Studies on the NADH-Menaquinone Oxidoreductase Segment of the Respiratory Chain in *Thermus thermophilus* HB-8*

(Received for publication, February 1, 1989)

Steven W. Meinhardt†, Da-Cheng Wang, Koyu Hon-nami‡, Takao Yagi‡, Tairo Oshima‡, and Tomoko Ohnishi**

From the Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, the Technology R & D Division, Alcohol Production Head Office, Chiba 281, Japan, the Department of Molecular and Experimental Medicine, Division of Biochemistry, Research Institute of Scripps Clinic, La Jolla, California 92037, and the Department of Life Science, Faculty of Science, Tokyo Institute of Technology, Yokohama 227, Japan

Five distinct low potential iron-sulfur clusters have been identified potentiometrically in the membrane particles from *Thermus thermophilus* HB-8. Three of these clusters (designated as [N-1], [N-2]), and [N-3]) exhibit the following midpoint redox potentials and g values: ([E₉₅₀] = 40, -100, -160, -300 mV) and ([E₉₅₀] = 1.93, 1.94, 2.02), ([E₉₅₀] = -304 mV, [g₉₅₀] = 1.99, 1.96, 2.04), and ([E₉₅₀] = -289 mV, [g₉₅₀] = 1.80, 1.83, 2.06), respectively. These clusters, one binuclear and two tetranuclear, have been shown to be components of the energy coupled NADH-menaquinone oxidoreductase complex (NADH dh I). They are reducible in NADH in the piericidin A inhibited aerobic membrane particles as well as in the purified NADH dh I complex. Two additional very low potential iron-sulfur clusters (one binuclear, [N-1]), and one tetranuclear, [N-2]) were observed in membrane particles. These clusters possess the following physicochemical properties: ([E₉₅₀] = -418 mV, [g₉₅₀] = 1.93, 19.5, 2.02) and ([E₉₅₀] = -317 mV, [g₉₅₀] = 1.80, 1.96, 2.04), respectively. No high potential tetranuclear cluster equivalent to the mitochondrial iron-sulfur cluster ([N-2]) was found in this bacterial system.

In membrane particles isolated from *T. thermophilus* HB-8 cells, four different semiquinone species have been identified based on their redox midpoint potentials: ([E₉₅₀(Q/QH₂)] = 40, -100, -160, -300 mV) and sensitivity to the quinone analogue inhibitor, 2-heptyl-4-hydroxy quinoline-N-oxide. Of these semiquinone species the -100 mV component has been suggested to be part of the NADH dehydrogenase.

Piericidin A sensitive Δψ formation has been demonstrated to be coupled to the NADH-MQ oxidoreductase in membrane vesicles of *T. thermophilus* HB-8.

Among the three energy transducing sites of the mitochondrial respiratory chain, the NADH-ubiquinone (Q) oxidoreductase (complex I) segment may be the most complex and least understood (1, 2). It consists of at least 25 different polypeptides with the total molecular mass of approximately 800 kDa, containing 16-24 non-heme iron atoms and equivalent acid labile sulfides, in addition to 1 FMN and 2-4 ubiquinone (3, 4). Much of the recent progress in our understanding of the mitochondrial complexes has come from the comparative studies of analogous complexes in bacterial systems which have a simpler subunit composition. Therefore, we have set out to characterize the NADH dehydrogenase complexes from several selected bacterial sources.

Previously, we have reported the thermodynamic and spectroscopic characterization of the iron-sulfur clusters of *Paracoccus denitrificans* (5, 6) in which we extended the results of previously published studies (7, 8). The NADH-ubiquinone oxidoreductase appears to be analogous to the bovine heart mitochondrial enzyme in containing five EPR detectable iron-sulfur clusters, two binuclear (N-1a and N-1b) and three tetranuclear clusters (N-2, N-3, and N-4), with some distinctive features. The NADH dehydrogenase complex isolated by T. Yagi from *P. denitrificans* membrane particles consisted of only 10 polypeptides (9). Therefore *P. denitrificans* possess a "mammalian-like" site I energy transducing enzyme which contains far fewer polypeptides than the mammalian counterpart.

*Escherichia coli*, on the other hand, possesses a very different NADH dehydrogenase system. It has been shown to consist of two separate dehydrogenase complexes, one, NADH dh I, is coupled to the formation of a proton electrochemical potential (Δψ) while the other, NADH dh II, is not energy-coupled (10). The latter enzyme most likely corresponds to the isolated NADH-ubiquinone oxidoreductase which consists of a single polypeptide with a non-covalently bound FAD and no detectable iron (11, 12). Recently, we have published spectroscopic and thermodynamic characterization of the *E. coli* NADH dh I complex in membranes and have shown it to contain at least two or possibly four different iron-sulfur clusters (13, 14). This complex proved to be very difficult to work with due to its lability, although it does provide an interesting system for further study.

