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Purification and Characterization of Two Types of NADH-Quinone Reductase from *Thermus thermophilus* HB-8†

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ABSTRACT: Two types of the NADH-quinone reductase were isolated from *Thermus thermophilus* HB-8 membranes, by use of the nonionic detergent, dodecyl β-maltoside, and NAD-agarose affinity, DEAE-cellulose, hydroxyapatite, and Superose 6 column chromatography. One of these (NADH dehydrogenase 1) is a complex composed of 10 unlike polypeptides, and the other (NADH dehydrogenase 2) exhibits a single band (M_r 53 000) upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The NADH-ubiquinone-1 reductase activity of the isolated NADH dehydrogenase 1 was about 14 times higher than that of the dodecyl β-maltoside extract and partially rotenone sensitive. The NADH-ubiquinone-1 reductase activity of the isolated NADH dehydrogenase 2 was about 30-fold as high as that of the dodecyl β-maltoside extract and rotenone insensitive. The purified NADH dehydrogenase 1 contained noncovalently bound FMN, non-heme iron, and acid-labile sulfide. The ratio of FMN to non-heme iron to acid-labile sulfide was 1:11-12:7-9. The high content of iron and labile sulfide is suggestive of the presence of several iron-sulfur clusters. The purified NADH dehydrogenase 2 contained noncovalently bound FAD and no non-heme iron or acid-labile sulfide. The activities of both NADH dehydrogenases were stable at temperatures of ≥80 °C. The occurrence of two distinct types of NADH dehydrogenase as a common feature in the membranes of various aerobic bacteria is discussed.

Among the enzyme complexes that comprise the mitochondrial oxidative phosphorylation system, the least studied is the NADH-ubiquinone oxidoreductase complex (complex I). The reason is perhaps associated with the fact that this enzyme has a highly complex structure. It is comprised of ≥25 unlike polypeptides (Heron et al., 1979; Hatefi, 1985) and eight iron-sulfur (FeS) clusters (Ohnishi et al., 1985; Hatefi, 1985). Three of the FeS clusters are EPR[1]-silent, and one has an apparent E_m of about −400 mV (Hatefi et al., 1985), leaving four clusters that can be reduced by NADH. The NADH-Q reductase segment of aerobically grown *Paracoccus denitrificans* membranes contains coupling site 1 (Stouthamer, 1980) and exhibits NADH-reducible EPR signals similar to those of the mitochondrial complex I (Albracht et al., 1980; Meinhardt et al., 1987). However, by comparison to mammalian complex I, the NADH dehydrogenase complex from *Paracoccus* membranes is composed of only 10 unlike polypeptides (Yagi, 1986), suggesting that the *Paracoccus* NADH dehydrogenase complex is simpler than its mammalian counterpart in terms of its polypeptide composition.

Thermus thermophilus HB-8, isolated from a hot spring in Japan, is a strictly aerobic and extremely thermophilic bacterium [Oshima & Imahori, 1971, 1974; also see Fee et al. (1986)]. The respiratory chain of *T. thermophilus* HB-8 contains the energy coupling site 1 (Ohnishi et al., 1987), and its NADH-Q reductase activity is sensitive to rotenone and piericidin A (Yagi, 1987; Ohnishi et al., 1987). In addition,

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[1] Abbreviations: EPR, electron paramagnetic resonance; E_m, oxidation-reduction potential; Q, quinone; NDH-1, NADH dehydrogenase 1; NDH-2, NADH dehydrogenase 2; EDTA, ethylenediaminetetraacetate; Q_1, ubiquinone 1; deamino-NAD, nicotinamide hypoxanthine dinucleotide; TCD buffer, buffer composed of 10 mM Tris-HCl (pH 7.5) and 0.1% dodecyl β-maltoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; M_r, molecular weight estimated from relative mobility in SDS-PAGE; TLC, thin-layer chromatography; R_f, relative mobility; NMR, nuclear magnetic resonance; DEAE-cellulose, (diethylaminoethyl)cellulose; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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N,N'-dicyclohexylcarbodiimide inhibits the NADH-Q reductase activity of *T. thermophilus* HB-8 (Yagi, 1987) as it does the NADH-Q reductase activity of complex I and *Paracoccus* membranes. However, EPR studies have suggested that the composition of the FeS clusters of the NADH-Q reductase segment of this thermophilic bacterium is simpler than that of mammalian mitochondria or *P. denitrificans* membranes (Ohnishi et al., 1987). It was of interest, therefore, to attempt the isolation of the NADH-Q reductase of this bacterium in order to compare its composition, structure, and function with those of the NADH-Q reductases of mammalian mitochondria and *Paracoccus* membranes.

