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The Role of Lipid in the Energy-Dependent Transhydrogenase Systems of *Escherichia coli*

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Abstract

The energy-dependent and independent transhydrogenase activities and the NADH oxidase of membrane particles of *Escherichia coli* WS1 were inactivated by phospholipase A from *Crotalus terrificus*. Ca^{2+}-activated ATPase was stimulated by this treatment. Although these results suggest that phospholipid is involved in the transhydrogenase systems, trypsin treatment produced similar results. Proteolytic activity was not detected in the phospholipase preparation but its presence could not be ruled out. Membranes containing different unsaturated fatty-acid components were obtained by growing the fatty-acid auxotroph, *E. coli* K1060, on linoleic, oleic, or elaidic acids. Discontinuities in the Arrhenius plots of the activities of NADH oxidase, Ca^{2+}-activated ATPase, energy-dependent and independent transhydrogenases, were observed at definite temperatures ("transition temperatures"). With the exception of NADH oxidase, the transition temperatures could not be correlated with those expected for phase changes in the phospholipids of the membranes. Transition temperatures were also found when a lipid-free, purified ATPase was used. It is concluded that phase changes in the bulk of the phospholipids do not effect transhydrogenase and ATPase activities, and that there is no evidence that the bulk of the phospholipid is involved in the activity of these enzymes. However, we cannot exclude the possibility that a limited amount of lipid in immediate contact with the enzyme protein is essential for its activity.

Introduction

The energy-dependent transhydrogenase catalyzes the transhydrogenation of $NADP^+$ by NADH. Input of energy results both in an enhancement in the rate of the energy-independent reaction as well as an increase in the apparent equilibrium constant from 1 to about 500 [1]. Energy appears to be supplied as an energized state formed either by substrate oxidation through the respiratory chain or from ATP by a reversal of the reactions of oxidative phosphorylation. The mechanism of the reaction is unknown but may involve either the obligatory coupling of a proton pump to the transhydrogenase so that energization involves translocation of protons across the membrane [2] or energization may cause a conformational change in the transhydrogenase from an inactive to an active form [3]. In either case, the environment of the membrane-bound enzyme may contribute to its properties. In this paper we have studied the role of phospholipids in transhydrogenase activity.

The only previous work which bears on this is that of Luzikov et al. [4, 5] who showed that treatment of submitochondrial particles with phospholipase A from *Crotalus terrificus* resulted in loss of both respiration- and ATP-driven transhydrogenase activities but with less effect on the energy-independent transhydrogenase activity. Phospholipases C and D had essentially no effect on these systems. Since free fatty-acids and lysophophatides formed as digestion products might be inhibitory to the transhydrogenase system, we have studied the involvement of phospholipids in this system by examining the effect of temperature on the enzyme system using membrane particles prepared from an unsaturated fatty-acid mutant [6] of *E. coli* in which the lipid composition of the membrane was selectively altered. This technique depends on the phase transitions which occur in a phospholipid bilayer on heating. These transitions can be detected as temperatures at which there are changes in the slopes of the Arrhenius plot of the effect of temperature on the behavior of a spin label in the membrane [7] or on other physical properties of the membrane [8]. Generally, two temperatures can be detected. Above the higher of these temperatures (t_h) the lipids of the membrane are in the liquid phase while below the lower transition temperature (t_l) the lipids are in the solid phase. At temperatures between t_l and t_h, liquid and solid phases are in equilibrium [7]. There is good agreement between transition temperatures determined by physical methods and those observed for certain biological processes such as active transport of β-glucosides and β-galactosides [6, 7, 9, 10]. The temperatures at which phase changes occur in the phospholipids depends on the nature and degree of unsaturation of the fatty acid side chains of the phospholipid. Thus, mutants unable to form or degrade unsaturated fatty-acids are particularly useful for these studies since the transition temperatures

can be systematically varied by altering the extent of unsaturation of the fatty-acids which are incorporated into the membrane phospholipids.

