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The Second Coenzyme Q_1 Binding Site of Bovine Heart NADH: Coenzyme Q Oxidoreductase

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The rotenone sensitivity of bovine heart NADH: coenzyme Q oxidoreductase (Complex I) depends significantly on coenzyme Q_1 concentration. The rotenone-insensitive Complex I reaction in Q_1 concentration range above 300 μM indicates an ordered sequential mechanism with Q_1 and reduced Q_1 (Q_1H_2) as the initial substrate to bind to the enzyme and the last product to be released from the enzyme product complex, respectively. This is the case in the rotenone-sensitive reaction although both K_m and V_{max} values of the rotenone-insensitive reaction for Q_1 are significantly higher than those of the rotenone-sensitive reaction (Nakashima *et al*., 2002, *J. Bioenerg. Biomemb.* **34**, 11–19). This rigorous control mechanism between the nucleotide and ubiquinone binding sites strongly suggests that the rotenone-insensitive reaction is also physiologically relevant.

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[2]) is at the entrance of the mitochondrial respiratory chain (Hatefi, 1985; Walker, 1992; Weiss *et al.*, 1991) and catalyzes the reduction of coenzyme Q (Q) via a mechanism involving NADH coupled to the translocation of protons to the intermembrane space in a ratio of $4H^+/2e^-$ (Di Virgilio and Azzone, 1982; Esposti and Ghelli, 1994; Scholes and Hinkle, 1984; Weiss and Friedrich, 1991; Wikström, 1984). Complex I is an extremely large membrane protein complex with a flavin (FMN) (Hatefi, 1985; Walker, 1992; Weiss *et al.*, 1991) and several iron–sulfur clusters acting as redox active catalytic centers (Ohnishi, 1998; Ohnishi *et al.*, 1985). Due to difficulties encountered in the isolation of the large membrane-bound protein, both the structure and the reaction mechanism of this enzyme are poorly understood.

In our previous paper, we reported a steady state kinetic study on the rotenone-sensitive reaction which indicates an ordered sequential mechanism with the order of the substrate binding and product release as follows: Q_1—NADH—NAD^+—Q_1H_2 (Nakashima *et al.*, 2002). The mechanism suggests a long-range interaction between the NADH binding site and the Q_1 binding site. An increase in the rotenone-insensitive enzyme activity is observed with an increase in Q_1 concentration above the saturating Q_1 concentration for the rotenone-sensitive reaction. Thus we investigated the steady state kinetics of the rotenone-insensitive reaction, for elucidation of the function of this enzyme, particularly for understanding the mechanism of the rotenone inhibition. A complete steady state kinetic analysis of the reaction (Cleland, 1977) shows an ordered sequential mechanism with Q_1 and Q_1H_2 as the initial substrate to bind to the enzyme and the last product released, respectively. The rigorous control in the mechanism suggests that the rotenone-insensitive reaction is also physiologically relevant.

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Key to abbreviations: Complex I, NADH: coenzyme Q oxidoreductase; Qn, coenzyme Q (ubiquinone) with *n* isoprenoid units; Q_1H_2, reduced coenzyme Q_1; NMR, nuclear magnetic resonance.

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EXPERIMENTAL PROCEDURES

Materials and enzyme reaction assay methods are as described in the previous paper (Nakashima *et al.*, in press).

The NMR relaxation time (T_1) of Q_1 was measured with a JEOL Excalibur 400 spectrometer. NMR samples were measured in D_2O in the presence of dodecyl maltoside, ethanol, and phosphate buffer at the same concentrations as in the enzyme reaction mixture. Deuterated ethanol was used to remove the background due to ethanol —OH. A PW_2 (180° pulse) of 34 μs and PW_1 (90° pulse) of 17 μs were applied sequentially at a fixed 10-s interval while the pulse sequence repetition interval ranged from 0.1to 5 s. Data were averaged over 40–400 runs depending on the concentration of Q_1.