This paper describes purification of two types of NADH-Q reductase from *T. thermophilus* HB-8. One type (designated as NDH-1) is a multisubunit complex, and the other (NDH-2) exhibits a single band on SDS–PAGE. NDH-1 contains FMN, non-heme iron, and acid-labile sulfide as prosthetic groups, and NDH-2 contains FAD and no iron and labile sulfide. The enzymatic activities of both NDH-1 and NDH-2 increase with increase of temperature up to 80 °C. The possible involvement of NDH-1 in the energy coupling site 1 is discussed.

Materials and Methods

Isolation of T. thermophilus HB-8 Membranes. *T. thermophilus* HB-8 (ATCC27634) cells were grown aerobically at 75 °C according to Hon-nami and Oshima (1977) and harvested at late exponential phase. Membranes were prepared as follows. The cells, suspended in a buffer containing 50 mM Tris–SO_4 (pH 7.5), 10 mM $MgSO_4$, and 1 mM EDTA, were passed through the French press cell at a shear force of 16000 psi (110 MPa). The suspension was centrifuged at 31500*g* for 30 min. The supernatant was centrifuged at 130000*g* for 90 min. The pellet was washed by recentrifugation and suspended with the same buffer (30–40 mg/mL). The suspension was frozen in liquid nitrogen and stored at −70 °C.

Analytical Procedures. Protein was estimated by the method of Lowry et al. (1951) or by biuret in the presence of 1 mg of sodium deoxycholate/mL (Gornall et al., 1949). The nature of the flavins was determined according to Yagi (1962) and Faeder and Siegel (1973). Non-heme iron and acid-labile sulfide were estimated according to Doeg and Ziegler (1962) and Fogo and Popwski (1949), respectively. Enzymatic assays were basically carried out according to Yagi (1986, 1987). Unless otherwise indicated, NADH-$K_3Fe(CN)_6$ reductase activity was assayed spectrophotometrically at 420 nm in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 0.25 mM NADH at 60 °C. The concentration of $K_3Fe(CN)_6$ was 1 mM. NADH-ubiquinone-1 (Q_1) reductase activity was measured at 340 nm in the same buffer, except for the addition of 50 μM Q_1 instead of $K_3Fe(CN)_6$. The reaction was started by the addition of the enzymes. Deamino-NADH-Q_1 and NADPH-Q_1 reductase activities were determined at 340 nm in the same medium at that used for measurement of NADH-Q_1 reductase activity, except that 0.25 mM NADPH or 0.25 mM deamino-NADH was added in place of 0.25 mM NADH. The extinction coefficients used for activity calculations were $\Delta\epsilon_{340}$ = 6220 M^{-1} cm^{-1} for NADH, NADPH, and deamino-NADH and $\Delta\epsilon_{420}$ = 1000 M^{-1} cm^{-1} for $K_3Fe(CN)_6$.

SDS gel electrophoresis was performed by the method of Laemmli (1970). Marker proteins of known molecular weight were purchased from Sigma.

Materials. NADH, NADPH, and dodecyl β-maltoside were obtained from Behring Diagnostics; FMN, FAD, and deamino-NADH were from Sigma. The TLC sheet (silica gel 60 F-254) was obtained from Merck. Ubiquinone 1 was a kind gift from Eisai Chemical (Tokyo, Japan). All other chemicals used were of reagent grade or of the highest quality available.

