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Effect of the Side Chain Structure of Coenzyme Q on the Steady State Kinetics of Bovine Heart NADH: Coenzyme Q Oxidoreductase

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Steady state kinetics of bovine heart NADH: coenzyme Q oxidoreductase using coenzyme Q with two isoprenoid unit (Q_2) or with a decyl group (DQ) show an ordered sequential mechanism in which the order of substrate binding and product release is NADH-Q_2 (DQ) -Q_2H_2 (DQH_2)-NAD^+ in contrast to the order determined using Q_1 (Q_1-NADH-NAD^+-Q_1H_2) (Nakashima et al., *J. Bioenerg. Biomembr.* **34**, 11–19, 2002). The effect of the side chain structure of coenzyme Q suggests that NADH binding to the enzyme results in a conformational change, in the coenzyme Q binding site, which enables the site to accept coenzyme Q with a side chain significantly larger than one isoprenoid unit. The side chains of Q_2 and DQ bound to the enzyme induce a conformational change in the binding site to stabilize the substrate binding, while the side chain of Q_1 (one isoprenoid unit) is too short to induce the conformational change.

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

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 electron transfer chain (Hatefi, 1985; Walker, 1992; Weiss *et al.*, 1991). The structure and function of complex I, the largest electron transfer complex in the mitochondrial respiratory chain (total mass of about 1000 kDa) (Buchanan and Walker, 1996), have been extensively studied by many workers (Buchanan and Walker, 1996; Hatefi, 1985; Ohnishi, 1998; Walker, 1992; Weiss *et al.*, 1991) since the discovery of this complex by Hatefi about 40 years ago (Hatefi *et al.*, 1961).

One of the most fundamental functional studies for an enzyme system is the initial steady state kinetic analysis under turnover conditions. The results of these kinetic analysis provide unique functional information, such as the order of substrate binding and product release from the enzyme. The information is difficult to obtain by other methods (Cleland, 1977). However, the first complete initial steady state kinetic analysis (including product inhibition analysis) was not accomplished until 2002 (Nakashima *et al.*, 2002a). In the previous analysis, Q_1 (coenzyme Q with only one isoprenoid unit) was used instead of the natural substrate Q_{10}, because of the low solubility of Q_{10} in aqueous buffers (Nakashima *et al.*, 2002a). The kinetic results indicate an ordered sequential mechanism with Q_1 being the first substrate to bind to the enzyme and Q_1H_2 the last product to be released. The kinetic results are strongly indicative of rigorous control between the bindings sites for NADH and Q_1, which has been suggested to be separated from each other at a great distance within the enzyme structure.

To study further the mechanism of the interaction between the two substrate binding sites, the role of the side chain structure of coenzyme Q with regard to the steady state kinetic properties of complex I was examined using Q_2 and decy1 coenzyme Q (DQ) as the substrates. The

Abbreviations used: Complex I, NADH:coenzyme Q oxidoreductase; Qn, coenzyme Q (ubiquinone) with *n* isoprenoid units; QnH_2, reduced coenzyme Qn; DQ, decylubiqinone; DQH_2, reduced decylubiquinone.

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present results indicate that the order of interaction of the reactants with the enzyme is NADH-Q_2 (or DQ)-Q_2H_2 (or DQH_2)-NAD^+, in contrast to the previously reported order, Q_1-NADH-NAD^+-Q_1H_2 (Nakashima *et al.*, 2002a,b).

EXPERIMENTAL PROCEDURES

Piericidine A was prepared by the method of Tamura *et al.* (1963). The reduced forms of Q_2 and DQ were prepared by the method of Rieske (1967). Other materials and enzyme reaction assay methods are as described in our previous paper (Nakashima *et al.*, 2002a).

RESULTS

Steady State Kinetic Analysis Employing Q_2 as a Coenzyme Q Substrate

The stoichiometric reduction of Q_2 with NADH under the present experimental conditions was monitored via comparison of the absorbance changes at 340 and 290 nm as described in the previous paper (Nakashima *et al.*, 2002a). At a saturating concentration of NADH (10 μM), the enzyme activity increases with increasing Q_2 concentration in a rectangular hyperbolic relationship, which yields a K_M value of 4.0 μM. No further activity increase with Q_2 concentrations up to 200 μM was observed in clear contrast to the enzyme assay system using Q_1 instead of Q_2 as reported in the previous paper. A total of 95–90% of the activity was inhibited by piericidin A in the concentration range of Q_2 up to 100 μM. Thus, below 100 μM of Q_2 concentration, piericidin A-insensitive reaction is negligible.

