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Steady-State Kinetics of NADH:coenzyme Q Oxidoreductase Isolated from Bovine Heart Mitochondria

Yumiko Nakashima,[1] Kyoko Shinzawa-Itoh,[1] Kenji Watanabe,[1] Kazuki Naoki,[1] Nobuko Hano,[1] and Shinya Yoshikawa[1,2]

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Steady-state kinetics of the bovine heart NADH:coenzyme Q oxidoreductase reaction were analyzed in the presence of various concentrations of NADH and coenzyme Q with one isoprenoid unit (Q_1). Product inhibitions by NAD^+ and reduced coenzyme Q_1 were also determined. These results show an ordered sequential mechanism in which the order of substrate binding and product release is Q_1–NADH–NAD^+–Q_1H_2. It has been widely accepted that the NADH binding site is likely to be on the top of a large extramembrane portion protruding to the matrix space while the Q_1 binding site is near the transmembrane moiety. The rigorous controls for substrate binding and product release are indicative of a strong, long range interaction between NADH and Q_1 binding sites.

KEY WORDS: NADH:coenzyme Q oxidoreductase; complex I; membrane protein; steady-state kinetics; ordered sequential mechanism; mitochondrial respiration; coenzyme Q.

INTRODUCTION

NADH:coenzyme Q oxidoreductase (EC 1.6.99.3) (complex I) catalyzes the reduction of coenzyme Q (Q) by NADH at the entrance of the mitochondrial respiratory chain (Hatefi, 1985; Walker, 1992; Weiss *et al.*, 1991). The presence of this complex has been proven by isolation from mitochondrial preparations almost 40 years ago by Hatefi (Hatefi *et al.*, 1961). It has been proposed that this process is coupled to translocation of protons to the intermembrane space with stoichiometry of $4H^+/2e^-$ (Di Virgilio and Azzone, 1982; Esposti and Ghelli, 1994; Scholes and Hinkle, 1984; Weiss and Friedrich, 1991; Wikstrom, 1984). Complex I, the largest electron transfer complex in the mitochondrial respiratory chain with a total molecular mass of about 1000 kDa, contains 41–43 different subunits (Buchanan and Walker, 1996; Walker, 1992). Seven of these are hydrophobic subunits encoded by mitochondrial genes (Chomyn *et al.*, 1986). Many redox active sites have been reported, including four to six (electron spin resonance) EPR detectable iron–sulfur clusters (Ohnishi, 1998; Ohnishi *et al.*, 1985), one flavin mononucleotide (Hatefi, 1985; Walker, 1992; Weiss *et al.*, 1991), and several tightly bound Q molecules (Suzuki and Ozawa, 1986).

Extensive analysis of the initial steady-state of an enzyme under turnover conditions provides unique information that describes the function of the enzyme under the turnover conditions such as the order of substrate binding and product release from the enzyme. This information is difficult to obtain by any other method (Cleland, 1977). Thus, an extensive initial steady-state kinetic analysis is indispensable for elucidation of the reaction mechanism of complex I. Availability of a stable and active enzyme preparation is critical for successful analysis of the initial steady-state kinetics. Because of difficulties in purification, steady-state properties of complex I have often been analyzed using mitochondrial or submitochondrial particle preparations in the presence of various inhibitors used to block electron transfer from the electron transfer complexes downstream of the respiratory chain (Esposti *et al.*, 1996; Fato *et al.*, 1996; Kotlyar and Vinogradov, 1990).

Key to abbreviations: complex I, NADH: coenzyme Q oxidoreductase; Q_n, coenzyme Q (ubiquinone) with *n* isoprenoid units; Q_1H_2, reduced coenzyme Q_1; EPR, electron spin resonance.

[1]Department of Life Science, Himeji Institute of Technology and CREST, Japan Science and Technology Corporation, Kamigohri, Akoh Hyogo 678-1297, Japan.

[2]To whom all correspondence should be addressed; e-mail: yoshi@sci.himeji-tech.ac.jp.

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However, special caution is required because of potential inhibitory effects on complex I from the inhibitors used to block electron transfer complexes downstream from the complex I reaction.

For the initial steady-state analysis of complex I, artificial electron acceptors such as ferricyanide (Hatefi, 1968; Hatefi *et al.* 1962; Leif *et al.*, 1995) and 2,6-dichlorophenol indophenol (Dooijewaard and Slater, 1976) are often used for the enzyme activity assay. However, these artificial electron acceptors appear to react with the NADH binding site. In fact, the electron transfer reactions between NADH and the electron acceptors are not inhibited by rotenone. The inhibition of the enzyme reaction by rotenone has been used as a standard for integrity of the complex I reaction system (Buchanan and Walker, 1996; Hatefi, 1968). On the other hand, Q with one isoprenoid unit (Q_1) is used as the electron acceptor (Estornell *et al.*, 1993; Ragan, 1978; Yagi, 1986), because the natural substrate, Q_{10} (with 10 isoprenoid units), in the bovine heart mitochondrial respiratory chain (Szarkowska, 1966), is essentially insoluble in aqueous buffer solutions, and the NADH–Q_1 reaction is rotenone sensitive if the enzyme is intact.