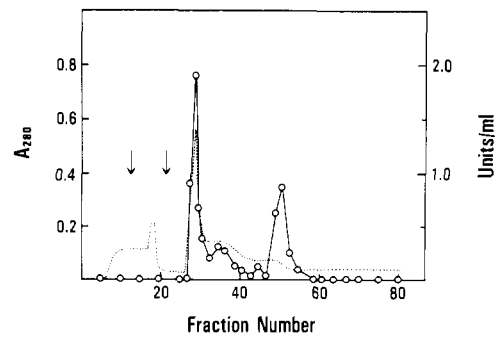

FIGURE 1: Hydroxyapatite column chromatography of fractions from the DEAE-cellulose column exhibiting NADH dehydrogenase activity. The fractions from the DEAE-cellulose column showing NADH-$K_3Fe(CN)_6$ reductase activity were pooled and applied to a 1.5 × 13 cm hydroxyapatite column. The column was washed with 40 mL of TCD buffer (first arrow) and eluted with a gradient of 0–350 mM potassium phosphate (pH 7.5) in TCD buffer (second arrow). Absorbance was monitored at 280 nm (dotted trace), and fractions of 5 mL each were collected. NADH-$K_3Fe(CN)_6$ reductase activity (○) was measured according to Yagi (1986).

Results

Purification of Two Types of NADH-Q Reductase. *T. thermophilus* HB-8 membranes (5 mg/mL) suspended in a buffer containing 0.25 M sucrose and 10 mM Tris-HCl buffer (pH 7.8) were incubated for 30 min on ice in the presence of 1% dodecyl β-maltoside. All steps were carried out at 4 °C unless otherwise indicated. After incubation with the dodecyl β-maltoside, the suspension was centrifuged for 30 min at 50000 rpm in a No. 60 Ti rotor of a Spinco Model L8-70M ultracentrifuge. The supernatant was applied to a NAD–agarose column (2.5 × 10 cm), and the column was washed with 2 column volumes of 10 mM Tris-HCl (pH 7.5) containing 0.1% dodecyl β-maltoside (TCD buffer) and the eluted with a 0–1 M NaCl linear gradient. The fractions containing NADH-$K_3Fe(CN)_6$ reductase activity were collected and loaded on a DEAE-cellulose column (1 × 8 cm). The column was washed with 2 column volumes of TCD buffer as described above and then eluted with a 0–500 mM NaCl linear gradient. The active fractions were pooled and applied to a hydroxyapatite column (1.5 × 13 cm). The column was washed with 40 mL of TCD buffer and the eluted with a 0–350 mM potassium phosphate (pH 7.5) linear gradient. There were two peaks of NADH-$K_3Fe(CN)_6$ reductase activity (Figure 1). The first and second peaks were designated respectively NADH dehydrogenase 1 (NDH-1) and NADH dehydrogenase 2 (NDH-2). These two fractions were pooled separately and concentrated by Centricon-30. Each concentrated sample was loaded on a Superose 6 column HR 10/30 (1 × 30 cm) of FPLC at room temperature and eluted with the TCD buffer. The NDH-1 and NDH-2 were eluted at 24 min and 35–37 min, respectively (see Figure 2). The elution profile of NDH-2 at 280 nm exhibited two peaks. However, SDS–PAGE experiments indicated that both fractions were composed of the same single subunit; therefore, the fractions were combined. The active fractions were concentrated by Centricon-30. The concentrated NDH-1 and NDH-2 were stored at −70 °C. When NDH-1 was rechromatographed on a hydroxyapatite column, the specific activity and FMN content of the NDH-1 were remarkably decreased. Addition

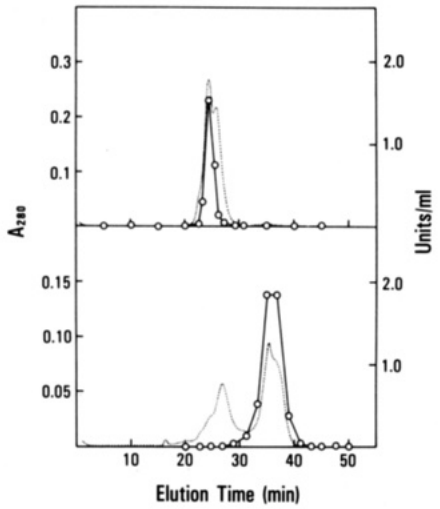

FIGURE 2: Superose 6 column chromatography of the first peak (upper) and second peak fractions (lower) from hydroxyapatite column chromatography. The column was eluted with TCD buffer at a flow rate of 0.5 mL/min. Absorbance was monitored at 280 nm (dotted trace), and fractions of 0.5 mL each were collected. NADH-$K_3Fe(CN)_6$ reductase activity was assayed as described in Figure 1.