Figure 1 shows the enzyme activity increase with increasing NADH concentration at various fixed concentrations of Q_2. At each Q_2 concentration a simple rectangular hyperbolic curve was obtained. The V_{max} and K_M values are given in Table I. The data yield a set of straight double reciprocal plots (1/v versus 1/NADH) intersecting at a single point as shown in the inset. The results indicate that this enzyme binds both substrates to form a ternary complex before releasing products in a sequential mechanism. For determination of the order of substrate binding and product release, inhibitory effects of products (Q_2H_2 and NAD^+) were examined for all substrate–product combinations at a fixed concentration of the second substrate. The results are given in Fig. 2 and Table I. In any substrate–product combination, a set of simple hyperbolic relationships was obtained with intersecting straight double reciprocal plots as shown in the insets. The values of K_M and V_{max} of each

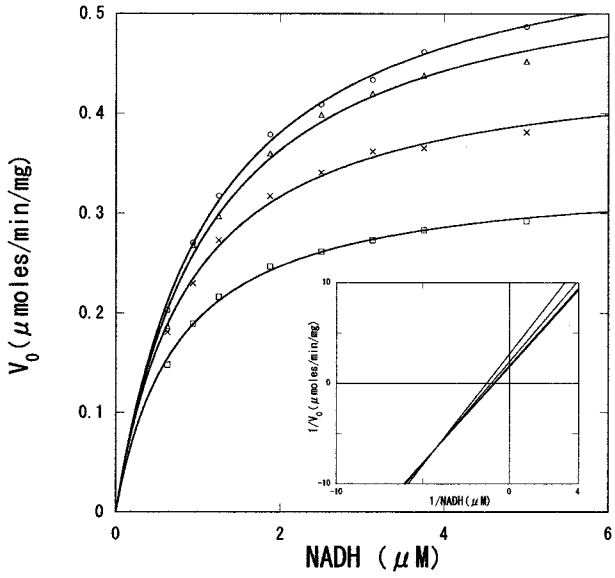


Fig. 1. NADH-dependent oxidation rates at various fixed concentrations of Q_2 in the absence of products. The Q_2 concentrations were 50 μM (circle), 25 μM (triangle), 10 μM (cross), and 5 μM (square). The enzyme concentration was 11 μg/mL, in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.2% dodecylmaltoside. Solid curves were obtained by fitting the data to the Michaelis–Menten equation. Inset: The double reciprocal plots of the NADH dependent rates obtained by V_{max} and K_M values, which give the rectangular hyperbolic curves in the main panel.

line are given in Table I. Only the NADH-NAD^+ combination yields an interesting point at the ordinate indicating competitive inhibition, while the other combinations indicate noncompetitive inhibition. These results indicate an ordered sequential mechanism with NADH as the initial substrate to bind to the enzyme, and NAD^+ the last product to be released from the enzyme.

Steady State Kinetic Analysis Employing DQ as a Coenzyme Q Substrate

The molar equivalents of oxidized NADH and reduced DQ during the enzyme reaction, under the present initial steady state conditions (DQ concentration below 200 μM) as determined by the method described above, indicate the stoichiometric oxidation of NADH with DQ. Below 200 μM DQ concentration, the enzyme activity increases in a simple rectangular hyperbolic fashion with respect to DQ concentration with K_M of 70 μM at 10 μM NADH. However, above 300 μM DQ, a significant deviation to lower values (an apparent substrate inhibition) is detectable. In the concentration range where the apparent substrate inhibition is detectable, the amount of DQ reduced was lower than 70% of the amount of NADH

Table I. Kinetics Parameters for NADH-Q_2 Reductase

Varying substrate	Fixed substrate (μM)		Product (μM)		V_{max} (μmoles/min/mg) (SE)	K_M(μM)(SE)
NADH	Q_2	5		0	0.340 (0.004)	0.751 (0.034)
		10		0	0.466 (0.015)	0.947 (0.097)
		25		0	0.567 (0.019)	1.097 (0.114)
		50		0	0.598 (0.022)	1.112 (0.120)
	Q_2	10	NAD^+	0	0.451 (0.010)	0.936 (0.062)
				100	0.435 (0.011)	1.032 (0.077)
				200	0.441 (0.016)	1.209 (0.125)
				400	0.436 (0.015)	1.356 (0.129)
	Q_2	25	Q_2H_2	0	0.562 (0.013)	1.013 (0.075)
				25	0.452 (0.012)	0.855 (0.077)
				50	0.389 (0.007)	0.780 (0.062)
Q_2	NADH	5	NAD^+	0	0.545 (0.009)	5.071 (0.516)
				50	0.516 (0.006)	5.158 (0.277)
				200	0.491 (0.005)	5.365 (0.228)
				400	0.486 (0.007)	5.526 (0.331)
	NADH	5	Q_2H_2	0	0.538 (0.007)	4.391 (0.265)
				25	0.479 (0.008)	7.129 (0.420)
				35	0.440 (0.013)	7.964 (0.777)
				50	0.413 (0.012)	8.319 (0.807)

oxidized during the initial NADH oxidation by the enzyme system. Thus, the present kinetic analysis was performed with DQ concentration below 200 μM.

