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MEMBRANE LIPID FLUIDITY AND ITS EFFECT ON THE ACTIVATION ENERGY OF MEMBRANE-ASSOCIATED ENZYMES

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Summary

1. The fatty acid composition of mitochondrial membranes from sheep and rats was altered by feeding these animals diets which were rich in unsaturated fatty acids. Changes in membrane lipid fluidity resulting from the altered membrane lipid composition were assessed by determining the upper temperature limit of the disorder-order transition (T_f) and the Arrhenius activation energy (E_a) of succinate oxidase.

2. After feeding the unsaturated fatty acid-rich diet to sheep the E_a, in the temperature range above T_f, increased from 8 to 63 kJ · mol^{-1} while T_f decreased from 32 to 15°C. Rats fed an unsaturated fatty acid-rich diet exhibited an increase in E_a from 17 to 63 kJ · mol^{-1} and a decrease in T_f from 23 to 4°C.

3. This decrease in T_f was related to an increase in the ratio of linoleic acid to stearic acid in the membrane lipid. T_f was not related to the proportion of unsaturated fatty acids in the membrane lipids, although an increase in unsaturation usually led to a decrease in T_f.

4. The results show that membrane lipid fluidity has a direct influence on the conformation of the active site of some membrane-associated enzymes, with the result that such enzymes display a higher E_a when the membrane lipids are comparatively more fluid. The increase in E_a of membrane-associated enzymes which accompanies changes in the physical state of membranes suggests that some proteins may phase separate with the more fluid lipids at low temperatures.

Introduction

Lipids from a variety of membranes undergo a thermotropic, disorder-order transition within the temperature range of 40—0°C [1]. At temperatures above

T_f, the upper limit of the transition, membrane lipids are considered to be predominantly in a fluid, liquid crystalline phase. At temperatures below T_s, the lower limit of the transition, membrane lipids are considered to be in a solid or gel phase [2]. Within the transition the lipids are in a mixed phase. These changes in lipid structure, induced by lowering the temperature, alter the kinetics of enzymes associated with membranes and can, in some instances, have a critical effect on various physiological functions [3,4].

With membranes from homeothermic (warm-blooded) animals the Arrhenius activation energy (E_a) of membrane-associated enzymes increases below the temperature limits of the lipid transition [1]. Similar increases in E_a occur with membrane-associated enzymes of yeast [5] and *Escherichia coli* [6]. These observations suggest that an increase in the molecular order of membrane lipids induces changes in the structure of membrane-associated enzymes consistent with an increase in E_a.

A comparison of the E_a values for succinate oxidase of liver mitochondrial membranes in equivalent structural phases, derived from homeothermic and poikilothermic (cold-blooded) animals is, however, not consistent with this relationship. The membrane lipids of poikilotherms contain relatively higher proportions of unsaturated fatty acids [7], and thus would be more disordered than the lipids in membranes of homeotherms. Furthermore, no disorder-order transition is observed with membrane lipids of poikilotherms in the temperature range of 30—0°C, and the E_a of associated enzymes is constant over this temperature range [4]. For the homeotherm, the structural phase equivalent to the membrane lipids of poikilotherms in the above temperature range, is the fluid phase above T_f. The E_a values for succinate oxidase for toad and fish liver mitochondria [4,8] are far greater than those observed with the same membranes from homeotherms which are considered to be comparatively more ordered. This contradicts the situation brought about by changes in membrane physical state as a result of temperature in which comparatively higher E_a values are observed when the membrane lipids become more ordered. Thus while it is apparent that the conformation of membrane-associated enzymes is influenced by membrane lipid structure, there appear to be differences in the response of these proteins to structural changes in lipids induced by compositional changes on the one hand and changes in physical state on the other.

To resolve this question, the effect of an altered membrane lipid composition on the E_a of the membrane-associated succinate oxidase system was investigated using sheep and rats. Upon altering the fatty acid composition of mitochondrial membranes a decrease in both T_f and T_s, consistent with an increase in membrane fluidity, was observed. The increase in E_a observed in the more ordered phase, i.e. in the temperature range below T_f, can be explained on the basis that the membrane proteins phase separate together with the more fluid lipids at low temperatures.

