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Phase Transitions in Yeast Mitochondrial Membranes

THE EFFECT OF TEMPERATURE ON THE ENERGIES OF ACTIVATION OF THE RESPIRATORY ENZYMES OF *SACCHAROMYCES CEREVISIAE*

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The effect of temperature on the activation energies of mitochondrial enzymes of the yeast *Saccharomyces cerevisiae* was examined. Non-linear Arrhenius plots with discontinuities in the temperature range 14–19°C and 19–22°C were observed for the respiratory enzymes and mitochondrial ATPase (adenosine triphosphatase) respectively. A straight-line Arrhenius plot was observed for the matrix enzyme, malate dehydrogenase. The activation energies of the enzymes associated with succinate oxidation, namely, succinate oxidase, succinate dehydrogenase and succinate–cytochrome *c* oxidoreductase, were in the range 60–85kJ/mol above the transition temperature and 90–160kJ/mol below the transition temperature. In contrast, the corresponding enzymes associated with NADH oxidation showed significantly lower activation energies, 20–35kJ/mol above and 40–85kJ/mol below the transition temperature. The discontinuities in the Arrhenius plots were still observed after sonication, treatment with non-ionic detergents or freezing and thawing of the mitochondrial membranes. Discontinuities for cytochrome *c* oxidase activity were only observed in freshly isolated mitochondria, and no distinct breaks were observed after storage at −20°C. Mitochondrial ATPase activity still showed discontinuities after sonication and freezing and thawing, but a linear plot was observed after treatment with non-ionic detergents. The results indicate that the various enzymes of the respiratory chain are located in a similar lipid macroenvironment within the mitochondrial membrane.

A considerable number of studies on membrane-bound enzymes and transport systems in mutant organisms of *Escherichia coli* (Wilson *et al.*, 1970; Overath *et al.*, 1970; Esfahani *et al.*, 1971) and *Acholeplasma laidlawii* (Steim *et al.*, 1969; Engelman, 1970, 1971) have shown discontinuities in Arrhenius plots with an increase in activation energy at lower temperatures. Physical techniques have indicated that the discontinuities are paralleled by a change in the physical state of the membrane lipids from a liquid-crystalline state above the transition temperature to a gel state below the transition temperature (for review see Oldfield & Chapman, 1972).

Discontinuities or 'breaks' in Arrhenius plots of membrane-bound enzymes have been reported in mammalian mitochondria (Raison *et al.*, 1971*a*,*b*; Lenaz *et al.*, 1972). Several laboratories have shown that phase-transition temperatures in yeast cells (Eletr & Keith, 1971) and in isolated yeast mitochondria (Ainsworth *et al.*, 1972; Watson *et al.*, 1973*a*) could be correlated with the fatty acid composition of the membrane lipids. The availability of mutants and the ready manipulation of growth conditions have made studies on the yeast system particularly attractive in investigations related to membrane structure and function.

The purpose of the present work was to examine, in some detail, the effect of temperature on the activation energies of mitochondrial enzymes in wild-type *Saccharomyces cerevisiae*. Preliminary accounts of parts of the present work have been published (Watson *et al.*, 1973*b*,*c*).

Methods

Growth of organism

S. cerevisiae, strain N.C.Y.C. 239 (National Collection of Yeast Cultures, Brewing Industry Research Foundation, Nutfield, Surrey, U.K.), was used in all studies. Cells were grown in 10-litre New Brunswick Fermenters (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) at 30°C in media containing 1% yeast extract, 0.2% peptone and mineral salts (Wickerham, 1946). The carbon source was 0.5% ethanol. Stirring speed was 500 rev./min and sterile air was passed through the culture at 1 litre/min. A 1% inoculum from a starter culture grown for 24h on 0.5% ethanol was used. The cells were harvested at the beginning of stationary growth phase which corresponded to a growth of around 6mg dry weight of cells/ml of medium.