In the present experiments using a fatty-acid auxotroph, strain K1060 of *E. coli,* we have examined the effect of temperature on the membrane-bound respiration- and ATP-driven transhydrogenase activities, and on the activities of the Ca^{2+}-activated ATPase, NADH oxidase, and energy-independent transhydrogenase, which are components of one or both of the energy-dependent transhydrogenase systems. With the exception of NADH oxidase, the discontinuities observed in the Arrhenius plots for these enzymes do not correlate with the temperature expected for a phase change in the phospholipids. It is concluded that the bulk of the phospholipids do not directly influence the transhydrogenase and ATPase activities. However, we cannot exclude the possibility that the fraction of the phospholipid immediately in contact with these enzymes is involved in their activity. The inhibitory action of phospholipase A could be interpreted as support for this hypothesis.

Materials and Methods

Bacteria and Media

E. coli K1060 (F^-, thi^-, $fabB^-$, old E^-) an unsaturated fatty-acid auxotroph was used in most experiments [6]. In some experiments wild type *E. coli* WS1 (F^-, Pro^-, Lac_1^-, Gal_2^-, His^-, Ara^-, Xyl^-, Man^-, B_1^-, StrR) was used [11]. Bacteria were grown in M63 medium supplemented with 0.1% casamino acids. Glycerol (0.5%) was used as a carbon source. Brij 35 (0.04%) was added to solubilize the fatty-acid supplements except for elaidic acid when 0.1% was used. Fatty-acids were used at a final concentration of 0.02% [12]. Casamino acids were omitted from the growth medium of cells grown for the transhydrogenase assay. Cells were grown with aeration at 37° C in 1-l flasks containing 250 ml of the medium. Growth was estimated by measuring the absorbance at 660 nm with a Coleman model 124 spectrophotometer.

Preparation of membrane particles

Cells were harvested at the end of the exponential phase of growth and washed twice at room temperature with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.04% Brij 35, and twice at 4° C with the above buffer but lacking Brij 35. The washed cells were resuspended at a concentration of 1 g wet weight per 10 ml 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$. The cells were broken by a single passage through a French pressure cell and the membrane particles were prepared as described previously [13].

Enzyme assays

Ca^{2+}-activated ATPase, energy-independent and energy-dependent transhydrogenases, and NADH oxidase were assayed by published procedures [14, 15]. The temperature of the cuvette compartment of a Coleman 124 spectrophotometer was controlled with a Haake thermostat, and the temperature of the reaction mixture was checked with a thermistor probe. Since most of these assays were carried out in Tris buffer which is known to be affected by temperature, the pH of a typical reaction mixture was measured at 0° C and at 37° C. A difference of 0.5 pH units, which would not significantly affect the activity of these enzymes, was observed between these two temperatures.

Treatment of membrane particles with phospholipase A

Membrane particles (0.1–0.6 mg protein) were treated for 5 min at 37° C with phospholipase A (3–200 μg) in 50 mM glycylglycine buffer, pH 7.5, containing 0.5% bovine serum albumin and 5 mM $CaCl_2$. Samples of 20–200 μl were removed after treatment was over and diluted to 1.0 ml with either 50 mM Tris-HCl buffer, pH 7.8, containing 0.25 M sucrose, 5 mM $MgCl_2$, and 0.01 mM DTT (for aerobic and ATP-driven transhydrogenase) or with glycylglycine buffer (for energy-independent transhydrogenase, NADH oxidase, and ATPase). Samples were assayed immediately.

Treatment of membrane particles with trypsin

Membrane particles (0.2–0.8 mg protein) were treated at 37° C for 5 min with various concentrations of trypsin (1–200 μg) in 50 mM Tris-HCl buffer, pH 7.8, containing 0.25 M sucrose, 5 mM $MgCl_2$, and 0.01 mM DTT. The reaction of trypsin was terminated by the addition of trypsin inhibitor at a concentration 5 times that of the trypsin. In the control samples trypsin inhibitor was added before the trypsin.