Materials and Methods

Two sheep, aged 16 weeks, were supplied by the CSIRO Division of Animal Production. One sheep was fed a formaldehyde-treated, protein-coated, sunflower oil diet for 4 weeks [9], which is a diet known to produce a marked

increase in the level of unsaturated fatty acids in tissue lipids [9]. The other sheep was maintained at pasture. Rats were maintained from weaning on either an unsaturated fatty acid-rich diet, made by soaking commercial pellets in sunflower oil (fatty acid composition, 75% linoleic and 15% oleic acid), or on the pellets alone (control group).

Mitochondrial membranes were isolated by methods previously described [4], from the liver, kidney and heart tissue of rats from both groups after varying periods on the diet and from sheep after 28 days on the diet.

Succinate oxidase activity was measured polarographically as previously described [10].

Electron spin resonance (ESR) spectra were recorded with a Varian E4 spectrometer fitted with a temperature-controlled cell housing which maintained the sample at ±0.1°C of the set temperature. The temperatures of T_f and T_s were determined from the change in the temperature coefficient of spin label motion as previously described [1].

Fatty acid analysis was performed on the phospholipid fraction isolated from mitochondrial lipid extracts. Mitochondria were extracted with 20 vols. of chloroform/methanol (2 : 1, v/v) in the presence of the antioxidant, butylated hydroxytoluene. Non-lipid contaminants were removed by passage through Sephadex G-25. Phospholipids were separated from non-polar lipids by column chromatography on acid-washed Florisil. Fatty acids were analysed by gas-liquid chromatography as methyl esters.

Results

The Arrhenius activation energy (E_a) for succinate oxidation by mitochondria from homeothermic animals increases as the temperature is lowered and an Arrhenius plot of this activity in the temperature range of 0—40°C is triphasic [1]. The three values for E_a correspond with the three structural phases induced by lowering the temperature, and the temperatures at which these changes in E_a occur are coincident with the temperatures at which changes in the molecular ordering of membrane lipids are detected by monitoring the motion of spin-labelled fatty acids infused into the membrane [1].

As shown in Fig. 1A, the E_a for succinate oxidation by liver mitochondria from a control sheep increases from 8 to 50 kJ · mol^{-1} when the temperature is reduced below 32°C (T_f). At this temperature the membrane lipids change from a disordered or fluid phase, to a mixed disordered-ordered phase. The E_a increases again to 173 kJ · mol^{-1} below 14°C (T_s). At this temperature the lipids change from the mixed disordered-ordered phase to a predominantly ordered or solid phase [1]. As shown in Fig. 1C the E_a for succinate oxidase activity of kidney mitochondria from a control sheep also increases below T_f (29°C). (As this study was primarily concerned with changes in E_a in the temperature range where membrane lipids are in the disordered phase (above T_f), or mixed disordered-ordered phase (between T_f and T_s), succinate oxidase activity was not usually measured below T_s.)

In contrast to the control animal, mitochondria from sheep fed the unsaturated fatty acid-rich diet showed two important differences: an increase in the E_a for succinate oxidase activity above T_f, and a lowering of the tempera-

