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**Purification and properties of soluble NADH
dehydrogenase from a psychrophilic bacterium,
Vibrio sp. strain ABE-1**

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A soluble form of NADH dehydrogenase (EC 1.6.99.3) was purified about 940-fold from cytoplasmic fraction of a psychrophilic bacterium *Vibrio* sp. strain ABE-1. Apparent molecular weight of the purified enzyme was estimated to be about 35,000 by gel filtration. The enzyme was specific for NADH as an electron donor, and utilized 2,6-dichlorophenolindophenol, ferricyanide and menadione as an electron acceptor, but could not reduce cytochrome *c*, lipoamide, FMN and FAD. *o*-Phenanthroline partially inhibited the enzyme activity but *p*-chloromercuribenzoate, Rotenone and Amytal did not. The enzyme exhibited maximum activity at 25°C. About 70% of the maximum activity was observed at 5°C and *K_m* value for NADH progressively increased with the elevation of temperature, suggesting that the enzyme sufficiently functions at low temperatures.

A variety of enzymes, which catalyze the reduction of different electron acceptors with NADH and/or NADPH as an electron donor(s), have been found in many organisms (DIXON and WEBB, 1979). These are often detected by the reduction of artificial electron acceptors, and designated as diaphorase (SINGER, 1963). However, some of them have not yet been elucidated in their physiological functions.

We previously reported that NADH dehydrogenase (NADH-DH) is almost equally distributed in cytoplasmic and membrane fractions of a psychrophilic bacterium, *Vibrio* sp. strain ABE-1 (*Vibrio* ABE-1) (TAKADA *et al.*, 1981). The NADH-DH activities in the both fractions are considerably high at low temperatures, that is, they exhibited about 70% of the respective maximum activities even at 5°C. However, comparative studies on the activities under different physical and chemical conditions such as pH, salts and temperature suggested that the cytoplasmic and membrane-bound enzymes were presumed to be distinct from each other.

To evaluate the nature of the cytoplasmic NADH-DH, we attempted to purify the enzyme and to investigate some characteristics of the purified enzyme. Furthermore, the relationship of the purified enzyme to other homologous enzymes was also discussed.

Materials and Methods

Bacterial strains and growth condition

Vibrio ABE-1 (TAKADA *et al.*, 1979) was grown at 10°C for 24 h with vigorous shaking in a nutrient medium consisting of 1% peptone, 1% meat extract and 0.5 M NaCl (pH 7.0). Cells were harvested and washed as described previously (TAKADA *et al.*, 1981). The washed cells were frozen at -20°C until use.

Preparation of cytoplasmic fraction

Cytoplasmic fraction of *Vibrio* ABE-1 was prepared by the previous procedures (TAKADA *et al.*, 1981) with the following modification. After the removal of unbroken cells, the sonic extract was centrifuged at $78,500 \times g$ for 2 h or $105,000 \times g$ for 1 h at 2°C, and the supernatant was used as the cytoplasmic fraction.

Enzyme assay

Unless otherwise stated, NADH-DH activity was assayed spectrophotometrically at 20°C as reported previously (TAKADA *et al.*, 1981). Standard reaction mixture contained 0.1 M Tris-HCl (pH 7.5), $134 \mu\text{M}$ NADH, 10 mM KCN, $35 \mu\text{M}$ 2,6-dichlorophenolindophenol (DCIP) and an appropriate amount of enzyme in a final volume of 2 ml. Menadione reductase activity was assayed by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH. One unit of the enzyme activity was defined as the amount of enzyme catalyzing the conversion of $1 \mu\text{mol}$ of substrates per min. The enzyme activities were calculated by the following molecular extinction coefficients: NADH, 6.3×10^3 at 340 nm (McCOMB *et al.*, 1976; ZIEGENHORN *et al.*, 1976); DCIP, 20.6×10^3 at 600 nm (ARMSTRONG, 1964); ferricyanide, 1×10^3 at 420 nm (MINAKAMI *et al.*, 1962).

