RESPIRATORY CHAIN ASSEMBLY AND THEIR PROTEOLYTIC ELIMINATION

A. V. GALKIN, T. V. TSOI and V. N. LUZIKOV
Department of Subcellular Biogenesis, Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Lomonosov State University, Moscow 117234, USSR

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1. Introduction

The possible regulatory role of proteolytic enzymes in mitochondrial development has been discussed [1–3]. Nevertheless, it remained unknown whether such regulation was executed simply through coordination of synthesis and breakdown of mitochondrial proteins or whether more subtle mechanisms could be involved. On the other hand, the stringent dependence of the stability of the mitochondrial oxidative phosphorylation machinery on the efficiency of its functioning, evidenced by model in vitro and in vivo experiments in this laboratory [4], gave grounds for a hypothesis [4,5] that lytic enzymes may control mitochondrial assembly by selectively eliminating components which for some reason fail to perform properly. This idea seemed to be supported by [6]. It was shown that at the earlier stages of respiratory development during aerobic growth of *Saccharomyces cerevisiae* on galactose an excess of mitochondrial cytochromes is formed which proves to be unnecessary for the maintenance of a constant respiration level and is eliminated in the course of further yeast growth. Elimination appeared to be proteolytic since it was suppressed by phenylmethyl sulfonyl fluoride (PMSF) and pepstatin that are known to inhibit yeast proteinases [7–9]. However, the actual causes of the apparent inactivity of the 'excess' components remained obscure.

This work demonstrates that such 'excess' entities, albeit potentially functional, are indeed ineffective as members of the respiratory chain, most probably due to their improper integration into the mitochondrial inner membrane.

2. Materials and methods

Yeast growth conditions were the same as in [10,11]. PMSF and pepstatin were added portionwise, beginning from the mid-exponential growth phase, to cumulative concentrations of 2×10^{-4} M and 1.5×10^{-6} M, respectively [6].

Cell homogenates were prepared according to [12] in a medium containing 0.6 M sorbitol, 1 mM EDTA, 0.25% bovine serum albumin, 20 mM Tris-maleate, 10 mM potassium phosphate (pH 6.7). Membrane fractions were obtained by centrifuging the homogenates at 70 000 × *g* for 30 min. Cytochrome contents and enzymatic activities were quantitated with respect to unit weight of cells from which the fractions had been isolated.

3. Results and discussion

Membrane fractions were obtained from mid-exponential control cells shown to possess the highest cytochrome content [6], from stationary control cells and from cells grown from mid-exponential to stationary phase in the presence of PMSF and pepstatin. These fractions contained the entire mitochondrial populations of the corresponding cells, as judged by determinations of cytochrome contents and inner membrane marker enzymes in cell homogenates, 70 000 × *g* fractions and supernatants. The data presented in table 1 are in good accord with the results obtained for intact cells [6]. The overall cytochrome content in mitochondrial populations from stationary control cells is 30–40% lower than that in

Table 1
Characteristics of mitochondrial populations isolated from *S. cerevisiae* cells

Source of preparation	Cytochrome content (nmol .g dry wt cells^{-1})			Activities (μg-atoms O. (min .g dry wt cells)$^{-1}$)			
	cyt. $c + c_1$	cyt. b	cyt. aa_3	succinate oxidase + 5 μM cyt. c		ubiquinol oxidase	ascorbate oxidase
Mid-exponential phase, control	48.7	59.3	13.0	26.2	38.3	95.0	278
Stationary phase, control	26.8	39.1	8.6	27.1	38.9	94.3	261
Stationary phase, inhibitor-grown	43.7	57.3	12.4	26.8	37.8	95.4	263

Cytochrome contents were calculated from the difference (reduced minus oxidized) room-temperature spectra of the membrane fractions. The following wavelength pairs and extinction coefficients (mM^{-1} .cm^{-1}) were used: cytochromes $c + c_1$ (551–540 nm), 20; cytochromes b (562–575 nm), 19.1; cytochromes aa_3 (605–630 nm), 24 [14]. Activities were measured at 30°C in 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, with the following substrates: succinate oxidase, 20 mM succinate; ubiquinol oxidase, 16 μM reduced CoQ_2; 4 μM cytochrome c; ascorbate oxidase, 20 mM ascorbate; 1 mM TMPD; 20 μM cytochrome c

preparations from exponential cells; practically no decrease in the cytochrome contents is observed in the presence of proteinase inhibitors. A similar relation was found for the contents of succinate-reducible flavoproteins (not shown). At the same time, succinate oxidase activities of all three preparations are identical and are equally stimulated by cytochrome c. The activities of shorter respiratory chain segments (ubiquinol oxidase and ascorbate oxidase) also prove to be similar, regardless of the concentration of substrates and exogenous cytochrome c (table 1). Thus the lack of surplus activity in preparations containing excess amounts of respiratory chain components is not due to their lower affinity for substrates or to a deficiency in the 'mobile linkages' (i.e., coenzyme Q and cytochrome c) between electron transfer complexes. Moreover, as follows from table 2, even such short segments as succinate:ubiquinone reductase, ubiquinol:cytochrome c reductase and cytochrome c oxidase display almost identical activities when these are assayed in intact membranes ('basal' activities). Therefore it can be suggested that the excess components cannot participate in mitochondrial oxidation.