Fato *et al.* proposed a ping-pong mechanism for the NADH–Q_1 reductase reaction in investigations employing submitochondrial particle preparations in the presence of inhibitors for blocking electron transfer between the electron transfer complexes downstream of the respiratory chain (Fato *et al.*, 1996). The method, however, appears to lack the sensitivity required to support the conclusion. Furthermore, the product inhibition analysis, which is required for a complete steady-state kinetic analysis, has not been reported.

Here, we report a complete steady-state kinetic analysis of the NADH–Q_1 reductase reaction catalyzed by isolated bovine heart complex I. The results show an ordered sequential mechanism including Q_1 as the initial substrate and reduced coenzyme Q_1 (Q_1H_2) as the last product.

EXPERIMENTAL PROCEDURES

Materials

Q_1 was synthesized from coenzyme Q_0 and 3-methyl-2-buten-1-ol via an electrophilic replacement reaction. Q_1H_2 was prepared by reduction of Q_1 employing the method of Rieske (1967). Q_1 and Q_1H_2 were determined using their extinction coefficient (in ethanol); ε_{275nm} of 14.02 mM^{-1} cm^{-1} (Dawson *et al.*, 1968) and ε_{290nm} of 4.14 mM^{-1} cm^{-1} (Kubota *et al.*, 1992), respectively. Protein concentration was determined using the method of Markwel (Markwel *et al.*, 1981). Other reagents were of the highest grade commercially available.

Complex I was purified from bovine heart muscle using the method of Hatefi (1978) with the following modifications: the final complex I fraction in the Hatefi procedure, from 700 mL of mitochondrial inner membrane fraction (23 mg protein/mL), obtained as precipitate by 35% saturation of ammonium sulfate, was collected by centrifugation. The precipitate was dissolved in 40 mM potassium phosphate buffer (pH 8.0) containing 0.2% (w/v) dodecyl maltoside and 10% glycerol in a total volume of approximately 10 mL. The complex I fraction was layered on the top of a stepwise sucrose gradient in a centrifuge tube from 0.4 to 1.0 M in 0.1 M interval and centrifuged for 16 h at 40,000 rpm with a Beckman L60S rotor. Each layer in the tube contained 0.2% dodecyl maltoside and 40 mM potassium phosphate buffer (pH 8.0). After centrifugation, the complex I fraction was found at the bottom of the tube leaving two colored layers containing cytochrome bc_1 complex and other unidentified colored proteins above the bottom layer. The bottom layer, which still showed residual amounts of cytochrome bc_1 complex in the absorption spectrum, was collected and diluted with the same volume of 40 mM potassium phosphate buffer (pH 8.0) containing 0.2% dodecylmaltoside in order to lower the liquid density of the fraction caused by the high concentration of sucrose. The diluted fraction was applied to a Q-sepharose column (Pharmacia, fast flow) equilibrated with 40 mM potassium phosphate buffer (pH 8.0) containing 0.2% dodecylmaltoside and 10% glycerol, and eluted with a linear concentration gradient of potassium phosphate buffer (pH 8.0) from 40 to 400 mM, at a fixed concentration of 0.2% dodecylmaltoside and 10% glycerol. Complex I was eluted at a gradient concentration between 270 mM and 300 mM potassium phosphate. The complex I fraction was dialyzed for 2 h against 40 mM potassium phosphate buffer (pH 8.0) containing 10% glycerol. A second 2-h dialysis was then done against a new external medium. Nonprotein constituents in the purified sample are 1 mol of FMN, 3 mol of Q_{10}, 28 $\pm$ 0.5 atoms of iron and approximately 280 phospholipid-derived phosphorous atom equivalents. Visible-Soret spectra (Fig. 1) indicates that the purified preparation is essentially free from any hemoprotein. The enzyme preparation (8–10 mg protein/mL) can be stored at $-80^{\circ}C$ for at least 1 year without any change in its enzymatic activity and absorption spectral properties.