Materials

Trypsin (crystalline) and phospholipase A (*Crotalus terrificus*) were purchased from Calbiochem, La Jolla, California. Elaidic acid (*trans*-Δ^9-$C_{18:1}$) linoleic acid (*cis, cis*-Δ^9, ^{12}-$C_{18:2}$), and oleic acid (*cis*-Δ^9-$C_{18:1}$) were obtained from Sigma Chemical Company, St. Louis.

Results and Discussion

Effect of phospholipase A on the energy-dependent transhydrogenase and related reactions

In agreement with the results of Luzikov et al. [4] with submitochondrial particles, treatment of membrane particles of *E. coli*

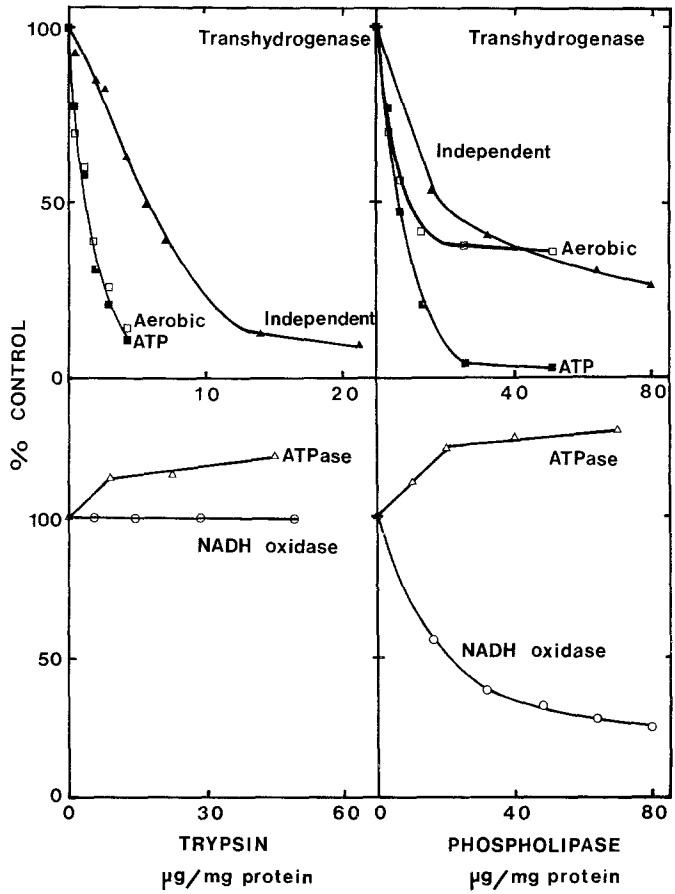

Figure 1. Effect of trypsin and phospholipase A on NADH oxidase, Ca^{2+}-activated ATPase, energy-independent, and aerobic- and ATP-driven-transhydrogenase activities of membrane particles of *E. coli* WS1. The particles were treated as described in *Materials and Methods.* The control (100% value) specific activities of NADH oxidase, Ca^{2+}-activated ATPase, energy-independent, aerobic- and ATP-driven transhydrogenases were 1200, 233, 658, 73, and 78 nmole/min/mg protein for the phospholipase experiment, and 205, 300, 341, 32, and 88 nmole/min/mg protein for the trypsin experiment, respectively.

WS1 with phospholipase A resulted in inhibition of both respiration- and ATP-driven energy-dependent transhydrogenase activities (Fig. 1). The energy-independent transhydrogeanse was also inhibited, in contrast to the results with submitochondrial particles. Loss of energy-dependent transhydrogenase activities was obtained at slightly lower ratios of

phospholipase A to membrane protein than were required for inhibition of the component reactions (energy-independent transhydrogenase, NADH oxidase, ATPase) of these systems. This suggests that the most sensitive site of action is at the level of energy-coupling to the transhydrogenase reaction.

Free fatty-acids which are products of phospholipase A digestion are known to be uncoupling agents and might be responsible for this effect. The presence of bovine serum albumin which can bind fatty-acids in our reaction mixtures would only be effective in preventing uncoupling if the fatty-acids were released from the membrane.