Determination of molecular weight

The molecular weight of NADH-DH was estimated from the elution volume of gel filtration on a Sephadex G-100 column (1.5×100 cm) equilibrated with 10 mM Tris-HCl, 2 mM MgCl_2 , 2 mM 2-mercaptoethanol, 8.7% glycerol and 50 mM NaCl (pH 7.5). Cytochrome *c* (M_r 12,500), chymotrypsinogen A (25,000), ovalbumin (45,000), bovine serum albumin (68,000) and aldolase (158,000) were used as marker proteins.

Protein determination

Protein concentration was determined by the method of LOWLY *et al.* (1951) with bovine serum albumin as a standard and, in some cases, by KALCKAR's equation (1947).

Electrophoresis

A polyacrylamide disc gel electrophoresis was carried out at 4°C by the method of DAVIS (1964) and WILLIAMS and REISFELD (1964) with minor modification. Proteins were run at a constant current of 2 mA/gel tube. After electrophoresis, protein bands were stained with 0.25% Coomassie brilliant blue and the band of NADH-DH was identified by staining for the enzyme activity. The reaction mixture for the activity staining consisted of 40 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 0.27 mM NADH and 0.5 mM nitro blue tetrazolium in a final volume of 10 ml.

For a preparative thin-layer electrophoresis on Sephadex gels, Sephadex G-200 (superfine) were swollen in 50 mM Tris-glycine (pH 8.3) as an electrode buffer. The slurry was spread to a 1.2 mm thick on a glass plate of 10 × 12 cm. The gel plate was set in a flat bed electrophoresis apparatus and both ends of the plate were connected to the electrode buffer with a filter paper strip. Sample and small volume of 0.5% bromophenol blue solution as a marker dye were mixed and applied on a starting line of about 1 cm from cathode end of the gel plate. After electrophoresis, the gel plate was covered for several minutes with a filter paper (Toyo filter paper No. 2), which had been soaked in the activity staining mixture as described above. A blue band for NADH-DH activity was appeared on the filter paper after for a while. On the other hand, proteins on the plate were transferred into a dry filter paper and stained with Coomassie brilliant blue as reported by RADOLA (1968).

Chemicals

Horse heart cytochrome *c* was obtained from Boehringer Mannheim. NAD, NADH and NADPH were obtained from Oriental Yeast Co., Ltd. and Boehringer Mannheim. FMN and menadione were purchased from Nakarai Chemicals, Ltd. DCIP, lipoamide and potassium ferricyanide were from Merck, Sigma Chemical Co. and Wako Pure Chemical Ind. Ltd., respectively. Blue dextran 2000, Sepharose 4B, DEAE-Sephadex A-25 and other Sephadex gels were product of Pharmacia. NAD-Sepharose was prepared by the method of HOCKING and HARRIS (1973). Procedures for the preparation of calcium phosphate gel was based on the method of MEISTER (1952). All other reagents were of analytical grade.

Results

Purification of soluble NADH-DH

Unless otherwise stated, all purification procedures were carried out below 4°C.

Cytoplasmic fraction of *Vibrio* ABE-1 was prepared from the washed

cells as described in Materials and Methods. Two percent protamine sulfate in a buffer A, which consisted of 10 mM Tris-HCl, 2 mM MgCl₂, 2 mM 2-mercaptoethanol and 8.7% glycerol (pH 7.5), supplemented with 0.5 M NaCl was added slowly with stirring to the cytoplasmic fraction to give a final concentration of about 0.38 mg protamine sulfate/mg protein. After further stirring for 20 min, the mixture was centrifuged and the precipitate was discarded.

The supernatant was diluted with 9 volumes of buffer A and mixed with 170 g wet weight of calcium phosphate gel with further stirring for 60 min. The gel was then collected by centrifugation and the supernatant was discarded. NADH-DH was eluted from the gel by mixing with 0.3 M potassium phosphate (pH 7.5) containing 2 mM MgCl₂, 2 mM 2-mercaptoethanol and 8.7% glycerol, stirring for 60 min and centrifugation. The collected supernatant fluid was concentrated to about 50 ml with polyethylene glycol No. 6000 or 20000.

