RELATION OF ARRHENIUS DISCONTINUITIES OF NADH DEHYDROGENASE TO CHANGE IN MEMBRANE LIPID FLUIDITY OF *BACILLUS CALDOTENAX*

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1. Introduction

In [1], NADH dehydrogenase (EC 1.6.99.3) was highly purified from *Bacillus caldotenax*, and the effects of membrane lipids on the enzyme examined. The purified enzyme exhibited a linear Arrhenius plot and enzyme in membranes or with membrane lipids exhibited a discontinuous Arrhenius plot [1]. The Arrhenius discontinuities of the enzyme in membranes were suggested to be triggered by a change in some physical state of membrane lipids. The effects of various phospholipid species on the Arrhenius plot of the enzyme were studied here with special reference to the correlation of the Arrhenius discontinuities to the fluidity change with temperature of the lipids.

2. Materials and methods

NADH dehydrogenase was purified from *B. caldotenax* YT-G to a 80% purity, according to [1].

Membranes were prepared from the freshly cultured cells according to [2], and lipids were extracted according to [3]. Phospholipids were separated from neutral lipids by acetone precipitation [4]. Phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidyl glycerol (PG) were separated from the phospholipids according to [5,6]. Each isolated phospholipid was checked for purity by thin-layer chromatography according to [2].

The enzyme was reconstituted with membrane lipids or phospholipids essentially according to [7], as described below. The purified enzyme (0.1–0.2 mg) was precipitated by 60% $(NH_4)_2SO_4$, dissolved in 10 mM Tris–HCl buffer (pH 7.2) containing 20% glycerol, 2% sodium cholate and 3 mg membrane lipids/ml or 1 μmol phospholipid/ml, and dialyzed against 10 mM Tris–HCl buffer (pH 7.2) containing 20% glycerol at 4°C overnight.

Assay of NADH dehydrogenase was carried out in 20 mM Tris–HCl buffer (pH 7.5) by measuring NADH-2,6-dichlorophenol indophenol (DCIP) reductase activity [8] according to [1]. The enzyme reaction proceeded linearly with time for at least 2–3 min under all the assay conditions used here. The relation of s/V to s for NADH was plotted at various fixed concentrations of DCIP, and the velocities (V) with various concentrations of DCIP at infinitive concentrations of NADH were estimated. From the relation of s/V to s for DCIP, the maximum velocity (V_{max}) with DCIP was determined.

Fluorescence polarization measurements were carried out with an instrument constructed in Mitsubishi-Kasei Institute of Life Science [9]. Membrane lipids or phospholipids were dissolved in 20 mM Tris–HCl buffer (pH 7.5) containing 2% sodium cholate and dialyzed against 20 mM Tris–HCl buffer (pH 7.5) at 4°C overnight. To 3 ml lipid suspension thus prepared (0.4 mg lipid/ml) or the membrane suspension in 20 mM Tris–HCl buffer (pH 7.5) (0.24 mg protein/ml) was added 10 μl of 1,6-diphenyl-1,3,5-hexatriene (DHP) (final conc. 1 μM) in tetrahydrofuran. The fluorescence anisotropy (r) was calculated according to [9].

Calorimetric data were obtained using a Perkin Elmer DSC2 differential scanning calorimeter. Heating rate was of 5°C/min and sensitivities of 0.2 mcal/s. The sample volume was 50 μl. Samples in 20 mM phosphate buffer (pH 7.5) were prepared according to [10].

Protein was determined according to [11] and membrane protein according to [12]. Lipids were determined by the P_i method in [13].

Dipalmitoyl phosphatidylcholine (DPPC) was purchased from Sigma, dipalmitoyl phosphatidylglycerol (DPPG) from Funakosi Co. and DPH from Tokyo Kasei Co.

3. Results and discussion

As reported in [1], NADH dehydrogenase purified from *B. caldotenax* exhibited a linear Arrhenius plot, while Arrhenius plot for the enzyme in membranes exhibited two discontinuities at ~50°C and ~22°C (see also fig.1). When the enzyme was mixed with membrane lipids prepared from the cells grown at 65°C (65°C-lipids), the Arrhenius plot for this lipid-bound enzyme was reported to exhibit a single discontinuity at ~50°C [1]. Re-examination of the Arrhenius plot for the lipid-bound enzyme revealed two discontinuities at ~48°C and ~22°C (fig.1). These results suggest that the Arrhenius discontinuities of the membrane- or lipid-bound enzyme are related to changes in some physical state of membrane lipids.