It has been shown previously that trypsin treatment will activate the ATPase of *E. coli* [16, 17]. Since phospholipase A activated the ATPase of the membrane particles, this suggested that the apparent effects of the phospholipase might be due to the presence of trypsin-like enzymes in the preparation. Examination of the phospholipase A for proteolytic enzymes by the method of Kučera and Lysenko [18] gave negative results. Moreover, treatment of alcohol dehydrogenase with phospholipase A under the same conditions as used with the membrane particles did not result in any loss of dehydrogenase activity. However, the striking similarity in the relative sensitivity of some of the membrane enzymes to trypsin (Fig. 1) and to phospholipase A makes it difficult to exclude the possible presence of a low amount of proteolytic activity in our preparation. This possibility, together with the uncertainty about whether the products of phospholipase A digestion are inhibitory, suggests that the conclusion that phospholipids are involved in the activity of the transhydrogenase systems should be made with reservations.

The possible involvement of lipids in the activity of NADH oxidase is supported by the fact that trypsin did not inhibit this enzyme in contrast to phospholipase A. Moreover, Mavis et al. [19] were able to correlate the loss of NADH oxidase activity with the extent of digestion of membrane phospholipids by phospholipase C.

Transition temperature studies

To overcome the uncertainties when phospholipase A was used to study the role of phospholipids in the transhydrogenase reaction, we examined the effect on these enzymes of changing the lipid composition of the membranes of the unsaturated fatty-acid auxotroph *E. coli* K1060 by growing it on media containing either elaidic (*trans*-Δ^9-$C_{18:1}$), oleic (*cis*-Δ^9-$C_{18:1}$), or linoleic (*cis*-Δ^9, ^{12}-$C_{18:2}$) acids. Mavis and Vagelos [20] have shown that the total fatty-acids of membrane particles of this strain contain about 65% elaidic acid, or 54% oleic acid, or 53% linoleic acid, when the growth medium is supplemented with the respective fatty-acid. In each case no other unsaturated fatty-acid is present in the membrane.

TABLE I. Transition temperatures and energies of activation derived from Arrhenius plots of selected enzyme activities of membrane particles of *E. coli* K1060 grown with linoleic (L), oleic (O), or elaidic (E) acid, or of *E. coli* WS1[a]

Enzyme	Particle	E_L	t_l	E_H	t_h
NADH oxidase	L	25.7	8	15.7	29
	O	23.7	14	12.4	37
	E	28.0	22	20.6	40
	WS1	18.8	15	12.1	29
Aerobic TH	L	22.0	18	12.0	30
	O	20.4	20	9.9	30
	WS1	17.4	24	12.4	
Independent TH	L	20.0	14	12.1	31
	O	24.9	13	14.9	25
		(25.2)	(16)	(14.7)	(26)
	E	26.7	15	19.5	35
	WS1	17.6	25	10.0	40
		(22.9)	(25)	(10.0)	
ATP TH	L	17.7	17	11.1	32
	O	26.6	18	15.1	32
	WS1	30.9	20	14.2	29
ATPase	L	25.0	25	8.6	
	O	20.5	25	8.0	
	E	21.6	25	11.5	
	WS1	18.4	24	5.5	

[a] The lower and higher transition temperatures, t_l and t_h, are expressed in °C. The energies of activation E_L and E_H refer to the reactions below and above t_l, respectively. The assays were carried out as described in *Materials and Methods*. Energy-independent-transhydrogenase activity was measured by reduction of 3-acetylpyridine-NAD^+ by NADPH or by reduction of $NADP^+$ by NADH. The values obtained with the latter assay are shown in parentheses.

Consequently, in these membranes, phase changes in the phospholipids occur at different transition temperatures.

