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THE BIOGENESIS OF MITOCHONDRIAL MEMBRANES IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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SUMMARY

Membrane lipids of yeast mitochondria have been enriched by growing yeast cells in minimal medium supplemented with specific unsaturated fatty acids as the sole lipid supplement. Using the activity of marker enzymes for the outer (kynurenine hydroxylase) and inner (cytochrome *c* oxidase and oligomycin-sensitive ATPase) mitochondrial membranes, Arrhenius plots have been constructed using both promitochondria and mitochondria obtained from O_2-adapting cells in the presence of a second unsaturated fatty acid (i.e. linoleate (N_2) to elaidic (O_2)). Transition temperatures which reflect the unsaturated fatty acid enrichment of the new membranes reveal interesting features involved in the mechanism of the assembly of these two mitochondrial membranes. This approach was further enforced with both lipid depletion and mitochondrial protein inhibition studies. Kynurenine hydroxylase which does not require fatty acid for its continued synthesis during aerobiosis seems to be incorporated into the preformed linoleate–anaerobic outer membrane. The newly synthesized activities of inner mitochondrial membrane enzymes on the other hand, appear to integrate their activity into newly formed aerobic–elaidic-rich inner membrane. These latter enzymes show a distinct dependence on fatty acid supplement for their continued synthesis during their aerobic phase. This suggests that O_2-dependent proteo-lipid precursors are formed before these enzymes are integrated into their membrane mosaic. Two separate models are proposed to explain these results, one for the lipid-rich outer mitochondrial membrane and another for the protein-rich inner mitochondrial membrane.

INTRODUCTION

Early studies on the biogenesis of mitochondria from yeast utilized such tech-

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niques as the effect of growth environment, genetic manipulation and the use of inhibitors of protein synthesis (for review see refs 1 and 2). These studies have provided a basis for understanding the mechanisms involved in the production of catalytic proteins during the formation of functional mitochondria. It is desirable, however to correlate lipid and protein synthesis in order to gain an insight into the assembly of mitochondrial membranes. Hence, we have adopted a new approach to this problem in order to shed light on the biogenesis and assembly of mitochondrial membranes. This approach makes use of the interesting property that lipids of biological membranes undergo a thermotropic transition [3].

The temperature-transition (T_t) studies as indicated by breaks in Arrhenius plots have been made use of by a number of early workers to study the structure–function relationship in biological membranes [4, 5]. These transition temperatures reflect a transformation of the membrane lipid bilayer from a melted gel–crystalline (ordered) state to a gel–liquid (disordered) state and seem to be influenced by the fatty acid composition of the membrane phospholipids. These transitions are also accompanied by changes in the activation energies of membrane-bound enzymes [3, 6–8], changes which have been attributed to a restriction in the mobility of the protein molecule caused by a liquid–gel transition in the lipid components of the membranes. Initial reports from our laboratory [6, 9, 10] have shown that several mitochondrial enzyme activities are lipid-dependent and that their Arrhenius transition points are characteristic of the fatty acid supplement in the growth medium. Furthermore, it was shown that the newly synthesized enzyme was probably incorporated into newly formed mitochondrial membranes.

It is well known that biosynthesis and subsequent incorporation of lipids into phospholipid–protein complexes play an important role in the formation and integrity of biological membranes. Yeast cells offer a useful system for the study of these constructions, since the relative structure and hence the function associated with yeast mitochondrial membranes can be manipulated by changing conditions of growth and/or the nutritional lipid supplement. The isolation of unsaturated fatty acid yeast auxotrophs by Resnick and Mortimer [11] and the subsequent exploitation of these strains by different workers has helped in the demonstration that alterations in fatty acid moiety of membrane phospholipids lead to changes in many of the membrane-dependent functions [3, 4, 7–9].

The results presented in this communication deal with the elaboration of marker enzymes of both the outer and inner mitochondrial membranes (kynurenine hydroxylase; cytochrome *c* oxidase and the oligomycin-sensitive ATPase respectively) under conditions of change in both the growth environment and lipid supplementation. Such a study was undertaken to delineate the mechanisms involved in the assembly of these two membranes. The results indicate that there is a requirement of free lipid precursors for the assembly to take place. These results are discussed in terms of two models for the membrane assembly: one for lipid-rich outer mitochondrial membrane and one for protein-rich inner membrane.

METHODS

Saccharomyces cerevisiae, strain 77 (our standard laboratory strain) was grown on either complete medium supplemented with ergosterol and Tween 80 [12] or on

minimal medium supplemented with ergosterol and one of a series of unsaturated fatty acids (oleic, linoleic, linolinic or elaidic acid) [6, 9, 10]. For aerobic-growth experiments 3 % galactose was used as a carbon source and the cells were harvested an hour prior to the onset of stationary phase. For anaerobic–aerobic transition experiments, cells were anaerobically grown on 3 % glucose minimal medium, supplemented with ergosterol and 0.02 % linoleic acid, in a commercial fermentor with constant N_2 sparging. Cells were harvested prior to stationary phase, washed free of the anaerobic lipid supplement, transferred to fresh minimal medium containing a new fatty acid and simultaneously induced with O_2 by vigorous aeration with air. The cell mass increased from 1.5 to 3.0 mg dry wt per ml of culture during this latter 2-h aerobic adaptation period. Samples of these adapting cells were taken as indicated in the text and mitochondria from them were prepared as described below. The unsaturated fatty acid auxotroph KD-20 (kindly supplied by Dr S. Fogel, University of California, Berkeley) grown on 3 % glucose minimal medium under both anaerobic and aerobic conditions, was supplemented with 0.15 % unsaturated fatty acids.