Ca^{2+}-ATPase of sarcoplasmic reticulum was reported to exhibit a triphasic Arrhenius plot, but the Arrhenius plot of microviscosity for sarcoplasmic reticulum was linear [14]. The change with temperature of the fluorescence anisotropy (r) of DPH in membranes or membrane lipids of *B. caldotenax*, however, showed two distinct inflexion points at ~50°C and ~23°C or ~52°C and ~32°C, respectively, which were approximately coincident with the Arrhenius discontinuities of the corresponding enzyme activities (fig.1,2).

Membrane lipids undergo a thermotropic, disorder

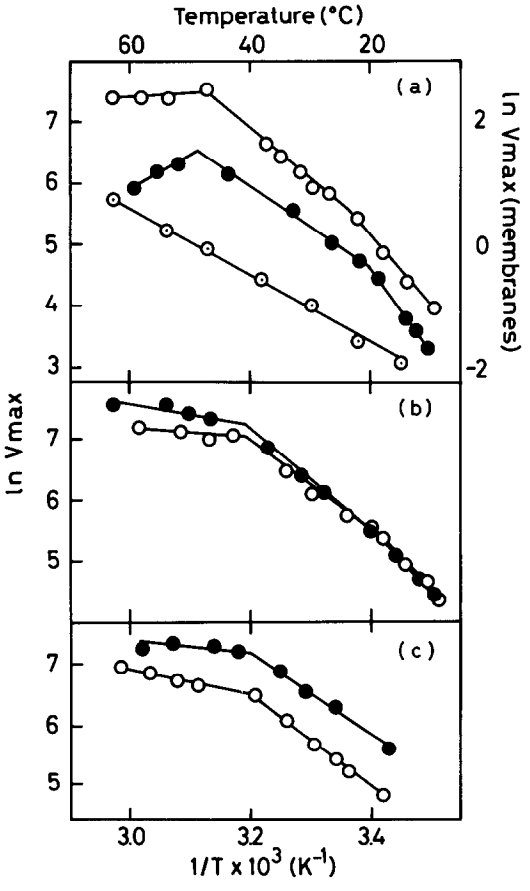

Fig.1. The Arrhenius plots for NADH dehydrogenase. (a) in membranes from the cells growth at 65°C (•), with 65°C-lipids (○) and in a purified state (⊙); (b) with PG (•) and CL (○) from 65°C-lipids; (c) with DPPG (•) and DPPC (○). V_{max} is expressed as units/mg protein.

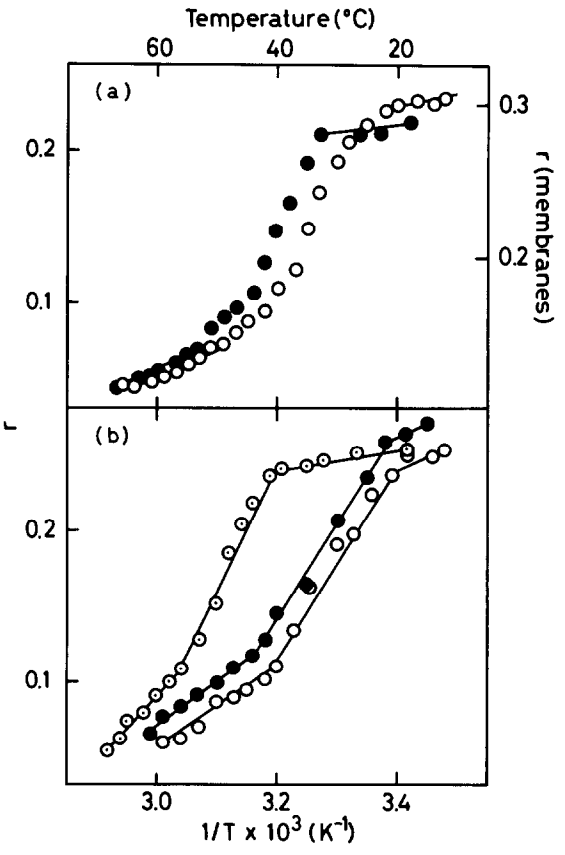

Fig.2. The fluorescence anisotropy (r) vs temperature (T) plots: (a) membranes from the cells grown at 65°C (○) and 65°C-lipids (•); (b) PE (⊙), PG (•) and CL (○) from 65°C-lipids.

