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PHYSICAL STATE OF THE MITOCHONDRIAL INNER MEMBRANE AS A FACTOR CONTROLLING THE PROTEOLYSIS OF MITOCHONDRIAL TRANSLATION PRODUCTS IN YEAST

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The degradation of mitochondrial translation products has been studied in *Saccharomyces cerevisiae* yeast. A high rate of degradation is observed in the early exponential phase of aerobic growth. Maturation of the yeast and glucose repression suppress the degradation. Anaerobic growth is also marked by a low breakdown rate of mitochondrial translation products. These variations did not correlate with the cytochrome *c* hydrolase activity of sonic submitochondrial particles (this activity was shown to reflect the general state of the proteolytic system of the inner mitochondrial membrane that is responsible for the breakdown of mitochondrially made polypeptides; see Novikova, L.A., et al. (1981) FEBS Lett. 135, 245–248). Experiments with lipid-soluble paramagnetic probes revealed significant variations in the physical state of the mitochondrial inner membrane, as judged from the comparison of the temperature-dependence plots of structural parameters obtained from the EPR spectra of the probes. The breakdown of mitochondrial translation products was, in general, the more rapid the lower was the temperature of the structural transition in the mitochondrial inner membrane and the higher was the relative content of unsaturated fatty acyl chains in the membrane phospholipids.

Introduction

The inner mitochondrial membrane is known to be made up of polypeptides synthesized both on mitochondrial and on cytoplasmic polysomes (see references in Refs. 1 and 2). The mitochondrially made polypeptides are found in some enzymic complexes of the membrane, e.g., in coenzyme Q-cytochrome *c* reductase (cytochrome *b*), cytochrome *c* oxidase (subunits I, II, and III), H^+-ATPase (several subunits of the membrane sector). This means that the mitochondrial translation products are indispensable for the formation of respiring and energy-transducing mitochondria.

Several years ago [3], we developed a procedure for determining the rate of proteolysis of mitochondrially made polypeptides in vivo that takes into account the recycling and postincorporation of the label. Using this method, we were able to establish that the half-lives of the mitochondrial translation products can be as short as 15–20 min under certain conditions of *Saccharomyces cerevisiae* growth [4]. As shown through experiments done later [5,6], the proteolysis of these polypeptides is carried out by proteinase(s) localized in the inner mitochondrial membrane.

Bearing in mind the potentially high activity of these proteinases towards the mitochondrially made polypeptides, one could suppose that the assembly of the inner mitochondrial membrane is proteolytically controlled (through maintaining a certain level of the above-mentioned enzymic com-

plexes or removing unassembled polypeptides of mitochondrial origin).

The present work is devoted to elucidating the principles governing the proteolysis of mitochondrially made polypeptides. The data presented suggest that the rate of this process in yeast under various physiological conditions depends on the physical state of the mitochondrial inner membrane. In particular, it may be controlled by the order and mobility of hydrocarbon chains of membrane phospholipids which, in their turn, determine the lateral mobility of membrane proteins.

Materials and Methods

The strain of *S. cerevisiae* and growth conditions have been described [7]. The yeast was grown aerobically to the early exponential or early stationary phase at 35°C in a semisynthetic medium containing 0.25% glucose. In experiments with glucose repression, the yeast was grown aerobically to the early stationary phase and then thickened to 5% (wet wt.). The cells, resuspended in the growth medium and supplemented with 10% glucose, were incubated for 0.5, 1.0, 1.5, and 2.0 h. In other experiments, the yeast was grown anaerobically for 26 h at 35°C (to the early stationary phase), with argon (oxygen content below 0.01%) bubbled through the cell suspension. All the procedures used for harvesting and washing the cells were carried out in the flow of argon below 5°C.

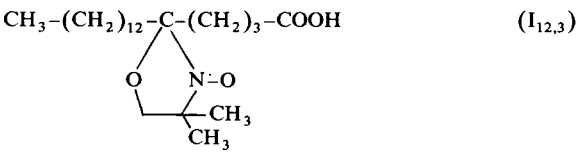
The mitochondrial translation products were selectively labelled with [^{3}H]leucine in the presence of cycloheximide, and their proteolysis was followed as described previously [3]. With the repressed yeast, [^{3}H]leucine was incorporated into these polypeptides 30 min before the completion of each period of incubation.