Thermophilic bacteria, on the other hand, have provided interesting and useful ATP synthetase and electron transport systems, due to the extremely stable protein structure, as elegantly demonstrated by Kagawa's (15) and Fee's groups (16, 17), respectively. Yagi et al. (18) have reported the isolation of two separate membrane bound NADH dehydrogenase complexes from *T. thermophilus* HB-8, a bacterium containing only menaquinone (19). This represents a system which is similar to that found in *E. coli* (10, 14). In *T. thermophilus* HB-8, NADH dh I is composed of 10 different polypeptides which contain 10-12 non-heme iron atoms, 7-9 acid labile sulfides, and one FMN. The other enzyme, NADH dh II, consists of a single polypeptide with a non-covalently linked...
FAD. The isolated complexes were found to be extremely thermostable and were isolated with relative ease. Here we have characterized by EPR spectroscopy the NADH dh I complex in membrane particles from *T. thermophilus* HB-8 as well as the partially purified NADH dehydrogenase complex. We have found that this complex contains at least three different iron-sulfur clusters, one binuclear and two tetranuclear. We have also shown that electron transfer in this enzyme is coupled to the formation of a membrane potential, \( \Delta \psi \). We have identified at least four different semiquinone species in membrane particles, one of which may arise from the NADH dh I complex.

**MATERIALS AND METHODS**

*T. thermophilus* HB-8 (ATCC27634) were grown aerobically at 75 °C and harvested in the late exponential phase as described in Ref. 20. Membrane particles were prepared as described by Yagi et al. (18). The flavin concentration of the isolated NADH dehydrogenase complex was determined fluorometrically according to Refs. (21, 22). EPR measurements were performed with a Varian E109 spectrometer. The sample temperature in the range of 50 K and below was controlled by a variable temperature cryostat (Air Products LTD-3-110). The temperature was measured using an Allen-Bradley type carbon resistor placed approximately 1 cm beneath the sample in a flowing cold helium gas stream. Temperatures above 50 K were attained with a nitrogen gas flow system in which the temperature was regulated by a Johnson Foundation digital temperature controller. The temperature was monitored by an Omega Engineering Inc. (Stamford, CT) copper-constantan T subminiature thermocouple probe. The magnetic field was calibrated with a proton NMR probe or, on a routine basis, using a Varian g value standard, weak pitch. Frequency of the microwave source was measured using an EIP model 545 microwave frequency meter. Double integration and subtraction of EPR spectra were performed with a dedicated IBM PC microcomputer. Spectra simulations were obtained by numerical convolution over the angles \( \theta \) and \( \Phi \) as described previously in Ref. 23. Spectral simulations were calculated using the University of Pennsylvania, School of Medicine VAX-6000 (Digital Electronics Corporation, Maynard, MA). Transition probability corrections were made according to Assa and Vägård (24). The spectral line shape was assumed to be gaussian. Potentiometric titrations were conducted according to Dutton (25). All redox midpoint potentials stated in the text and titration curves plotted in the figures were calculated on an IBM-PC using a fitting program written by E. A. Berry, Lawrence Berkeley Laboratory, Berkeley, CA.

Generation of the membrane potential (\( \Delta \psi \)) in the inside-out membrane vesicles was measured by following the fluorescence quenching of bis-(1,3-diethylbarbituric acid) pentamethine oxonol (dBA-C5) (12). The reaction mixture (2 ml total) contained 50 mM K-phosphate, pH 7.5, 5 mM MgSO4, 2 \( \mu \)M dBA-C5 (5). Reactions were carried out at 50 °C and fluorescence emission was monitored at 614 nm with excitation at 588 nm. Protein concentrations were determined by a modification of the method of Lowry et al. (27). The NADH dh I complex was partially purified according to the method of Yagi et al. (18) using the DEAE-Sepharose and hydroxyapatite chromatography steps.