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Isolation of mitochondria

Mitochondria were prepared by disruption of cells with glass beads (0.45–0.5mm diameter) for 15s in a Braun shaker (B. Braun Apparatebau, Melsungen, Germany) at speed 2 (4000rev./min). The isolation buffer contained 0.25M-sorbitol–10mM-Tris–HCl (pH7.4)–2mM-EDTA and 0.1% bovine serum albumin. Mitochondria were isolated from the homogenate by differential centrifugation and purified on a discontinuous sucrose gradient consisting of a cushion of 6ml of 70% (w/v) sucrose and 3ml each of 60, 50, 30 and 15% (w/v) sucrose. The sucrose was buffered with 20mM-Tris–HCl (pH7.4)–1mM-EDTA. The gradients were run at 24000rev./min for 2.5h in the SW 25.1 rotor of a Beckman model L2 ultracentrifuge. Two bands on the sucrose gradient were always observed. In all cases the lower band (density 1.190) was the major component and the mitochondria from this band were collected and suspended in the isolation buffer at a concentration of 10–15mg of protein/ml. The upper band which contained oligomycin-sensitive ATPase* activity (R. L. Houghton & K. Watson, unpublished work), was found to be a mixture of intact and broken mitochondria when examined in the electron microscope (K. Watson, unpublished work). The properties of the upper band were not further investigated. In some experiments, mitochondria were also prepared by enzymic digestion of the cell wall with snail gut enzyme and disruption of the protoplasts in a French Pressure Cell as previously described (Watson *et al.*, 1971).

Fatty acid analysis

Fatty acids in cells and mitochondria were assayed essentially as previously described (Watson *et al.*, 1973*a*). The methyl esters of the fatty acids were prepared by refluxing extracts for 2h in methanolic HCl. The methyl esters were extracted by the addition of *n*-hexane and the sample was evaporated to a small volume under reduced pressure in a rotary evaporator. Samples were analysed by g.l.c. on a Perkin–Elmer F-11 gas chromatograph, by using a flame-ionization detector. The column was a 2m polyethylene glycol succinate column operating at 180°C, and a carrier-gas (N_2) flow rate of 35ml/min was used. The relative percentages of the fatty acids were determined by measurement of the peak area estimated from peak height multiplied by one-half peak base.

Enzyme assays

Succinate oxidase and NADH oxidase activities were measured polarographically by using a Rank oxygen electrode (Rank, Cambridge, U.K.) connected to a Churchill Thermocirculator (Churchill Instruments Co., Perivale, Middx., U.K.). The assay medium contained, in a final volume of 3.1ml, 0.25M-sorbitol–5mM-potassium phosphate–1mM-EDTA–10mM-Tris–maleate (pH6.5), 0.1% bovine serum albumin and 1–3mg of mitochondrial protein. The reaction was started by the addition of 0.5mM-ADP and substrate, either 20mM-succinate or 2mM-NADH. O_2 concentration in the buffer at the different temperatures was calculated as described by Estabrook (1967).

All other enzyme activities were measured with a Cary 14 spectrophotometer fitted with a Churchill Thermocirculator. The temperature in the reaction cuvette was continuously monitored by means of an immersible thermocouple (±0.1°C). Dry N_2 gas was passed through the cell compartment to prevent condensation at the lower temperatures. A 1cm-light-path cell and a final volume of 1.1ml was used in all assays.

Succinate dehydrogenase (*EC* 1.3.99.1). Succinate dehydrogenase was measured by a modification of the method of Arrigoni & Singer (1962). The reaction mixture contained 50mM-potassium phosphate buffer pH7.2, 1mM-KCN and 50–200μg of mitochondrial protein. The mixture was incubated for 5min and the reaction was started by the rapid addition of 0.05mM-dichlorophenol-indophenol and 0.5mM-phenazine methosulphate. Absorbance changes were measured at 600nm and a standard extinction coefficient of 21 $mM^{-1} \cdot cm^{-1}$ for dichlorophenol-indophenol was used (King & Howard, 1967).

NADH dehydrogenase (*EC* 1.6.99.3). The reaction mixture contained 50mM-potassium phosphate buffer, pH7.2, 1.5mM-potassium ferricyanide and 50–200μg of protein. The reaction was started by the addition of 0.6mM-NADH and absorbance changes were followed at 420nm. An extinction coefficient of 1.03 $mM^{-1} \cdot cm^{-1}$ for potassium ferricyanide was used (King & Howard, 1967).