Isolation of mitochondria

Harvested cells were washed once with ice-cold distilled water and twice with ice-cold 0.04 M phosphate buffer, pH 7.4, containing 0.1 % bovine serum albumin, chloramphenicol (4 mg/ml) and cycloheximide (25 μg/ml). The resulting washed cells were suspended in 1.0 M sorbitol containing 0.1 M Tris–HCl buffer, pH 8.0, 1 mM EDTA and 0.1 % bovine serum albumin. After 30 s homogenization in a Braun shaker, a mitochondrial fraction was obtained by differential centrifugation after the procedure of Henson et al. [13]. The final mitochondrial pellet was suspended in 0.25 M sucrose containing 10 mM Tris–HCl buffer (pH 7.4) to yield a suspension containing 5–10 mg protein per ml.

Enzyme assay

Oligomycin-sensitive ATPase was determined by the method of Tzagoloff [14] while kynurenine hydroxylase activities were measured by the procedure outlined by Schott et al. [15]. Temperatures were varied from 2 to 37 °C during various incubation periods by use of a constant temperature refrigerated bath. Cytochrome oxidase activity was measured spectrophotometrically as described before using thermojacketed cuvettes and immersible thermocouples to monitor reaction temperatures [10].

Fatty acid analysis

Lipids were extracted from 0.2 to 0.5 ml of mitochondrial suspension by the method of Folch et al. [16]. Methyl esters of the extracted fatty acids were prepared by the BF_3 method and were resolved on an EGSS-X column using a Beckman GC-45 chromatograph employing standard gas-chromatographic procedures. Temperature was programmed from 160 to 190 °C at a rate of 2 °C per min over a period of 16 min with N_2 as carrier gas.

Respiration was monitored polarographically using a Clark-type O_2 electrode and protein concentration was determined using the procedure of Lowry et al. [17].

RESULTS

Enzymes characteristic of both the outer and inner mitochondrial membrane, kynurenine hydroxylase (outer membrane); cytochrome *c* oxidase and oligomycin-

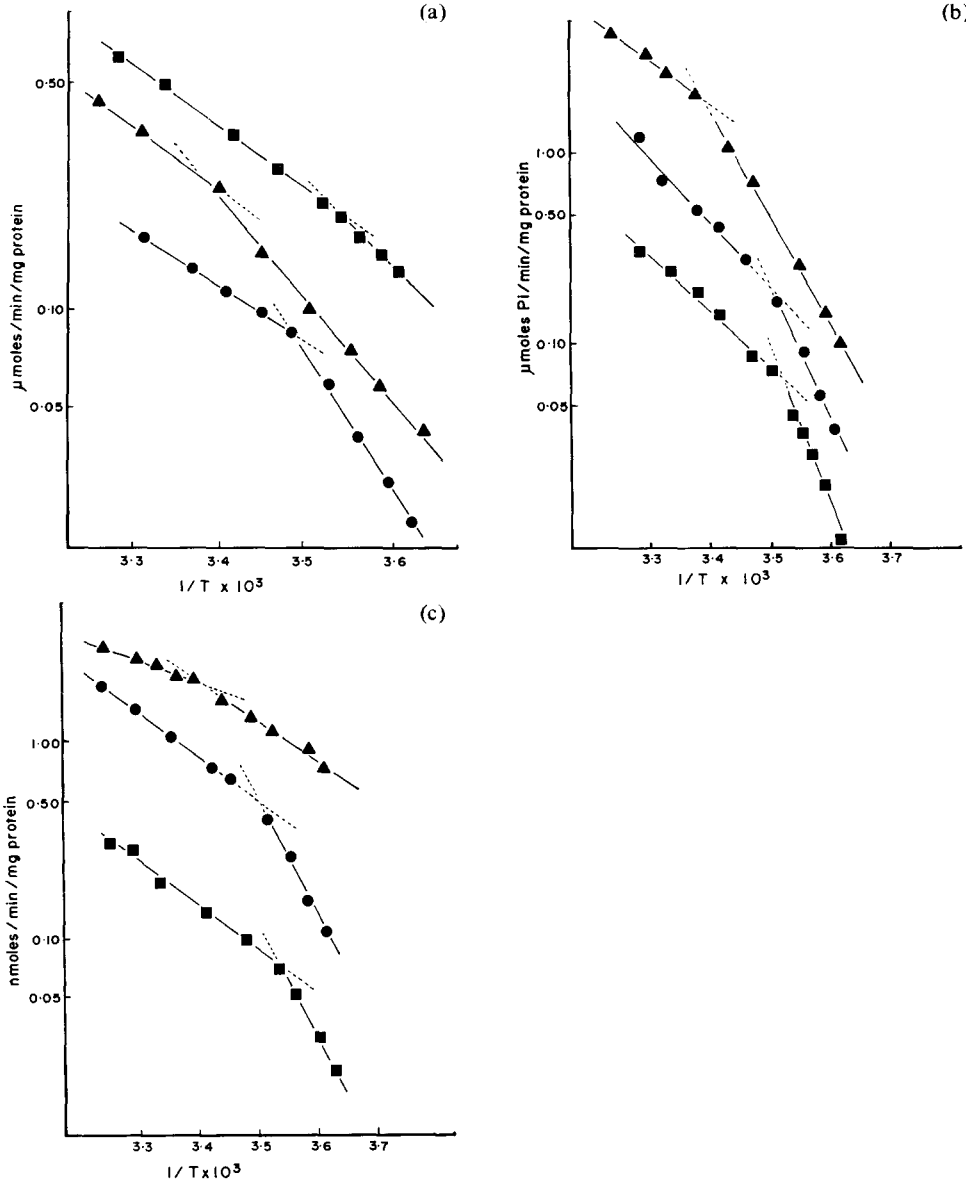

Fig. 1. Arrhenius profiles of mitochondrial enzymes. The enzyme activities were assayed from mito, chondria isolated from late log phase aerobic galactose cultures supplemented with elaidic (▲—▲)-linoleic (■—■) or oleic (●—●) acids (0.02 %). The lines represent best fits (linear regression analysis) and the curves have been frame shifted for clarity. a, cytochrome *c* oxidase; b, oligomycin-sensitive ATPase; c, kynurenine hydroxylase.