The concentrated sample was applied to a Sephadex G-150 column (6 × 60 cm) previously equilibrated with buffer A supplemented with 50 mM NaCl and eluted with the same buffer at a flow rate of 40 ml/h. Fractions containing the dehydrogenase activity were pooled and concentrated several fold with polyethylene glycol.

Next, the concentrated enzyme was applied to a DEAE-Sephadex A-25 column (OH⁻ form; 2.5 × 38 cm) equilibrated with buffer A supplemented with 0.15 M NaCl. The column was then washed with about 450 ml of the same buffer and the adsorbed enzyme was eluted with a linear gradient of 0.15 M to 0.6 M NaCl in 450 ml of buffer A at a flow rate of 23 ml/h. NADH-DH was eluted at about 0.4 M NaCl. Active fractions were combined, concentrated to about 30 ml with polyethylene glycol and dialyzed overnight against buffer A.

The dialyzed sample was applied to a NAD-Sepharose 4B column (1.5 × 8.5 cm) previously equilibrated with buffer A and the column was washed with three bed volume of buffer B, which was buffer A containing 0.2 M NaCl. Then, the enzyme was eluted stepwisely with 45 ml of buffer B containing 2 mg/ml NAD, 30 ml of buffer B containing 4 mg/ml NAD and 30 ml of buffer A containing 0.6 M NaCl and 4 mg/ml NAD at a flow rate of 23 ml/h. The enzyme activity was found in the both eluates of buffer B containing different concentrations of NAD. Fractions containing high enzyme activity were combined, concentrated to 1.5 ml by polyethylene glycol and dialyzed for 3 h against buffer A.

The dialyzed enzyme was further purified with a thin-layer electropho-

Table 1. Purification of soluble NADH-DH

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Cytoplasmic fraction	370	2,919	1,466	0.50	1.0	100
Protamine sulfate	425	2,321	1,386	0.60	1.2	95
Calcium phosphate gel	325	1,755	1,136	0.65	1.3	77
Sephadex G-150	363	588	677	1.15	2.3	46
DEAE-Sephadex A-25	111	148	543	3.67	7.3	37
NAD-Sepharose	51	7.0	351	50.1	100	24
Electrophoresis on Sephadex G-200	28	1.5	281	187	374	19
Sephadex G-100	11.5	0.42	198	471	942	14

NADH-DH activity was assayed with the standard reaction mixture. Details of each purification step are given in the text. About 90 g fresh weight of the washed cells was used for the purification.

Table 2. Kinetic constants for soluble NADH-DH

	Km	Vmax
Electron acceptor	(μ M)	(units/mg)
Ferricyanide	217	1,587
DCIP	25	714
Menadione	21	943
Electron donor		
NADH	100	667

For the determination of kinetic constants for electron acceptors, enzyme assay was carried out with the standard reaction mixture except for using variable concentrations of electron acceptors. When kinetic constants for NADH (DCIP as an electron acceptor) was examined, 134 μ M NADH in the standard reaction mixture was replaced by different concentrations of NADH.

resis on Sephadex G-200. Electrophoresis was carried out for 2 to 3 h at a constant voltage of 120 V as described in Materials and Methods. After electrophoresis, positions of the enzyme and proteins on the gel plate were determined by the staining. Migration distance of NADH-DH was close to