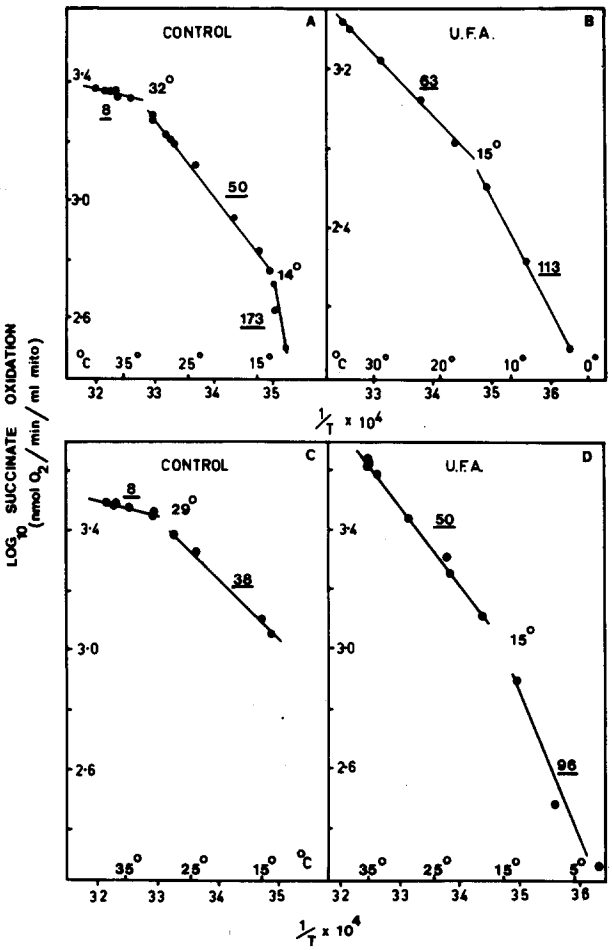

Fig. 1. Arrhenius plots of succinate oxidase activity of mitochondria from sheep liver (A and B) and kidney (C and D). The numbers underlined, adjacent to the straight lines, are the Arrhenius activation energies in kJ · mol^{-1} for the temperature range indicated by the straight line. Plots (A) and (C) are for mitochondria from control sheep and plots (B) and (D) for sheep fed the unsaturated fatty acid (UFA)-rich diet. The straight lines were fitted by the method of least squares [16].

ture of T_f. Figs. 1A and B show the increase in E_a from 8 to 63 kJ · mol^{-1} and the decrease in the temperature of T_f from 32 to 15°C for liver mitochondria from sheep fed the unsaturated fatty acid-rich diet. An increase in E_a and a decrease in the temperature of T_f was also observed with kidney mitochondria from these sheep. For both liver and kidney mitochondria the temperature of T_s was detected at about 4°C by spin labelling [11] indicating that the change at 15°C (Fig. 1B and D) represents the upper limit of the transition, T_f. As shown in Fig. 1, the E_a for succinate oxidase activity in the transition range between T_f and T_s also increases in these sheep, from 50 to 113 kJ · mol^{-1} in liver mitochondria and from 38 to 96 kJ · mol^{-1} in kidney mitochondria.

Feeding sheep the unsaturated fatty acid-rich diet results in an increase in the cellular level of unsaturated fatty acids [9]. It was therefore important to determine if this diet also increased the proportion of unsaturated fatty acids in

TABLE I

FATTY ACID COMPOSITION OF SHEEP MITOCHONDRIAL PHOSPHOLIPIDS

Fatty acids are designated by the number of carbon atoms followed by the number of double bonds and are expressed as mol%.

Fatty acid	Liver		Kidney	
	Control diet	Unsaturated fatty acid-rich diet	Control diet	Unsaturated fatty acid-rich diet
14 : 0	6.6	0.8	0	0.8
14 : 1	0	0	0.8	0
16 : 0	8.3	5.5	7.4	4.6
16 : 1	0	0	0	0.6
17 : 0	2.2	0.8	0.8	0.9
16 : 2	2.8	0	4.9	0
18 : 0	18.1	28.5	10.4	8.7
18 : 1	16.3	5.0	7.5	5.3
18 : 2	8.1	33.9	10.2	39.1
20 : 3	2.3	0	5.1	0.8
20 : 4	13.0	17.9	15.7	30.1
20 : 5	5.7	0	4.0	0
22 : 4	1.1	1.1	8.0	2.2
22 : 5	8.6	2.8	5.2	2.9
22 : 6	7.0	3.8	19.8	3.8
Σ unsaturated fatty acids	64.9	64.5	81.2	84.8

the lipids of mitochondrial membranes. Table I compares the fatty acid composition of the phospholipids from the liver and kidney mitochondria of control and sheep fed the unsaturated fatty acid-rich diet, With both liver and kidney, dietary supplementation of linoleic acid (18 : 2) led to a marked increase in the proportion of this and arachidonic acid (20 : 4) such that the sum of these two acids constitute 52% and 69% of the total phospholipid fatty acids of liver and kidney mitochondria, respectively. Associated with this decrease in heterogeneity of fatty acids is a narrowing of the range of the disorder-order transition. For example in the membranes from sheep liver mitochondria the transition range was reduced from 18 Celsius degrees (32—14°C) in the control to about 11 Celsius degrees (15—4°C) in the diet-treated sheep.