The cell homogenate was obtained by the method of Tzagoloff [8], and mitochondria and sonic submitochondrial particles (the inner mitochondrial membrane preparation) were isolated according to Schatz [9]. The submitochondrial particles were not contaminated with either outer mitochondrial membranes, endoplasmic reticulum, or vacuoles, as evidenced by the assays of kynurenine hydroxylase, antimycin-insensitive NADH-cytochrome *c* reductase, and proteinases A and B.

Cytochrome *c* hydrolase activity was assayed as described in Ref. 6. Protein content was measured by the method of Lowry et al. [10] with bovine serum albumin as standard.

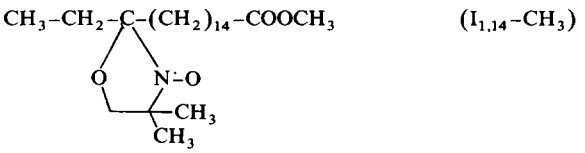
Lipids were extracted from submitochondrial particles as described by Folch et al. [11]. Methyl esters of fatty acids were prepared by the method of Metcalfe et al. [12]. The content of fatty acids was determined in a Varian model 3700 gas chromatograph, column 0.4 cm × 100 cm, phase SP-2330 on Chromosorb W (100–200 mesh), column temperature 150–200°C, carrier He at 2 ml/min, injection and detector temperature 250°C.

Lipid-soluble spin-labeled derivatives of stearic acid:



($I_{12,3}$)

and



($I_{1,14}$-CH_3)

were used as probes for thermoinduced structural rearrangements in the submitochondrial particles [13–15]; both were manufactured by Syva (Palo Alto, CA, U.S.A.). The probes were dissolved in ethanol; their final concentration in the submitochondrial particle suspension was $1 \cdot 10^{-4}$ M. The molar ratio of the spin probe to the submitochondrial particle lipid never exceeded 1 : 30. The final concentration of ethanol did not exceed 1%; a previous study has shown that ethanol in this concentration does not create any side effects which might be associated with modification of the submitochondrial particle membrane [14–16]. Our experiments have shown that EPR spectra were not distorted owing to intermolecular interactions of the probes. This followed from the fact that varying the probe concentration ($2 \cdot 10^{-5}$ to $4 \cdot 10^{-4}$ M) did not affect the EPR spectra.

The EPR spectra of the spin labels were taken in a Varian E-4 EPR spectrometer with the follow-

ing instrument settings: field modulation frequency, 100 kHz; microwave power, 10 mW; microwave frequency, 9.17 GHz; modulation amplitude, 1 G; time constant, 0.1–0.3 s; scan rate, 12.5 G/min. Sample temperature was regulated with a variable temperature controller accurate within 0.5°C.

The rotational correlation time (τ_c) of the paramagnetic group of the $I_{1,14}$–CH_3 probe was calculated by Eqn. 1 according to McConnell and McFarland [17]:

$$\tau_c = 0.65 H_0 \left(\sqrt{\frac{I_0}{I_{-1}}} - 1 \right) \quad (1)$$

The parameter characterizing the degree of anisotropy of the rotation of this probe was the value ε determined, as described in Ref. 18, by Eqn. 2:

$$\varepsilon = \left(\sqrt{\frac{I_0}{I_1}} - 1 \right) \Big/ \left(\sqrt{\frac{I_0}{I_{-1}}} - 1 \right) \quad (2)$$

where I_1, I_0 and I_{-1} are the amplitudes of the superfine structure components of the EPR spectrum of $I_{1,14}$–CH_3, and H_0 is the width of the central component.

The EPR spectrum of the $I_{12,3}$ probe immersed in the membrane makes it possible to determine parameter S, which characterizes the ordering of the lipid bilayer surrounding the paramagnetic group of the probe [13–16, 18–21]. For our aims, parameter $2T'_{\parallel}$ (proportional to S) was more expedient. Indeed, to determine S one has to know, in addition to $2T'_{\parallel}$ (the distance between extrema 1 and 5 of the EPR spectrum of the $I_{12,3}$ probe, see Fig. 2a), parameter $2T'_{\perp}$ (the distance between extrema 2 and 4 of the spectrum, see Fig. 2a). The latter cannot be determined accurately at temperatures below 10°C when the probe in the membrane moves slowly and extremum 4 is absent.

The plots of the EPR spectral parameters vs. temperature were approximated with straight lines by the least squares method using the Hewlett-Packard model 9825A computer.