**RESULTS**

**Piericidin A Sensitivity**—The NADH-Q1 oxidoreductase and the NADH oxidase activities observed in *T. thermophilus* HB-8 membrane particles are highly sensitive to the quinone analogue site 1 inhibitor, piericidin A. Fig. 1 presents a plot of the NADH-Q1 oxidoreductase and the NADH oxidase activities measured in *T. thermophilus* HB-8 membrane particles in the presence of varying concentrations of piericidin A. In each case the initial rate was monitored as the absorbance change at 340 nm due to the oxidation of NADH, with either Q1 (O) (in the presence of 5 mM KCN) or oxygen (A) as the terminal electron acceptor. Both activities exhibited a maximal inhibition of approximately 80% and an \( I_0 \) value near 0.25 nmol of piericidin A/mg protein. This relatively low \( I_0 \) and the incomplete inhibition by piericidin A suggests that more than one NADH dehydrogenase complex is concomitantly functioning in the membranes as in the case of *Escherichia coli* (10). Recently Yagi et al. (18) have isolated two separate NADH dehydrogenase complexes from *T. thermophilus* HB-8. One of these complexes, NADH dh II, was completely insensitive to another site I inhibitor, rotenone. The other NADH dh I partially retained sensitivity to this inhibitor and was 30% inhibited by 25 \( \mu \)M rotenone. This confirms the presence of two separate NADH dehydrogenase complexes and that only one is sensitive to inhibition by piericidin A.

**The Iron-Sulfur Clusters**—In all of the systems previously studied, all iron-sulfur clusters present in the NADH-Q oxidoreductase segment of the respiratory chain have been shown to be located on the substrate side of the piericidin A inhibition site (28-30). Therefore, we have recorded the spectra of the EPR detectable iron-sulfur clusters in NADH-reduced *T. thermophilus* HB-8 membrane particles which have been pretreated with piericidin A. The spectra from these particles recorded under several EPR conditions are shown in Fig. 2. In Fig. 2A are shown spectra obtained with membrane particles reduced with NADH in the presence of piericidin A (solid line) and with no reductant added (dotted line) at a temperature of 26 K and a microwave power level of 2 mW. Under these conditions signals from binuclear type iron-sulfur clusters are predominant in the spectrum. The addition of NADH to the EPR detectable iron-sulfur clusters in NADH-reduced T. thermophilus membrane particles was suspended in 50 mM Tris-HCl buffer, pH 7.5, 10 mM MgSO4, and 1 mM EDTA at a protein concentration of 0.19 mg/ml. The initial rate of NADH-Q1 reductase activity (O) was measured with 160 \( \mu \)M NADH and 150 \( \mu \)M Q1, in the presence of 5 mM KCN. Electron transfer activity from NADH to oxygen (A) was also measured spectrophotometrically under the same condition as Q1 reductase activity, in the absence of Q1, and KCN. Control activity of NADH-Q1 reductase was 3.0 \( \mu \)mol/min·mg protein, and NADH oxidation was 0.3 \( \mu \)mol/min·mg. The electron transfer activities were measured at 50 °C.

**Fig. 1. Inhibition of electron transfer from NADH to Q1 and from NADH to oxygen by piericidin A.** *T. thermophilus* HB-8 membrane particles were suspended in 50 mM Tris-HCl buffer, pH 7.5, 10 mM MgSO4, and 1 mM EDTA at a protein concentration of 0.19 mg/ml. The initial rate of NADH-Q1 reductase activity (O) was measured with 160 \( \mu \)M NADH and 150 \( \mu \)M Q1, in the presence of 5 mM KCN. Electron transfer activity from NADH to oxygen (A) was also measured spectrophotometrically under the same condition as Q1 reductase activity, in the absence of Q1, and KCN. Control activity of NADH-Q1 reductase was 3.0 \( \mu \)mol/min·mg protein, and NADH oxidation was 0.3 \( \mu \)mol/min·mg. The electron transfer activities were measured at 50 °C.

1 The abbreviations used are: dBA-C5, (1,3-diethylbarbituric acid) pentamethine oxonol; HOQNO, 2-heptyl-4-hydroxyquinoline N-oxide; mW, milliwatts; Hepes, 4-(2-hydroxyethyl)piperazineethane sulfonic acid.

2 Measurements of the reduction of menaquinone-1 and ubiquinone-1 gave similar \( K_m \) and \( V_{max} \) values. Therefore, these quinones were used interchangeably during activity assays.
chrome c oxidase. This signal is seen in the absence of any reductant and after the addition of NADH to the piericidin A-pretreated particles. This clearly demonstrates that the electron transport chain has remained oxidized from the piericidin A inhibition site to cytochrome c oxidase. This also demonstrates that this binuclear cluster is on the substrate side of the piericidin A inhibition site and constitutes a part of the NADH dh I complex.