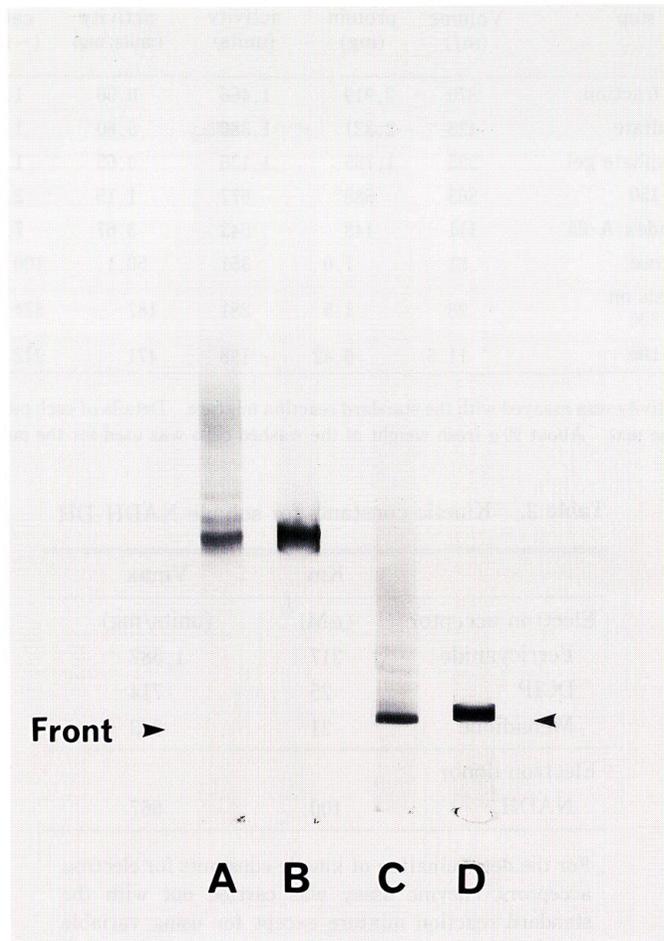


Fig. 1. Polyacrylamide disc gel electrophoresis of the purified soluble NADH-DH. Electrophoresis was carried out with a separating gel of pH 7.5 (A and B) and pH 8.9 (C and D) as reported by WILLIAMS and REISFELD (1964) and DAVIS (1964), respectively. 15 μ g of the purified NADH-DH was applied on each gel. After electrophoresis, gels were stained with Coomassie brilliant blue (A and C) or for the enzyme activity (B and D) as described in Materials and Methods.

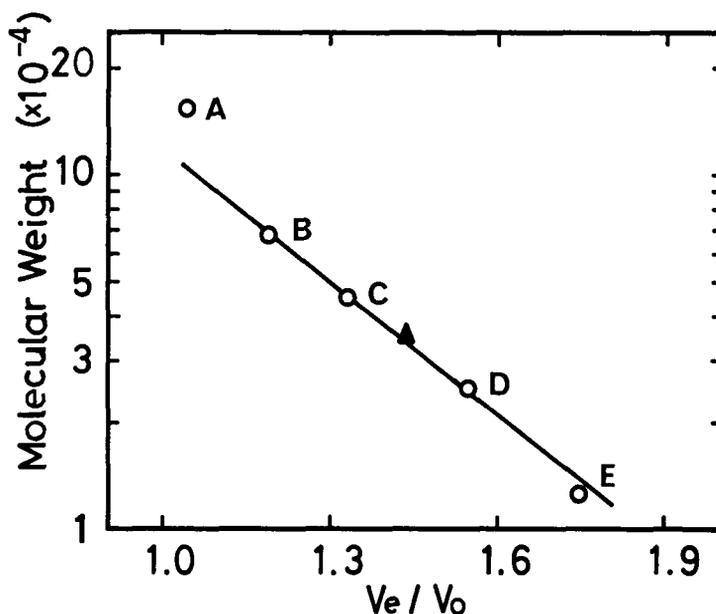


Fig. 2. Determination of molecular weight of soluble NADH-DH by gel filtration. Details of the experiment are described in Materials and Methods. The void volume of the column was measured by the elution of blue dextran 2000. ▲, purified enzyme. Marker proteins (○): A, aldolase; B, bovine serum albumin; C, ovalbumin; D, chymotrypsinogen A; E, cytochrome *c*.

that of marker dye and far from those of many other proteins (data not shown). The gels containing NADH-DH activity were scraped off and the enzyme was recovered from the gels with small volume of buffer A. The enzyme solution was concentrated with polyethylene glycol.