Succinate–(*EC* 1.3.99.1) *and NADH–*(*EC* 1.6.99.3) *cytochrome c oxidoreductase.* The reaction mixture contained 50mM-potassium phosphate buffer, pH7.2, 1mM-KCN, 0.08mM-cytochrome *c* and 50–200μg of protein. The reaction was started by the addition of substrate, either 20mM-succinate or 0.6mM-NADH. Absorbance changes were measured at 550nm and an extinction coefficient of 19.2 $mM^{-1} \cdot cm^{-1}$ for cytochrome *c* was used (King & Howard, 1967).

Cytochrome c oxidase (*EC* 1.9.3.1). Cytochrome *c* oxidase was measured by the method of Smith (1955). Reduced cytochrome *c* was prepared as described by Wharton & Tzagoloff (1967). The reaction mixture contained 50mM-potassium phosphate buffer, pH7.2, and 0.1mM-reduced cytochrome *c*. The reaction was started by the addition of 50–200μg of mitochondrial protein. Absorbance decrease was measured at

* Abbreviation: ATPase, adenosine triphosphatase.

550nm and an extinction coefficient of 19.2mM^{-1}·cm^{-1} for cytochrome *c* was used.

Malate dehydrogenase (*EC* 1.1.1.37). Malate dehydrogenase was measured by the method of Ochoa (1955). The reaction mixture contained 50mM-potassium phosphate buffer, pH7.2, 0.15mM-NADH and 50–100μg of mitochondrial protein. The reaction was started by the addition of 1mM-oxaloacetate. Absorbance decrease was measured at 340nm and an extinction coefficient of 6.22mM^{-1}·cm^{-1} for NADH was used (Bergmeyer, 1965).

ATPase (*EC* 3.6.1.3). Mitochondrial ATPase was measured by a coupled enzyme system (Pullman *et al.*, 1960). The reaction mixture contained 50mM-Tris–sulphate (pH9.4)–5mM-phosphoenolpyruvate–5mM-$MgCl_2$–2mM-ATP, pyruvate kinase (10μg; Sigma type II), lactate dehydrogenase (20μg; Sigma type II) and 50–100μg of mitochondrial protein. The reaction was started by the addition of 0.15mM-NADH. Absorbance decrease was measured at 340nm and an extinction coefficient of 6.22mM^{-1}·cm^{-1} for NADH was used. Sensitivity to the F_1 inhibitor prepared from ox heart mitochondria was assayed as described by Pullman & Monroy (1963). The ATPase activity of the isolated mitochondria was inhibited 70–80% by 150μg of F_1 inhibitor/mg of protein.

Sonication

Sonication was carried out in an MSE Ultrasonicator operating at 1.5A. The sample, which was kept immersed in an ice-bath, was sonicated for 20s periods with cooling between. Sonication time varied from 1 to 5min.

Dry-weight and protein determinations

Dry weights were determined by filtration on to Whatman GF/C glass-fibre paper (diameter 2.4cm) and drying in an oven at 110–120°C for 24h. Protein was determined by the procedure of Lowry *et al.* (1951). Bovine serum albumin was used as standard.

Materials

ADP, ATP, NADH, phenazine methosulphate, dichlorophenol-indophenol, oligomycin, bovine serum albumin (fraction V, essentially fatty acid-free) and Triton X-100 were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Methyl ester standards of fatty acids were obtained from Applied Science Laboratories Inc., State College, Inglewood, Calif., U.S.A. Tergitol-NPX and Nonidet P40 were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. All other chemicals were analytical-reagent grade where available and obtained from BDH Chemicals.

Results

The fatty acid composition of the mitochondrial membranes isolated from *S. cerevisiae* cells grown on 0.5% ethanol to early stationary phase was approx. 45% palmitoleic acid and 35% oleic acid, i.e. about 80% of the total fatty acid was unsaturated fatty acid. The remainder of the fatty acids consisted of a mixture of stearic acid (5%), palmitic acid (5–10%) and fatty acids of carbon chain length C_{10}–C_{14}. All experiments described in this paper were carried out on mitochondrial membranes with this approximate fatty acid composition. Preparations that had fatty acid compositions of less than 70% or greater than 90% of the total fatty acid as unsaturated fatty acid were discarded.