Enzyme Activity Assay

The rate of NADH oxidation by complex I was determined by monitoring the absorbance decrease at 340 nm

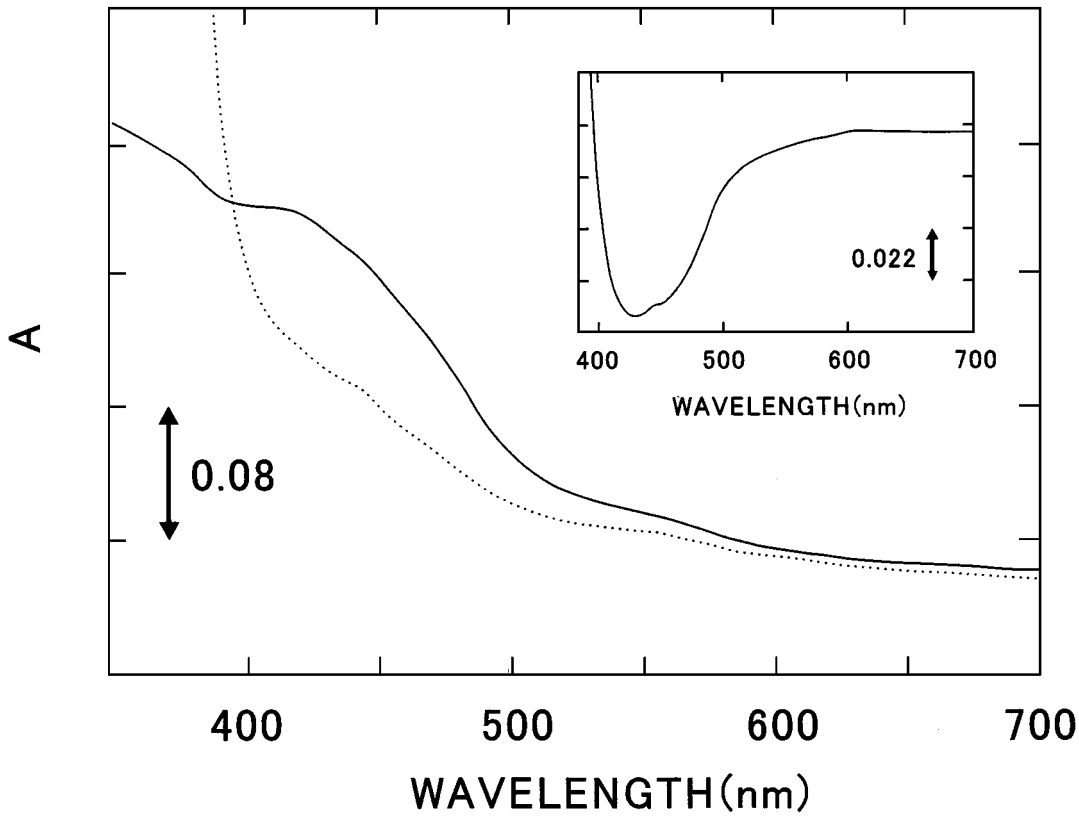


Fig. 1. Absorption spectra of the purified complex I. The spectrum of the reduced form (a dotted line) was taken 15 min after addition of a slightly excess amount of solid dithionite to the oxidized form as prepared (its spectrum in a solid line), placed in a cuvette of 1 cm light path at the enzyme concentration of 1.88 mg protein/mL in 50 mM Tris-HCl buffer (pH 8.0) containing 0.2% dodecyl maltoside and 660 mM sucrose. The significant absorbance increment below 410 nm in the spectrum of the reduced form is due to the absorbance of dithionite. The inset shows a difference spectrum of the reduced form vs the oxidized form, given in the main panel.

at $20 \pm 0.1^{\circ}$C. A water jacket for the cell holder and a small stir bar at the bottom of the cuvette were required for sufficient removal of temperature gradients. Typically the reaction was initiated by addition of 41 μg of complex I (in volume of 5 μL) to a reaction mixture (2 mL) containing 10 μM NADH, 25 μM Q_1, and 0.1% dodecylmaltoside in 100 mM potassium phosphate buffer (pH 8.0) as shown in Fig. 2. The initial rate was determined by calculating the slope of the initial linear absorbance decrease at 340 nm. Because a reliable extinction coefficient is not available for complex I, it was quantified by determination of protein concentration assuming a molecular mass of 1000 kDa. The accuracy of the protein determination is lower than that of the enzyme activity measurement. The enzyme activity assays performed herein were done using an identical stock solution (8.2 mg/mL) divided into about 200 portions of 100 μL, and frozen at -80°C. Each portion once thawed was stored at 0°C for 8 h without causing detectable changes in the enzyme activity. The thawed enzyme solution was used for enzyme activity assays without dilution. Under these experimental conditions, the error in the enzyme activity assay due to enzyme concentration was negligible compared to the error due to the enzyme activity assay method. Each data point given in this paper is the average of duplicate determinations. Kaleida Graph (Synergy Software) was used for curve fitting of the enzyme assay data.