As a final step, the concentrated sample was applied to a Sephadex G-100 column (1.5×100 cm) equilibrated with buffer A supplemented with 50 mM NaCl and eluted with the same buffer at a flow rate of 10 ml/h. Active fractions were pooled and used as the purified soluble NADH-DH in the following experiments.

Table 1 shows a summary of a typical purification of soluble NADH-DH. NADH-DH was finally purified 940-fold with a recovery of 14%.

When dialyzed cytoplasmic fraction was directly applied to a DEAE-Sephadex A-25 column, about 30% of the enzyme could not be adsorbed even if excessive amount of the gel was used. Since over 50% of the unadsorbed enzyme was eluted at void volume of a gel permeation chromatography with

Table 3. Effect of flavin nucleotides on soluble NADH-DH activity

Electron acceptor	Flavin nucleotide	Concentration	Relative activity
		(μM)	(%)
DCIP	None	—	100
	FMN	20	100
		100	94
		20	100
	FAD	100	99
Ferricyanide	None	—	100
	FMN	20	113
	FAD	20	108
Menadione	None	—	100
	FMN	50	98
	FAD	50	91

The enzyme activity was assayed with the standard reaction mixture except that $35\ \mu\text{M}$ DCIP, $0.607\ \text{mM}$ ferricyanide or $0.1\ \text{mM}$ menadione was used as an electron acceptor. Each activity was expressed as a relative value to the enzyme activity without flavin nucleotides.

Table 4. Effect of various compounds on the activity of soluble NADH-DH

Compound	Concentration	Remaining activity		
		DCIP	Ferricyanide	Menadione
	(mM)	(%)	(%)	(%)
None	—	100	100	100
<i>o</i> -Phenanthroline	1	65	60	69
<i>p</i> -Chloromercuribenzoate	0.1	95	97	97
Rotenone	0.08	101	98	ND
Amytal	1	102	104	97

The enzyme activity was assayed as described in Table 3 except that $10\ \text{mM}$ KCN was excluded from the reaction mixture. Before starting the reaction, the enzyme was incubated for 3 min with each compound in the reaction mixture without NADH. ND, not determined.

Sephadex G-150 (data not shown), it was supposed that the unadsorbed enzymes formed an agglomerate due to aggregation with each other and/or other macromolecules in cytoplasm. However, because such aggregates

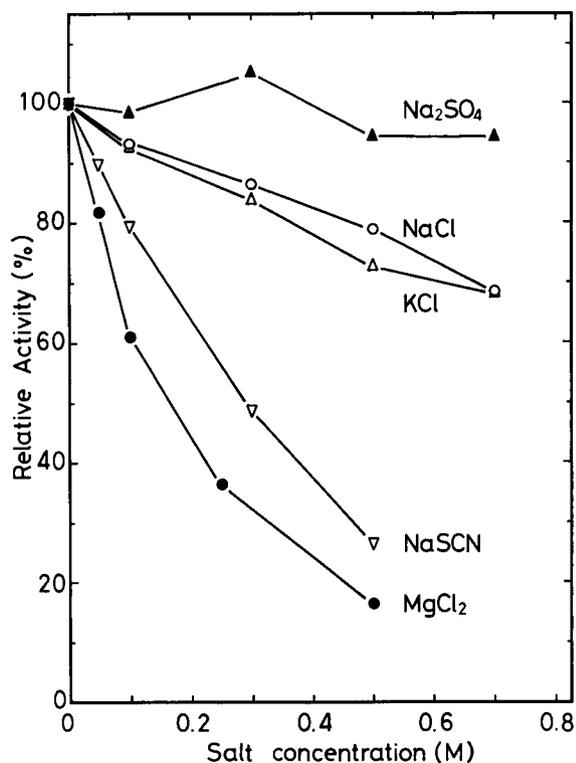


Fig. 3. Effect of several salts on soluble NADH-DH activity. The enzyme activity was assayed with the standard reaction mixture containing the indicated concentrations of salts.