NADH oxidase and succinate oxidase

Fig. 1 shows Arrhenius plots in which the logarithm of enzyme activity is plotted against the reciprocal of the absolute temperature. With both NADH oxidase and succinate oxidase activities there was a 2–3-fold increase in activation energy in the temperature range 14–19°C. The energy of activation of the NADH oxidase, above the transition temperature, was consistently observed to be severalfold lower than that of the succinate oxidase. The higher activation energy of the latter system was reflected in a rapid decrease

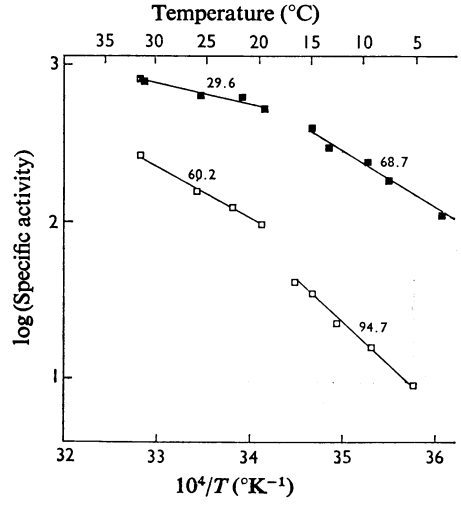

Fig. 1. *Arrhenius plots of oxidation of NADH and succinate*

O_2 uptake was measured polarographically as outlined in the Methods section. The specific activities are expressed as ng-atoms of O taken up/min per mg of protein. The numbers on the graphs are the activation energies expressed as kJ/mol. ■, NADH oxidase; □, succinate oxidase.

in activity with temperature, until at temperatures below 5–10°C very low succinate oxidase activity was observed. Conversely, the lower activation energy of the NADH oxidase was reflected in a less rapid decrease in activity with temperature and considerable enzyme activity was still measurable at temperatures below the transition temperature. The discontinuities in the Arrhenius plots were reversible and analogous transition temperatures were obtained when experiments were conducted by changing the reaction temperature from high to low temperatures and then, with the same stock mitochondrial suspension, changing the reaction temperatures from low to high temperatures. There was little change in enzymic activity in the time required to measure these changes (approx. 6h).

Sonication or storage at −20°C (2–4 weeks) of the mitochondrial membranes did not abolish the breaks in the Arrhenius plots, although a loss (up to 50%) in enzyme activity was noted after cold storage. The effect of the non-ionic detergents Triton X-100, Tergitol and Nonidet P40 on enzyme activity was complex. Concentrations of detergent (0.025–0.05%) which were just sufficient to abolish the stimulation of respiration by ADP (state 3 of Chance & Williams, 1956) led to a variable degree of inhibition of respiration. However, breaks in Arrhenius plots were still clearly observed (Fig. 2). Higher concentrations of detergent (0.1–0.2%) lead to a marked inhibition of respiration (50–80%) and restricted the accuracy of measuring respiratory activity at temperatures below 15°C. On the other hand, control experiments on rat liver mitochondria treated with low concentrations of Nonidet P40 (0.025%) gave straight-line Arrhenius plots with a constant energy of activation with succinate as substrate. These latter results were in agreement with the results of Raison *et al.* (1971*a*).

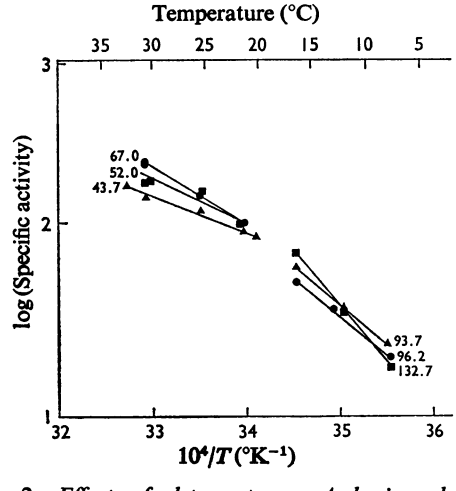

Fig. 2. *Effect of detergents on Arrhenius plots of succinate oxidase activity*

O_2 uptake was determined polarographically and specific activities are expressed as ng-atoms of O taken up/min per mg of protein. The numbers on the graphs are the activation energies expressed as kJ/mol. The detergents Nonidet P40 (0.05%, v/v) and Tergitol (0.05%, v/v) were incubated with buffer and mitochondria at the appropriate temperatures and the reaction was started by the addition of 20 mM-succinate and 0.5 mM-ADP. ●, Control; ▲, +Nonidet P40; ■, +Tergitol.