The percentage of unsaturated fatty acids in the membrane phospholipids of liver mitochondria was not altered by the dietary treatment, however a threefold increase in the ratio of linoleic (18 : 2) to stearic acid (18 : 0) consistent with an increase in membrane fluidity was observed (Table II). With kidney and heart mitochondria the mol% of unsaturated fatty acids increased from 81% to 85% and from 71% to 81%, respectively, while the 18 : 2/18 : 0 ratio increased 4.5 and 1.4-fold, respectively. With all mitochondrial preparations, T_t, as measured by spin labelling, decreased by about 15 Celsius degrees as a result of the dietary treatment (Table II).

The changes induced in both the temperature of T_t and the E_a of succinate oxidase of mitochondria from the sheep fed the unsaturated fatty acid-rich diet were also observed with liver mitochondria from rats fed a similar diet. Two

TABLE II

RELATIONSHIP BETWEEN THE TEMPERATURE OF T_f, THE PROPORTION OF UNSATURATED FATTY ACIDS AND THE RATIO OF LINOLEIC ACID (18 : 2) TO STEARIC ACID (18 : 0) IN THE PHOSPHOLIPIDS OF MITOCHONDRIA FROM SHEEP FED CONTROL OR UNSATURATED FATTY ACID-RICH DIETS

	Control diet			Unsaturated fatty acid-rich diet		
	T_f (°C)	Mol% lipid unsaturation	18 : 2/18 : 0	T_f (°C)	Mol% lipid unsaturation	18 : 2/18 : 0
Liver	32	65	0.4	15	65	1.2
Kidney	29	81	1.0	15	85	4.5
Heart	30	71	2.4	15	81	3.4

experimental approaches were used for rats. In the first, adult male rats were fed the unsaturated fatty acid-rich diet for periods of up to 20 days. The greatest lowering of T_f was from 24°C for rats fed the control diet, to 17°C for rats fed the experimental diet. This decrease in the temperature of T_f was accompanied by a change in E_a for succinate oxidase activity from 12 kJ · mol^{-1} to 29 kJ · mol^{-1} (results not shown). In a second series of experiments young rats, from patents maintained on the unsaturated fatty acid-rich diet, were fed this same diet from the end of weaning for periods of up to 272 days. Table III shows the temperature of T_f and the E_a in the temperature ranges above and below T_f for liver mitochondria from these rats and from rats fed a control diet. The T_f of 17°C for 30-day-old rats is lower than the 22—24°C routinely observed for adult rats, i.e. 60 days and older. The temperature of T_f was further reduced in the membranes of young rats maintained for the same period on the unsaturated fatty acid-rich diet. Concomitant with this lowering of the temperature of T_f, the E_a for succinate oxidase increased both above and below T_f. Rats maintained on this diet and which were tested at age 180 days or older, exhibited similar value for T_f and E_a as that of control rats (Table III).

Rats fed the unsaturated fatty acid-rich diet and which exhibited a lowering of T_f showed a relatively greater lipid unsaturation due to elevated levels of

TABLE III

THE RELATIONSHIP BETWEEN THE TEMPERATURE OF T_f AND THE E_a OF SUCCINATE OXIDASE OF MITOCHONDRIA FROM RATS OF VARIOUS AGES FED CONTROL OR UNSATURATED FATTY ACID-RICH DIETS

Age (days)	Control diets			Unsaturated fatty acid-rich diet		
	T_f (°C)	E_a (kJ · mol^{-1})		T_f (°C)	E_a (kJ · mol^{-1})	
		Above T_f	Below T_f		Above T_f	Below T_f
30	17	38	96	4	63	—
60	24	17	50	15	42	92
106	22	17	71	16	46	—
150	24	21	59	17	33	75
180	22	21	63	23	13	58
272	23	17	59	23	21	63

arachidonic (20 : 4) and docosahexaenoic (22 : 6) acids and decreased levels of palmitic (16 : 0) and stearic (18 : 0) acids. However, no clear relationship was observed between the percentage of unsaturated fatty acids and the temperature of T_f. In addition, for mitochondrial membranes isolated from liver, kidney and heart of control rats, which showed a similar value for T_f of 23°C, the level of unsaturation in the fatty acids of the phospholipid fraction ranged from 68% for liver to 89% for kidney and heart mitochondria, respectively (results not shown).