Arrhenius plots for the effect of temperature on the respiration- and ATP-driven transhydrogenase systems, and on the energy-independent transhydrogenase, NADH oxidase, and Ca^{2+}-activated ATPase activities are shown in Figs. 2–6. The transition temperatures and the activation energies for these reactions are summarized in Table I. Data obtained with wild-type *E. coli* WS1 are also included. It was not possible to obtain values for the respiration- and ATP-driven transhydrogenase reactions for cells grown on elaidic acid. For growth with this fatty-acid the medium required supplementation with amino acids. As found previously, the presence of amino acids during growth represses the formation of the energy-dependent transhydrogenases [14].

The same two transition temperatures (17–20° C, 29–32° C) were

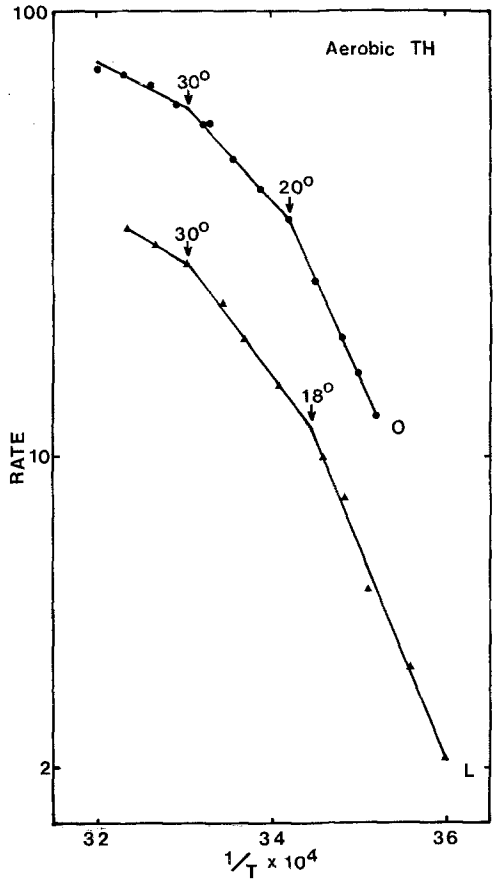

Figure 2. Arrhenius plot of effect of temperature on the activity of respiration-driven transhydrogenase in membrane particles from cells of strain K1060 grown with oleic (O) or linoleic (L) acid. The rate of the reaction is expressed as nmole/min/mg protein. The assay was carried out as described in *Materials and Methods* using 0.31 and 0.66 mg particle protein for the assays with O and L particles, respectively.

detected for both respiration- and ATP-driven transhydrogenase activities and were not dependent on the fatty-acid composition of the membrane (Figs. 2 and 3). In a similar manner the energy-independent transhydrogenase showed two transition temperatures (13–15° C, 25–35° C) which did not seem to be related in any systematic way to the expected fatty-acid composition of the particles (Fig. 4). This result differs from that of Sweetman and Griffiths [21] who were not able to find a transition temperature in Arrhenius plots of the activity of the

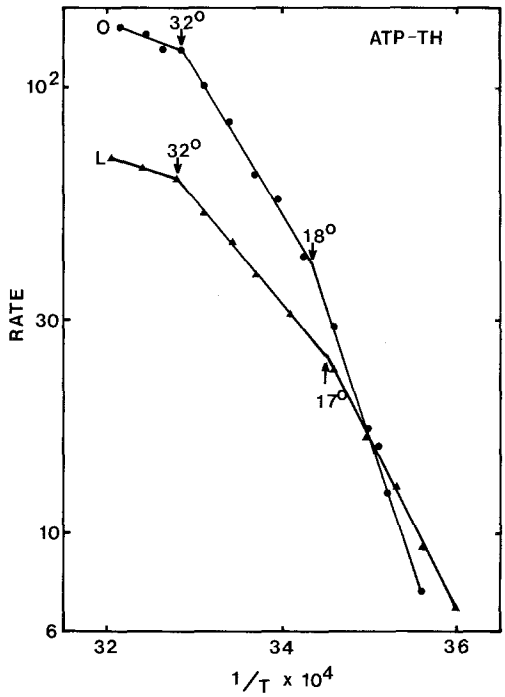

Figure 3. Arrhenius plot of effect of temperature on the activity of ATP-driven-transhydrogenase in membrane particles from cells of strain K1060 grown with oleic (O) or linoleic (L) acid. The rate of the reaction is expressed as nmole/min/mg protein. The assay was carried out as described in *Materials and Methods* using 0.31 and 0.66 mg particle protein for the assays with O and L particles, respectively.