RESULTS

Dependence of Rotenone-Sensitivity on Q_1 Concentration

At a saturating concentration of rotenone significantly higher than the enzyme concentration (Nakashima *et al.*, in press), the rotenone inhibition was influenced strongly by Q_1 concentration. As shown in Fig. 1, the fractional inhibition of rotenone at 150 nM in the presence of 10 μM NADH decreased significantly with Q_1 concentration above 50 μM. At a Q_1 concentration below 20 μM Q_1, rotenone inhibition did not exceed 85%. On the other hand, above 400 μM, the rotenone-sensitive activity was much lower than that of the total activity (lower than 7%). In the high Q_1 concentration range, the saturation level of rotenone concentration was essentially the same as those in the Q_1 concentration range below 50 μM. Increasing the rotenone concentration above 150 nM up to 1 μM did not increase the rotenone inhibition in the presence of high concentrations of Q_1.

In the Q_1 concentration range for the previous and present experimental conditions (i.e. below 600 μM), NADH oxidation in the initial linear portion of the reaction was stoichiometric to the reduction of Q_1, monitored by the absorbance change at 290 nM, as described in the previous paper (Nakashima *et al.*, in press). The molar ratio of NADH oxidized to Q_1 reduced did not deviate from unity more than 0.05.

Steady State Kinetic Analysis

Steady State properties were examined mainly above 400 μM Q_1 where the rotenone-insensitive reaction accounted for more than 90% of the total activity. As shown in Fig. 2, simple rectangular hyperbolic curves were obtained at three fixed Q_1 concentrations (400, 500, and 600 μM). The statistically calculated values for V_{max} and K_m are given in Table I. The K_m values are identical within experimental error, indicating that the three double reciprocal plots ($1/v_0$ versus 1/NADH) intersect at the abscissa as shown in the inset. The solid lines in the main panel are rectangular hyperbolic curves constructed using the V_{max} and K_m values given in Table I. No systematic deviation is detectable from the theoretical curves. The results indicate a sequential mechanism for substrate binding and product release which includes an enzyme—Q_1—NADH ternary complex.

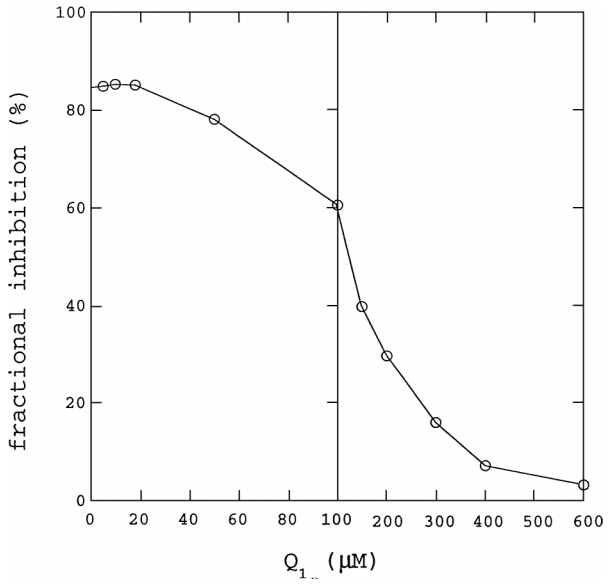


Fig. 1. Effect of Q_1 concentration on the rotenone sensitivity of Complex I. Fractional rotenone-inhibition values (percent decrease in the enzyme activity) of Complex I preincubated with an excess amount of rotenone as described in the text are plotted against the Q_1 concentration in the reaction mixture. The final concentrations of Complex I, rotenone, and NADH in the reaction mixture were 1.84 μg/mL, 150 nM, and 10 μM, respectively, in 0.1 M potassium phosphate buffer, pH 8.0, containing 0.1% dodecyl maltoside.