Figures 3 and 4 show the initial steady state kinetic results under various substrate concentrations in the absence and presence of products, respectively. The kinetic parameters obtained from these results are given in Table II. In the absence of products, the double reciprocal plots give a set of parallel lines (Fig. 3B). Because of the level of experimental accuracy for the kinetic measurements, it is impossible to identify the difference in the slopes of the double reciprocal lines statistically. However, the apparent parallel lines, which are suggestive of a ping-pong-type substrate binding and product release mechanism, are not confirmed by the product inhibition results. A ping-pong mechanism with two substrates and two products would provide two cases of competitive inhibition and two cases of noncompetitive product inhibition (Cleland, 1977). However, the present results show one case of competitive inhibition (for the NADH-NAD^+ combination) and three cases of noncompetitive product inhibition. Furthermore, in the ping-pong mechanism, NAD^+ cannot competitively inhibit the enzyme activity versus NADH. Thus the product inhibition results indicate that, in the absence of products, the apparent parallel lines intersect at a point far from the original point (Fig. 3). It is well known that only a set of parallel double reciprocal plots is not sufficient for elucidation of an enzyme reaction mechanism as a ping-pong type (Cleland, 1977). The order of substrate binding and product release deduced from these results is an ordered sequential mechanism as follows; NADH-DQ-DQH_2-NAD^+, which is identical to that for the system including Q_2 as given above. It should be noted that K_M values for DQ are about one order higher than those for Q_2 (Table I) and Q_1 (Nakashima *et al.*, 2002a).

Product Inhibition for the Rotenone-Sensitive Reaction by Q_1H_2 Against NADH in the Enzyme System Including Q_1 as the Coenzyme Q Substrate

Under the previous experimental conditions for the analysis of product inhibition by Q_1H_2 against NADH (Nakashima *et al.*, 2002a), the determination of the product inhibition was not accurate enough for identification of the class of inhibition, since the Q_1H_2 concentration is limited to prevent occurrence of the rotenone-insensitive reaction. In the present work, by increasing the enzyme concentration, the product inhibition was reexamined accurately enough to conclude that the product inhibition is noncompetitive as given in Fig. 5. The kinetic parameters are shown in Table III.

Steady State Kinetic Analysis Using Q_0 as a Coenzyme Q Substrate

The determination of reaction stoichiometry during the course of the reduction of Q_0 with NADH using the extinction coefficients of ubiquinone determined for Q_1