The triphasic nature of the Arrhenius plots for succinate oxidase activity can be abolished by perturbing the membrane lipids with non-ionic detergents [10], indicating that the changes in E_a as a function of temperature are a consequence of an alteration in lipid structure and are not an intrinsic property of the membrane-associated enzyme. As shown in Fig. 2, treatment of liver mitochondria from control rats with a non-ionic detergent, resulted in a linear Arrhenius plot for succinate oxidase with an E_a of 33 kJ · mol^{-1}. This approximates the mean of the values for the E_a above and below T_f obtained with untreated mitochondria. Detergent treatment also produced a constant E_a for mitochondria from rats fed the unsaturated fatty acid-rich diet. The E_a of 54 kJ · mol^{-1} is intermediate in value between the E_a obtained above and below T_f; 42 and 92 kJ · mol^{-1}, respectively, for untreated mitochondria. Thus from the data of Fig. 2 it is obvious that even after detergent treatment the E_a for succinate oxidation increased from 33 kJ · mol^{-1} to 54 kJ · mol^{-1} as a result of the dietary treatment, consistent with a general increase in lipid fluidity.

To determine if other enzyme systems were affected by the changes induced by the unsaturated fatty acid-rich diet, the E_a above T_f was determined for the oxidation of both citrate and ascorbate. The E_a for all three oxidase systems was greater in mitochondria from these rats indicating that the change in mem-

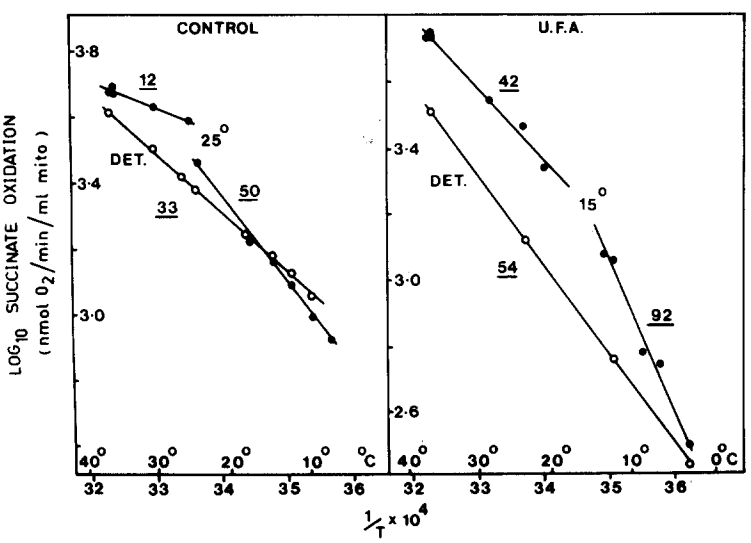

Fig. 2. The effect of detergent treatment on the E_a of succinate oxidase of mitochondria from rats fed a control or unsaturated fatty acid (UFA)-rich diet. Where indicated the non-ionic detergent Teric X-8 (Nonidet P-40, Shell Chem. Co. Australia) was added to a final concentration of 0.1% (v/v) during measurement of oxygen uptake.

brane structure resulting from the unsaturated fatty acid-rich diet affects membrane-associated enzymes other than succinate oxidase (results not shown).