RESULTS

Measurement of NADH:Q_1 Reductase Activities

Addition of 0.1% dodecylmaltoside stimulates the enzyme activity by 20–30% and provides a proportional increase in NADH oxidation with respect to the enzyme concentration. In the absence of dodecylmaltoside, activity determinations were less reproducible and a significant

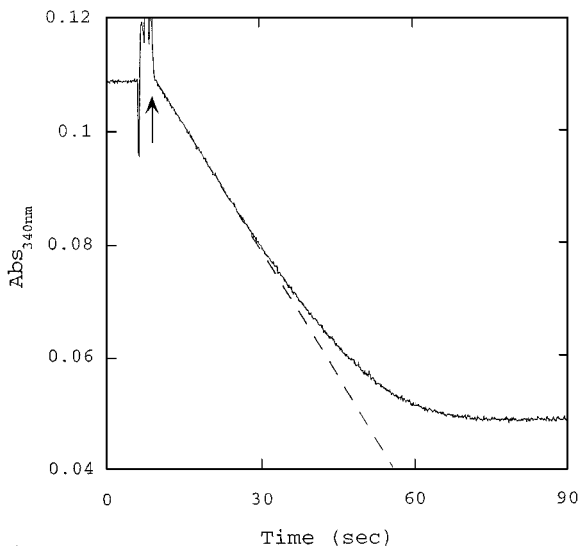


Fig. 2. Absorbance decrease at 340 nm due to the NADH:Q_1 reductase reaction. The enzyme reaction at $20 \pm 0.1^{\circ}$C was initiated by addition of enzyme at the point indicated by the arrow. The broken line shows the slope of the initial linear absorbance change. The reaction mixture contained 10 μM NADH, 25 μM Q_1, and 20.5 μg complex I/mL in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.1% dodecylmaltoside.

deviation from proportional increase was detected between the enzyme reaction rate and the enzyme concentration in the range of 0–51 μg/mL. The enzyme activity was independent of the concentration of dodecylmaltoside between 0.05 and 0.3%. Ethanol was used to dissolve Q_1 and rotenone. Reaction mixtures contained various amounts up to 2.0% (v/v) ethanol depending on the Q_1 and rotenone concentrations and its presence did not affect the enzyme reaction rate and the rotenone-sensitivity.

Under the present experimental conditions described above, enzyme activity was determined using the linear portion of the NADH oxidation curve. In order to determine the amount of Q_1 reduced during the linear portion, the absorbance change at 290 nm was also determined and the contribution due to the oxidation of NADH at this wavelength was subtracted. No deviation from unity in the molar ratio of NADH oxidized to Q_1 reduced in the traces was higher than 0.05 under these experimental conditions, indicating that NADH is oxidized stoichiometrically with Q_1 in the initial linear portion of the curve. This result indicates that all of the electrons from NADH are transferred to Q_1 without leakage to exogenous oxidants such as O_2. Determination of the dependence of NADH concentration (0.625–10 μM) on NADH–Q_1 reductase activity at a fixed Q_1 concentration of 25 μM was repeated eight times. Data were fit to the Michaelis–Menten equation without any systematic deviation and provided a V_{max} value of 0.516 ± 0.016 μ mol/(min mg) protein and a K_m value of 1.949 ± 0.069 μM. The standard errors for the two values are about 3% of the averaged values.

Rotenone Sensitivity

In the presence of 1 μM rotenone, NADH was no longer linearly oxidized in the initial 15 s portion of the enzyme reaction, since rotenone binding to the enzyme is not instantaneous. Preincubation of the enzyme with rotenone in the same medium as the substrate-free reaction mixture at 20°C for 30 s provided a linear absorbance decrease at 340 nm. The slope was identical to the slope attained after the initial decrease in the slope when the enzyme was added without preincubation with rotenone. The 30 s incubation at 20°C appears to be sufficient for attainment of equilibrium between the enzyme and the inhibitor since incubation for 1 h at 0°C gave no further decrease in the enzyme activity. The rotenone sensitivity was examined at various concentrations of rotenone as shown in Fig. 3 after the preincubation. In this experiment, a 50 μL portion of the preincubation mixture (75.6 μg enzyme/mL) in the presence of rotenone was added to the rotenone-free reaction mixture of 2 mL containing 10 μM NADH and 18 μM Q_1. The initial rate was plotted against the final concentration of rotenone in the reaction mixture. The results indicate that rotenone cannot completely

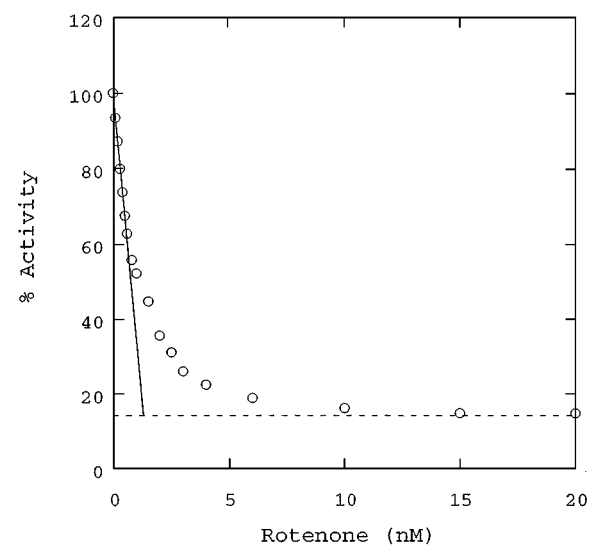


Fig. 3. Effect of rotenone on the enzyme activity of complex I at fixed substrate concentrations. The enzyme activities of complex I at 10 μM NADH and 18 μM Q_1 are plotted against the rotenone concentrations in the reaction mixture. The reaction mixture contained 10 μM NADH, 25 μM Q_1, and 1.84 μg complex I/mL in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.1% dodecylmaltoside. The dotted line denotes the enzyme reaction rate at 150 nM rotenone.