NADH- and succinate-cytochrome c oxidoreductase

A similar pattern was seen when these two enzyme systems were examined. Arrhenius plots showed breaks between 14° and 19°C, and the activation energy of the NADH system, particularly above the transition temperature, was noticeably lower than that of the succinate system (see Table 1). The enzyme activities survived mild sonication and freezing at −20°C and thawing, and non-linear Arrhenius plots were still evident. The effects of non-ionic detergents were not investigated.

NADH dehydrogenase and succinate dehydrogenase

A feature of the succinate dehydrogenase activity of the yeast mitochondria was the relatively steep decrease in activity with temperature resulting in high activation energies both above and below the transition temperatures (Fig. 3). The contrast with the NADH dehydrogenase activity in this case was especially marked, since the NADH-linked enzyme was characterized by a relatively low activation energy. Mild sonication and freezing at −20°C (up to 1 month) did not abolish the non-linearity of the Arrhenius plot for the succinate dehydrogenase activity, but discontinuities in the NADH dehydrogenase were ill-defined after these treatments.

Cytochrome c oxidase

Fig. 4 shows Arrhenius plots of cytochrome *c* oxidase activities in freshly isolated mitochondria and mitochondria that had been stored at −20°C for 24h. The latter treatment led to a 30–50% loss in activity and, further, abolished the non-linearity of the Arrhenius plot. Discontinuities with an increase in activation energy were only observed for cytochrome *c* oxidase in freshly isolated mitochondria. Even in these latter experiments, two out of the six mitochondrial preparations examined for cytochrome *c* oxidase activity showed straight-line Arrhenius plots. Our results may explain the linear Arrhenius plots for cytochrome *c* oxidase reported by Lenaz *et al.* (1972), who used ox heart mitochondria which had been previously stored in a frozen state.

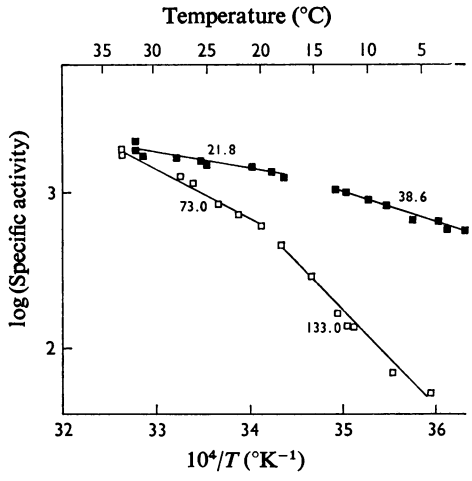

Fig. 3. *Arrhenius plots of succinate dehydrogenase and NADH dehydrogenase activities*

Enzyme activities were measured as outlined in the Methods section. Specific activity was expressed as nmol of substrate transformed/min per mg of protein. The numbers on the graphs are the activation energies in kJ/mol. ■, NADH dehydrogenase; □, succinate dehydrogenase.

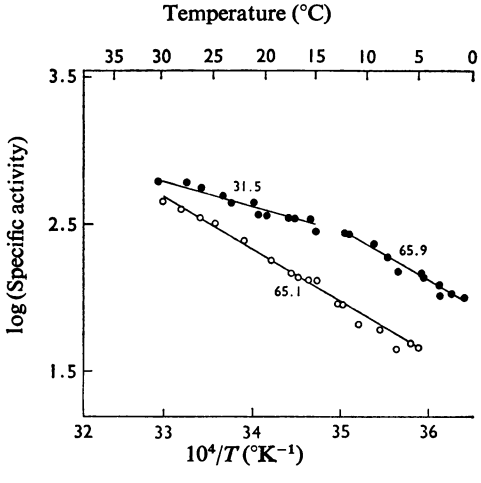

Fig. 4. *Arrhenius plots of cytochrome c oxidase activity in fresh and frozen mitochondria*

Enzyme activity was measured as outlined in the Methods section. Specific activity was expressed as nmol of cytochrome *c* oxidized/min per mg of protein. The numbers on the graphs are the activation energies in kJ/mol. ●, Cytochrome *c* oxidase activity in freshly isolated mitochondria. ○, Cytochrome *c* oxidase activity in mitochondria stored at −20°C for 24 h.