sensitive ATPase (inner membrane) demonstrate a break in their Arrhenius plots which is related to the fatty acid supplement of their constituent cell population (cf. Fig. 1, a, b and c). It is to be noted that these transition temperatures are very close to those reported in the literature for membrane-bound enzymes containing these fatty acids [18, 19]. The curves in these figures and those in subsequent figures were obtained by lines of best fit using a linear regression program to mechanically fit the raw data by use of the least-square theorem. (This meant fitting a line through points 1–4, 1–5, 4–9, 5–9 etc., and then compare the goodness of fit obtained by using such criteria as correlation coefficient and standard deviation of the slope and y intercept.) As may be seen from Fig. 1a this procedure gave unambiguous results for each fatty acid used for growth. Oleate having the best fit lines for points 1–5 and 5–9 with a transition temperature of about 12 °C. Similarly for the elaidic curve, points 1–3, and 4–8 gave the best fit, resulting in an intercept giving a transition temperature of about 22 °C. By this criteria the transition temperature for linoleate was 8 °C. The transition-temperature profiles for the other two enzymes were similarly characteristic of the different fatty acid supplement.

One can ask whether this change in transition temperature is manipulated directly by the lipid environment and a number of observations suggests that it is. To date only membrane-bound enzymes have been observed to exhibit transition in their Arrhenius profiles [3, 5, 20, 21]. In cell-free extracts we have not been able to demonstrate a temperature transition for the soluble glucose-6-phosphate dehydrogenase and when Arrhenius kinetics were carried out on acetone-extracted or Triton X-100-treated mitochondria the three mitochondrial enzymes studied here gave straight line plots.

The data presented in Table I gives further support to the fact that membrane lipids become enriched with the fatty acid supplemented in the growth medium. These data obtained from aerobically grown wild type cells show that more than 70 % of the total unsaturated fatty acid of the organelle is incorporated from the growth medium. This incorporation is carried out at the expense of the internally synthesized unsaturated fatty acids of similar chain length. The $C_{16:1}$ unsaturated fatty acids are only partially repressed by the free C_{18} acids and are hardly affected by Tween 80, an esterified oleic acid.

Fig. 2 shows that although all three enzyme activities are lipid-dependent, cf. Fig. 1, only cytochrome oxidase and the ATPase are dependent upon free lipid precursors for their continued synthesis during growth under aerobic conditions. In this experiment the unsaturated fatty acid requiring mutant strain KD-20 was used. Yeast was grown anaerobically on minimal medium supplemented with oleate, harvested, washed free of fatty acids and then reinoculated into fresh aerobic, minimal medium with or without oleic acid supplementation. The absence of free oleic acid prevented the synthesis of the two inner membrane enzymes but did not affect the synthesis of the outer membrane enzyme, kynurenine hydroxylase.

Both kynurenine hydroxylase and ATPase, in contrast to cytochrome oxidase, were synthesized by anaerobically grown yeast (cf. Fig. 4, b and c). Data from previous anaerobic to aerobic transfers had led us to believe that some precursor(s) of cytochrome oxidase accumulated under anaerobic conditions [10]. We therefore carried out the following experiment, analogous to that reported by Mason and Schatz [22]. Yeast was grown anaerobically on fully supplemented medium, harvested in