inhibit the enzyme activity. The concentration of rotenone binding sites determined from the decrease in the activity at rotenone concentrations below 0.9 nM as shown in Fig. 3 suggests that molecular weight of complex I is 1300 kDa. The inhibition of rotenone at 150 nM in the presence of 10 μM NADH (approximately 85% inhibition) was essentially independent of Q_1 concentration below 50 μM. However, the rotenone sensitivity decreased significantly with Q_1 concentration above 100 μM. Thus, in the present investigation, Q_1 concentration was limited below 50 μM. The rotenone sensitivity at a fixed Q_1 concentration was independent of rotenone concentrations above 20 nM–1 μM.

Steady-State Kinetic Analysis in the Absence of Products

The initial rates of NADH dependent (up to 10 μM NADH) oxidation by complex I (v_0) were determined at various fixed concentrations of Q_1 as shown in Fig. 4. At a fixed Q_1 concentration, each curve shows a simple rectangular hyperbolic relationship. The statistically calculated values for V_{max} and K_m are given in Table I. These parameters indicate that double reciprocal plots ($1/v_0$ vs. 1/NADH) would intersect at the abscissa, since the K_m values given in each concentration range are identical to each other within experimental error. These double reciprocal plots are given in the inset of Fig. 3. The rectangular hyperbolic curves giving these double reciprocal plots are shown in the main panels. No systematic deviation from the calculated rectangular hyperbolic curves is detectable.

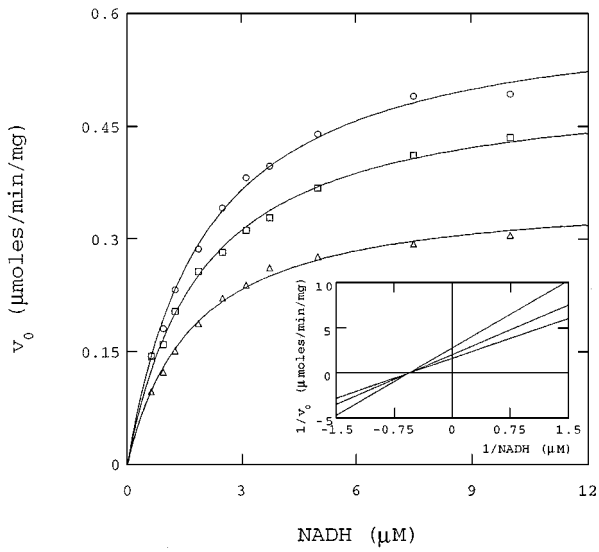


Fig. 4. NADH-dependent oxidation rates at various fixed concentrations of Q_1 in the absence of products. The Q_1 concentrations were 50 μM (circle), 25 μM (square), and 10 μM (triangle). The enzyme concentration was 20.5 μg/mL, in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.1% dodecylmaltoside. Solid curves were obtained by fitting the data to the Michaelis–Menten equation. Inset is the double reciprocal plots of the NADH dependent rates obtained by the V_{max} and K_m values which give the rectangular hyperbolic curves in the main panel.

The reliability of a data point in a double reciprocal plot has a strong dependence on the v_0 value. (The smaller the v_0 value, the lower the reliability of $1/v_0$.) Thus, fitting a straight line to a double reciprocal plot without statistical treatment is very likely to result in an erroneous fitting. Each straight line is transformed to the corresponding hyperbolic curve to show the extent of deviations of

Table I. Kinetic Parameters for NADH–Q_1 Reductase

Varying substrate	Fixed substrate	Fixed substrate concentration (μM)	Product	Product concentration (μM)	V_{max} (μ mol/min mg) (SE)	K_m(μM) (SE)
NADH	Q_1	10	–	0	0.365 (0.007)	1.720 (0.098)
		25		0	0.513 (0.012)	1.954 (0.124)
		50		0	0.613 (0.012)	2.051 (0.112)
	Q_1	25	NAD^+	0	0.513 (0.012)	1.954 (0.124)
				20	0.471 (0.005)	1.885 (0.053)
				80	0.458 (0.011)	2.433 (0.148)
				100	0.436 (0.009)	2.478 (0.136)
Q_1	NADH	6	NAD^+	0	0.794 (0.016)	12.915 (0.841)
				100	0.604 (0.012)	9.751 (0.694)
				200	0.537 (0.006)	9.374 (0.353)
				400	0.469 (0.008)	10.164 (0.561)
	NADH	6	Q_1H_2	0	0.794 (0.016)	12.915 (0.841)
				25	0.794 (0.013)	15.277 (0.767)
				75	0.778 (0.021)	18.674 (1.260)