The next step in probing the nature of the

Table 2
Activation of the individual respiratory chain complexes in solubilized membrane preparations

Source of preparation	Succinate:ubiquinone reductase (μmol CoQ_2 reduced .min^{-1})		Ubiquinol:cytochrome c reductase (μmol cyt. c reduced .min^{-1})		Cytochrome oxidase, (μmol ferrocyt. c oxidized .min^{-1})	
	basal	activated	basal	activated	basal	activated
Mid-exponential phase, control	32.1	44.0	42.0	77.3	19.0	56
Stationary phase, control	29.2	32.5	41.4	58.1	18.4	32.5
Stationary phase, inhibitor-grown	30.4	44.7	40.5	75.0	18.2	52.6

Activities were assayed in intact membranes (basal) and after membrane disruption by freezing–thawing in isolation medium with 0.05% Lubrol WX. The detergent was also present in the assay media for activated samples (concentrations in brackets). Succinate:ubiquinone reductase, 20 mM succinate, 6.7 μM CoQ_2 (0.01% Lubrol); ubiquinol:cytochrome c reductase, 14.5 μM reduced CoQ_2, 18 μM cytochrome c (0.03% Lubrol); cytochrome oxidase, 11 μM ferrocytochrome c, 0.24 mM dissolved oxygen (0.06% Lubrol). Activities given per g dry wt. cells (see section 2)

observed phenomena was the evaluation of the potential catalytic capacity of the individual electron transfer complexes. To this end, mitochondrial membranes were disrupted by freezing–thawing in the presence of a non-ionic detergent, and succinate: ubiquinone reductase, ubiquinol:cytochrome *c* reductase and cytochrome oxidase activities were determined in a range of substrate concentrations under conditions ensuring maximal activation. As exemplified in table 2, the activities of the 3 constituent respiratory chain complexes in solubilized preparations from exponential and inhibitor-grown cells exceed those of the stationary control. Furthermore, the relation of activities is nearly proportional to the contents of the corresponding components, and app. K_m values are rather similar (e.g., fig.1; analogous results were obtained for the other two complexes). All this gives grounds for concluding that the 'excess' components are essentially normal per se but they fail to form a conjoint catalytic system (i.e., respiratory chain) most probably due to improper alignment within the membrane.

Such non-integrated elements are obviously not

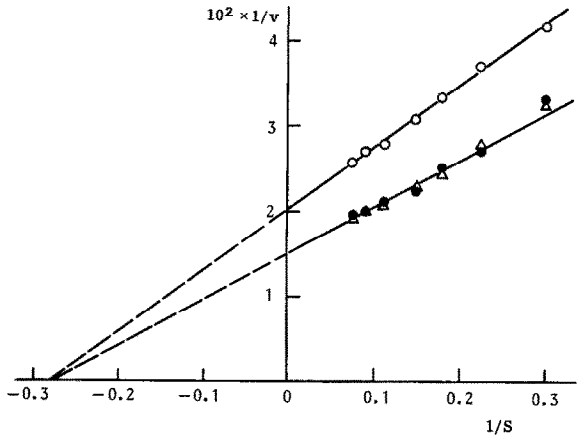

Fig.1. Succinate:ubiquinone reductase activities in solubilized mitochondrial preparations from exponential (△), stationary control (○) and stationary inhibitor-grown (●) cells. The assay medium contained 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 20 mM succinate, 4×10^{-7} M antimycin, 1 mM KCN, 0.01% Lubrol WX; temp. 30°C. Membrane fractions were preincubated with 20 mM succinate to activate succinate dehydrogenase. Reaction was started by adding CoQ_2 in a minimal volume of methanol. Initial rate (v) is expressed as μmol CoQ_2 reduced $.min^{-1}$ .g dry wt cells^{-1} (see section 2); S is expressed as μM CoQ_2. Straight lines are least square fittings.

the sequelae of the presence of PMSF and pepstatin, since they are also observed in the mitochondria of the exponential control cells. Their elimination in the course of further respiratory system development can be prevented by proteinase inhibitors. The reported phenomena are not the unique property of yeast growth on galactose: accumulation of 'excess' inactive components has also been found in yeast growing in a glucose medium with PMSF or pepstatin [13]. The above argues in favour of such abnormalities arising from naturally occurring errors in mitochondrial assembly, and non-functioning entities being selectively eliminated by proteolytic destruction, in accordance with the hypothesis [4,5].

A number of questions like the nature of the proteinases responsible or the possible involvement of other enzymes need further clarification. Nevertheless, the proposed principle of 'selection by a performance criterion' already seems to be worth consideration, at least because it explains the apparent high precision of the immensely complex process of mitochondrial assembly.

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