Table I: Purification of Two Types of NADH-Q Reductase from *T. thermophilus* HB-8 Membranes

purification step[a]	μmol of NADH oxidized min^{-1} (mg of protein)$^{-1}$		protein (mg)
	NADH-Q_1 reductase	NADH-$K_3Fe(CN)_6$	
dodecyl β-maltoside extract	0.13	0.58	262
NAD–agarose	0.10	0.99	66.5
DEAE-cellulose	0.13	1.35	37.7
NDH-1	1.8	21	1.2
NDH-2	4.0	100	0.25

[a] For purification details, see text.

of 20 μM FMN did not result in recovery of enzymatic activity.

NDH-1 had a $K_3Fe(CN)_6$ reductase activity of 21 μmol of NADH oxidized min^{-1} (mg of protein)$^{-1}$ and a Q_1 reductase activity of 1.8 μmol of NADH oxidized min^{-1} (mg of protein)$^{-1}$ (Table I). The NADH-Q_1 reductase activity of NDH-1 was about 14 times as high as that of the dodecyl β-maltoside extract. By comparison, NDH-2 had a $K_3Fe(CN)_6$ reductase activity of 100 μmol of NADH oxidized min^{-1} (mg of protein)$^{-1}$ and a Q_1 reductase activity of 4 μmol of NADH oxidized min^{-1} (mg of protein)$^{-1}$. In terms of NADH-Q_1 reductase activity, NDH-2 was purified about 30 times.

Figure 3 shows the absorption spectra of NDH-1 and NDH-2 in the wavelength range 400–650 nm. Addition of NADH to NDH-1 caused a smaller absorbance decrease than that of NDH-2 throughout the wavelength range examined. The effect of NADH on the absorbance of NDH-1 is similar to the effects of observed with the isolated NADH dehydrogenase complex from *Paracoccus* (Yagi, 1986) and *Candida utilis* (Tottmar & Ragan, 1971), which also contain iron–sulfur clusters and flavin. When $Na_2S_2O_4$ was added to NDH-1, a small absorbance increase at around 550 nm was observed. This absorbance increase may be due to marginal amounts of contaminating cytochromes which, at any rate, could not be greater than about 0.016 nmol/mg of protein. This value is about 2% of the FMN content of NDH-1 (see Table III). Addition of $Na_2S_2O_4$ to NDH-2 resulted in a large absorbance decrease around 450 nm, and there was no suggestion of contaminating cytochromes (Figure 3B). The absorbance changes of NDH-2 are characteristic of flavoprotein reduction (Hisae et al., 1983; Bergsma et al., 1982; Saeki et al., 1985; Haber & Brandt, 1985).