the data points from the calculated curve. A standard error for determination of a v_0 value at a certain substrate concentration is determined mainly by the accuracy of the apparatus for the determination, not by the absolute value of v_0. Thus, it is reasonable to assume that the sizes of error bars for all the data points in a set of v_0–NADH determinations are essentially identical. Thus, deviations of the data points from the theoretical curve would be shown most clearly in the hyperbolic relation between v_0 and NADH concentration.

Product Inhibition for the Complex I Reaction

Product inhibition was examined for determination of the binding order of the two substrates and the order of release of the two products in the sequential complex I reaction for all substrate–product combinations. Inhibition of the enzyme by NAD^+ was investigated for both NADH and Q_1, giving noncompetitive inhibition patterns, as shown in Figs. 5A and B. The product inhibition of Q_1H_2 against Q_1 was examined in the presence of Q_1 below 50 μM as shown in Fig. 5C. The highest total concentration of Q_1 and Q_1H_2 was 125 μM. Under these conditions, Q_1H_2 is unlikely to induce the rotenone insensitive reaction. No reverse reaction was detectable in the electron transfer from NADH to Q_1 by this enzyme even when Q_1 was in large excess compared to NADH in the reaction mixture. Thus, the product inhibition by Q_1H_2 is likely to inhibit only the release of Q_1H_2 from the active site. On the other hand, in the concentration range of Q_1 below 50 μM, essentially no rotenone insensitive electron transfer would occur. Thus, Q_1H_2 at this concentration is likely to inhibit only the rotenone sensitive pathway. The parameters shown in Table I indicate competitive inhibition of Q_1H_2 against Q_1. The inhibition of Q_1H_2 against NADH showed that even 100 μM Q_1H_2 did not provide any statistically significant product inhibition under the

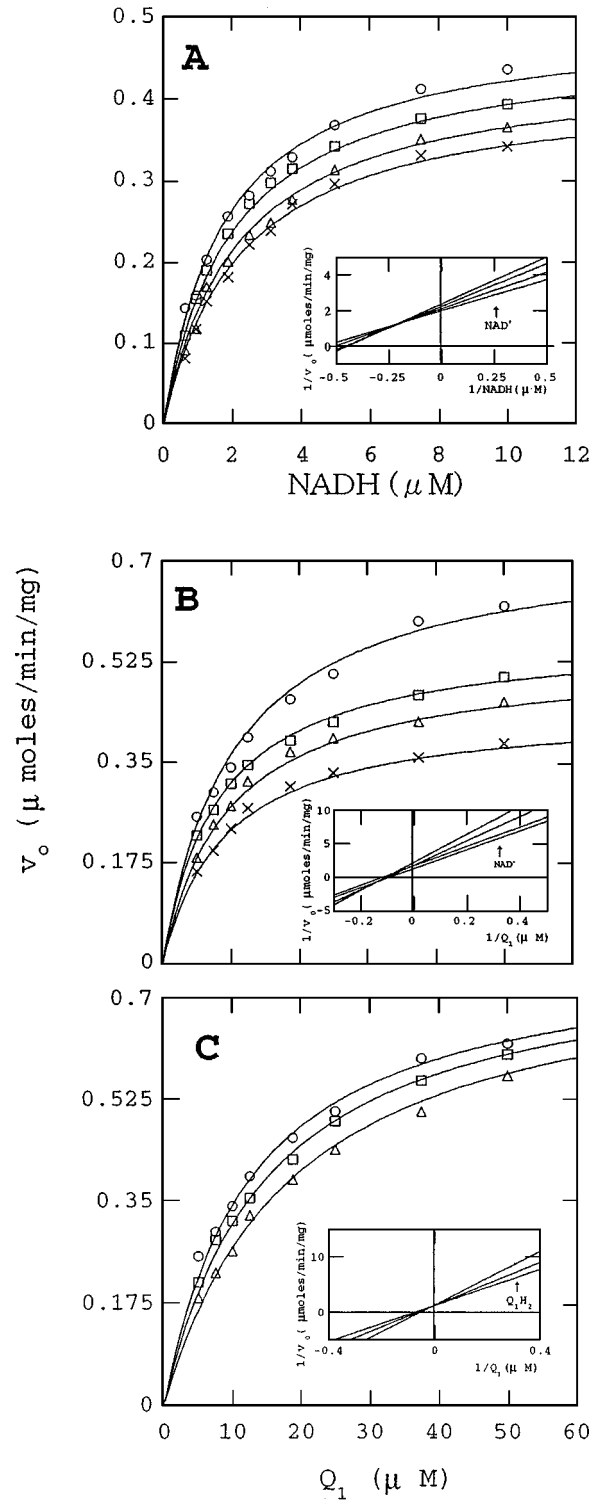
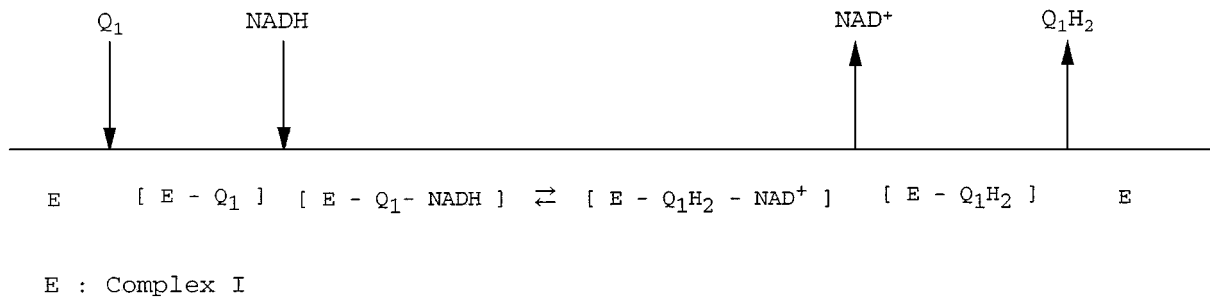