Malate dehydrogenase

In contrast with the membrane-bound enzymes, the mitochondrial matrix enzyme malate dehydrogenase exhibited a straight-line Arrhenius plot with a constant activation energy of about 58 kJ/mol (see Table 1). These results are in agreement with those reported for ox heart mitochondria by Lenaz *et al.* (1972).

Mitochondrial ATPase

The ATPase of yeast mitochondria showed discontinuities in Arrhenius plots with an increase in activation energy at temperatures somewhat higher, 19–22°C, than those observed for the enzymes of the respiratory chain. There was an approximate threefold increase in activation energy below the transition temperature (Fig. 5). Controlled sonication for periods of 1–2min led to a threefold increase in

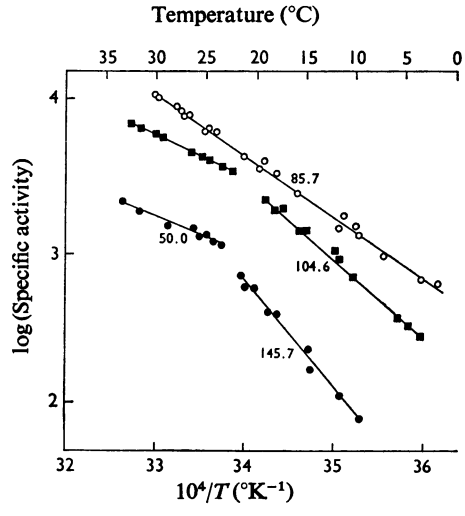

Fig. 5. *Effect of Triton X-100 and sonication on Arrhenius plots of ATPase activity*

Enzyme activity was measured as outlined in the Methods section. Specific activity was expressed as nmol of NADH utilized/min per mg of protein. Sonication: mitochondria were sonicated for 2 min at 1.5 A and then centrifuged at 10000*g* for 15min. The pellet was discarded and the supernatant centrifuged at 50000*g* for 45min. The submitochondrial particles were suspended in isolation buffer at a concentration of 10–15mg of protein/ml. Triton X-100: mitochondria were suspended in isolation buffer in the presence of 0.1% (v/v) Triton X-100 for 15min at 0–4°C. The solution was centrifuged at 10000*g* for 15min and the pellet discarded. The supernatant was centrifuged at 100000*g* for 60min and the pellet suspended in isolation buffer at a protein concentration of 10–20 mg/ml. ●, Control; ■, sonication; ○, Triton X-100.

Table 1. *Transition temperatures and activation energies of yeast mitochondrial enzymes*

The results are the average of six to eight determinations for each enzyme and are expressed as the means ± S.E.M.

	Transition temperature (°C)	Activation energy (kJ/mol) Above	Below
Succinate oxidase	16.8±1.8	61.7±5.8	100.4±4.2
Succinate–cytochrome *c* oxidoreductase	16.1±1.8	61.0±3.1	93.7±7.4
Succinate dehydrogenase	17.3±2.3	84.0±6.4	159.6±8.9
NADH oxidase	17.1±1.1	34.4±3.8	75.7±6.2
NADH–cytochrome *c* oxidoreductase	16.4±1.9	31.9±2.4	83.2±4.8
NADH dehydrogenase	16.2±1.3	23.5±1.8	39.5±2.1
ATPase	20.2±1.4	53.0±4.9	148.7±7.4
Cytochrome *c* oxidase	15.8±1.9	31.5±1.2	68.4±4.3
Malate dehydrogenase	—	58.0±4.1	58.0±4.1

specific activity. The discontinuities were still observed, although they were less well defined compared with control mitochondria (Fig. 5). Prolonged sonication for 3–5 min led to a progressive decrease in the non-linearity of the Arrhenius plots. Detergent treatment of the mitochondrial membranes with Triton X-100 (0.1%) gave a fivefold increase in specific activity, and in this case a straight-line Arrhenius plot was obtained. The mitochondrial ATPase was particularly stable to freezing, and storage at −20°C for several months still resulted in ATPase activities which exhibited non-linear Arrhenius plots.

Table 1 summarizes the activation energies, above and below the transition temperatures, for mitochondrial ATPase and the enzymes of the respiratory chain of yeast mitochondria.