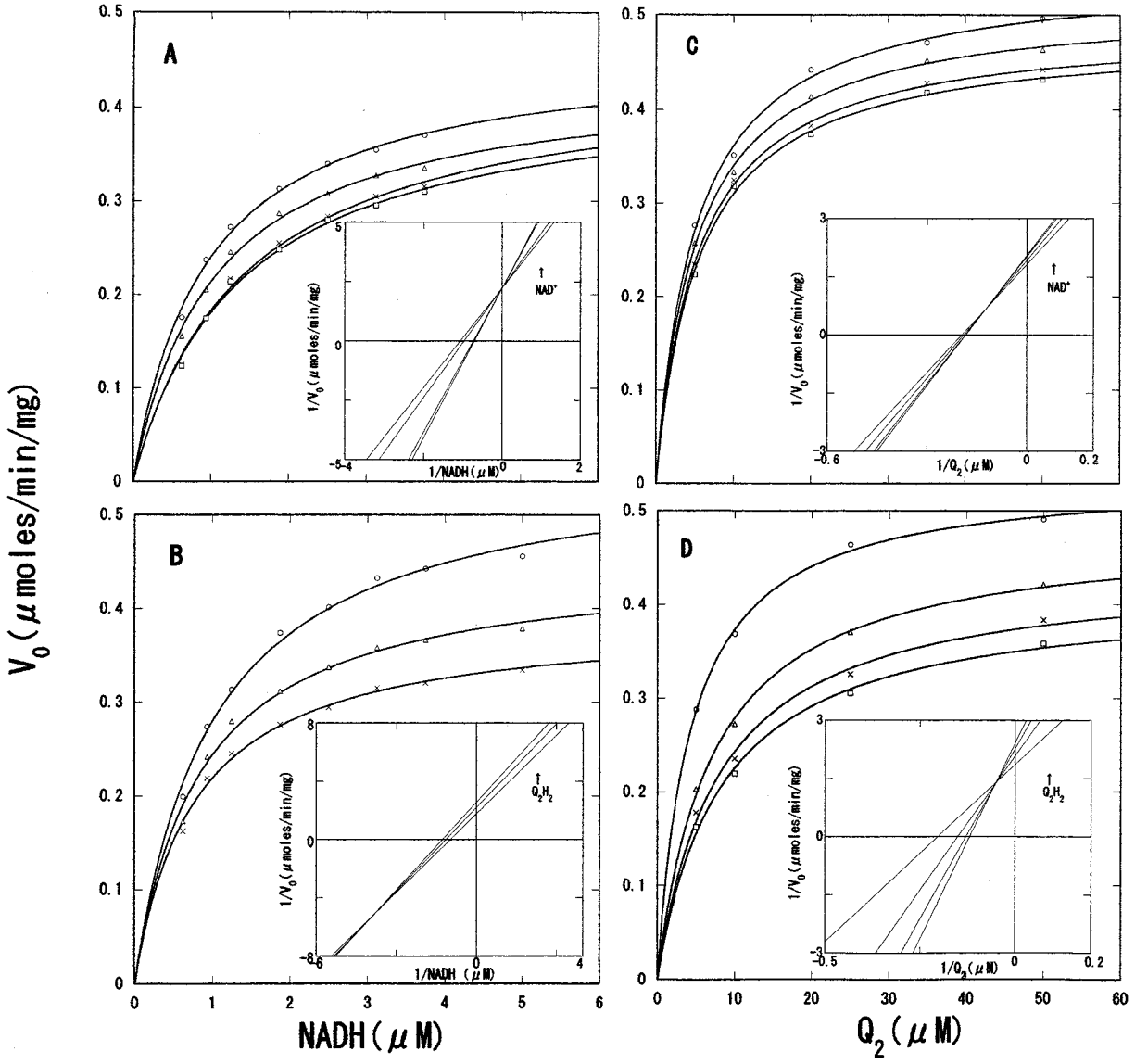


Fig. 2. Product inhibition for the NADH:Q_2 reductase reaction with the total Q_2 concentration range below 100 μM. Solid curves were obtained by fitting the data to the Michaelis–Menten equation. The insets are the double reciprocal lines of the solid curves. (A) NAD^+ concentrations are 0 μM (circle), 100 μM (triangle), 200 μM (cross), and 400 μM (square) at a fixed Q_2 concentration of 10 μM. (B) Q_2H_2 concentrations are 0 μM (circle), 25 μM (triangle), and 50 μM (cross) at a fixed Q_2 concentration of 25 μM. (C) NAD^+ concentrations are 0 μM (circle), 50 μM (triangle), 200 μM (cross), and 400 μM (square) at a fixed NADH concentration of 5 μM. (D) Q_2H_2 concentrations are 0 μM (circle), 25 μM (triangle), 35 μM (cross), and 50 μM (square) at a fixed NADH concentration of 5 μM. The enzyme concentration was 11 μg/mL, in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.2% dodecylmaltoside.

indicated that only about 30% of electrons from NADH were transferred to Q_0. This effect has been determined as due to the instability of Q_0H_2 under the present reaction media, which was confirmed by significant absorbance change of Q_0H_2 in aqueous media. The instability of Q_0H_2 is likely to be due the structure of Q_0 in which the isoprenoide chain is replaced with a hydrogen atom. In one electron reduced state of Q_0 (the semiquinone state), the radical at the carbon atom with the hydrogen atom provides a very reactive carbon atom, since the radical is not protected by any alkyl group as in Q_1, Q_2, and DQ. Thus, this reactive carbon atom could initiate various irreversible side reactions. Attempts to stabilize Q_0H_2 via manupulation of the composition of the reaction media, temperature, and anaerobiosis were unsuccessful. However, in the initial steady state kinetic analysis in the absence of products,