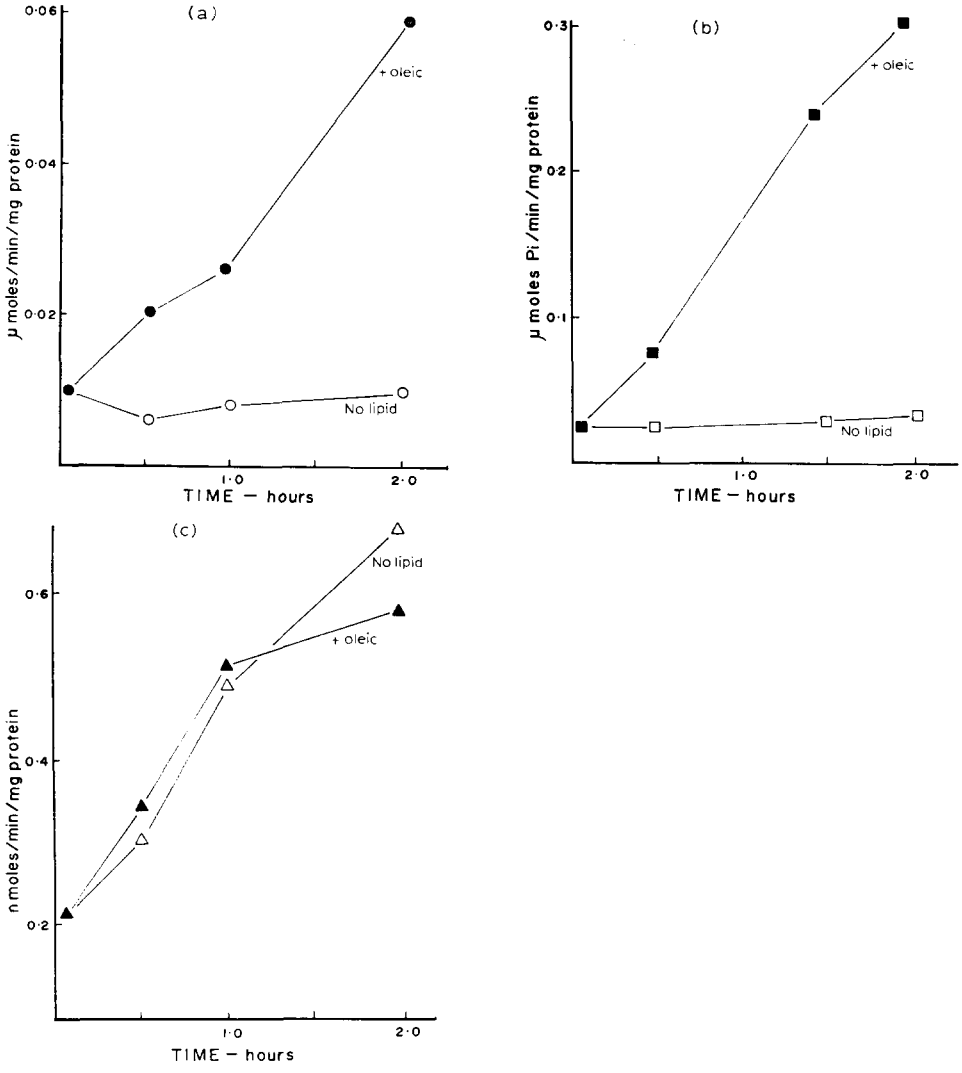

Fig. 2. Effect of free lipid on induced enzyme synthesis. Yeast, mutant strain KD-20, ol 1-2, was grown anaerobically on glucose minimal medium supplemented with 0.15 % oleic acid, harvested, washed and reinoculated into fresh medium plus (closed symbols) or minus (open symbols) oleic acid (0.15 %). a, cytochrome *c* oxidase; b, ATPase; c, kynurenine hydroxylase.

late exponential phase, washed and then inoculated into fresh aerobic medium containing either (1) chloramphenicol (4.0 mg/ml), (2) cycloheximide (50 μg/ml) or (3) no addition (control). These results are shown in Fig. 3. These indicate that some chloramphenicol-sensitive precursor(s) accumulate under anaerobic conditions (Curves 1 and 3), but that no excess cycloheximide-sensitive precursors are produced prior to induction by O_2 (Curves 2 and 3). To explore this hypothesis further, the experiment was repeated except that this time chloramphenicol (4.0 mg/ml) was added to the anaerobic culture 2 h prior to harvesting. The cells were harvested, washed thoroughly,

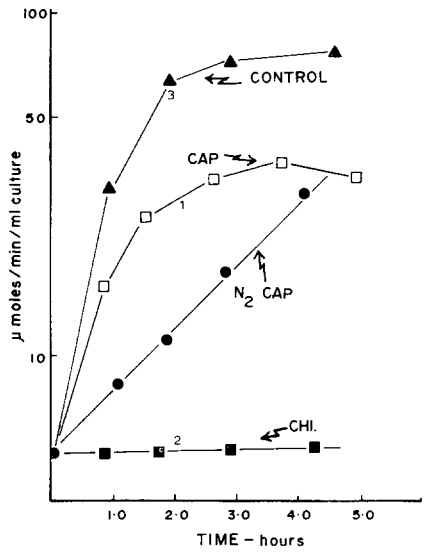

Fig. 3. Effect of inhibitors on cytochrome *c* oxidase induction. Yeast were grown anaerobically on fully supplemented 3 % glucose medium, harvested and transferred to fresh medium under aerobic conditions. Mitochondria were isolated from aerobic cells at times indicated and cytochrome *c* oxidase activity from these organelles was monitored as described in the text. Cells were aerobically grown in the presence of the following inhibitors: (1) □—□ chloramphenicol, 4 mg/ml; (2) (■—■) cycloheximide, 50 μg/ml; (3) (▲—▲) none; In a second experiment chloramphenicol (4 mg/ml) (●—●) was added to the anaerobic culture 2.0 h before harvesting. The cells were then harvested. washed and transferred to fresh medium with no inhibitor added.

and then adapted in the absence of chloramphenicol. This pretreatment abolished the auto-assembly-like burst of synthesis exhibited by the control culture and restored the normal [23] exponential synthesis of cytochrome oxidase.

TABLE I

EFFECT OF LIPID SUPPLEMENT ON THE FATTY ACID COMPOSITION OF MITOCHONDRIAL MEMBRANES

Yeast were grown aerobically on either 3 % galactose complete medium containing ergosterol and Tween 80 or on a series of minimal media containing 0.02 % fatty acids listed below. The cells were harvested in their late exponential phase, mitochondria were prepared and the fatty acid content determined by gas–liquid chromatography as described in Methods. Fatty acids denoted by the convention, number of carbon atoms:number of unsaturated linkages.

Lipid in growth medium	Percent fatty acid							
	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Tween 80	1	2	13	33	3	33	trace	nil
Oleic acid	> 1	4	19	18	6	40	trace	nil
Linoleic acid	> 1	2	20	14	8	9	42	nil
Linolenic acid	> 1	3	21	10	7	7	trace	44
Elaidic acid	2	3	26	16	9	39	nil	nil

Knowing from the above that the three enzymes vary in their dependence upon lipid for synthesis (Fig. 2) that the various components of the inner membrane enzymes respond differentially to O_2 induction (ref. 33 and Fig. 3) and that external lipid supplements are preferentially incorporated into mitochondrial membranes (Table I), it is now possible to use the Arrhenius profiles to investigate the process of mitochondriogenesis in yeast using O_2 induction to initiate this process in anaerobically grown cells.