were scarcely found on a gel filtration of the enzyme sample after treatment with calcium phosphate gel and the enzyme was then completely adsorbed to DEAE-Sephadex A-25, protamine sulfate and/or calcium phosphate gel seems to prevent the formation of the aggregates. Therefore, these purification techniques were employed in spite of their poor efficiencies of the purification.

Polyacrylamide disc gel electrophoresis of the purified enzyme under different pH conditions shows that the preparation was composed of a major protein band with NADH-DH activity and several inactive minor bands (Fig. 1). These results indicate that the purification is still incomplete. As seen in a thin-layer electrophoresis on Sephadex G-200, the enzyme protein also exhibited very high mobility on the electrophoresis by the method of DAVIS (1964) (Fig. 1, C and D).

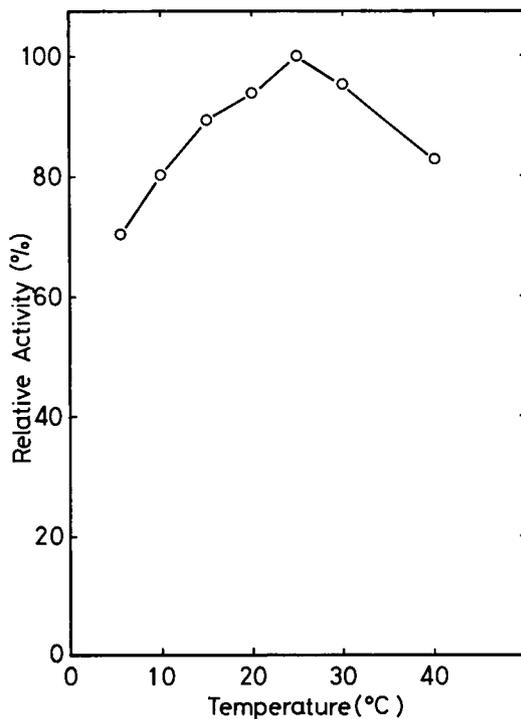


Fig. 4. Effect of temperature on soluble NADH-DH activity. The enzyme activity was assayed at the indicated temperatures with the standard reaction mixture except that $134 \mu\text{M}$ NADH and $35 \mu\text{M}$ DCIP was replaced by 1.34 mM NADH and 1.215 mM ferricyanide, respectively.

By gel filtration on a Sephadex G-100 column, apparent molecular weight of the purified enzyme was estimated to be about 35,000 (Fig. 2).

Substrate specificities and kinetic properties

As an electron donor, the purified NADH-DH exclusively depended on NADH, which could be scarcely replaced with NADPH (less than 2% with $537 \mu\text{M}$ NADPH). V_{max} and apparent K_{m} value for NADH (DCIP as an electron acceptor) are shown in Table 2.

On the other hand, the purified enzyme reduced ferricyanide, DCIP and menadione. Affinity for menadione was almost the same as that for DCIP and was higher than that for ferricyanide. However, V_{max} was over twice higher with ferricyanide than with DCIP (Table 2). Horse heart cytochrome *c* (0.074 mM), FAD (0.21 mM), FMN (1 mM) and lipoamide (4.82 mM) could not act as an electron acceptor.

The enzymes similar to the purified enzyme from *Vibrio* ABE-1 in catalytic properties have been found to be flavoproteins (DIXON and WEBB, 1979). It was reported that some flavoproteins lost flavin cofactors during the purifications (DANCEY and SHAPIRO, 1976; PETITDEMANGE *et al.*, 1980; BORNELEIT and KLEBER, 1983). Therefore, enzyme activity was assayed with different electron acceptors in the presence of flavin nucleotides (Table 3). The purified enzyme exhibited essentially the same activity in regardless of the presence of flavin nucleotides.