In Fig. 2B are shown spectra from the same samples as used in Fig. 2A which were obtained under EPR conditions in which tetranuclear type iron-sulfur signals predominate, i.e. 12 K, 20 mW. In the absence of added reductants, the signal due to CuA is clearly seen. Upon the addition of NADH to the piericidin A-pretreated sample, several signals are seen. Still present is the partially saturated signal at \( g = 1.94 \) due to the binuclear cluster described in Fig. 2A. In addition, a rhombic spectrum is now discernible at \( g_{\text{av}} = 1.89, 1.95, 2.04 \). In the difference spectrum a small very broad signal is seen near \( g = 1.80 \) at 0.37 tesla magnetic field position. In our preliminary study (6), this signal was overlooked. This signal is further resolved when the sample temperature is lowered and the microwave power increased (Fig. 2C). This extremely rapidly relaxing signal, presumably due to a second tetranuclear iron-sulfur cluster, is seen at \( g_i = 1.80 \) and \( g_j = 2.05 \) (the \( g \) value will be more clearly defined later). From these results we have been able to identify at least three distinct iron-sulfur clusters as part of the NADH dh I complex. All of the signals reported here were observed in a single sample in which electron transport chains have remained oxidized beyond piericidin A inhibition sites. Therefore, all three of the iron-sulfur clusters identified here are on the substrate side of the piericidin A inhibition site and are part of the NADH dehydrogenase complex. To further characterize the redox properties of these iron-sulfur clusters and to determine whether additional low potential iron-sulfur clusters are present in the membrane, we have performed potentiometric titrations of the membrane particle and have measured the spectra under several different EPR conditions.

Fig. 3 shows the results of a potentiometric titration of the binuclear and tetranuclear clusters present in the membrane. Fig. 3A shows an oxidative titration of the binuclear clusters. In reductive titrations of the iron-sulfur clusters, we obtained redox midpoint potentials which varied from 20 to 50 mV higher than those obtained from oxidative titrations. The range of the hysteresis is the result of several oxidation and reduction titrations. We have not been able to resolve the reason for this somewhat large hysteresis at this time. This titration has been resolved into three different components. The highest potential component, with an \( E_0 \) of -35 mV (\( n = 1 \)) is that of the binuclear cluster [S-1] of the succinate dehydrogenase complex. As reported in Refs. 6, 31, this iron-sulfur cluster possesses a spectrum of axial symmetry (Fig. 4A) which differs from the spectrum obtained for the cluster S-1 in most organisms (1, 2). Previously, we have shown that this iron-sulfur cluster is reducible by succinate and is part of the succinate dehydrogenase. In Fig. 4B, the spectrum of the second component, which has a midpoint potential of -274 mV (\( n = 1 \)) is shown. This component exhibits a spectrum similar to the binuclear cluster of the NADH dehydrogenase which we have resolved in Fig. 2A. Therefore, we have assigned this [2Fe-2S] type cluster with an \( E_0 \) value of -274 mV to the NADH dehydrogenase and have labeled it [N-1]\. The final component which we have labeled [N-1]L, has a midpoint of -418 mV (\( n = 1 \)) and a rhombic spectrum (Fig. 4C). Preliminary experiments indicate that this component has a pH-dependent midpoint potential, similar to the cluster [N-1] in the bovine heart mitochondrial complex I (32, 33) but is present at a much lower spin concentration than that of [N-1]L. Thus, at this moment, this cluster has not been established as a component of the T. thermophilus NADH dehydrogenase.

The oxidative titration of the \( g = 1.89 \) signal measured at 11 K, 20 mW is shown in Fig. 3B. In this case the signal titrated as two \( n = 1 \) components with midpoint potentials of -304 and -437 mV, labeled [N-2]H and [N-2]L, respectively. These components appear to have very similar spectral (Fig. 5A) which were obtained under EPR conditions with the following parameters: microwave frequency 9.323 GHz; modulation amplitude 12.5 x 10^-4 tesla; time constant 0.128 s; scan rate, trace A, 1 x 10^-4 tesla/min; traces B and C, 2.5 x 10^-4 tesla/min. Traces A and C are an average of four scans.
Fig. 3. Oxidative potentiometric titration of the iron-sulfur clusters in T. thermophillus HB-8 membrane particles, pH 8.0. Membrane particles were suspended in buffer (100 mM Hapes, 50 mM KCl, pH 8.0) at a protein concentration of 27 mg/ml. The following mediators were then added: 40 μM of each 2,3,5,6-tetramethylphenylenediamine, 1,2-naphthoquinone, 1,2-naphthoquinone-4-sulfonate, 1,4-naphthoquinone, duroquinone, indigotetrasulfonate, indigodisulfonate, benzylviologen, methylviologen, 60 μM phenosafranine, and 20 μM pyocyanin. The binuclear type iron sulfur clusters were measured by the peak to peak amplitude of the g = 1.94 signal recorded at 26 K and a microwave power of 2 mW. The tetranuclear clusters were titrated monitoring the g = 1.89 and g = 1.81 peak amplitudes relative to the high field base line under the EPR conditions of sample temperature, 12 K; microwave power 20 mW and 8 K, 50 mW, respectively.