The experimental design and premises were as follows: yeast would be grown anaerobically on minimal medium supplemented with linoleic acid (8 °C transition), harvested, washed and then exposed to O_2 challenge in fresh elaidic acid-supplemented minimal medium (transition around 22 °C). If the activities of membrane-bound enzymes are specifically modified by the lipids present during the assembly and/or synthesis during aerobic phase [5]then one would expect the following results. The ATPase and kynurenine hydroxylase, which are also synthesized anaerobically, should show two transitions in early aerobic samples. One for the population of anaerobically synthesized enzyme molecules (around 8 °C) and one for the newly synthesized aerobic population (around 22 °C). For cytochrome oxidase one might hypothesize either one or two transitions depending upon the role assigned to the anaerobically synthesized chloramphenicol-sensitive precursors (Fig. 3). This premise of course makes the assumption that the lipids and proteins to not migrate or laterally exchange within the mitochondrial membranes [24].

The results of such experiments are shown in Fig. 4, a, b and c. The curves have not been frame-shifted so that the changes in specific activity reflect changes in mitochondrial composition.

The cytochrome oxidase results (Fig. 4) seem to follow the hypothesis outlined above. The 0.5-h curve showed two transition points (as determined from the correlation coefficients and standard errors of the slope and intercept as outlined for Fig. 1) one at a low temperature (linoleic) and one at a high temperature (elaidic). The 1.0-h data were plotted as a curve because it was not possible to fit either one, two or three lines to this data with statistical accuracy comparable to the other data of Figs 1 and 4 (coefficients ranged from 0.87 to 0.93 vs > 0.99 for all solid lines, Fig. 4). The standard errors of the slopes and intercepts for the possible lines through the 1.0-h data were also large in comparison with the other data. After 2.0 h of O_2 induction cytochrome oxidase showed only one clear transition that is typical of enzyme isolated from aerobic, elaidic acid-grown yeast.

The oligomycin-sensitive ATPase showed a different response from that of the oxidase. Again there was induction under aerobic conditions (cf. Fig. 4) but this time superimposed on a background of anaerobically synthesized enzyme. In none of these curves could one detect a two-phase transition shifted continuously from a linoleic type transition (anaerobic sample) through several intermediary stages towards a higher temperature transition (16 °C, 2.0 h). These single transitions indicate that the ATPase molecules constitute a homogeneous population in terms of their lipid environment.

On the other hand, the kynurenine hydroxylase data (Fig. 4c) show an entirely different response to aerobiosis. The specific activity at 1.0 and 2.0 h decreased and the transition point remained at a low temperature (linoleic type). The reason for this decrease is not immediately clear, but is being investigated further. Not until the yeast have passed through 1.75 aerobic generations (5.0 h) did the kynurenine hydro-