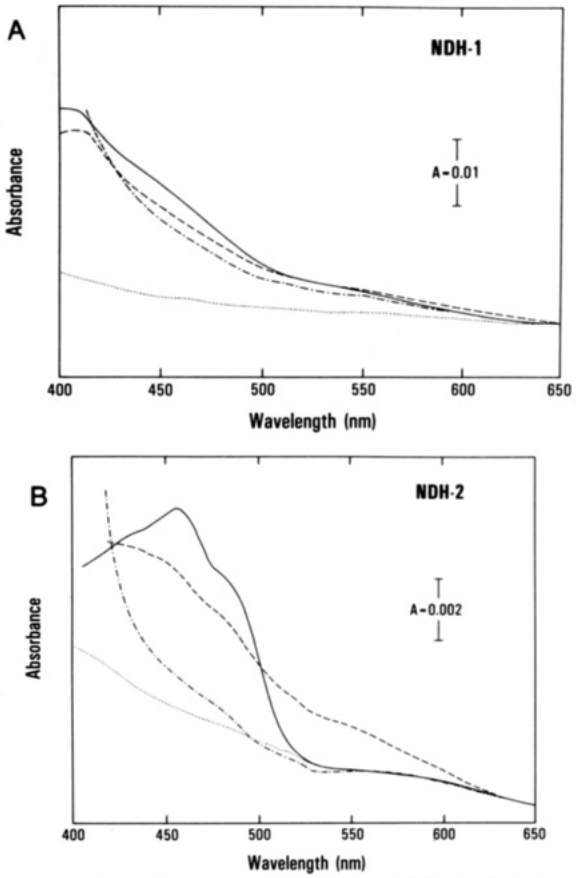

FIGURE 3: Absorption spectra of NDH-1 and NDH-2. The isolated NDH-1 and NDH-2 were diluted in 50 mM potassium phosphate (pH 7.5) containing 1 mM EDTA to 1.11 and 0.027 mg of protein/mL, respectively. Oxidized enzymes (—); after addition of 83 μM NADH (– –); after addition of solid $Na_2S_2O_4$ (–·–). The base lines are indicated by the dotted traces.

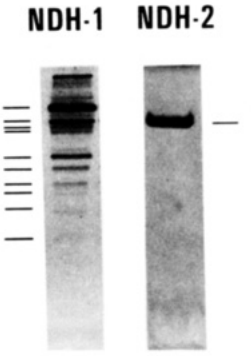

FIGURE 4: Polypeptide composition of NDH-1 and NDH-2. Isolated NDH-1 (10 μg) and NDH-2 (3 μg) denatured with 6% SDS were each electrophoresed on a SDS–14% polyacrylamide gel according to the procedure of Laemmli. The gels were stained with 0.05% Coomassie blue R-250 in the presence of 25% 2-propanol and 10% acetic acid and destained with 7% acetic acid. Dashes along the gels show polypeptides. The molecular weights of the polypeptides are given in the text.

Polypeptide Composition. NDH-1 is composed of ten unlike polypeptides (Figure 4). The polypeptides have apparent molecular mass values of 70, 58, 53, 49, 39, 34, 26, 24, 20, and 16 kDa. NDH-2 showed a single band on SDS–PAGE with apparent M_r 53K. The NADH dehydrogenases isolated from various bacteria have been reported to show a single band on SDS–PAGE [e.g., *Escherichia coli* (M_r 38K) (Dancey et al., 1976), *Bacillus subtilis* (M_r = 63K) (Bergsma et al., 1982),

Table II: Nature of the Flavin Extracted from NDH-1 and NDH-2

	FMN	FAD	NDH-1 flavin	NDH-2 flavin
relative mobility (R_f)[a]				
solvent A	0.11	0.05	0.11	0.04
solvent B	0.41	0.59	0.37	0.58
relative fluorescence intensity[b]				
pH 7.7	1.00	0.80	0.76	0.38
pH 2.6	0.72	2.17	0.57	1.06
fluorescence ratio				
pH 2.6/7.7	0.72	2.71	0.75	2.79

[a] Thin-layer chromatography was carried out on precoated TLC sheets (silica gel 60 F254). Flavin was extracted with 4% trichloroacetic acid as described previously (Yagi, 1986). Solvent A: 1-butanol–acetic acid–H_2O, 4:1:5. Solvent B: 5% Na_2HPO_4. [b] Measurements were made according to Faeder and Siegel (1973). Excitation and emission wavelengths were 450 and 520 nm, respectively. Concentrations of FMN and FAD were 3.6 and 16 nM, respectively.

alkalophilic *Bacillus* (M_r 65K) (Hisae et al., 1983), and *Halobacterium cutirubrum* (M_r 64K) (Hochstein & Dalton, 1973)].

Components. (*A*) *Flavin.* The nature of flavin in NDH-1 and NDH-2 was determined fluorometrically and by thin-layer chromatography. The enzyme was incubated in the dark in the presence of 4% trichloroacetic acid for 15 min on ice and centrifuged in an Eppendorf centrifuge, and the supernatant removed. The supernatant was washed 2 times with ether and lyophilized. The lyophilized sample was dissolved in water. In the two different solvent systems used, the extracted flavins gave a single spot each on TLC. The R_f values of the flavin of NDH-1 and NDH-2 were the same as those of authentic FMN and FAD, respectively (see Table II). The fluorescence of the extracted flavin of NDH-1 decreased upon medium pH change from 7.7 to 2.6 in the same manner as that of FMN. However, the fluorescence of the extracted flavin of NDH-2 was enhanced about 3-fold by a similar medium acidification. This characteristic is consistent with the behavior of authentic FAD. These results indicate that NDH-1 and NDH-2 of *T. thermophilus* HB-8 contain noncovalently bound FMN and FAD, respectively, as prosthetic groups. This difference in the nature of flavin indicates that NDH-2 is not a fragment of NDH-1.