(fluid)–order (solid) transition. At temperatures above T_f, the upper limit of the transition, or at temperatures below T_s, the lower limit of the transition, membrane lipids are considered to be in a fluid or solid state, respectively [15]. The thermogram of membranes or membrane lipids of *B. caldotenax* showed a phase transition of the upper (T_f) and lower (T_s) boundaries at ~53°C and ~25°C or at ~52°C and ~23°C, respectively. The discontinuities in the r vs T plot for membranes or membrane lipids, therefore, are considered to be the transition from a phase-separation state to a fluid state and from a solid state to the phase-separation state, respectively. The Arrhenius discontinuities of NADH dehydrogenase in membranes or with membrane lipids of *B. caldotenax* may be related to the phase transition of membrane lipids.

The values of T_f of membranes or membrane lipids of *B. stearothermophilus* [16] and *Thermus thermophilus* [9] were almost the same as those of the growth temperatures of the bacteria, respectively. T_f of membranes or membrane lipids of *B. caldotenax* (~50–~52°C), however, was lower than the growth temperature (65°C). T_f of membrane lipids from the cells grown at 45°C (45°C-lipids) estimated by DSC and r analyses was ~40°C. This value was lower than that of 65°C-lipids, as suggested from the change in chemical composition of membrane lipids on changing the growth temperature from 65 to 45°C [2]. The T_f value of 45°C-lipids (~40°C), although slightly lower than the growth temperature (45°C), was almost coincided with the upper discontinuity at ~42°C in the Arrhenius plot for the enzyme with 45°C-lipids. These results may indicate that the membranes or membrane lipids of *B. caldotenax* at the growth temperature (45–65°C) is in a fluid state. The membranes or membrane lipids of *Acholeplasma laidlawii* [17] and *Escherichia coli* [18] have been reported to be in a fluid state at the growth temperature.

Molar ratio of PE, PG and CL in *B. caldotenax* grown at 65°C was 58%, 27% and 14%, when expressed as the percent of total phospholipids [2]. The enzyme was stimulated by PG or CL (fig.1), but not by PE, a major phospholipid component. The stimulation of the enzyme activity by membrane lipids [1] may be due to PG and CL. When the enzyme treated with PG (CL) was centrifuged at 250 000 × g for 5 h, 40% or 60% of the total activity was observed with the supernatant or precipitate, respectively. When the enzyme treated with PE was centrifuged, all the activity was observed with the supernatant, although precipitate was formed. The results shown above suggest that the enzyme did not bind to PE.

The Arrhenius plot for the enzyme with PG (or CL) exhibited two discontinuities at ~42°C and ~21°C, which were coincident with the discontinuities in the r vs T plot for PG (CL) at ~44°C and ~22°C, respectively (fig.1,2). DPPC or DPPG exhibits a phase transition from a solid to fluid state at 42°C [19] or 41°C [20], respectively. The Arrhenius plot for the enzyme with DPPC or DPPG exhibited a single discontinuity ~40°C (fig.2). These results also support that NADH dehydrogenase of *B. caldotenax* is strongly affected by the lipid fluidity change.

The Arrhenius activation energies (E_a) of the enzyme with phospholipids, which are in a fluid state, were very small, and almost the same for the enzyme samples with the phospholipids examined here (fig.1). In membranes or membrane lipids, the enzyme appeared to be thermophobic. The E_a increased greatly or slightly as phospholipids changed from a fluid state to a phase-separation state or from the phase-separation state to a solid state, respectively (fig.1), as reported with other organisms [21–23]. An increase in the molecular order of membrane lipids causes changes in the conformation of NADH dehydrogenase consistent with an increase in E_a.

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