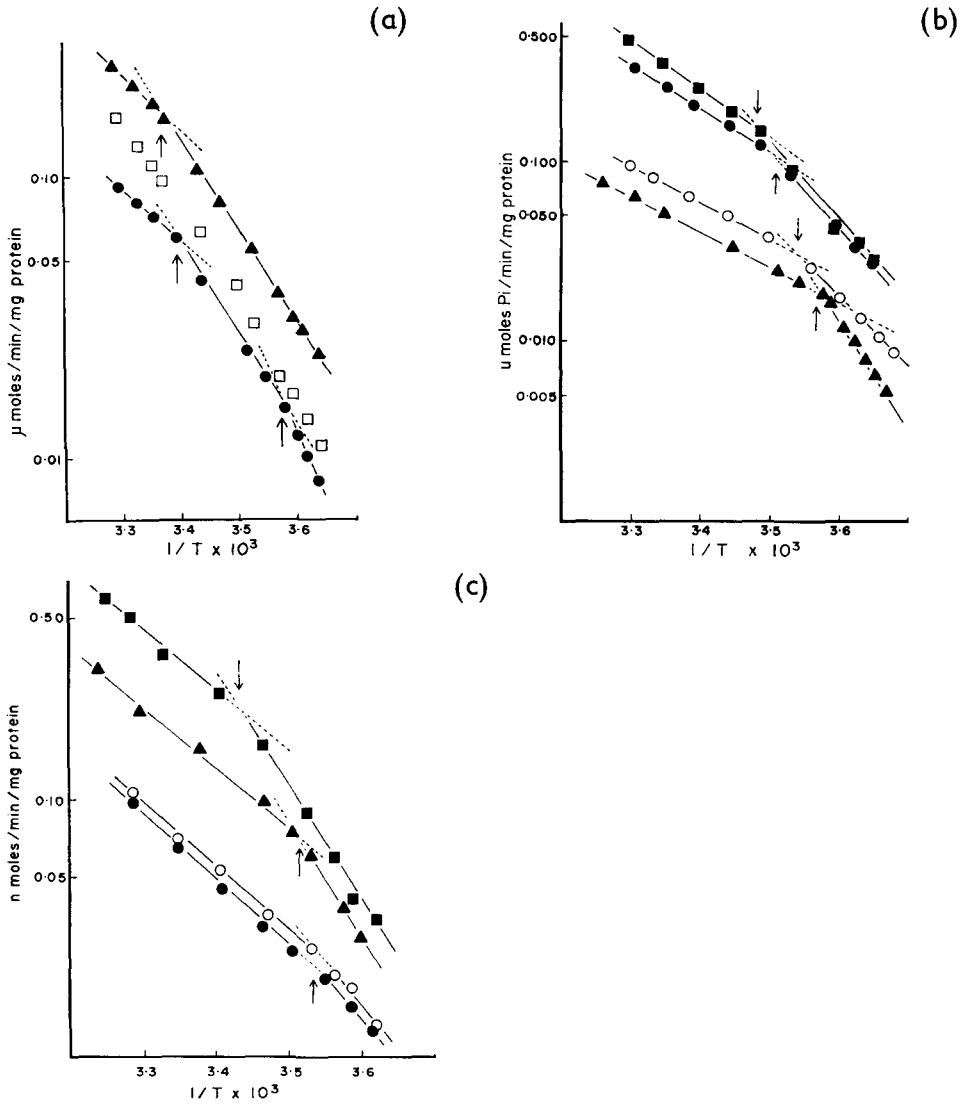

Fig. 4. Arrhenius profiles for enzyme activity determined from a linoleic (N_2) → elaidic (O_2) transfer. Yeast were grown anaerobically on glucose minimal medium plus linoleic acid (0.02 %) to late exponential phase, harvested, washed and exposed to O_2 in fresh glucose minimal medium plus elaidic acid (0.02 %). Isolated mitochondria were assayed for activity at various temperatures and Arrhenius profiles determined by linear regression analysis. Arrows indicate the most probable transition point. Curves are not frame-shifted. a, Cytochrome *c* oxidase: aerobic, +0.5 h (●—●); +1.0 h (□—□); +2.0 h (▲—▲). b. ATPase: anaerobic (▲—▲); aerobic, +0.5 h (○—○); +1.0 h (●—●); +2.0 h (■—■). c. Kynurenine hydroxylase: anaerobic (●—●); aerobic, +1.0 h (○—○); +2.0 h (▲—▲); +5.0 h (■—■).

xylase transition point shift to the higher temperature characteristic of elaidic type transition (Fig. 1c).

To entertain the possibility that the above changes can be due to restructuring

TABLE II

FATTY ACID COMPOSITION OF MITOCHONDRIA OBTAINED FROM YEAST CELLS UNDERGOING AEROBIC ADAPTATION

Yeast were grown anaerobically on 3 % glucose minimal medium supplemented with ergosterol and 0.02 % linoleic acid to a point 1 h prior to stationary phase and then adapted in minimal medium containing 0.02 % elaidic acid as described in Methods. Control cells were adapted in fresh 0.02 % linoleic acid medium. Cells were harvested at times indicated, mitochondria isolated and the fatty acid content determined by gas–liquid chromatography. Fatty acids denoted by the convention, number of carbon atoms:number of unsaturated linkages.

Condition of growth	Percent fatty acid Test: linoleic (N_2) → elaidic (O_2)								Control: linoleic (N_2) → linoleic (O_2)							
	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
N_2	6.8	9.7	26.2	10.6	6.0	4.0	35.9	0	5.1	8.7	25.2	12.1	4.9	3.2	41.2	0
O_2+1/2 h	3.0	9.4	20.8	15.4	5.9	13.3	24.9	0	2.5	5.6	27.7	16.7	6.0	2.4	40.7	0
O_2+1 h	4.3	7.7	24.4	18.5	6.7	14.3	21.6	0	4.5	9.7	20.8	18.2	4.7	2.5	40.2	0
O_2+2 h	4.3	5.1	21.6	24.7	5.8	17.7	20.6	0	3.3	5.4	23.0	16.6	4.2	3.0	42.7	0

of the lipid composition in the mitochondrial membranes we present the data in Table II. The left hand side of Table II shows the fatty acid composition of the mitochondria from which the enzyme data of Fig. 4 were derived. Clearly linoleic acid ($C_{18:2}$) has been replaced (decrease from 35.9 to 20.6 %) by elaidic acid ($C_{18:1}$) which is either incorporated or exchanged in the membranes (increased to 17.7 %). In the control experiment, linoleic–N_2 to linoleic–O_2, the changes in the C_{18} lipids were not apparent and the remainder of the changes taking place were similar. The unsaturated fatty acid $C_{16:1}$ increased from 10.6 to 24.7 % in the elaidic experiment but only reacts 16.6 % in the control (cf. elaidic and linoleic, Table I). All other changes e.g. $C_{14:0}$ and $C_{16:0}$ were more or less the same. In collaboration of this the Arrhenius profiles remained linoleic in type throughout the control experiment (N_2–linoleic to O_2–linoleic).

DISCUSSION

The results of Fig. 4 can only be interpreted in terms of the data presented in Figs 1–3, Tables I and II and extant in the literature. The work of Esfahani et al. [25] and Overath and Traüble [26] clearly show that for *Escherichia coli* membranes, transition in the Arrhenius plot of enzyme activity (T_t) largely coincide with the physical change of the membrane lipids from the crystalline to the melted state [25–27]. Fig. 1 shows for aerobically grown wild type yeast that the lipid supplement causes characteristic changes in the T_t and that this transition temperature (T_t) is essentially the same for each enzyme for any one supplement. That this is a causal relationship is strongly suggested by the data of Table I.

When yeast are grown in this manner one must expect that the phospholipid which contain unsaturated fatty acids will predominantly contain one kind of fatty acid only (see Table I). This will cause the membrane to have properties not usually found in "wild type" or "natural" membranes. The most important being that such a relatively homogeneous membrane will undergo freezing over a very narrow temperature range [26, 27]. As a corollary to this, such relative lipid homogeneity will surround the population of enzyme molecules being assayed for activity with a uniform environment, thus amplifying the effects of the liquid–gel transition on the activation energy (see Fig. 1, a, b and c and Table I). The above data and arguments also imply that this saturation effect of the fatty acid supplement is the same in both the inner membrane (Fig. 1, a and b) and the outer membrane (Fig. 1a).

Anaerobic mitochondria have more saturated fatty acids than do aerobic mitochondria (ref. 7 and Table II). This decrease in unsaturated fatty acids, together with the slight increase in $C_{16:1}$ acid should cause the overall T_t of the membranes to fall slightly in the first hour and then rise again in later samples as the elaidic acid comes to dominate the membrane structure. This effect is only seen in the case of kynurenine hydroxylase (cf. Fig. 4c: 1.0 and 2.0 h vs anaerobic curve).