Effect of inhibitors on soluble NADH-DH activity

Sensitivity of the purified enzyme to several inhibitors was examined with different electron acceptors (Table 4). All inhibitors tested had the

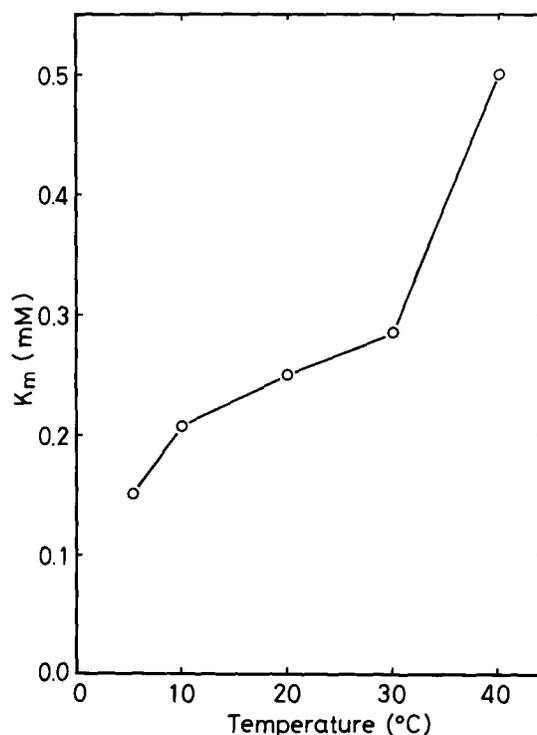


Fig. 5. Effect of temperature on K_m for NADH. The enzyme activity was assayed as described in legend of Fig. 4 except that various concentrations of NADH and 0.9 mM ferricyanide were used.

same effect on the activities with different acceptors. A sulphhydryl reagent, *p*-chloromercuribenzoate, and respiratory inhibitors such as Rotenone and Amytal did not affect on the enzyme activity at all. The enzyme activity was partly inhibited by a chelating reagent, *o*-phenanthroline, indicating that some metal ions might be necessary for the activity.

Effect of salts on the activity

Because *Vibrio* ABE-1 is a slightly halophilic bacterium (TAKADA *et al.*, 1979) and many enzymes of halophiles have been reported to exhibit salt-dependence (LARSEN, 1962; LANYI, 1974), effects of various salts on the activity of the purified enzyme were investigated (Fig. 3). Although Na₂SO₄ hardly affected the activity within the concentrations tested (up to 0.7 M), the other salts inhibited the activity with different extents, and in particular, NaSCN and MgCl₂ strikingly inhibited the activity. Among three chloride salts, Na⁺ and K⁺ exhibited almost the same inhibitory effect and a divalent cation Mg⁺⁺ was found to most strongly inhibit the enzyme activity. On the other hand, the extents of inhibitory effect of three anions were in the following order; SCN⁻ > Cl⁻ > SO₄⁻⁻.

Effect of temperature on the activity and Km for NADH

Ferricyanide reductase activities of the purified enzyme at different temperatures are shown in Fig. 4. The enzyme exhibited maximum activity at 25°C and about 70% and over 80% of the maximum activity was observed at 5 and 40°C, respectively. This result indicates that the activity of the soluble NADH-DH from *Vibrio* ABE-1 does not strongly depend on the temperature.

Furthermore, effect of temperature on Km for NADH was examined with ferricyanide as an electron acceptor (Fig. 5). The Km value for NADH was minimum at the lowest temperature tested, 5°C, and progressively increased with the elevation of assay temperature. Particularly, the value increased strikingly over 30°C. These results suggest that this enzyme can function actively at low temperatures.