Discussion

Feeding rats and sheep a diet rich in unsaturated fatty acids results in the lowering of the upper temperature limit, T_f, of the order-disorder transition for membrane lipids and an increase in the E_a of membrane-associated enzymes both above and below T_f. The lower temperature limit, T_s, is also lowered by this diet [11]. The decrease in T_f is most likely the result of a decrease in molecular ordering of the fatty acid chains of the membrane and is considered an indication of an increase in membrane lipid fluidity. Fluidity is a descriptive term used to convey the idea that membrane lipids and proteins are in rapid lateral motion and that the overall structure of the membrane is dynamic rather than static [12]. The term fluidity is also used when comparing the molecular ordering of membrane lipids in relation to physiological function [13]. In the present study the term fluidity is used to describe the molecular disorder of fatty acid chains in the membrane as influenced by both fatty acid composition and temperature. As the temperature of T_f reflects in part the fatty acid composition of the membrane, it is therefore taken as a comparative measure of membrane fluidity.

The E_a of an enzyme-catalysed reaction is an indication of the conformation of the active site. Therefore the increase in the E_a of succinate oxidase which accompanies both the increase in the proportion of linoleic and arachidonic acids and the lowering of the temperature of T_f, shows that the tertiary structure of some intrinsic membrane proteins is influenced by the fluidity of membrane lipids. The extent of this influence is illustrated in Fig. 3 which relates the E_a of succinate oxidase for the various mitochondrial preparations with membrane fluidity as indicated by the temperature of T_f. For the mitochondrial enzyme system from rat tissue the E_a increases as membrane fluidity increases, i.e. as T_f decreases, reaching an apparent maximum as T_f approaches 0°C. The validity of this relationship using mitochondrial membranes from rats of different ages and from different tissues with different fatty acid compositions, shows that T_f is a good indicator of the relative fluidity of membrane lipids.

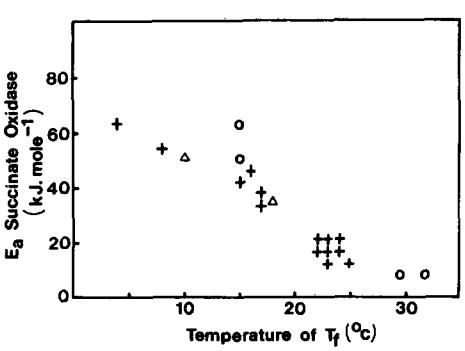

Fig. 3. The relation between E_a of succinate oxidase and the T_f of mitochondrial membranes from rat (+) and sheep (○) liver. The E_a was determined in the temperature range above T_f. The values of the E_a for echidna liver mitochondria (△) are taken from the data of McMurchie and Raison [14].

This is further supported by the observation that rat liver mitochondria show the same relationship between the E_a of succinate oxidase and membrane lipid fluidity as sheep mitochondria. This same relationship is observed with mitochondria from fatty acid auxotrophs of yeast [5]. In addition, values for the E_a of succinate oxidase for mitochondria from poikilothermic animals fit the general relationship shown in Fig. 3. Mitochondrial membranes of poikilotherms are relatively rich in unsaturated fatty acids [7] and changes in molecular ordering as determined by spin-labelling techniques, have not been observed in the temperature range 0—30°C [4]. With these membranes it is assumed that T_f is at, or below, 0°C and for these mitochondria the E_a of succinate oxidase is usually in excess of 50 kJ · mol^{-1} [4,8], which approximates the values expected from the relationship shown in Fig. 3. Between the extremes of membrane fluidity exhibited by the homeotherms and poikilotherms these are examples of animals whose membranes show a T_f at a temperature midway between these two extremes. Mitochondria from the echidna (*Tachyglossus aculeatus*) for example show a T_f at 17°C and the E_a for succinate oxidase for these mitochondria is 34 kJ · mol^{-1} [14] a value approximating that of rat liver mitochondria with a similar temperature for T_f (Table III).