However, when such mixed membranes are allowed to freeze, for example, during an assay to determine the T_t, one would expect the kinds of monotonic glycerides induced by the experimental conditions (see above) to crystallize out into more or less pure zones of lipid. This would produce a mosaic membrane [28] which should give rise to two populations of proteins in the membrane. In the transfer experiments one would expect to find one population at about 15 °C frozen into elaidic zones and

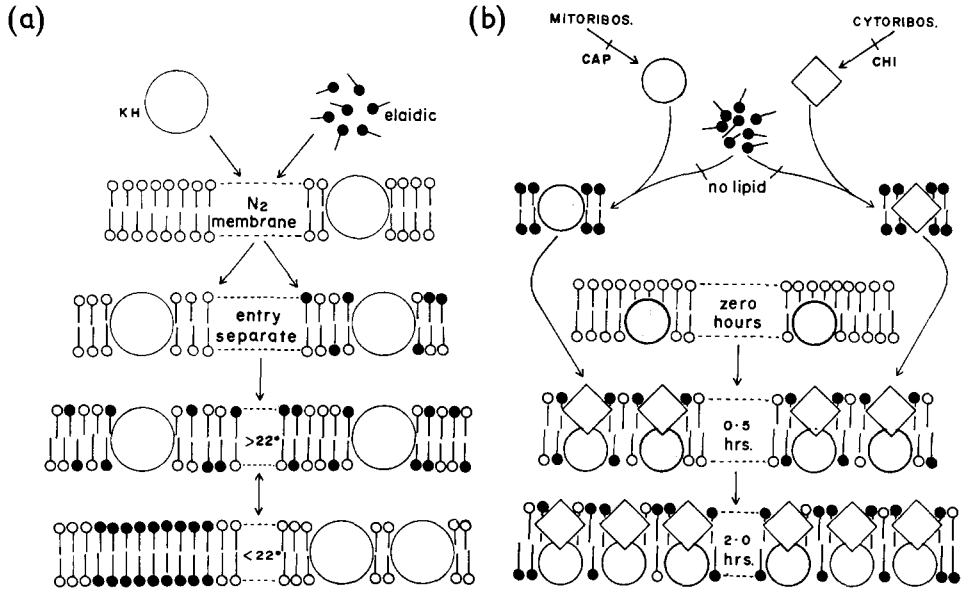

Fig. 5. Models of membrane assembly mechanisms. a. Lipid-rich membrane (kynurenine hydroxylase, outer membrane). Lipids (●) and enzyme dissolve separately into the membrane. Cooling of the membrane, <22 °C, causes mosaics to form with the enzyme retreating into the liquid zones (see text for details). b. Protein-rich membrane (cytochrome *c* oxidase, ATPase: inner membrane). Lipids bind to hydropholic mitochondrial and cytoplasmic precursors of the enzyme complexes before assembly takes place. Integration of these assemblies into the respiratory chain complex creates the inner membrane. Stipled circles indicate that these polypeptides are synthesized during the anaerobic linoleic phase.

another free to move in the predominantly linoleic zone. Kynurenine hydroxylase does not exhibit the double transition that would result from such populations.

Fig. 5a is a diagrammatic explanation for the synthesis and assembly of kynurenine hydroxylase into outer mitochondrial membranes. This enzyme is synthesized on cytoplasmic ribosomes (and not affected by chloramphenicol) [29] and does not require free lipid precursors for its continued synthesis (cf. Fig. 2c). It does however need to be embedded in a membrane before its activity can be expressed [6]. We therefore illustrate kynurenine hydroxylase dissolving into the outer membrane independently of newly synthesized (elaidic) lipids. Once dissolved into the membrane lateral diffusion (at 30 °C) would cause rapid mixing of the components. The outer membranes of mitochondria are relatively lipid rich [30, 31], so that one would expect proteins to be quite mobile within this membrane. From Table II and Fig. 1c it is reasonable to assume that by 2.0 h the outer membrane is mixed (as depicted in Fig. 5a) yet this sample gives one clear-cut transition which is characteristic of linoleic-type membrane. This can be explained by assuming that during the assay as the elaidic lipids freeze out kynurenine hydroxylase is excluded from these frozen zones and dissolved into the still liquid linoleic zones, eventually giving rise to a linoleic-type T_t. After 5.0 h there is no longer a large proportion of low melting point zone available to the enzyme and the elaidic transition comes to dominate the Arrhenius profile.

The second model, Fig. 5b, is designed to accomodate the data for both inner membrane enzymes. From Figs 2a and b it is obvious that both of these enzymes

require free lipid precursors before the constituent proteins can assembly to form active enzyme complexes. These data however do not indicate whether it is the mitochondrial (chloramphenicol-sensitive) peptides which bind the lipid, or the cytoplasmic (cycloheximide-sensitive) peptides or both. It is known from the work of Schatz [32, 33] and Tzagaloff [34] that both cytochrome oxidase and the ATPase are assembled from both mitochondrially and cytoplasmically derived components and this is illustrated in Fig. 5b. From Figs 2a and 3 it is possible to deduce that both the chloramphenicol-sensitive and cycloheximide-sensitive precursor(s) of the oxidase must bind lipid before assembly can take place. By comparing the control, chloramphenicol-inhibited and the chloramphenicol-pretreated curves in Fig. 3 it can be seen that anaerobic promitochondria contain chloramphenicol-sensitive components which can be activated by proteins synthesized on cytoplasmic (cycloheximide-sensitive) ribosomes in the absence of continued mitochondrial protein synthesis (cf. Mason and Schatz [22]). A similar phenomenon has been demonstrated for the ATPase by Kim and Beattie [35].

Looking at Fig. 2a, at zero time the anaerobic cells possess polypeptides which contain an excess of chloramphenicol-sensitive oxidase precursors (Figs 3 and 5b) which should be activated by cytoplasmically derived peptides (cycloheximide-sensitive). That this does not occur in the absence of free lipid argues that the cytoplasmically derived precursor(s) must bind free lipids before activation of the promitochondrial precursors can take place (cf. Fig. 5b, right hand side).