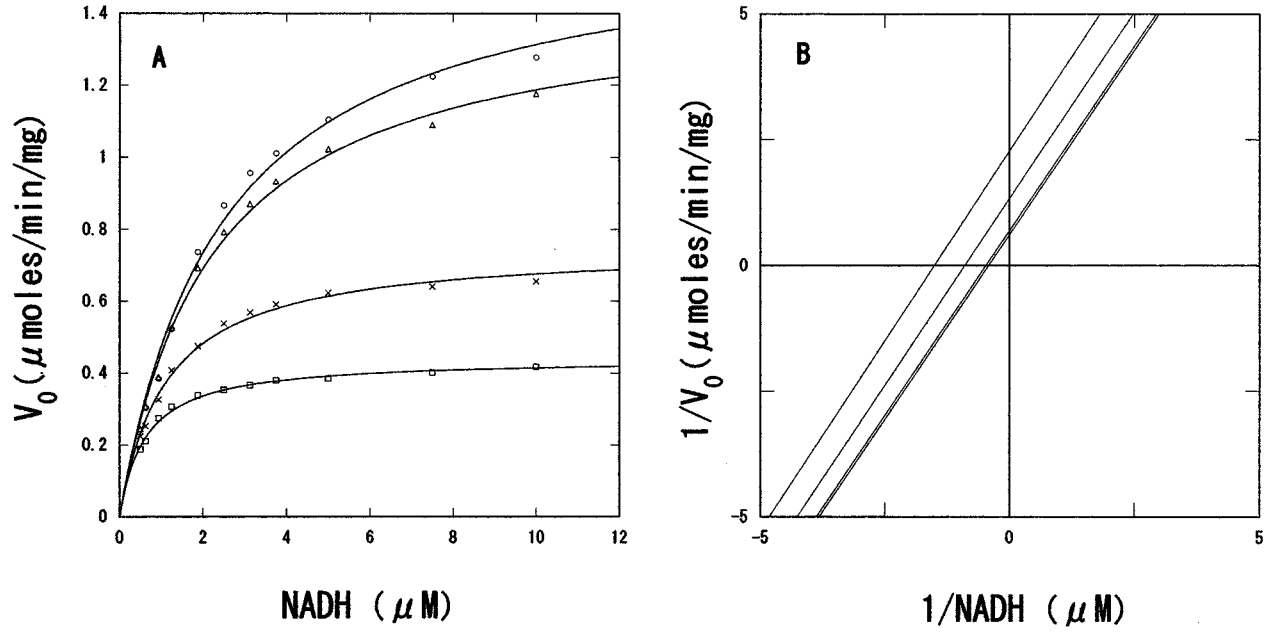
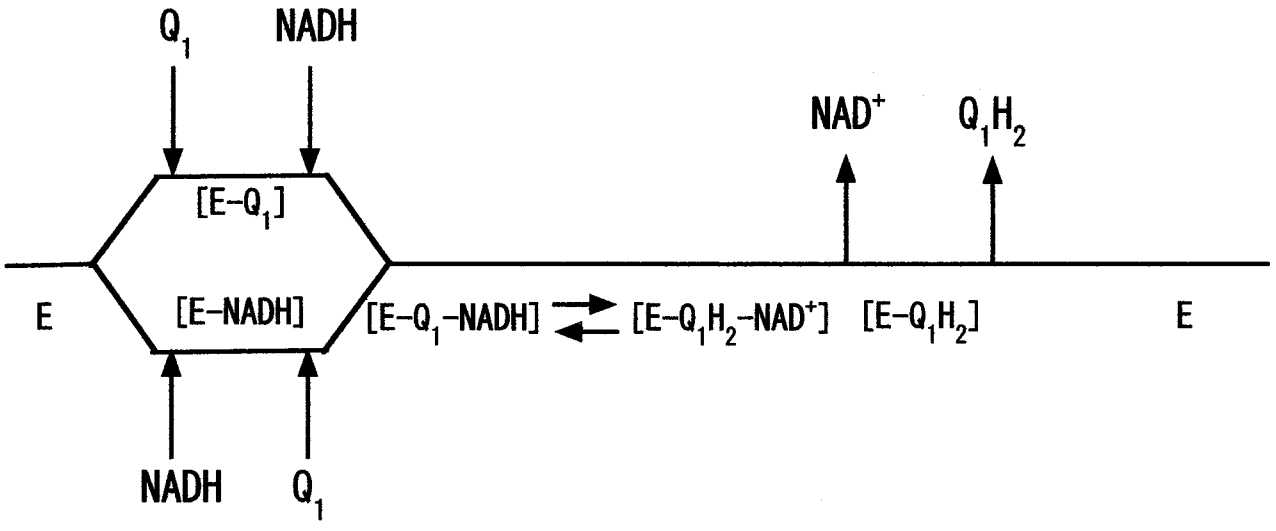
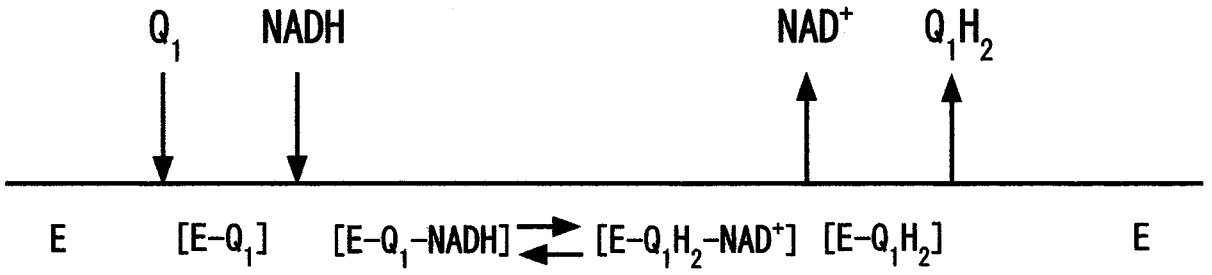


Fig. 3. NADH-dependent oxidation rates at various fixed concentrations of DQ in the absence of products. The DQ concentrations were 200 μM (circle), 100 μM (triangle), 50 μM (cross), and 25 μM (square). The enzyme concentration was 6 μg/mL, in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.2% dodecylmaltoside. Solid curves in A were obtained by fitting the data to the Michaelis–Menten equation. The double reciprocal plots of the NADH dependent rates in B were obtained by the V_{max} and K_M values, which give the rectangular hyperbolic curves in A.

effects of the possible degraded Q_0 are expected to be negligible. Piericidine A inhibition was only about 60% even at Q_0 concentrations below 10 μM, while the inhibition did not decrease significantly with Q_0 concentration up to 600 μM where about 40% inhibition was observed. This result suggests that Q_0 has essentially identical affinity for the sites of the piericidine A-sensitive and -insensitive reactions. This indicates that the piericidine A-sensitive and- insensitive reactions coexist with the whole Q_0 concentration range where the enzyme reaction is measurable



Scheme 1



Scheme 2

accurately. These two observations indicate that the complex I enzyme system, which employs Q_0 as the coenzyme Q substrate, is not suitable for the complete steady state kinetic analysis.