Fig. 5. Product inhibition for the NADH:Q_1 reductase reaction at Q_1 concentrations below 50 μM. Solid curves were determined by fitting the data to the Michaelis–Menten equation. The insets are the double reciprocal lines of the solid curves. (A) NAD^+ concentrations are 0 μM (circle), 20 μM (square), 80 μM (triangle) and 200 μM (cross) with varying concentrations of NADH at a fixed Q_1 concentration of 25 μM. (B) NAD^+ concentrations are 0 μM (circle), 100 μM (square), 200 μM (triangle) and 400 μM (cross) with varying concentrations of Q_1 at a fixed NADH concentration of 6 μM. (C) Q_1H_2 concentrations are 0 μM (circle), 25 μM (square) and 75 μM (triangle) with varying concentrations of Q_1 at a fixed NADH concentration of 6 μM. The enzyme concentration is 20.5 μg/mL in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.1% dodecylmaltoside.



Scheme 1.

present experimental conditions. However, the order of substrate binding and product release is able to be determined without knowing the effect of Q_1H_2 against NADH concentration. The above results indicate an obligatory order of substrate binding and product release as follows: Q_1–NADH–NAD^+–Q_1H_2 (Scheme 1) (Cleland, 1977).

DISCUSSION

Enzyme Activity

Under the standard assay conditions for the initial steady-state rate of complex I, NADH and Q_1 have been used as the two substrates, in the presence of phospholipids (asolectin) sonicated with cholate (Hatefi, 1978; Leif *et al.*, 1995). The sonicated phospholipids significantly stimulate the rotenone sensitive activity, depending on the structure of the acceptor Q or its analogues (Ragan, 1978). However, we found that the sonicated phospholipids cause deterioration of the reproducibility of the enzyme activity measurement. Instead, we used a nonionic detergent, dodecylmaltoside (0.1%) for stabilization and homogeneous dispersion of the enzyme preparation in the reaction mixture. The specific activity value obtained under these experimental conditions is reasonably consistent with specific activity values obtained at 24°C for the purified complex I (Ragan, 1978). However, a significantly higher value for a purified preparation has been reported at 37°C. The discrepancy is likely to be due to the fairly high activation energy of the NADH–Q_1 reductase reaction catalyzed by complex I, suggesting about 7 times increase in the enzyme activity as a result of changing the temperature of the reaction system from 20 to 37°C (Ragan, 1978). Thus, our determination of enzyme activity under similar experimental conditions corresponds to about half of the reported value (Yamaguchi *et al.*, 1998) ($69\ s^{-1}$ vs $145\ s^{-1}$). Our enzyme assay system did not contain phospholipids, which act as effective stimulants for complex I, while dodecylmaltoside in our assay system stimulates the enzyme function by only 20–30% as stated above. Thus, our enzyme preparation is apparently as active as Hatefi's preparation. Fato *et al.* recently reported enzyme activities for mitochondrial and submitochondrial preparations, which are about 3 times larger than that of Hatefi's report (Fato *et al.*, 1996). However, they estimated the amount of complex I in the membrane preparations via an NADH–ferricyanide reductase activity (Cremona and Kearney, 1964).