Product inhibition was examined for determination of the order of the substrate binding and the product release for all substrate–product combinations, as given in Fig. 3. A set of intersecting double reciprocal plots was obtained for each combination. Only the Q_1–Q_1H_2 combination gives the intersecting point at the ordinate showing competitive inhibition of Q_1H_2 against Q_1. Because of the large K_m values for Q_1, curves in Fig. 3(C) and (D) appear to be linear. However, Q_1 concentration ranges for these data points are not much lower than K_m values. In fact, the v_0 values on each solid line are not proportional to Q_1 concentration. Thus, it is reasonable to assume a

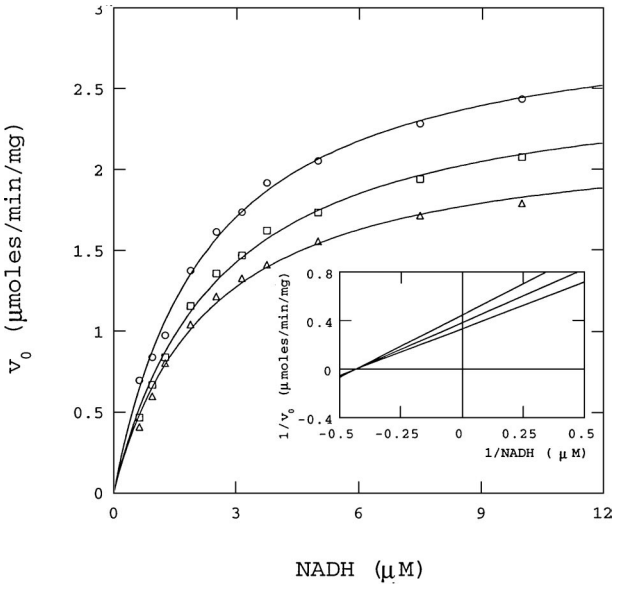


Fig. 2. NADH-dependent oxidation rates at various fixed concentrations of Q_1 in the absence of products. The Q_1 concentrations were 600 μM (circle), 500 μM (square), and 400 μM (triangle). The enzyme concentration was 10.25 μg/mL in 0.1 M potassium phosphate buffer, pH 8.0, containing 0.1% dodecyl maltoside. Solid curves were obtained by fitting the data to the Michaelis–Menten equation. 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 panels, are shown in the insets.

rectangular hyperbolic relationship (i.e. Michaelis–Menten mechanism) for each dataset. On the basis of the standard errors for K_m and V_{max}, it is statistically reasonable to conclude the competitive and noncompetitive inhibition by Q_1H_2 and NAD^+ respectively (Panels D and C). Experimental results obtained at Q_1 concentration below 400–300 μM are also included in Fig. 3(C) and (D). The data points above 400 μM Q_1 provide rectangular hyperbolic curves, each identical to the one given in the figures within the experimental error. This shows that the influence of the rotenone-sensitive reaction to the rotenone-insensitive reaction is negligible below 400 μM down to 300 μM Q_1.

This steady state kinetic analysis indicates an ordered sequential mechanism with the order of substrate binding and product release as Q_1—NADH—NAD^+—Q_1H_2, as given in Scheme 1 (Cleland, 1977).

Examination of a Possible Secondary Effect on the Enzyme Preparation due to the High Concentration of Q_1

The Q_1 concentration range used in the present work is much higher (up to 600 μM) than those in the previous reports (below 120 μM) (Esposti *et al.*, 1996; Fato *et al.*, 1996). It might be expected that high concentrations of Q_1 (which is at least partly hydrophobic) might cause denaturation of the purified Complex I which is not very stable. Thus, we examined the effect of high concentrations of Q_1 on enzyme activity. Complex I (10.25 μg/mL) was preincubated with 600 μM Q_1 at 20°C for 30 s before addition of the 0.2 mL portion of the preincubation mixture to 1.8 mL of the reaction mixture containing 11.1 μM NADH. The addition of the enzyme solution yields 60 μM Q_1 and 10 μM NADH in the initial state of