DISCUSSION

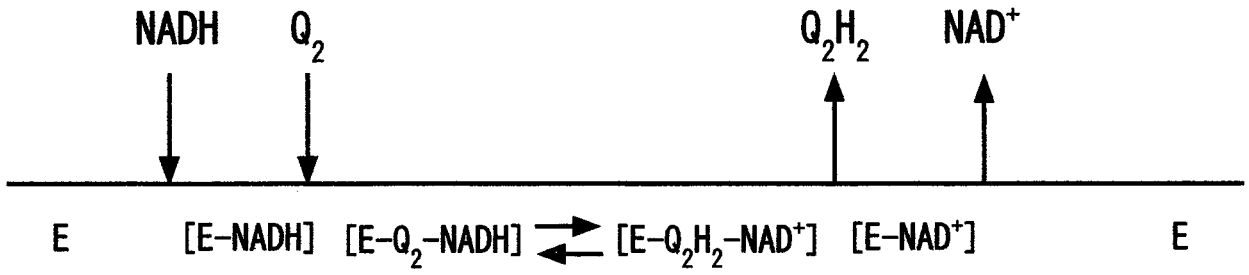
Steady State Kinetic Analysis of the Enzyme System Including Q_1 as the Coenzyme Q Substrate

In our previous paper, the kinetic analysis in the absence of products and product inhibition for the three combinations, NADH-NAD^+, Q_1-NAD^+, and Q_1-Q_1H_2 resulted in the conclusion that the enzyme follows an ordered sequential mechanism with Q_1 as the first substrate and Q_1H_2 as the last product (Nakashima *et al.*, 2002a). Product inhibition for the NADH-Q_1H_2 combination could not be accurately determined. However, competitive product inhibition for the combination would give a mechanism composed of random substrate binding and ordered product release (Scheme 1), while noncompetitive inhibition would provide an ordered sequential mechanism (Scheme 2). The results of this present work exclude the possibility of Scheme 1.

Mechanism of Coenzyme Q Binding to the Protein Pocket

Addition of one isoprenoid unit to Q_1 increases greatly the hydrophobicity of the molecule. However, only about a 60% decrease in K_M value is observed (12.9 μM vs. 5.1 μM) (Table I and Table I in (Nakashima *et al.*, 2002a)). On the other hand, DQ, which has hydrophobicity similar to Q_2, exhibits 16.7-fold increase in K_M value over Q_2. These results clearly indicate a highly specific interaction between the isoprenoid moiety and the protein pocket during the process of coenzyme Q binding.

As shown in Schemes 2 and 3, the structure of the side chain of coenzyme Q greatly influences the order of substrate binding and product release. Namely, in the Q_1 reaction system, Q_1 is the first substrate to bind to the enzyme and Q_1H_2 is the last product released (Nakashima *et al.*, 2002a; and the present work), while in Q_2 or DQ reaction systems investigated in the present work, NADH is the initial substrate to bind to the enzyme and NAD^+ is the last product released. The simplest interpretation for the results is as follows: the enzyme free from both substrates and products (E) has a Q_1 binding pocket that is too small to accept Q_2 or DQ. However, when NADH binds at a distinct site from the Q_1 binding pocket, a conformational