Rotenone Sensitivity

About 85% of the total activity was abolished by rotenone above 20 nM regardless of the Q_1 concentration below 50 μM. The decrease in the enzyme activity with rotenone concentration up to 20 nM indicates an extremely strong affinity of rotenone to the enzyme. However, rotenone does not completely inhibit the enzyme activity. The residual activity (about 15% of the total activity) is strictly constant from 20 nM to 1 μM at a constant Q_1 concentration below 50 μM. It is quite unlikely that the isolated complex I preparation used for this investigation is a mixture of two types (rotenone sensitive and insensitive) of complex I.

The observation that rotenone sensitive activity at low Q_1 concentrations which accounts for 85% of the total activity is consistent with Ragan's results (Ragan, 1978). Based on the effects of phospholipids and the quinone analogue structure on the rotenone sensitive and insensitive activities, Ragan proposed that the rotenone sensitive electron transfer path includes the Q_{10} binding site in the transmembrane area while the Q_1 binding site is outside of the membrane in the rotenone insensitive pathway. However, these results cannot exclude the possibility that the rotenone insensitive activity is due to a residual activity of the rotenone-bound enzyme, since no structural evidence has been obtained for the presence of the two quinone binding sites. Even if the Ragan's proposal is valid, the contributions of the rotenone insensitive reaction

to the overall reaction do not seem significant under the present experimental conditions. In the presence of saturating amounts of rotenone, the rotenone insensitive activity shows about 15% of the enzyme activity in the absence of rotenone. However, the two electron transfer pathways are common in most parts except for those near the Q_1 binding sites. Much faster rotenone sensitive electron transfer occupies the common pathway to inhibit the slower rotenone insensitive pathway. Thus, contribution of the rotenone insensitive electron transfer in the absence of rotenone seems significantly lower than 15%. In fact, no kinetic result thus far suggests the presence of the second enzyme reaction.

Reaction Mechanism

As shown in Fig. 3 inset, a set of intersecting lines for the double reciprocal plots of $1/v_0$ vs. 1/NADH at various fixed Q_1 concentrations indicates a sequential mechanism involving ternary complex including the enzyme and the two substrates. The product inhibition pattern identifies the order of the substrate binding and product release. Namely, for an enzyme system with two products and two substrates, an ordered sequential mechanism shows a competitive inhibition by the second product released from the enzyme against the first substrate to be bound to the enzyme. On the other hand, noncompetitive inhibition is observed for any other substrate–product combination (Cleland, 1977). In a random sequential mechanism, a ternary complex including the enzyme and the two substrates is formed as an intermediate species, but the two substrates bind to the enzyme randomly and the two products are released from the ternary complex without a specific order. This provides competitive inhibition for all substrate–product combinations (Cleland, 1977). Thus, a noncompetitive product inhibition for a substrate–product combination indicates an ordered sequential mechanism and disproves a random sequential mechanism. In addition to the noncompetitive inhibition, a competitive inhibition for a substrate–product combination determines the order of the substrate binding and the product release in the enzyme system. The product inhibition by Q_1H_2 against NADH is not large enough for determination of the inhibition pattern even at 100 μM Q_1H_2 under the present experimental conditions. Rotenone inhibition decreases significantly with increases in Q_1 concentration above 100 μM, suggesting that Q_1 at the concentration above 100 μM induces rotenone insensitive activity (unpublished results). Thus, the total concentration of Q_1 and Q_1H_2 was kept below 125 μM and Q_1 concentration was kept below 50 μM in the present work. An ordered sequential mechanism indicates ternary complex formation with a strong interaction between the NADH and Q_1 binding sites. Binding of Q_1 induces a conformational change in the NADH binding site to allow access of NADH. The resulting ternary complex, Q_1–enzyme–NADH, dissociates after electron transfer from NADH to Q_1. In this process, release of NAD^+ from the complex stimulates the dissociation of Q_1H_2.

It should be noted that until now, no extensive initial steady-state kinetic analysis including product inhibition analysis has been reported for this enzyme system. Thus, no information for the order of substrate bindings and product releases has been reported. Fato *et al.* reported a steady-state kinetic analysis for complex I using mitochondrial membrane and submitochondrial preparations in the Q_1 concentration range below 120 μM. They propose a ping-pong mechanism, that is, parallel double reciprocal plots (Fato *et al.*, 1996). However intersecting lines could be fitted to their results (see Fig. 2 in Fato *et al.*, 1996). No product inhibition for the system has been reported.

According to the widely accepted view, the NADH binding site is at the end of the large extramembrane portion protruding to the matrix space and Q_1 binding site is very near the transmembrane region (possibly ND1 subunit or PSST subunit in bovine enzyme) (Prieur *et al.*, 2001; Roth and Hägerhäll, 2001; Shuler *et al.*, 1999). Thus, the present ordered sequential mechanism indicates a long range interaction between the two binding sites, induced by tightly controlled conformational changes including many subunits, which may be one of the basic functions of this large multicomponent membrane protein.

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