These results demonstrate a remarkable consistency in the relationship between mitochondrial membrane fluidity as measured by T_f and the E_a of succinate oxidase even though the mitochondrial membranes which exhibit this relation have varying lipid compositions and are derived from different animals. Considering that membrane lipids do not participate in the reactions catalysed by membrane-associated oxidases but exert their influence by interacting at hydrophobic regions of the protein distinct from the active site, they can be considered allosteric effectors of membrane-associated enzymes. The relationship between E_a and membrane fluidity discussed above indicates that the particular physical parameter of the lipids measured by T_f, is, in effect, a measure of the capacity of the lipids to interact with membrane proteins and influence the tertiary structure. The concept of lipids acting as allosteric effectors of membrane protein conformation has important implications in studies involving the reconstitution of lipid-depleted, membrane-associated enzymes. In general the main criteria for assessing the specificity and suitability of lipids to combine with lipid-depleted enzymes is the regaining of activity. Although this provides some measure of the interaction between lipid and protein the E_a would be a more valid parameter for assessing the conformation of the active site providing measurements were made in a temperature range in which the lipids were in equivalent phases. The similar values for the E_a of D-β-hydroxybutyrate dehydrogenase either in the membrane-bound form or when reconstituted with either mitochondrial lipids or with dioleoylphosphatidylcholine [15] indicate that the lipid environment in either of these reconstituted systems maintains this enzyme in a conformation comparable to that in the membrane.

In addition to the increase in the E_a of succinate oxidase for membranes with increasing fluidity, the E_a also increases when the temperature is reduced below both T_f and T_s. This latter increase in E_a is induced by an alteration in the physical state of the membrane lipids [1,4,6,16]. Furthermore the change

in E_a is abolished when membrane lipids are perturbed by detergent (Fig. 2 and Ref. 10). Based on the observation that E_a decreases with decreasing disorder of the fatty acid chains (Fig. 3) the E_a, in the temperature ranges below T_f and T_s should decrease due to the greater order existing in the membrane lipids at low temperatures. The E_a however, increases with changes in molecular order induced by low temperature. This apparent anomalous response of the membrane-associated proteins can be explained in terms of changes in the lateral distribution of membrane lipids which occur at low temperatures. There is evidence to show that lowering the temperature results in solidification of high melting point lipids and the remaining fluid lipids, phase separate in the plane of the bilayer and form discrete domains [17,18]. Areas of solid and fluid phase lipids can be detected in freeze-fracture electron micrographs of phospholipids cooled from temperatures below the T_f [19]. Freeze-fracture electron micrographs of rat liver mitochondrial membranes, similarly frozen after equilibration at temperatures below T_f also exhibit large smooth areas of gel phase lipid with the protein particles aggregated into small areas of fluid phase lipids [20]. It is apparent from this evidence that membrane-associated protein particles phase separate and remain with the fluid phase lipids. The increase in E_a which occurs with rat and sheep liver mitochondria at low temperatures can thus be explained in terms of this type of phase separation. As the temperature is lowered, more of the higher melting lipids would form gel phase lipid and the remaining domains of fluid phase lipids would become progressively disordered due to enrichment by lipids of low melting point. A separation of membrane particles has been observed in membranes of rat liver mitochondria frozen from 7°C, a temperature corresponding to the transition exotherm for these membranes [20]. Thus below T_s, membrane-associated enzymes of rat liver mitochondria phase separate and the increase in E_a below T_s is consistent with this separation. The increase in E_a below T_f would thus indicate that phase separation also occurs at this temperature. The endothermic transition for rat liver mitochondrial membranes extends to about 30°C [21] and phase separation of membrane proteins could occur up to this temperature. The increase in E_a at T_f suggests that phase separation occurs only up to about 23°C.

The constant E_a for rat liver mitochondria treated with detergent (Fig. 2) shows that the detergent has decreased the lipid-lipid interactions of membrane lipids and abolished the phase separation in the temperature range studied. The detergent, however, did not appreciably affect the interaction between membrane lipid and membrane protein. This follows from the observation that with the more fluid membranes of mitochondria from rats fed the unsaturated fatty acid-rich diet, the E_a was greater after detergent treatment.

The results confirm that changes in membrane fluidity can affect the E_a of membrane-associated enzymes and since the rate constant is related to E_a, the lipids influence the rate of reactions catalysed by these enzymes. Thus some cellular reactions involving membrane-associated enzymes could be regulated by changing membrane fluidity. Such changes could be mediated both intrinsically by changes in membrane lipid composition or extrinsically by compounds which influence membrane lipid fluidity.

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