energy-independent transhydrogenase in membranes of wild-type *E. coli*. We detected a transition at 25° C in these cells (Table I) which would not have been found by Sweetman and Griffiths since their measurements were made over the range of 5–25° C. We do not know why the transition temperature is higher in membranes from wild-type cells compared to those of the fatty-acid auxotroph.

In contrast to the transhydrogenase activities, the activity of NADH oxidase (Fig. 5) showed low and high temperature transitions at 8° and 29° C for linoleic-acid-grown cells, 14° and 36° C for oleic-acid-grown cells, and 22° C and 38° C for elaidic-acid-grown cells, which correlated with the fatty-acid composition of the membranes. Linden et al. [7, 9], using spin probes, showed t_l and t_h to be at 9° and 29°, 16°, and 31°, and 31° and 38° C for linoleic acid, oleic acid, and elaidic-acid-grown

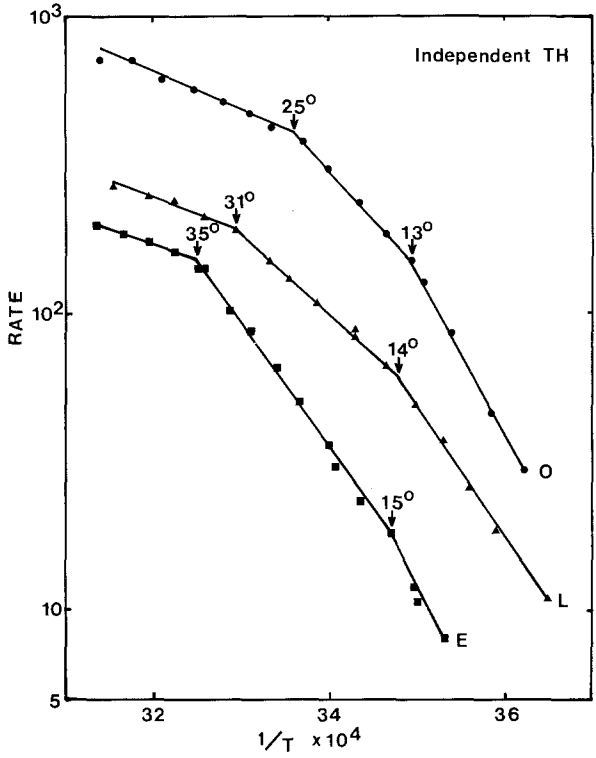

Figure 4. Arrhenius plot of effect of temperature on the activity of energy-independent-transhydrogenase in membrane particles from cells of strain K1060 grown with oleic (O), linoleic (L), or elaidic (E) acid. The rate of the reaction is expressed as nmole/min/mg protein. To facilitate plotting the rates for the E particles have been divided by two. The assay was carried out as described in *Materials and Methods* using 0.062, 0.26, and 0.14 mg particle protein for the assays with O, L, and E particles, respectively.

cells. Thus, the transitions observed in Arrhenius plots of the NADH oxidase are probably due to the effect of phase changes in the phospholipids of the membrane. This agrees with the results of Haest et al. [22] who showed a direct correlation between the temperature at which a phase change occurred in the phospholipids as measured by differential scanning colorimetry and the transition temperatures measured from Arrhenius plots of NADPH oxidase activity in membranes from *Staphylococcus aureus* and *Bacillus subtilis*. The transitions observed with the transhydrogenase systems do not appear to reflect phase changes in the membrane phospholipids and may indicate the existence of different conformations of the enzymes.