5A and B) and relaxation properties (not shown). At present we have not been able to resolve whether the lower Eₘ species is part of the NADH dehydrogenase or not. Fig. 3C shows a titration of the g = 1.81 signal of the extremely fast relaxing tetranuclear cluster which we have designated [N-3]ₜ. This signal titrates as a single n = 1 component with a midpoint potential of -289 mV. The resolved spectra of each of these components are shown in Figs. 4 and 5 with the simulations of each component. The simulation parameters are listed in the figure legend. Spin quantitation of these signals indicated that the N-1₁/N-2₁/N-3 ratios are 1.0:0.4:0.7:1.1:0.4. At this time, there appears to be at least five low potential iron-sulfur clusters, two binuclear and three tetranuclear, present in T. thermophilus membranes. Of the five clusters observed, at least three, one binuclear and two tetranuclear, appear to be NADH-reducible iron-sulfur clusters in the NADH dh I complex.

In order to further support the presence of these iron-sulfur clusters in the NADH dh I complex, we have isolated the NADH dh I complex by a simplification of the method of Yagi et al. (18). This two-step method resulted in a preparation of approximately 40% purity based on the flavin content, containing 25–30 different polypeptides rather than 10 (18). However, this preparation is relatively free from EPR active contaminants, as seen in Fig. 6 and yields a sufficient quantity of the in vitro dehydrogenase complex for EPR analysis. In this figure, we show spectra obtained from the NADH-reduced isolated complex. Again we were able to identify three distinct iron-sulfur clusters. In the isolated complex the binuclear cluster's spectrum is shifted to slightly higher magnetic fields and exhibits a somewhat more rhombic spectrum with g₁ = 1.90 signal seen at 0.352 tesla in Fig. 6A may be due to some other modified cluster. During the isolation procedure some protein modification may have occurred since the relaxation properties of the tetranuclear clusters have changes such that they are more slowly relaxing than the corresponding clusters in situ. The rhombic tetranuclear iron-sulfur cluster observed at 13 K and 20 mW, Fig. 6B, has essentially the same line-shape as seen in the membrane particles. The very rapidly relaxing tetranuclear iron-sulfur cluster is also essentially the same as in membranes. Spin quantitation of these signals indicated that they are present in a ratio of 1:9:83:0.91 for [N-1₁]/[N-2₁]/[N-3]₁.
Fig. 5. Redox-resolved and -stimulated spectra of the tetranuclear iron-sulfur cluster in *T. thermophilus* HB-8 membrane particles. Samples from the titration in Fig. 3 were used. The resolved spectra of \([N-2\alpha]_T\) and \([N-2\beta]_T\) were obtained from the difference of samples poised at \(-400\) mV minus \(-250\) mV and \(-525\) mV minus \(-400\) mV, respectively. The resolved spectra of \([N-3\gamma]_T\) was obtained from the difference of samples poised at \(-350\) minus \(-150\) mV. The EPR conditions were as follows: microwave frequency 9.320 GHz; modulation amplitude \(12.5 \times 10^{-4}\) tesla; scan rate of \(1 \times 10^{-4}\) tesla/min; average of four scans. The microwave power and temperature were as shown in the figure. The simulation parameters were as follows: \(A:\ g_{\alpha,\beta} = 1.891, 1.949, 2.043\) and \(L_{\alpha,\beta} = 18, 8.5, 17 \times 10^{-4}\) tesla; \(B:\ g_{\alpha,\beta} = 1.889, 1.947,\) and \(2.04\) and \(L_{\alpha,\beta} = 18, 8.5, 17 \times 10^{-4}\) tesla; \(C:\ g_{\alpha,\beta} = 1.801, 1.830,\) and \(2.059,\) and \(L_{\alpha,\beta} = 32, 28, 10 \times 10^{-4}\) tesla.

The presence of three NADH-reducible iron-sulfur clusters, one binuclear and two tetranuclear, results in 10 iron atoms per complex. This result is in close agreement with the measured number of 10-12 iron atoms/complex (18) but does not preclude the possibility of additional iron-sulfur clusters being part of the complex.