Table I. Kinetic Parameters for NADH-Q_1 Reductase

Varying substrate	Fixed substrate (μM)	Product (μM)	V_{max} (μmoles/min/mg)	K_m (μM)
NADH	Q_1 400	0	2.240 (0.060)	2.275 (0.159)
	500	0	2.613 (0.064)	2.494 (0.154)
	600	0	2.988 (0.065)	2.274 (0.128)
NADH	Q_1 400	NAD^+ 0	2.240 (0.060)	2.275 (0.159)
		100	1.545 (0.024)	1.721 (0.078)
		200	1.406 (0.020)	1.580 (0.069)
		400	1.025 (0.013)	1.535 (0.060)
NADH	Q_1 400	Q_1H_2 0	2.240 (0.060)	2.275 (0.159)
		200	1.707 (0.023)	1.633 (0.065)
		300	1.414 (0.025)	1.471 (0.082)
Q_1	NADH 6	NAD^+ 0	3.724 (0.149)	542.7 (39.6)
		100	2.913 (0.102)	534.9 (34.4)
		200	2.473 (0.092)	519.2 (36.1)
		400	1.980 (0.069)	504.3 (43.3)
Q_1	NADH 6	Q_1H_2 0	3.724 (0.149)	542.7 (39.6)
		200	3.805 (0.200)	696.5 (60.3)
		300	3.792 (0.162)	804.3 (53.6)

Note. Values given in parentheses are standard errors.