During the initial phases of aerobic adaptation (0–0.5 h) two processes are occurring in the inner membrane of the promitochondria. Initially, there is the activation of preformed (linoleic–chloramphenicol-sensitive) precursors by newly synthesized (elaidic–cycloheximide-sensitive) components to give rise to a potentially heterogeneous enzyme complex (linoleic–chloramphenicol-sensitive + elaidic–cycloheximide-sensitive). This, of course, assumes that cytochrome oxidase can bind lipids tightly enough to prevent mixing via lateral diffusion. The properties of this enzyme [32] and the recent results of Jost et al. [36], suggest that this is in fact true. At the same time (Fig. 3, chloramphenicol-pretreated) there will be a rapid synthesis and assembly of new cytochrome oxidase molecules (elaidic–chloramphenicol-sensitive + elaidic–cycloheximide-sensitive).

These two kinds of oxidase assemblages (Fig. 5b, 0.5 h) would constitute two different populations of cytochrome oxidase, each with its own historically determined lipid environment. During an assay to determine T_t these bound lipids would act as seed crystals, trapping the various molecules into the appropriate zones of the membrane mosaic, resulting in the two transitions (elaidic and linoleic) observed in the 0.5-h sample in Fig. 4a. If this hypothesis is true, then it also tells us that it is the chloramphenicol-sensitive component(s) which are critical to the oxidative activity measured in this assay.

The events taking place at 1.0 h are not clear. The mixed curve of Fig. 4a (1.0 h) could be due to an equimolar mixture of the two types (unlikely) or it could be caused by a slow exchange of elaidic for linoleic lipids at the lipid-binding sites of the preformed chloramphenicol-sensitive components. This latter process would give rise to a whole range of differentially mixed membrane environments. This would, of course, cause the transition points of the total membrane to be spread over the range from "pure" elaidic to an equal mixture of elaidic–linoleic and eventually to "pure" linoleic

membrane, giving a gently curving Arrhenius profile (Fig. 4a, 1.0 h). Also during this time a rapid and considerable increase in the total amount of inner membrane occurs (Figs 2, 3 and 4) and this synthesis must be supported by incorporation of more lipids (from elaidic precursors) into the promitochondrion (Fig. 2 and Table II). By 2.0 h one would expect the inner membrane to be clearly dominated by the elaidic lipids and that the number of linoleic–chloramphenicol-sensitive oxidase molecules would be very small. This is probably the situation which gives rise to the single well defined transitions of Figs 1a and 4a at 2.0 h.

That this same basic model can be applied to the ATPase is not certain. Because the ATPase is synthesized under anaerobic conditions it is hard to perform the experiments reported in Fig. 3 for this enzyme. However, because this enzyme complex responds to both biochemical and genetic manipulation in a way which is virtually identical to that documented for cytochrome oxidase, it seems reasonable to hypothesize that the lipid-binding parts of this model (Fig. 5b, top) will also hold true for the ATPase. In this case the anaerobic membrane (zero h) will contain complete, linoleic type ATPase (Fig. 4b, anaerobic) and the assembly process from 0.0 to 0.5 h should give rise to two discrete populations of molecules (linoleic–ATPase+elaidic–ATPase). But the ATPase Arrhenius profiles (Fig. 4b) are clearly bi-phasic with one clear-cut, statistically established T_t. Moreover these T_t values (0.5, 1.0 and 2.0 h, Fig. 4b) are not characteristic of the precursor lipids (elaidic, 22 °C and linoleic, 8 °C),

The melting points of pure glycerides are close to the melting point of the constituent fatty acid: if one constructs a mixed glyceride, the melting point is approximately halfway between the melting points of the respective pure glycerides [37]. If one could justify a similarly-mixed lipid environment for all of the ATPase molecules present at a particular time in the mitochondrial membrane then one could explain the biphasic, single T_t response of the ATPase (Fig. 4b). The single, precise T_t argues for one homogeneous population of ATPase molecules while the continuous upward shift of T_t with time argues for a lipid environment which is gradually changing from linoleic dominated to elaidic dominated. A possible explanation could be as follows: Some of the ATPase components do tightly bind lipids [34] such that could postulate a situation occurring midway between that for kynurenine hydroxylase (Fig. 5a) and cytochrome oxidase (Fig. 5b) for this enzyme. Relative slow exchange of lipids (in terms of lateral diffusion rates) at the lipid-binding sites of the ATPase would give rise to ATPase molecules with a mixed-lipid environment. (The single transition profiles of Fig. 1 vs Fig. 4a, 0.5 h, 2.0 h and Fig. 4c all argue that very few mixed glycerides are formed in the course of these experiments). There is a relatively large amount of ATPase in the promitochondrion (Fig. 4b, anaerobic) such that the postulated exchange of lipids (elaidic for linoleic) would shift the freezing point of the ATPase environment (T_t) only slowly towards the value characteristic of "elaidic membrane". This correlates well with the data of Fig. 4b.

The inner mitochondrial membrane contains much more protein than does the outer membrane [30]. This may account for the very different responses of kynurenine hydroxylase, ATPase and cytochrome oxidase. The former enzyme appears to dissolve into a preformed lipid-based membrane whereas the latter enzymes bind lipids and then form a membrane. Perhaps these phospholipids are bound in order to solubilize the hydrophobic inner membrane proteins until such time as they can precipitate out as part of the respiratory chain complex. The bound, and transported lipids then

associate to form the permeability barrier necessary for such membrane-dependent phenomena such as ion transport and membrane potential [38].

The models outlined above are an attempt to explain the mechanisms for membrane biogenesis in yeast mitochondria, are necessarily preliminary and do not pretend to account for all of the known facts. The models are testable and suggest further lines of investigation and are therefore submitted for further exploration and criticism.

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