Results

Degradation of mitochondrial translation products

The degradation of mitochondrial translation products was studied in pulse-chase experiments, in which changes in the [^{3}H]leucine content in the trichloroacetic acid-insoluble fraction of mitochondria were followed after selectively incorporating the label in vivo into these polypeptides. The procedure described in detail in Ref. 3 allows one to suppress the recycling of the label and to account for its postincorporation in the chase from the aminoacyl-tRNA pools and from slowly exchanging amino acid pools which accumulate in the cells during the pulse in the presence of cycloheximide. The value of $^3H_{m,synth}$, the changes of which are depicted in Fig. 1, represents, in each point, the recorded level of [^{3}H]leucine in the trichloroacetic acid-insoluble fraction of mitochondria minus the amount of the label that appeared in this fraction owing to postincorporation.

As follows from Fig. 1, the degradation of mitochondrial translation products is the fastest in the early exponential phase of yeast aerobic growth. With the maturation of the yeast culture, the rate of proteolysis of mitochondrially made poly-

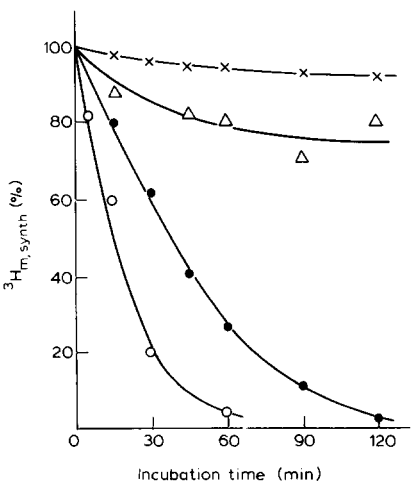


Fig. 1. Breakdown of mitochondrial translation products in yeast cultivated in various conditions. Degradation of mitochondrial translation products was assayed as in Ref. 3. (○) Yeast grown aerobically in a glucose (0.25%) medium to the early exponential phase; (●) yeast grown aerobically in glucose medium to the early stationary phase; (△) yeast grown anaerobically in glucose medium to the early stationary phase; (×) yeast grown aerobically in glucose (0.25%) medium to the early stationary phase and subjected to glucose repression (glucose concentration 10%). $^3H_{m,synth}$ was expressed as a percentage of the values corresponding to the time 0 of the chase.

peptides markedly decreases, so that their half-life in the early stationary phase amounts to 35–40 min vs. 20 min in the early exponential phase. The degradation of mitochondrially made polypeptides becomes still slower if the yeast is subjected to glucose repression in the early stationary phase. After a 2 h repression, the process virtually stops.

The low rate of degradation of these polypeptides is characteristic of the yeast grown anaerobically in a medium not supplemented with unsaturated fatty acids and ergosterol (Fig. 1). It should be noted that of the four above-mentioned cases, the last one shows a comparatively low level of [^{3}H]leucine incorporation (20% of that attained with aerobic yeast). This agrees with the data of Groot et al. [22]. Fig. 1 shows some difference in the proteolysis under glucose repression and under anaerobiosis; however, in both cases, the process is so slow as to be practically negligible.

Cytochrome c hydrolase activity of submitochondrial particles

To elucidate whether the variations in the rate of proteolysis of mitochondrial translation products are associated with the differences in the activity of the proteolytic system of the inner mitochondrial membrane, it was necessary to estimate this activity with an exogenous substrate. Cytochrome *c* may be used in such experiments, as its hydrolysis in the presence of submitochondrial particles, albeit relatively slow, is suppressed by the same inhibitors as the degradation of mitochondrial translation products [6].

Table I shows the cytochrome *c* hydrolase activities of submitochondrial particles prepared from early-exponential and early-stationary aerobically grown yeast, as well as from yeast subjected to glucose repression and those grown anaerobically in the absence of Tween-80 and ergosterol. The submitochondrial particles had close values of cytochrome *c* hydrolase activity assayed in the absence or in the presence of the activating detergent (SDS), with the exception of the early-exponential preparation in which the activity did not increase in the presence of 0.05% SDS. The data in Table I allow one to conclude definitely that in the early exponential phase maximal cytochrome *c* hydrolase activity did not exceed those in the other three cases, whereas the rate of proteolysis of mitochondrial translation products was much higher (see Fig. 1).