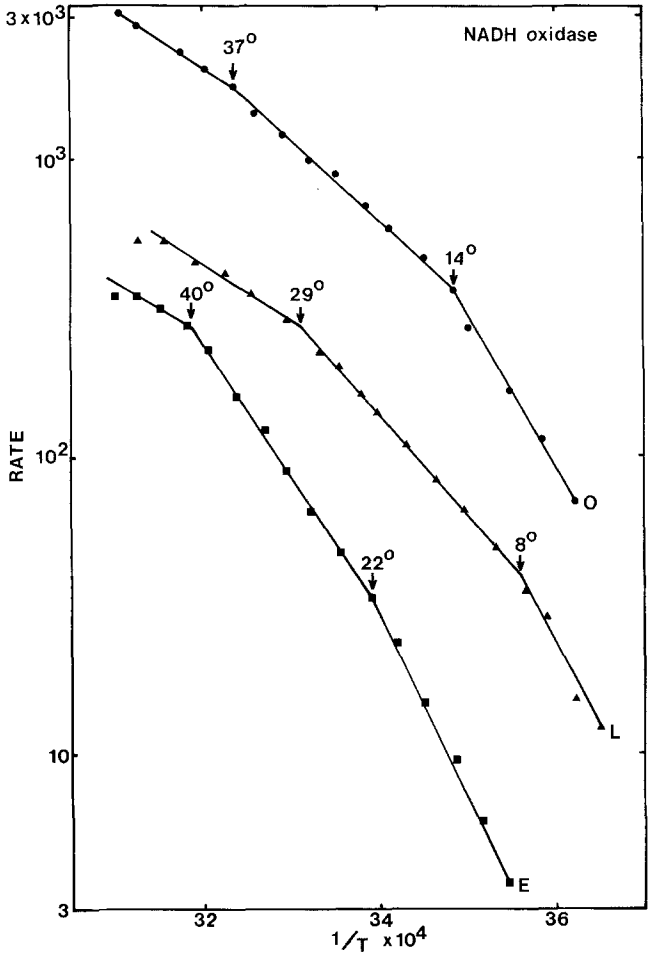

Figure 5. Arrhenius plot of effect of temperature on the activity of NADH oxidase in membrane particles from cells of strain K1060 grown with oleic (O), linoleic (L), or elaidic (E) acid. The rate of the reaction is expressed as nmole/min/mg protein. The assay was carried out as described in *Materials and Methods* using 0.062, 0.26, and 0.14 mg particle protein for the assays with O, L, and E particles, respectively.

The results obtained with the Ca^{2+}, Mg^{2+}-activated ATPase are shown in Fig. 6. The ATPase activity of membranes from cells grown on elaidic, oleic, and linoleic acids showed a fatty-acid-independent transition at 25° C. With linoleic acid there was an additional transition at 12° C. The lipid-free soluble ATPase purified to homogeneity also showed transition temperatures in the Arrhenius plot of 12°, 21°, and 31° C which must