Scheme 3

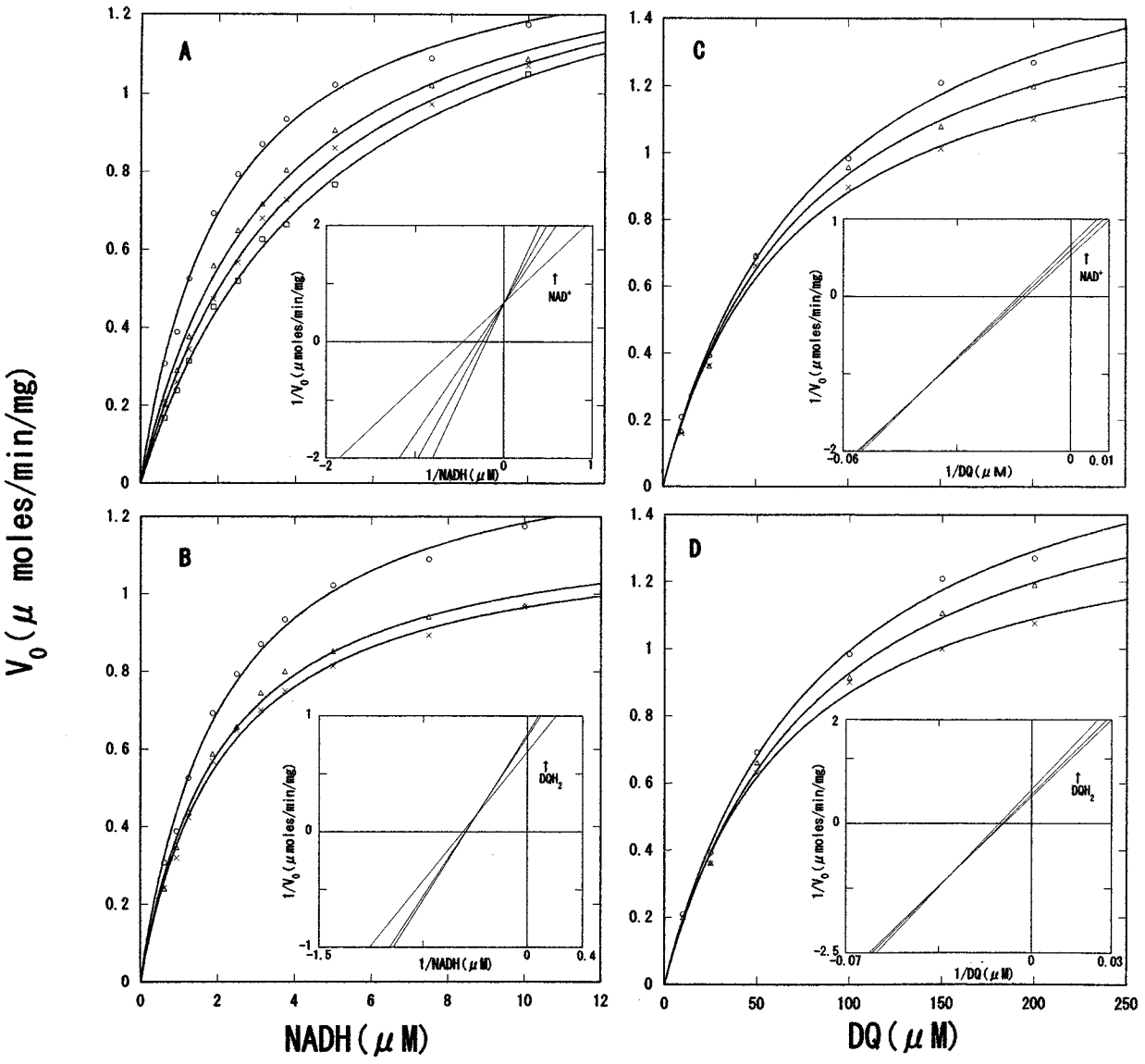


Fig. 4. Product inhibition for the NADH:DQ reductase reaction. Solid curves were obtained by fitting the data to the Michaelis–Menten equation. Inset: The double reciprocal plots of the NADH-dependent rates obtained by the V_{max} and K_M values which give the rectangular hyperbolic curves in the main panel. (A) NAD^+ concentrations are 0 μM (circle), 200 μM (triangle), 400 μM (cross), 600 μM (square) at a fixed DQ concentration of 100 μM. (B) DQH_2 concentrations are 0 μM (circle), 100 μM (triangle), and 200 μM (cross) at a fixed DQ concentration of 100 μM. (C) NAD^+ concentrations are 0 μM (circle), 400 μM (triangle), and 600 μM (cross) at a fixed NADH concentration of 10 μM. (D) DQH_2 concentrations are 0 μM (circle), 100 μM (triangle), and 200 μM (cross) at a fixed NADH concentration of 10 μM. The enzyme concentration was 6 μg/mL, in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.2% dodecylmaltoside.

change in the pocket is induced so that the pocket can accept Q_2 but it cannot accept Q_1. Thus, the NADH binding is required for the Q_2 binding. When Q_1 is used as one of the substrates, as shown in Scheme 2, Q_1 binds to E before NADH binding. As shown in Scheme 3, NADH is also able to bind to E. However, the conformational change induced by NADH inhibits Q_1 binding as stated above. In other words, E-NADH complex is a dead-end complex when Q_1 is used as one of the substrates, giving the ordered sequential mechanism as given by Scheme 2. Schemes 2 and 3 indicate that in the NADH-bound enzyme, Q_2 has much stronger affinity to the protein pocket than does Q_1, which is smaller than Q_2. The results strongly suggest that the second isoprenoid unit of Q_2 causes an "induced-fit"-type conformational change in the pocket to stabilize the Q_2 bound in the pocket (Koshland, 1973). However, because

Table II. Kinetics Parameters for NADH-Decylubiquinone Reductase

Varying substrate	Fixed substrate (μM)		Product (μM)		V_{max} (μmoles/min/mg) (SE)	K_M(μM)(SE)
NADH	DQ	25		0	0.441 (0.005)	0.617 (0.034)
		50		0	0.752 (0.014)	1.118 (0.070)
		100		0	1.447 (0.035)	2.185 (0.137)
		200		0	1.635 (0.059)	2.443 (0.224)
	DQ	100	NAD^+	0	1.484 (0.035)	2.142 (0.140)
				200	1.435 (0.047)	3.358 (0.243)
				400	1.521 (0.038)	4.094 (0.214)
				600	1.557 (0.046)	4.915 (0.275)
	DQ	100	DQH_2	0	1.484 (0.035)	2.143 (0.140)
				100	1.210 (0.032)	1.117 (0.150)
				200	1.177 (0.027)	2.193 (0.134)
DQ	NADH	10	NAD^+	0	1.852 (0.079)	85.836 (8.599)
				400	1.648 (0.107)	74.445 (12.03)
				600	1.491 (0.062)	68.787 (7.351)
	NADH	10	DQH_2	0	1.852 (0.079)	85.836 (8.599)
				100	1.695 (0.062)	82.602 (7.183)
				200	1.440 (0.059)	64.590 (6.924)