TABLE I

INFLUENCE OF YEAST GROWTH CONDITIONS ON THE CYTOCHROME *c* HYDROLASE ACTIVITY OF SUBMITOCHONDRIAL PARTICLES

In all experiments the incubation mixture contained 250 μg submitochondrial particle protein and incubation lasted 1 h. Cytochrome *c* hydrolase activity was measured in the absence or in the presence of 0.05% SDS.

Yeast growth conditions	Cytochrome *c* hydrolase activity (% substrate hydrolyzed)	
	+SDS	−SDS
Early exponential aerobic	5.0	6.5
Early stationary aerobic	12.0	3.0
Early stationary glucose-repressed	10.8	3.0
Early stationary anaerobic	12.3	–

Physical state of the inner mitochondrial membrane

Structural alterations in the inner mitochondrial membrane were to be studied as a probable cause of the different rates of proteolysis of mitochondrial translation products under varying growth conditions. Structural alterations in biological membranes can be studied with lipid-soluble spin probes [13–16,19–21].

Fig. 2a shows an EPR spectrum of the $I_{12,3}$ spin probe in the suspension of submitochondrial particles prepared from early-stationary aerobic yeast. The spectrum is a superposition of the spectra of the two portions of the probe: the major portion with the nitroxyl radical immersed into the membrane and having a hydrophobic environment (extrema 1–5), and the minor one with the paramagnetic group in a polar environment (extrema 6, 7). This is evident from the fact that addition to submitochondrial particles of hydrophilic paramagnetic agents (potassium ferricyanide, $1 \cdot 10^{-2}$ M; or chromium oxalate, $5 \cdot 10^{-3}$ M) weakly affects components 1–5, while components 6 and 7 of the spectrum are broadened owing to interaction of the probe with the water-soluble paramagnetic compounds (data not shown). Thus, it can be concluded that the EPR spectrum of the 'immobilized' type is determined by the movement

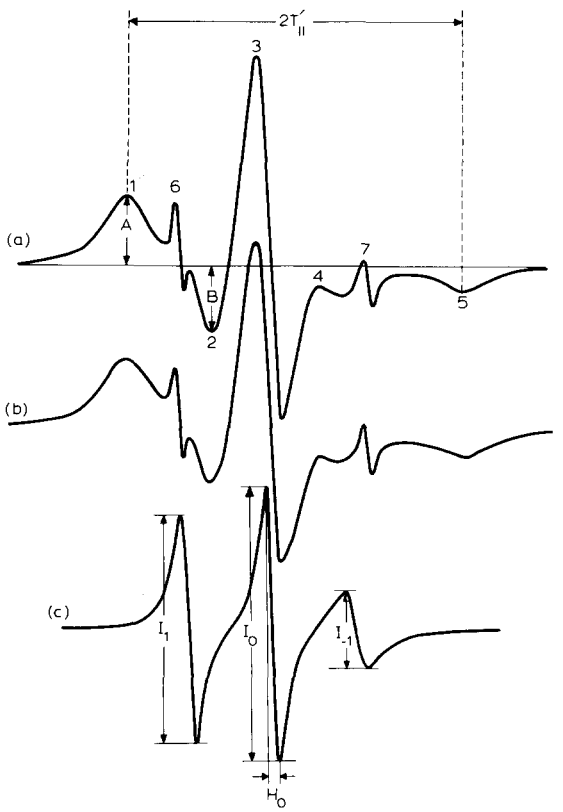


Fig. 2. EPR spectra of spin probes in submitochondrial particle suspensions. (a) $I_{12,3}$ in submitochondrial particles from nonrepressed yeast; (b) $I_{12,3}$ in submitochondrial particles from repressed yeast; (c) $I_{1,14}$-CH_3 in submitochondrial particles from nonrepressed yeast.