All the known isolated NADH dehydrogenases containing FAD as a prosthetic group and lacking FeS clusters from various microorganisms are composed of a single polypeptide [e.g., *E. coli* (Dancey et al., 1976), *Bacillus megaterium* (Saeki et al., 1985), alkalophilic *Bacillus* (Hisae et al., 1983), *H. cutirubrum* (Hochstein & Dalton, 1973), and *B. subtilis* (Bergsma et al., 1982)]. By contrast, the isolated NADH dehydrogenases containing FMN and FeS clusters are likely multisubunit complexes [e.g., bovine heart complex I (≥25 unlike subunits) (Hatefi, 1985), *C. utilis* NADH dehydrogenase complex (≥10 unlike subunits) (Tottmar & Ragan, 1971), and *Paracoccus* NADH dehydrogenase complex (10 unlike polypeptides) (Yagi, 1986)]. This tendency is consistent with NDH-1 and NDH-2.

The flavin contents of NDH-1 and NDH-2 were estimated fluorometrically after trichloroacetic acid extraction to be 0.75–0.85 and 18–19 nmol/mg of protein, respectively. The FMN content of NDH-1 is similar to that of complex I (0.98 nmol/mg) (Hatefi, 1985), higher than that of NADH dehydrogenase complex from *C. utilis* (0.6 nmol/mg) (Tottomer & Ragan, 1971), and lower than that of NADH dehydrogenase complex from *Paracoccus* (2.2–2.5 nmol/mg) (Yagi, 1986). Assuming one flavin per mole, the molecular weights of NDH-1 and NDH-2 calculated from their flavin contents would be (1.2–1.3) × 10^6 and (5.3–5.6) × 10^4, respectively. One copy each of the polypeptides of NDH-1 would give a M_r of 389 000. This value is around one-third of molecular weight estimated from the flavin content. This may mean that NDH-1 contains more than one copy each of the above polypeptides and/or partial loss of FMN from NDH-1 during purification. For NDH-2, the M_r of (5.3–5.6) × 10^4 is close to the apparent molecular weight of the polypeptide as suggested by SDS–PAGE.

Table III: Substrate and Inhibitor Specificities of NDH-1 and NDH-2[a]

	μmol of NADH oxidized min^{-1} (mg of protein)$^{-1}$	
	NDH-1	NDH-2
NADH-Q_1 reductase	1.78	3.83
+ 25 μM rotenone	1.13	3.90
deamino-NADH-Q_1 reductase	0.99	2.68
NADPH-Q_1 reductase	0.07	0
NADPH-$K_3Fe(CN)_6$ reductase	0.13	2.6

[a] Electron donors were added at 250 μM. $K_3Fe(CN)_6$ and Q_1 were 1 mM and 100 μM, respectively. The K_m values for NADH for NDH-1 and -2 are respectively 10 μM and 12 μM.

(*B*) *Non-Heme Iron and Acid-Labile Sulfide.* The contents of non-heme iron and acid-labile sulfide in NDH-1 were determined to be 8.0–9.0 nmol of Fe and 6.0–7.0 nmol of S^{2-} per milligram of protein, respectively. These values are lower than those of bovine heart complex I (24–26 nmol of Fe and 23–26 nmol of S^{2-} per milligram of protein) and *Paracoccus* NADH dehydrogenase complex (31 nmol of Fe and 27 nmol of S^{2-} per milligram protein). These results suggest that the ratio FMN:Fe:S^{2-} is 1:10–12:7–9 in NDH-1. According to EPR studies on *T. thermophilus* HB-8 membranes (Ohnishi et al., 1987), the NADH-Q reductase segment contains at least two FeS clusters. One is binuclear, and the other is tetranuclear. If that is the case and there are no EPR-silent FeS clusters, the FMN:Fe:S^{2-} ratio in the NDH-1 would have to be 1:6:6. The experimentally determined values allow, therefore, for the presence of additional FeS cluster(s) not detected in the above EPR studies. On the other hand, neither non-heme Fe nor acid-labile sulfide was found in the NDH-2. The absence of non-heme Fe and acid-labile sulfide has been reported for the NADH dehydrogenase isolated from *B. subtilis* (Bergsma et al., 1982), which does not conain the energy coupling site 1.