Discussion

NADH-DH in cytoplasmic fraction from *Vibrio* ABE-1 was purified by combination of gel filtrations, anionic ion-exchange and affinity chromatographies, and preparative electrophoresis. Because the cytoplasmic fraction prepared in this study contained about 75% of total cell proteins (data not shown), this enzyme was estimated to be purified about 1,250 fold from cell extract. In spite of the acquisition of high-fold purification, the preparation still contained some impurities (Fig. 1). This appears to be due to a small

intracellular content of the enzyme, in addition to poor recovery of the enzyme at the purification procedures.

Since the purified enzyme of *Vibrio* ABE-1 could not reduce lipoamide, FMN, FAD and cytochrome *c*, it can be classified into neither of the following enzymes: lipoamide dehydrogenase which is widely distributed in many organisms (WREN and MASSAY, 1966), NADH:flavin oxidoreductase in luminous (GERLO and CHARLIER, 1975) and intestinal bacteria (LIPSKY and HYLEMON, 1980), and NADH-cytochrome *c* reductase from bacteria (YAMAGUCHI and FUJISAWA, 1978). Furthermore, the enzyme does not also belong to NAD(P)H:quinone oxidoreductase (EC 1.6.99.2), which is commonly designated as menadione reductase, quinone reductase or DT-diaphorase (PETITDEMANGE *et al.*, 1980; SPITSBERG and COSCIA, 1982; PROCHASKA and TALALAY, 1986), because it scarcely oxidized NADPH while reduced menadione as well as the other electron acceptors (Table 2). Relation of the purified enzyme of *Vibrio* ABE-1 to other similar soluble enzymes could not be clarified. Although NADH-DH of respiratory chain has been considered to be membrane-bound (MACHTIGER and FOX, 1973), the corresponding enzymes of a few thermophilic bacteria become easily water-soluble by simple disruption of the cells with a French press or by washing the membranes with salt solution, showing that they are peripheral membrane proteins (MAINS *et al.*, 1980; WALSH *et al.*, 1983; WAKAO *et al.*, 1987). Furthermore, MATSUSHITA *et al.* (1987) recently reported that respiratory chain of *E. coli* membrane contains two distinct species of NADH-DH. It was reported that several enzymes of the mitochondrial matrix can bind to the mitochondrial inner membrane (D'SOUZA and SRERE, 1983; SUMEGI and SRERE, 1984). Therefore, it is also possible that the purified soluble enzyme of *Vibrio* ABE-1 may be loosely bound to the membrane and associated with the respiratory chain. Identification of natural electron acceptor(s) *in vivo* is prerequisite for complete understanding of physiological role of the purified enzyme. For that reason, the members of respiratory chain must be isolated and characterized, in addition to determination of prosthetic group of the enzyme.

The activity of the purified enzyme was inhibited with different extents by all salts tested in this study except Na₂SO₄ (Fig. 3). The salt-dependence of the enzyme activity was different from that of isozyme II of isocitrate dehydrogenase from this bacterium, which was strikingly stimulated by NaCl and KCl (OCHIAI *et al.*, 1979). Nevertheless, the NADH-DH seems to be sufficiently active *in vivo* because intracellular Na⁺ and K⁺ concentrations of the cells washed with a buffer containing 0.5 M NaCl were found within a range of less effective, that is, 68 and 377 mM, respectively (TAKADA *et al.*,

1988). Inhibition of the enzyme activity by a chaotropic anion SCN^- (Fig. 3) indicates that hydrophobic interaction plays an important role for a proper folding of the soluble NADH-DH protein like other halophilic enzymes (LANYI, 1974).

The purified enzyme exhibited high activity over a wide range of temperatures (Fig. 4). Furthermore, K_m value for NADH increased with the raise of temperature (Fig. 5). These properties accord with those of urocanase and histidine ammonia-lyase from a psychrotrophic *Pseudomonas putida* (HUG and HUNTER, 1974a and 1974b), which are known as typical psychrophilic enzymes, indicating that the soluble NADH-DH is an well-adapted enzyme to low-temperature environment.

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