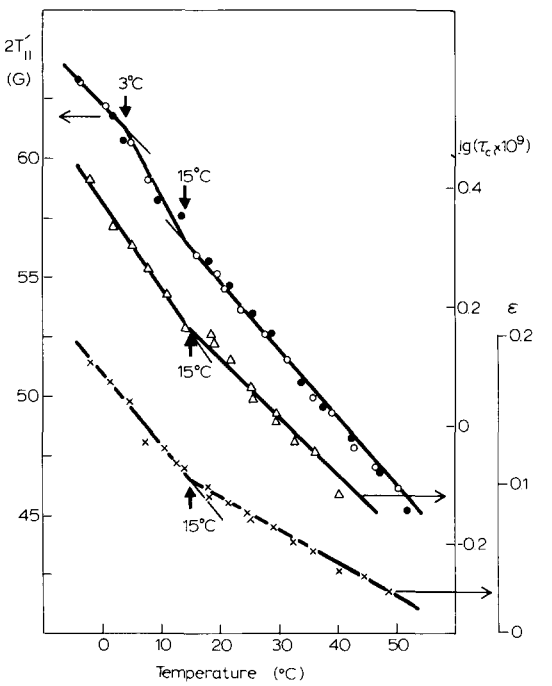


Fig. 3. Temperature dependences of EPR spectral parameters of spin probes in submitochondrial particle suspensions. (○,●) Parameter $2T'_{II}$ of $I_{12,3}$ (open and filled circles correspond to two experiments with different submitochondrial particle preparations); (△) parameter τ_c of $I_{1,14}$-CH_3; (×) parameter ε of $I_{1,14}$-CH_3. Submitochondrial particles were isolated from yeast grown to the early stationary phase.

of the $I_{12,3}$ probe in the submitochondrial particle membrane. Collation of the spectra obtained here with the data available in the literature [20,21] makes it possible to conclude that the probe undergoes fast anisotropic rotation.

Fig. 3 shows the typical pattern of the temperature dependence of parameter $2T'_{II}$ for the $I_{12,3}$ probe bound with submitochondrial particles isolated from early-stationary aerobic yeast. It can be seen that this dependence has two characteristic inflections (at 3 and 15°C) which are distinctly revealed by approximating the curve with straight lines. Temperature dependences like these are held to furnish information on the physical state of the lipid bilayer in biological membranes including submitochondrial particle membranes and membraneous preparations of respiratory complexes (for reviews see Refs. 13,19–21).

Similar results were obtained with the $I_{1,14}$-CH_3 spin probe. The EPR spectrum of this probe in submitochondrial particles (Fig. 2c) indicates that it undergoes restrained rotation in the hydrophobic region of the membrane (addition of potassium ferricyanide or chromium oxalate had no effect on the spectrum), the rotation being to a large extent isotropic. Since spectra of $I_{1,14}$-CH_3 bound to serum albumin, γ-globulins, and other serum proteins substantially differed (data not shown) from that represented in Fig. 2c, one may suggest that it is the movement of $I_{1,14}$-CH_3 in the membrane lipid phase that determines its EPR spectrum (cf. Ref. 20).

The $I_{1,14}$-CH_3 EPR spectrum makes it possible to determine, albeit formally but simply and accurately, the rotational correlation time τ_c (for $10^{-11} \ll \tau_c < 10^{-9}$ s). The lack of components of the spectrum indicating probe interaction with water as well as the lack of effect of paramagnetic broad-

eners on the spectrum testify that there is no partitioning of $I_{1,14}$-CH_3 between water and lipid which might have affected the apparent τ_c. Fig. 3 presents the temperature dependence of this parameter. It is also approximated by straight lines which intersect at 15°C for submitochondrial particles from early-stationary yeast. The inflection at 3°C in this case is not discerned, since in the low temperature region the movement of the probe is so retarded ($\tau_c > 10^{-9}$ s) that Eqn. 1 is inapplicable [17].

Fig. 3 shows the data obtained in studying the temperature dependence of the parameter ε which characterizes the degree of anisotropy of rotation of the $I_{1,14}$-CH_3 probe and, consequently, the degree of ordering of the hydrocarbon chains of membrane lipids. One can see that there is an inflection on the plot at 15°C like in the two previous cases (Fig. 3).

As already indicated, the temperature dependence of $2T'_{\parallel}$ for submitochondrial particles from stationary aerobic yeast has inflections at 3 and 15°C (see Fig. 3). For submitochondrial particles from exponential aerobic yeast (Fig. 4) the inflections are shifted to lower temperatures (2 and 5°C). On the other hand, for submitochondrial particles from stationary aerobic yeast subjected to a 2 h glucose repression and from anaerobically grown yeast (Fig. 4), the inflections are shifted to higher temperatures (13 and 23°C or 13 and 29°C, respectively).

Inflections on the plots for both spin probes were well reproducible. For instance, variations for five submitochondrial particle preparations from early-stationary aerobic yeast were ±1°C.