Semiquinone Radicals—Quinone molecules, ubiquinone or menaquinone, play an important role in the electron transport chain by shuttling reducing equivalents between complexes as well as in some cases carrying protons across the membrane as hydrogen. The difficulty of coupling the reduction or oxidation of an \(n = 2\) electron acceptor/donor such as ubiquinone with the oxidation or reduction of an \(n = 1\) electron acceptor/donor has been solved in several complexes through the use of stabilized semiquinone intermediates (34-38). In order to investigate whether such radicals exist in the electron transport chain of *T. thermophilus* HB-8 and to perform an initial characterization of these radicals we have conducted a potentiometric titration at pH 9.0 monitoring the amplitude of the \(g = 2.00\) signal. The result of this experiment is shown in Fig. 7, solid circles. From this titration it is evident that there are several free radical species in the *T. thermophilus* HB-8 membrane particles. All of the signals seen in the titration exhibited peak-to-trough widths \((\Delta H)\) of 0.7-1.0 \(\times 10^{-3}\) tesla (Fig. 8) which is indicative of a semiquinone radical (39, 40) rather than the flavin free radical (1.2-2.0 \(\times 10^{-2}\) tesla) (41).
transport chain with an inhibition point on the cytochrome c oxidase side of the piericidin A inhibition site. The presence of a Rieske-type iron-sulfur cluster (16) along with membrane-bound b and c cytochromes in T. thermophilus HB-8 (17) and the recent isolation of a bc complex from another thermophilic organism, PS3 (42), indicated that there may be a cytochrome bc complex in T. thermophilus HB-8. Therefore, we have investigated the effects of mitochondrial bc complex inhibitors on the NADH oxidase activity of membrane particles from T. thermophilus HB-8. Even at extremely high concentrations (200 nmol/mg protein) of the potent mitochondrial cytochrome bc complex inhibitors, antimycin (43) or stigmatellin (44), the NADH oxidase activity was inhibited by only 45 and 70%, respectively (Table I). UDHBTT and myxothiazol exerted no significant inhibitory effect. Even KCN at 3 mM was able to inhibit NADH oxidase activity by only 80%. The sole effective inhibitor found for this bacterial system was 2-heptyl-4-hydroxy quinoline-N-oxide (HOQNO). This compound was able to inhibit NADH oxidase activity by 90% at 20 nmol/mg protein. These results indicate that this complex contains considerably different quinone-binding sites from those found in the mitochondrial cytochrome bc complex. We have found that HOQNO has multiple inhibition sites in T. thermophilus HB-8 membrane particles as is the case of 3-undecyl-2-hydroxy-1,4-napthoquinone in the E. coli system (10). The primary site of inhibition is in the ubiquinol-cytochrome c oxidoreductase region. A secondary site was found to be in the NADH-Q oxidoreductase region. The latter is not inhibited by 25 nmol/mg protein and is only partially inhibited at much higher inhibitor concentrations.

Since HOQNO was found to be an effective inhibitor of the quinol oxidase we have investigated the effects of this compound on the multiple semiquinone species described above. When HOQNO was added at relatively low concentrations (50 μM), during a reductive titration, open squares, the loss of a semiquinone species with a midpoint potential of −160 mV, dotted line, was observed. Additionally an increase in the signal amplitude was observed for the semiquinone with an \( E_{1/2} \) value of −290 mV. This result represents the destabilization of a semiquinone species and the concomitant stabilization of the lower potential species as has been reported for the effect of antimycin on the \( Q_b \) or \( Q_c \) species, respectively, in the succinate-cytochrome c oxidoreductase (34). The addition of HOQNO at this concentration also makes evident that the central peak of the titration in the absence of inhibitor is composed of two overlapping titrations of different semiquinone species. At this concentration of HOQNO, the NADH-Q oxidoreductase activity was inhibited by only 10%. Therefore, it is unlikely that the \( E_{1/2} = -160 \) mV component is part of the NADH dh I complex. The highest potential semiquinone species, \( E_{1/2}(Q/QH_2) = 40 \) mV, ap-
peared to be only slightly increased in amplitude by the presence of 50 \( \mu \text{M} \) HOQNO. The semiquinone species in the central peak remaining in the presence of 50 \( \mu \text{M} \) HOQNO has an \( E_{\text{m}}(\text{Q/QH}) \) of -100 mV. When the concentration of HOQNO was further increased to 250 \( \mu \text{M} \), the \( E_{\text{m}} = -100 \text{ mV} \) semiquinone species was almost completely destabilized with no further changes in the \( E_{\text{m}} = -300 \text{ mV} \) species. These observations indicate that the thermodynamically stable semiquinone species with the \( E_{\text{m}} \) value of -100 mV may be part of the NADH dh I complex.