Enzymatic Activities. The NADH-Q_1 reductase activity of NDH-1 was partially sensitive to rotenone (Table III). This sensitivity was the same as that of the isolated rotenone-sensitive NADH-Q reductase from *Neurospora crassa* mitochondria (Ise et al., 1985). By contrast, rotenone did not inhibit the NADH-Q_1 reductase activity of NDH-2. Rotenone is known to inhibit the NADH-Q_1 reductase activity of enzyme systems containing the energy coupling site 1. Therefore, these results may suggest that NDH-1 contains the energy coupling site 1 of *T. thermophilus*.

In the case of the NADH-Q reductase segment of *E. coli* membranes, deamino-NADH was reported to be a substrate for the enzyme bearing the energy coupling site 1 but not for the enzyme lacking an energy coupling site (Owen et al., 1980a,b). Deamino-NADH was nearly as good a substrate as NADH for both NDH-1 and NDH-2 (see Table III). Therefore, with respect to the substrate specificity of NADH-Q reductase, *T. thermophilus* HB-8 differs from *E. coli*. NADPH was a poor substrate for both NDH-1 and NDH-2. In the case of bovine heart complex I and *Paracoccus* NADH

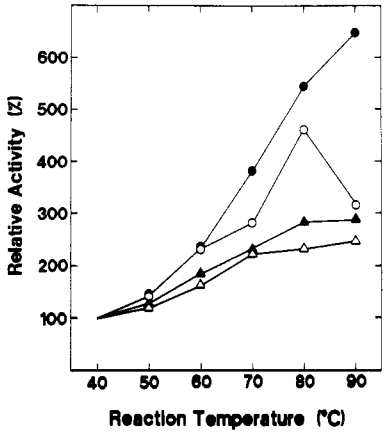

FIGURE 5: Effect of temperature on the NADH-Q_1 reductase and NADH-$K_3Fe(CN)_6$ reductase activities of NDH-1 and NDH-2. The NADH-Q_1 reductase activities of NDH-1 and NDH-2 are indicated by (○) and (●), respectively. The NADH-$K_3Fe(CN)_6$ reductase activities of NDH-1 and NDH-2 are marked by (△) and (▲), respectively. The protein concentrations of NDH-1 and NDH-2 used in the enzymatic assays were 4 and 2 μg/mL, respectively. The activities of 40 °C represented in the figure as 100% were 0.72 μmol of NADH oxidized min^{-1} mg^{-1} (○); 1.70 μmol of NADH oxidized min^{-1} mg^{-1} (●); 13 μmol of NADH oxidized min^{-1} mg^{-1} (△); 53 μmol of NADH oxidized min^{-1} mg^{-1} (▲). Details are described under Materials and Methods.

dehydrogenase complex, NADPH was also a poor substrate (Yagi, 1986).

Thermostability of NDH-1 and NDH-2. Figure 5 shows that the isolated NDH-1 and NDH-2 are extremely thermostable. The NADH-Q_1 reductase activity of NDH-1 was maximal at 80 °C, which coincides with the temperature for optimum growth (Oshima & Imahori, 1971, 1974). However, in the case of NDH-2, the peak of NADH-Q_1 reductase activity could not be found in the range 40–90 °C, suggesting that the maximum activity of NDH-2 is at a higher temperature than 90 °C. These results indicate that NDH-2 is more thermostable than NDH-1, which may be related to the fact that NDH-1 is a multisubunit complex and contains FeS clusters whereas NDH-2 is composed of a single polypeptide and lacks FeS clusters. By comparison, there was no significant thermostability difference between the NADH-$K_3Fe(CN)_6$ reductase activities of NDH-1 and NDH-2. This result seems reasonable also because the reaction between NADH and $K_3Fe(CN)_6$ occurs at the same or overlapping sites in NADH dehydrogenase and thus involves a simple structure (Dooijewaard & Slater, 1976a,b).