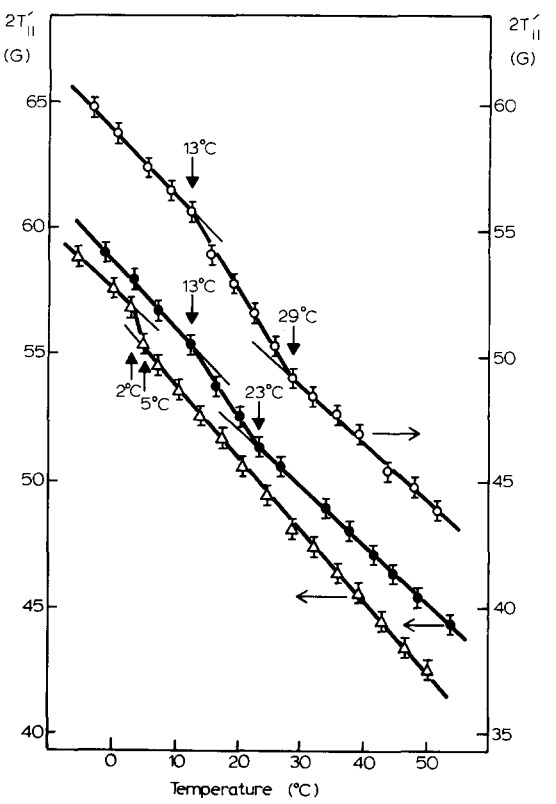


Fig. 4. Temperature dependence of the $2T'_{\parallel}$ parameter of the EPR spectrum of spin probe $I_{12,3}$ in submitochondrial particle suspension. (△) Submitochondrial particles of yeast grown to the early exponential phase; (●) submitochondrial particles of yeast after 2 h glucose repression in the early stationary phase; (○) submitochondrial particles of yeast grown anaerobically to the early stationary phase. To avoid superposition of curves and to show more clearly the inflections, the ordinate for the upper curve (submitochondrial particles from anaerobic yeast) is given on the right (follow horizontal arrows).

Variations in the relative content of unsaturated fatty acids in mitochondrial inner membrane phospholipids

It can be inferred from the preceding section that the phase-transition temperatures in submitochondrial particles increase in the following order: early exponential phase of aerobic growth < early stationary phase < glucose repression ⩽ anaerobic growth. In all four cases, chromatographic analysis of fatty acids in the submitochondrial particle phospholipid fraction was carried out. Table II shows that the particles from early-exponential yeast have the highest coefficient K (the ratio of the total amounts of unsaturated to saturated fatty acids). The coefficient is twice as low for the repressed yeast and more than 5-times lower for the anaerobically grown yeast. Thus, coefficient K decreases in the order: early exponentill phase of aerobic growth > early stationary phase > glucose repression > anaerobic growth.

It is to be mentioned that the yeast strain we were dealing with has only few fatty acids in the phospholipid fraction of the inner mitochondrial membrane, namely palmitic (16 : 0), stearic (18 : 0), palmitoleic (16 : 1), and oleic (18 : 1) acids. In the

TABLE II

INFLUENCE OF YEAST GROWTH CONDITIONS ON THE FATTY ACID COMPOSITION OF THE TOTAL PHOSPHOLIPID FRACTION OF SUBMITOCHONDRIAL PARTICLES

Preparation	Relative fatty acid content (% of total)							*K*
	$C_{12:0}$	$C_{14:0}$	$C_{14:1}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	
Early exponential aerobic	–	–	–	9.2	55.6	1.2	34.0	8.6
Early stationary aerobic	–	–	–	8.9	50.4	4.4	36.3	6.5
Early stationary glucose-repressed	–	–	–	11.8	48.1	8.0	32.0	4.0
Anaerobic	4.8	9.4	4.6	18.9	46.6	6.0	9.6	1.5
Glucose-repressed in the presence of Tween-80	–	–	–	10.5	40.7	7.5	41.3	4.0

early exponential phase, stearic acid was nearly absent from this fraction. In anaerobic yeast, besides the four above acids, lauric (12 : 0), myristic (14 : 0), and myristoleic (14 : 1) acids were also detected.

It seemed worthwhile to check whether coefficient *K* would increase on addition of unsaturated fatty acids to the yeast growth medium. In the experiment, the results of which are presented in Table II, the yeast was subjected to a 2 h glucose repression in the presence of Tween-80 (5 ml/l). One can see that coefficient *K* in this case was the same as for repression without Tween-80. It should be added that the relative content of individual unsaturated fatty acids was thereby appreciably altered.