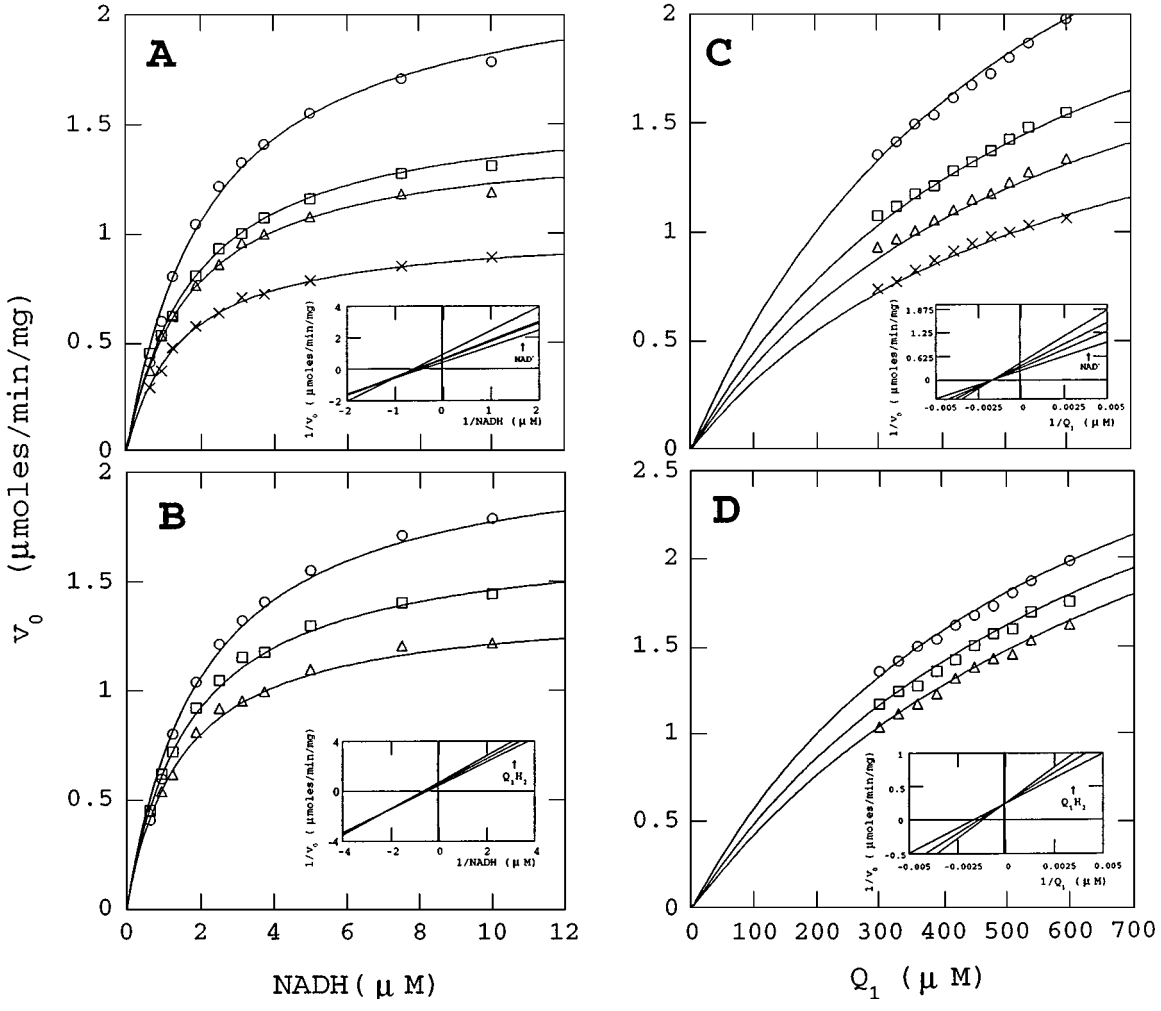


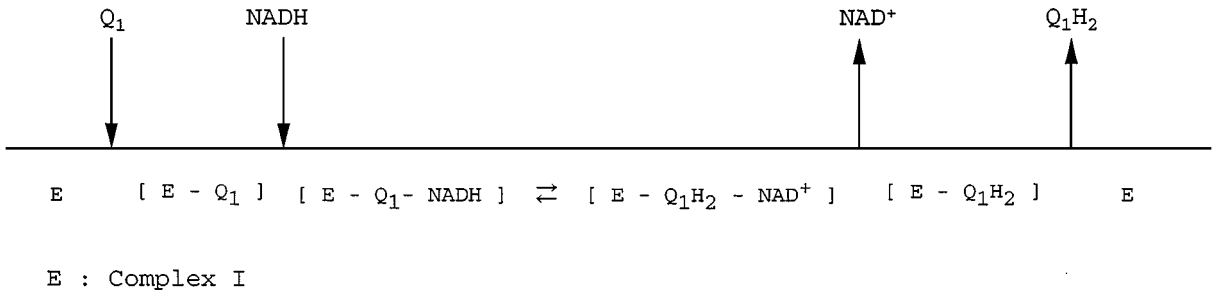
Fig. 3. Product inhibition for the NADH:Q_1 reductase reaction at Q_1 concentrations above 400 μM. Solid curves were determined by fitting the data to the Michaelis–Menten equation. The double reciprocal lines of the solid curves are shown in the insets. (A) NAD^+ concentrations are 0 μM (circle), 100 μM (square), 200 μM (triangle), and 400 μM (cross) with varying concentrations of NADH at a fixed Q_1 concentration of 400 μM. (B) Q_1H_2 concentrations are 0 μM (circle), 200 μM (square), and 300 μM (triangle) with varying concentrations of NADH at a fixed Q_1 concentration of 400 μM. (C) 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. (D) Q_1H_2 concentrations are 0 μM (circle), 200 μM (square), and 300 μM (triangle) with varying concentrations of Q_1 at a fixed NADH concentration of 6 μM. In C and D, data points in the presence of Q_1 below 400 μM are also included. The enzyme concentration was 10.25 μg/mL in 0.1 M potassium phosphate buffer, pH 8.0, containing 0.1% dodecyl maltoside.

the reaction mixture. The specific activity was identical to those determined without preincubation and with preincubation in the absence of Q_1, indicating that Q_1 at 600 μM does not affect the enzyme activity of our Complex I preparation.