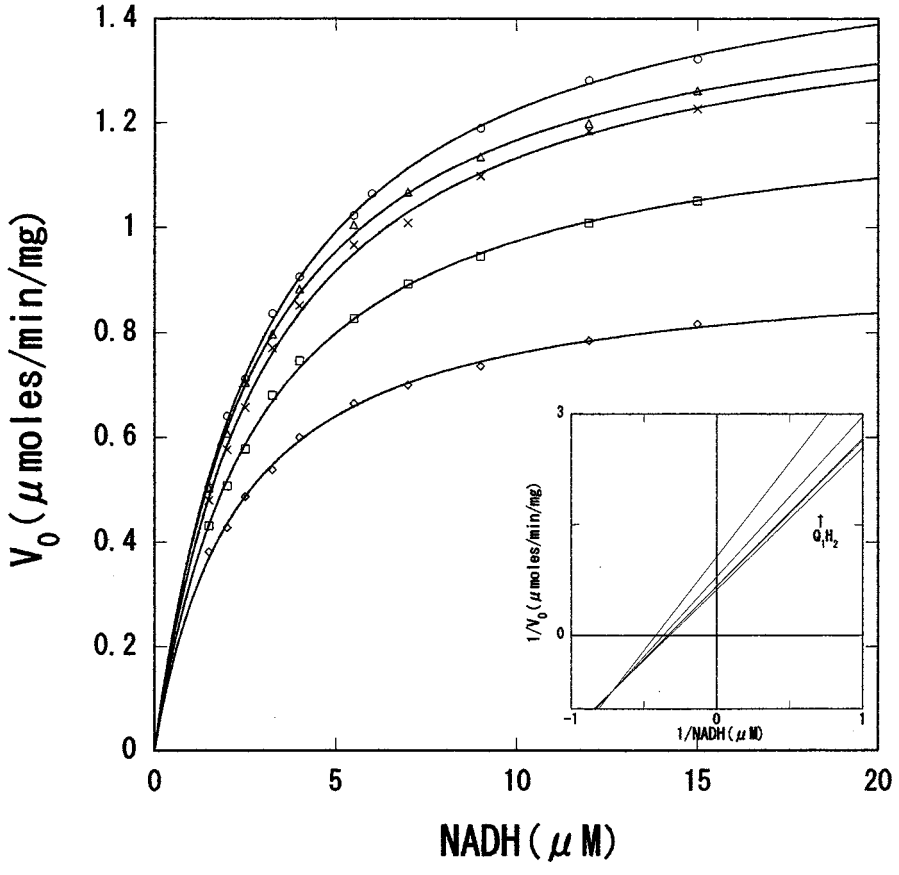


Fig. 5. Product inhibition for the NADH:Q_1 reductase reaction. Solid curves were obtained by fitting the data to the Michaelis–Menten equation. Inset: The double reciprocal plots of the NADH-dependent rates obtained by the V_{max} and K_M values which give the rectangular hyperbolic curves in the main panel. Q_1H_2 concentrations are 0 μM (circle), 15 μM (triangle), 30 μM (cross), 50 μM (square), and 80 μM (diamond) at a fixed Q_1 concentration of 20 μM. The enzyme concentration was 31.1 μg/mL, in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.2% dodecylmaltoside.

Table III. Kinetics Parameters for NADH-Q_1 Reductase in the Presence of Q_1H_2

Varying substrate	Fixed substrate (μM)	Product (μM)	V_{max} (μmoles/min/mg) (SE)	K_M(μM)(SE)
NADH	Q_1 20	Q_1H_2 0	1.604 (0.014)	3.008 (0.076)
		15	1.502 (0.013)	2.886 (0.072)
		30	1.481 (0.016)	3.071 (0.092)
		50	1.250 (0.010)	2.827 (0.068)
		80	0.934 (0.008)	2.292 (0.068)

Q_1 does not have the second isoprenoid unit which causes the "induced fit" for Q_2, Q_1 would not bind to the NADH-bound enzyme. The decyl group of DQ seems to be large (and flexible) enough for the "induced fit," although its K_M value is about one order higher than that of Q_2.

It is widely accepted that the NADH binding site is at the end of the large extramembrane moiety protruding to the matrix space and that the Q binding site is close to the transmembrane region. It should be noted that the strong and specific interaction appears to occur over a very long range. The occurrence of the "induced fit" in the Q-binding protein pocket could be coupled with the proton pump system of the enzyme.

The present results show that essentially no piericidine A-insensitive reaction is detectable when Q_2 and DQ were used for the coenzyme Q substrate. (It is well known that piericidine A-inhibition is essentially the same as that of rotenone.) On the other hand, approximately 50% of the reaction is piericidine A-sensitive in the Q_0 concentration range up to 600 μM. These results together with the presence of the rotenone-insensitive reaction at high Q_1 concentration confirm the previous proposal that this enzyme has two coenzyme Q binding sites, for the inhibitor-sensitive and -insensitive pathways (Nakashima *et al.*, 2002b). In the inhibitor-insensitive reaction, the affinity of coenzyme Q to the site decreases with increasing the size of the side chain. Thus, no significant piericidine A-insensitive reaction is detectable for the Q_2 or DQ enzyme reaction system. The rigorous control between the rotenone-insensitive Q binding site and the NADH binding site as shown in the previous paper suggests that the inhibitor-insensitive reaction is also physiologically relevant (Nakashima *et al.*, 2002b). The present results suggest that the inhibitor-insensitive electron transfer pathway is involved in a redox reaction including hydrophilic electron acceptors.

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