Discussion

As follows from this and a number of our previous works [3–5], the mitochondrial translation products are rapidly renewed proteins, the half-lives of which may, under certain physiological conditions, be as short as 15–20 min. As these half-lives were measured for polypeptides that had been synthesized under suppressed cytoplasmic translation, the physiological significance of such fast proteolysis may be the removal of the mitochondrially made polypeptides that have not been properly incorporated into the membrane because of the lack of cytoplasmically made partners [23].

The proteolysis of mitochondrial translation products is an intramembrane process. Indeed, both the digestible polypeptides and the digestive proteolytic system are localized in the inner mitochondrial membrane [6]. There may be, generally speaking, three main reasons for the altered rate of intramembrane proteolysis: (1) altered activity (or amount) of proteinase; (2) altered set of proteins to be hydrolyzed; and (3) altered fluidity of the membrane which determines the rate of lateral and rotational diffusion of membrane proteins [13] and, consequently, the conditions for their interaction.

It has been pointed out previously [6] that mitochondrial translation products are likely to be broken down by the same enzyme(s) as the externally added cytochrome *c*. Since the cytochrome *c* hydrolase activity of sonic submitochondrial particles was nearly the same under different physiological conditions, one may suggest that the substantial variations in the rate of proteolysis of mitochondrially made polypeptides could hardly be associated with alterations in the proteolytic system of the inner mitochondrial membrane.

The second suggestion also does not seem plausible. Indeed, the practically complete cessation of the proteolysis of mitochondrial translation products under glucose repression of yeast and the very slow proteolysis in anaerobically grown yeast (see Fig. 1) would imply that the normal set of hydrolyzable polypeptides is entirely (or almost entirely) replaced in these conditions. However, Ibrahim et al. [24] and Mian et al. [25] found that glucose repression induces in yeast only some increase in the relative content of high-molecular-weight mitochondrially made polypeptides without

changing their number of molecular weights. We have arrived at the same conclusion with our strain of yeast [26]. According to Groot et al. [22], aerobically and anaerobically grown yeast also show similar polypeptide patterns after separation of the mitochondrial translation products by SDS-polyacrylamide gel electrophoresis.

Most probably, the variations observed in the rate of the proteolysis were associated with alterations in the physical state of the inner mitochondrial membrane.

This follows from the results of our EPR study on the motion of spin probes in submitochondrial particles. As the temperature dependences of $2T'_{\parallel}$, τ_c, and ε for negatively charged $I_{12,3}$ and non-charged $I_{1,14}$-CH_3 showed inflections at the same temperature (15°C), one could suggest that this manifested some structural transition in the membrane. This transition can be considered cooperative. Indeed, it was detected with two probes whose paramagnetic groups were localized in different regions of the submitochondrial particle membrane as evidenced by the differences in their EPR spectra.

The thermoinduced structural transitions in biological membranes detectable with lipid-soluble probes are believed to be due to phase separation of lipids as well as to changes in lipid-protein interactions [13]. Then differences in the temperatures at which such transitions take place are indicative of the differences in the ordering and mobility of lipids and proteins in the membranes. This mobility is known to depend largely on the lipid composition of membranes, and in particular on the relative content of unsaturated fatty acyl chains in lipids [13,21].

In accord with this, comparison of Fig. 2 and Table III shows that, in general, the higher is the ratio of unsaturated to saturated fatty acids (K) the lower is the temperature of thermoinduced structural transition (which suggests higher mobility of lipids and proteins) in submitochondrial particles obtained from yeast grown under different conditions. Another correlation follows from the comparison of Fig. 1 and Table II; as can be seen, the higher is the relative content of unsaturated fatty acyl chains the higher, in general, is the rate of proteolysis of mitochondrially made polypeptides. This becomes understandable if one takes into account that both the digestible polypeptides and the digestive proteinases are localized in the same membrane (the inner mitochondrial membrane) and, therefore, they would interact the more easily the higher is their mobility in the membrane.

These correlations are of course only qualitative. They even seem not to hold for glucose repression versus anaerobiosis (cf. Fig. 1 and Table II); however, these are extreme cases in both of which the proteolysis is negligible, defying reliable matching. On the whole, the observed relationships indicate that the proteolysis in mitochondrial membranes may be naturally controlled through membrane fluidity.

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