As described in the previous paper (Nakashima *et al.*, in press), rotenone inhibition is not instantaneous. Thus, an initial burst in decrease of the slope of the NADH oxidation is observed during the initial 60 s. The initial burst is removed by preincubation of the enzyme with rotenone for 30 s at 20°C. No initial burst was detectable in the NADH oxidation when the enzyme preparation was preincubated with rotenone in the presence of 600 μM Q_1. This result shows that 600 μM Q_1 in the preincubation mixture does not inhibit the binding of rotenone to the enzyme.

NMR Relaxation of Q_1

It might be expected that high concentrations of Q_1 could form micelles or other aggregates in aqueous solution that could cause activity artifacts. However, the



Scheme 1.

structure of Q_1 indicates that it is unlikely to form micelles and is moderately hydrophilic. However, in order to show the homogeneous dispersion of Q_1 in aqueous solution most directly, we measured the NMR relaxation time (T_1) of protons of three methyl groups of Q_1, one at position 5 in the quinone ring and two in the isoprenoid group. These methyl protons exhibit three clear peaks at 1.80, 1.55, and 1.48 ppm for 600 μM Q_1 in 100 mM potassium phosphate buffer, pH 8.0, containing 1% deuterated ethanol and 0.1% dodecyl maltoside. The T_1 values of NMR relaxation for these signals were 0.77, 1.08, and 0.89 s, respectively. The T_1 values and peak positions of these signals were not influenced by Q_1 concentrations between 600 and 100 μM. Similarly ethanol did not significantly affect these NMR parameters. Furthermore, essentially identical NMR parameters were obtained for these three methyl signals in deuterated chloroform. These results indicate that Q_1 at concentrations below 600 μM is homogeneously dispersed in the reaction mixture without forming micelles or aggregates.

DISCUSSION

The Rotenone-Sensitive and Rotenone-Insensitive Reactions

Rotenone sensitivity was decreased with Q_1 concentration and only about 3% of the total activity was inhibited by addition of an excess amount of rotenone in the presence of 600 μM Q_1. On the other hand, about 80% of the total activity was abolished by rotenone at Q_1 concentrations below 50 μM. These results suggest that this enzyme catalyzes two types of reactions, a rotenone-sensitive reaction and a rotenone-insensitive reaction. The latter appears at high Q_1 concentrations because of the large K_m values for Q_1. The existence of the rotenone-insensitive reaction was also demonstrated by the enzyme activity increase in the Q_1 concentration range where the rotenone-sensitive reaction (K_m of 12 μM for Q_1) was fully saturated with Q_1 (data not shown). In contrast to the NAD^+ inhibition against NADH for the rotenone-sensitive reaction (Nakashima *et al.*, in press), NAD^+ decreases the apparent K_m value for NADH (Fig. 3(A) and Table I). In other words, the reactivities of NADH and NAD^+ to the enzyme are influenced by the Q_1 binding states, indicating a long-range interaction between the pyridine nucleotide and ubiquinone binding sites.

As described in the previous paper (Nakashima *et al.*, in press), this enzyme has only a single site for rotenone binding. The rate of the rotenone-insensitive reaction should decrease with a decrease in the concentration of Q_1 asymptotically to zero. However, as shown in Fig. 1, the fractional rotenone inhibition approached to about 85% with a decrease in the concentration of Q_1. This result suggests that rotenone cannot inhibit the enzyme activity completely even at low Q_1 concentrations. Thus, it is likely that the rotenone-insensitive activity at extremely low Q_1 concentrations compared with K_m value of the rotenone-insensitive reaction for Q_1 is due to residual activity of the rotenone-bound site, not due to the rotenone-insensitive reaction observed at high Q_1 concentrations.