**Membrane Potential Formation in the NADH dh I Segment of the Respiratory Chain**—In Fig. 9, we demonstrate that the piericidin A-sensitive NADH-MQ\(_1\) reductase activity of *T. thermophilus* membrane vesicles is coupled to the formation of a membrane potential (\( \Delta \psi \)). Formation of the membrane potential was measured using an oxonol fluorescent dye (diBA-C\(_7\)) which is quenched by positive charges moving into the inside of the membrane particle. In Fig. 9A we show that a membrane potential is generated by NADH oxidase activity (0.2–0.4 \( \mu \text{atoms} \text{O}_2/\text{min}. \text{mg protein} \)) which blocks the electron transfer from NADH to oxygen, with an inhibition site on the cytochrome c oxidase side of the quinone pool. NADH-Q oxidoreductase activity is not inhibited at this concentration of HOQNO. Thus, when MQ\(_1\) was added, the \( \Delta \psi \) formation resumed, coupled with the NADH-MQ\(_1\) reductase activity. The equilibrium level of \( \Delta \psi \) in the NADH-MQ oxidoreductase segment is close to that of the whole electron transfer from NADH to the terminal cytochrome c oxidase (Fig. 9A). In *T. thermophilus* HB-8 membrane preparations the turnover rate of the electron transfer from NADH to oxygen is about 10-fold faster than the rate of NADH oxidation through the whole respiratory chain. It has been reported that the cytoplasmic membrane of thermophilic bacteria grown at a high temperature appears to be more leaky to protons than that of the mesophilic bacterial membrane (16, 45). Thus, the relatively high \( \Delta \psi \) level in the NADH-MQ oxidoreductase segment simply arises from the balance of the high rate of the proton gradient formation against that of the proton leakiness of the membrane. The energy-coupled NADH-MQ\(_1\) oxidoreductase is inhibited by piericidin A. Therefore, in this figure we showed that the membrane potential formation coupled to the NADH-MQ\(_1\), oxidoreductase activity is also inhibited by piericidin A. Fig. 9D shows that the magnitude of the fluorescence quenching is greater when the energy-coupled reaction was initiated with NADH rather than MQ\(_1\). This indicates that, as in the case of the bovine heart submitochondrial particles (46) or the *E. coli* membrane particle system (10), contact with a relatively high concentration of NADH diminishes the \( \Delta \psi \)-generating activity. The addition of the ionophore valinomycin results in the complete dissipation of the membrane potential. We have not been able to show the total \( \Delta \psi \) formation by converting \( \Delta \psi \) to \( \Delta \psi \) in the presence of nigericin. In *T. thermophilus* HB-8 membrane particles nigericin at a concentration as low as 1 nM collapsed the \( \Delta \psi \) formation in the absence of added valinomycin. Similar phenomena have been previously observed in other bacterial systems (47, 48). The mechanism of this nigericin effect remains unresolved. *T. thermophilus* HB-8 membrane particles elicit only very low succinate oxidase activity (~0.03 nmoles of \( O_2 \) uptake/min. mg protein). If succinate is used as a reductant, the formation of a membrane potential is observed although at a much lower magnitude when compared with that of the NADII oxidase activity. This is inhibited by HOQNO (25 nmol/mg protein) as in the case of the NADH oxidase activity. Succinate-MQ\(_1\), reductase activity is not coupled to the \( \Delta \psi \) generation (Fig. 9C).

**DISCUSSION**

In this paper we have shown that *T. thermophilus* HB-8 possesses an energy-coupled iron sulfur containing NADH-Q oxidoreductase which is piericidin A-sensitive. This complex contains at least three NADH reducible iron-sulfur clusters, one binuclear and two tetranuclear. Based on the number of iron atoms present in the isolated complex (18) it is likely that an additional binuclear cluster, [N-1]\(_2\)\(_{17}\), is present. This cluster would be similar to the very low potential binuclear cluster [N-1a]\(_{16}\) of the bovine heart mitochondrial system (32, 33). Thus, the EPR detectable iron-sulfur composition of the *T. thermophilus* HB-8 NADH-Q oxidoreductase system is similar to the mammalian counterpart with one clear exception. The thermophilic system lacks an equivalent cluster to the mitochondrial or *P. denitrificans* cluster N-2 which shows a relatively high (~100 to -20 mV) and pH-dependent (~60 mV/pH) midpoint potential. The polypeptide composition of this system is much simpler than the mammalian counterpart, as is the case of the *P. denitrificans* NADH-Q oxidoreductase, consisting of only approximately 10 polypeptides (9). The stability of the isolated thermophilic complex and the relative ease of its isolation (18), makes this a very attractive system for the study of the mechanism of site I energy coupling.