Discussion

Thin-layer chromatography and fluorometric experiments have shown that NDH-1 contains noncovalently bound FMN while NDH-2 contains noncovalently bound FAD. This difference in the nature of flavin reveals that NDH-2 is not a fragment of NDH-1. Thus, at least two distinct types of NADH-Q reductase are present in *T. thermophilus* HB-8. Choc et al. (1982) have reported that a crude NADH dehydrogenase fraction lacking FeS clusters restored NADH oxidase activity after reconstitution with menaquinone, Rieske FeS protein, and cytochrome c_1aa_3 isolated from *T. thermophilus* HB-8. Therefore, NDH-2 seems to be the second NADH dehydrogenase of the respiratory chain in *T. thermophilus* HB-8. In *E. coli* and *Micrococcus lysodeikticus*, crossed immunoelectrophoresis experiments have suggested the existence of two types of NADH dehydrogenase also in these bacteria (Owen et al., 1980a,b; Owen & Salton, 1975). Up to now, one type of NADH dehydrogenase composed of a single subunit containing FAD has been isolated from *E. coli* (Dancey et al., 1976; Jaworowski et al., 1981) and *B. subtilis* (Bergsma et al., 1982) membranes. It has also been reported that preparations suggestive of another type of NADH dehydrogenase have been fractionated from *E. coli* (Hendler & Burgess, 1974; Bragg & Hou, 1967). However, the present work is the first isolation and characterization of two types of NADH-Q reductase from a single species.

E. coli membranes grown under sulfate-limited conditions have been reported to lack EPR signals due to FeS clusters in the NADH-Q reductase segment but to show higher electron-transport activity from malate to O_2 (Poole & Haddock, 1975). The possibilities considered were that sulfate limitation results in either (1) the loss of FeS clusters in NADH-Q reductase, which causes the decoupling of this enzyme complex normally associated with proton translocation, or (2) an increase in the second NADH-Q reductase composed of a single subunit and a decrease in the NADH-Q reductase containing energy coupling site 1. Unfortunately, the *E. coli* NADH-Q reductase segment that contains the energy coupling site 1 (like NDH-1) appears to be too labile to be isolated (Matsushita et al., unpublished data). The isolation of two types of NADH-Q reductase from *T. thermophilus* HB-8 membranes suggest that *T. thermophilus* HB-8 might be a useful system in which to investigate the reasons for changes in the properties of NADH-Q reductase induced by various growth conditions.

The known NADH-Q reductases which carry an energy coupling site share the following features. They are all multisubunit enzyme complexes, they all contain FMN and FeS clusters as prosthetic groups, and their Q reductase activity is inhibited by rotenone (Yagi, 1987; Hatefi, 1985; Ohnishi, 1973). Therefore, the partial rotenone sensitivity and the presence of FMN and FeS clusters in NDH-1 might mean that this multisubunit enzyme is the NADH-Q reductase complex of *T. thermophilus* which involves an energy-coupling site. On the other hand, NDH-2 might be the counterpart of the single-subunit NADH dehydrogenase of *B. subtilis* which lacks an energy coupling site.

NMR and Raman spectroscopic studies have indicated that the tertiary structure and heme iron–methionine ligation in cytochrome *c*-552 from *T. thermophilus* HB-8 are retained even at 87 °C, regardless of its redox state (Hon-nami et al., 1980). The isolated cytochrome oxidase (cytochrome c_1aa_0 type) of this bacterium has also been shown to be stable in the temperature range of 25–60 °C (Fee et al., 1980). F_1-ATPase isolated from thermophilic bacterium PS-3 is also stable at high temperatures, with the optimum activity being in the temperature range of 60–75 °C (Yoshida et al., 1975, 1977). As shown in Figure 6, the enzymatic activities of NDH-1 and NDH-2 preparations are also highly stable at temperatures as high as 80 °C. Therefore, these preparations should be useful materials for study of the structure and mechanism of action of NADH-Q reductases. Indeed, one might be able to resolve NDH-1 into its component flavoprotein and FeS protein subunits, characterize each subunit, and attempt a stepwise reconstitution. Such a study might provide information regarding the components required for ferricyanide reduction, Q reduction, rotenone sensitivity, and proton translocation.

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