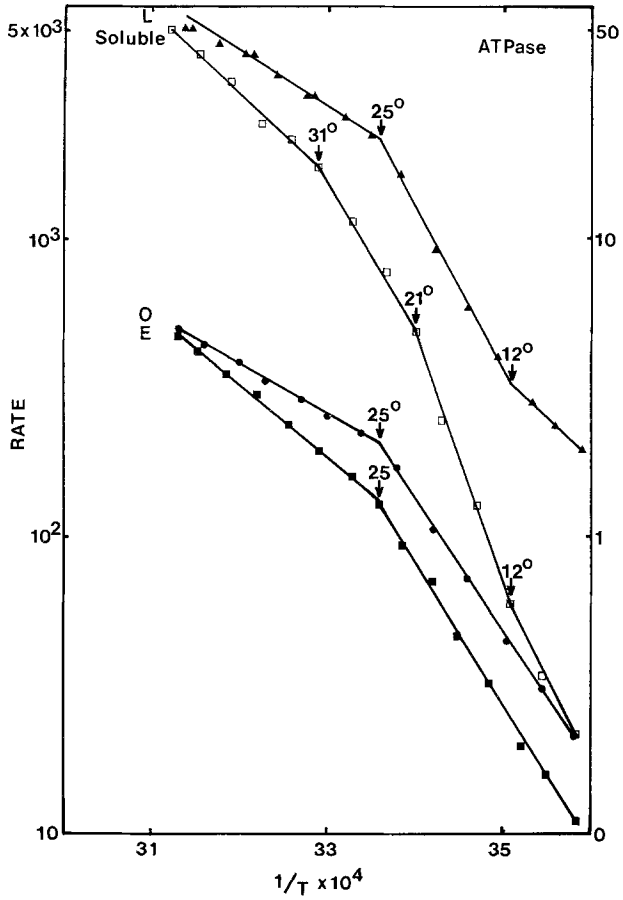

Figure 6. Arrhenius plot of effect of temperature on the activity of Ca^{2+}-activated ATPase in membrane particles from cells of strain K1060 grown with oleic (O), linoleic (L), or elaidic (E) acid, and on the activity of ATPase solubilized and purified from *E. coli* NRC482 by the method of Bragg and Hou [13] ("soluble"). The rate of the reaction is expressed as nmol/min/mg protein. To facilitate plotting the rates for the soluble ATPase have been divided by three. The scale on the ordinate at the right refers to the rate with L particles. The assay was carried out as described in *Materials and Methods* using 0.59, 0.58, 0.64, and 0.028 mg protein for the assays with the O, L, and E particles, and with the soluble enzyme, respectively.

reflect conformational changes in the enzyme protein. Thus, there is no evidence from our results for an interaction of phospholipid with this enzyme. A similar conclusion was reached by Siñeriz et al. [23] using another unsaturated fatty-acid-requiring strain of *E. coli*. These workers

found a single fatty-acid-independent transition at 32–34° C using membranes from oleic- and linolenic-acid supplemented cells. Sweetman and Griffiths [24] using wild-type cells detected a transition at 19° C whereas we found a transition temperature at 24° C with our wild-type cells. The reasons for these differences is not clear, although differences in the strain of the bacterium used and in the growth conditions might be responsible.

The mitochondrial ATPase of yeast is functionally similar to that of the membrane of *E. coli* in that both are involved in the formation of ATP by oxidative phosphorylation. However, in contrast to our results with *E. coli*, the yeast enzyme is sensitive to changes in fatty-acid composition of the membrane. Thus, Janki et al. [25] showed a change in the transition temperature from 8–12° to 22° C when the fatty-acid supplement during growth was changed from linoleic acid, to oleic acid, to elaidic acid, respectively. Furthermore, Haslam et al. [26] showed that transition temperatures of 8°, 27°, and 35° C were obtained when the content of unsaturated fatty-acids were 83%, 20%, and 13% of the total lipids, respectively. These results suggest that the sites of interaction of the ATPases of *E. coli* and of yeast with their membranes are different. This might account for the observed differences between the two enzymes, as in their different sensitivities to oligomycin. The ATPase from *E. coli* is not inhibited by oligomycin [27] whereas the sensitivity of the yeast enzyme is related to the degree of unsaturation of the fatty-acids in the membrane phospholipids [26]. Interesting in this context, the activity of the ATPase of another bacterium, *Rhodospirillum rubrum,* in contrast to *E. coli* is inhibited by oligomycin and is dependent on the presence of phospholipid [28, 29].

In conclusion, there is no clear evidence that the activities of the energy-dependent transhydrogenase systems and of the ATPase of *E. coli* are closely linked to the phospholipids of the membrane. The relationship suggested from experiments with phospholipase A may be explicable on the basis of inhibition by the products of digestion or be due to the presence of proteolytic enzymes in the phospholipase preparation. However, it is still possible that a limited number of phospholipid molecules directly in contact with the enzyme protein are essential for enzyme activity. These might be destroyed by phospholipase A with resulting inactivation of the enzymes but their response to changes in temperature might not be typical of the bulk of the phospholipid.

Acknowledgments

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