At present the anomaly of the substoichiometric amount of the cluster [N-3]\(_{17}\) seen in membranes is not readily explained. It may indicate the presence of some magnetic interaction with another component which remains for further studies. The presence of two tetranuclear clusters, [N-2a]\(_{17}\) and [N-
$E_m$, with the same spectral features but with different redox properties is somewhat puzzling. Preliminary redox titrations at several pH values suggest that this occurs only above pH 8 and that the $E_m$ values of the two clusters are not pH-dependent. This may indicate that the enzyme is unstable at higher pH values and results in a population of the cluster $2\&$, with the same spectral features but with different redox and that the $E_m$ values of the two clusters are not pH- at several pH values suggest that this occurs only above pH 8 two clusters, $[N-2\&$ and $[N-2\&$, with each other and with $[N-1\&$. This may indicate the presence of more than one copy of this cluster in the complex or that two separate enzymes present in the membrane use iron-sulfur clusters in very similar environments. The resolution of this question must await further study. The T. thermophilus HB-8 NADH- Q oxidoreductase system is very similar to that found in E. coli in several respects. The first is the existence of two distinct membrane-bound NADH oxidizing complexes, one of which is energy coupled. In both bacterial systems the non-energy conserving complex was found to be a single subunit, flavoenzyme containing FAD. On the other hand, the energy-coupled enzymes contain multiple iron-sulfur clusters and FMN. Preliminary studies of the Rhodobacter capsulatus indicate that it also contains two NADH-Q oxidoreductase complexes, one of which is very similar to the mammalian complex I and piericidin A sensitive, while the other is not. It raises an important question as to the control of the oxidation of NADH by these two enzymes in the membrane since both appear to be present on the same side, the cytoplasmic or negative side, of the cell membrane. It is also interesting that in each case the energy-coupled enzyme is sensitive to piericidin A, a quinone analogue site I inhibitor. This would suggest that the NADH-Q oxidoreductase complexes from all of the sources studied so far, ranging from the primitive thermophilic organisms to the mammalian enzymes, all contain a common structural site, presumably the quinone-binding site. It also suggests that in spite of the differing number of iron-sulfur clusters they may use a common means to pump protons. Determination of whether there is sensitivity to HOQNO would suggest that this complex is more complex mammalian systems. The number of stable semiquinone species in these membranes suggests that the T. thermophilus HB-8 is an excellent system for the study of how quinones react with the electron transport complexes. The species with midpoints of $-160$ and $-300$ mV are affected by concentration of HOQNO low enough to inhibit the ubiquinol oxidase but not the NADH-Q or the succinate-Q oxidoreductases. This implies that these species are part of a QH$_2$-cytochrome c oxidoreductase and lend credence to the concept of a cytochrome bc complex in T. thermophilus which is similar to the mammalian complex. Its resistance to inhibitors such as antimycin, myxothiazol, and stigmatellin along with its sensitivity to HOQNO would suggest that this complex is more similar to the cytochrome $b_6$ complex of higher plants (49, 50). Kutoh and Sone (42) have recently isolated from another thermophilic organism, PS-3, a cytochrome $bc_2$-type complex which exhibits inhibitor sensitivities similar to the cytochrome $b_6$ complex of higher plants. The $-100$ mV species is the most likely candidate for the NADH-Q oxidoreductase since it is affected by higher HOQNO concentrations. We are presently investigating the effects of various site I inhibitors on the semiquinone signals reported here, along with studies on the isolated complex, to determine if any of these signals can be assigned to the NADH dh I complex. We also hope to use this system to determine if there is more than one quinone reaction site in this complex.

We have shown the $\Delta\phi$ formation coupled with the NADH-MQ oxidoreductase in the T. thermophilus membrane particles, which contains a rotenone inhibition site, but not a high $E_m$ cluster equivalent to the [N-2]$\&$. Determination of the $H^+/2e^-$ stoichiometry in this NADH-MQ oxidoreductase system is a major prerequisite for determining the mechanism of proton and electron transfer in this complex.

Acknowledgments—We would like to thank Drs. S. DeVries and C. I. Ragan for their kind gifts of piericidin A, and Dr. A. Waggoner for pentamethine oxonol dye. We would also like to thank D. Chen and D. Gangi for their expert technical assistance. In addition, thanks are due to Kevra Co. Ltd. in Tokyo for generously providing us UQ, and MQ.

REFERENCES


5. D.-C. Wang and T. Ohnishi, unpublished data.
6. S. W. Meinhardt and T. Ohnishi, unpublished data.
Studies on the NADH-menaquinone oxidoreductase segment of the respiratory chain in Thermus thermophilus HB-8.
S W Meinhardt, D C Wang, K Hon-nami, T Yagi, T Oshima and T Ohnishi

Access the most updated version of this article at http://www.jbc.org/content/265/3/1360

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/3/1360.full.html#ref-list-1