The integrity of purified preparations of Complex I has been evaluated by investigation of the rotenone sensitivity of the enzyme activity (Hatefi and Stempel, 1969). The rotenone-insensitive activity observed at high Q_1 concentration is unlikely to be due to denaturation of the purified preparation since Q_1 at 600 μM did not cause denaturation of the Complex I preparation. Additionally, aggregates or micelles (which could affect the kinetics of the enzyme) were not detectable even at the highest concentration of Q_1 as revealed by the NMR relaxation measurement. These results indicate that the rotenone-insensitive activity observed here is not due to denaturation of the enzyme. Thus, for an accurate evaluation of the integrity of purified Complex I, the rotenone sensitivity should be examined at low Q_1 concentration where the rotenone-insensitive reaction is negligible (lower than 20 μM).

Physiological Significance of the Rotenone-Insensitive Reaction

The high K_m value for Q_1 in the rotenone-insensitive reaction seems consistent with nonphysiological Q_1 binding on the hydrophilic surface of the enzyme, as previously suggested (Ragan, 1978). However, the overall Q_{10} concentration in mitochondrial membrane is definitely within the millimolar range. It has been reported that Q_{10} content is 1.06–0.43% of the total lipid of bovine mitochondria (Fleischer *et al.*, 1967). The overall concentration of Q_{10} (1.06%) in bovine heart mitochondrial lipid phase corresponds to 11.6 mM (Fleischer *et al.*, 1967). Furthermore the effective concentration of Q_{10} to the enzyme could be higher than the overall concentration since the movement of Q_{10} is restricted significantly in the mitochondrial inner membrane. Thus, the rotenone-insensitive Q_1 binding site with the large K_m values for Q_1 (but lower than mM) is likely to be active in the enzyme reaction in the mitochondrial inner membrane. Furthermore, the rotenone-insensitive reaction indicates that a rigorous long-range control exists between the NADH binding site and the rotenone-insensitive Q_1 binding site in a manner similar to the rotenone-sensitive reaction. Such a rigorous control mechanism also strongly suggests that the rotenone-insensitive reaction is physiologically relevant.

On the other hand, it is well known that rotenone inhibits almost completely the Complex I reaction in intact mitochondrial innermembrane (Hatefi, 1985) in which Q_{10} concentration is about 10 mM (Fleischer *et al.*, 1967). A possible interpretation for the apparent discrepancy is as follows: the second ubiquinone (Q_{10}) binding site is composed of two subsites for the quinone moiety near the extramembrane surface of Complex I and for the isoprenoide chain moiety in the transmembrane hydrophobic surface. And rotenone binds only to the latter subsite. Rotenone binding to the latter subsite is sufficient for a complete inhibition of Q_{10} binding to the site in the mitochondrial membrane. However, the short and flexible isoprenoide tail of Q_1 is likely to have an additional binding site different from the isoprenoide subsite for Q_{10}, so that rotenone cannot inhibit Q_1 binding. Thus, NADH—Q_1 oxidoreductase reaction in the present enzyme assay system at high Q_1 concentration is rotenone-insensitive, while NADH—Q_{10} oxidoreductase reaction in the mitochondrial innermembrane is rotenone-sensitive. On the other hand, the rotenone-sensitive reaction in the present assay system at low Q_1 concentration is inhibited by binding of rotenone to the quinone binding site on the transmembrane surface. Thus, the reaction is sensitive to the rotenone in the isolated enzyme reaction system as well as in the intact mitochondrial membrane. Various alternative interpretations may be possible at present. However, as discussed above, it is unlikely that the rotenone-insensitive activity in the present assay system for the purified enzyme preparation is provided by some denatured enzyme fractions in the enzyme preparation, if any. The structural information at high resolution for the Q sites is required for solving the rotenone-inhibition mechanism.

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