Discussion

The membrane-bound enzymes of the respiratory chain of yeast mitochondria examined in this paper all showed non-linear Arrhenius plots with an increase in activation energy at temperatures between 14° and 19°C. For a given mitochondrial preparation, the transition temperatures for different respiratory enzymes were always determined to be within a narrow temperature range, usually 1–2°C. Transition temperatures varied from preparation to preparation, although large temperature differences, 3–5°C, between different preparations could normally be attributed to alterations in the fatty acid composition of the mitochondrial membranes.

Raison *et al.* (1971*a*,*b*) have shown that the respiratory enzymes of mitochondria from rat liver and chilling-sensitive plants have discontinuities in Arrhenius plots at 23–24°C and 10–12°C respectively. A reappraisal of the data of Lenaz *et al.* (1972) on ox heart mitochondria preparations shows that, apart from the anomalously high transition temperature reported for succinate oxidase (27°C), the transition temperatures for the enzymes examined all fall within the narrow range 18–20°C. These studies, taken in conjunction with the present observations on yeast mitochondria, support the conclusion that, for a given mitochondrial membrane, the enzymes of the respiratory chain have similar transition temperatures, and hence are probably in a similar lipid environment. Data on discontinuities in Arrhenius plots for mitochondrial enzymes cannot therefore be taken to support the concept of a heterogeneous distribution of lipids within mitochondrial membranes as suggested by Lenaz *et al.* (1972). It may well be that experiments linking transition temperatures in non-linear Arrhenius plots with membrane lipid composition may only yield information as to the macrolipid environment, that is, the membrane as a whole, rather than the microlipid environment of a particular membrane-bound enzyme. However, it was noteworthy in this context that yeast mitochondrial ATPase showed significantly higher transition temperatures, 19–22°C, than those observed for the enzymes of the respiratory chain, 14–19°C. There are several possible interpretations of this result. One is that the two enzyme systems undergo conformational changes at different temperatures. Thermally induced reversible changes in enzyme structure have been suggested to account for breaks in Arrhenius plots (Massey *et al.*, 1966; Zeylemaker *et al.*, 1971). Another interpretation is that the ATPase and the enzymes of the respiratory chain are localized in different lipid environments within the mitochondrial membrane. Differences in transition temperature, within the same membrane, have been reported for proline uptake and succinate dehydrogenase in *E. coli* (Esfahani *et al.*, 1971). These authors have interpreted their results as evidence for a heterogeneous distribution of membrane lipids in *E. coli*. Evidence for a heterogeneous distribution of lipids in *A. laidlawii* has also been presented by Oldfield *et al.* (1972).

Enzymes associated with the oxidation of NADH and succinate in yeast mitochondria showed essentially the same transition temperature in Arrhenius plots. These results indicate that the NADH- and succinate-oxidation systems are in a similar lipid domain within the mitochondrial membrane. On the other hand, all three enzymes of the NADH system examined, the NADH oxidase, NADH dehydrogenase and NADH–cytochrome *c* oxidoreductase, showed a 2–3-fold lower activation energy above the transition temperature, than the corresponding enzymes of the succinate system (Table 1). A similar phenomenon has been observed in mitochondria isolated from *Candida lipolytica* (Skipton *et al.*, 1973). One may speculate that the different activation energies may be expressions of different lipid–protein interactions within the mitochondrial membrane. Such a proposal gains support from the concept of mitochondrial membrane topography (for review see Racker, 1970). The localization of succinate dehydrogenase on the matrix side of the inner membrane of mitochondria appears to be well established. In the yeast system, two pathways for the oxidation of NADH have been identified (von Jagow & Klingenberg, 1970). One pathway, for the oxidation of endogenous NADH, is located towards the inner side (or matrix side) of the inner mitochondrial membrane, and a second pathway, for the oxidation of exogenous NADH, is located towards the outer surface of the inner membrane. An additional pathway for the oxidation of exogenous NADH, associated with the outer mitochondrial membrane, has been reported in plant mitochondria (Douce *et al.*, 1973). The presence of this latter pathway has, as yet, not been reported in yeast mitochondria. Nevertheless, it is clear that the location of the enzymes associated with succinate oxidation is different from that for the oxidation of exogenous NADH. The question as to whether mitochondrial membrane topography plays a role in activation energies for membrane-bound enzymes remains to be determined. The differences in activation energy between the succinate- and NADH-oxidation systems may simply reflect the independent nature of these systems and that the enzyme systems undergo conformational changes with different activation energies.

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