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Effect of pH on the Steady State Kinetics of Bovine Heart NADH: Coenzyme Q Oxidoreductase

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Complete initial steady state kinetics of NADH-decylubiquinone (DQ) oxidoreductase reaction between pH 6.5 and 9.0 show an ordered sequential mechanism in which the order of substrate bindings and product releases is NADH-DQ–DQH_2-NAD^+. NADH binding to the free enzyme is accelerated by protonation of an amino acid (possibly a histidine) residue. The NADH release is negligibly slow under the turnover conditions. The rate of DQ binding to the NADH-bound enzyme and the maximal rate at the saturating concentrations of the two substrates, which is determined by the rates of DQH_2 formation in the active site and releases of DQH_2 and NAD^+ from the enzyme, are insensitive to pH, in contrast to clear pH dependencies of the maximal rates of cytochrome *c* oxidase and cytochrome bc_1 complex. Physiological significances of these results are discussed.

KEY WORDS: NADH: coenzyme Q oxidoreductase; Complex I, membrane protein; steady state kinetics; ordered sequential mechanism; pH-dependency; mitochondrial respiration; proton-pump.

INTRODUCTION

Complex I (NADH: coenzyme Q oxidoreductase, EC1.6.99.3) reduces Coenzyme Q (Q) by NADH at the entrance of the mitochondrial electron transfer chain, coupled with proton pumping from the inside of mitochondrial inner membrane to the outside (Hatefi, 1985; Walker, 1992; Weiss *et al*., 1991). The structures of the redox sites and protein moiety of the largest mitochondrial electron transfer complex (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). Compared with these structural studies, the enzyme-kinetical studies have been done much less extensively, since the stable enzyme preparation is difficult to obtain. In fact, a complete initial steady state kinetics including the analysis of product inhibitions was reported in 2002 (Nakashima *et al*., 2002a, b). Following the reports, we have examined effect of the side chain structure of coenzyme Q on the steady state kinetics of the enzyme (Hano *et al*., 2003). These results indicate strong interactions between NADH-binding site and ubiquinone-binding site, which are likely to be far apart with each other in the large enzyme molecule.

Detailed analyses of the effect of pH on the steady state kinetic properties are indispensable for elucidation of the mechanism of this enzyme since this enzyme pumps protons. Therefore, we examined the effect of pH on the rates of reaction steps determinable in the initial steady state kinetic analysis. Unexpectedly, only the NADH binding to the enzyme is pH-dependent.

EXPERIMENTAL PROCEDURE

The reaction medium for the rate determinations contained 0.1 M pyrophosphate buffers and potassium phosphate buffers above and below pH 8.0, respectively. No significant effect due to buffer species was observed

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Key to abbreviations: Complex I, NADH-coenzyme Q oxidoreductase; Q_1 and Q_2, coenzyme Q with 1 and 2 isoprenoide units, respectively; DQ, decylubiquinone; DQH_2, reduced decylubiquinone; EPR, electron paramagnetic resonance.

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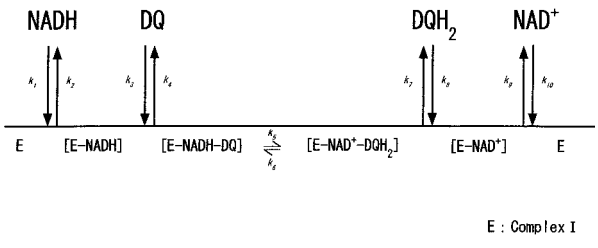
in the rate determinations at pH 8.0. Piericidin A was prepared with the method of Tamura *et al*. (1963). Other experimental conditions are as given in the previous papers (Nakashima *et al*., 2002a). The enzyme concentration was calculated assuming that the molecular mass of Complex I is 1000 kDa (Buchanan and Walker, 1996).

RESULTS

Effect of pH on the Initial Steady State Kinetic Pattern of NADH-DQ Oxidoreductase Reaction.

The stoichiometric reduction of DQ by NADH was confirmed with the experimental conditions at the highest and lowest pH studied in this work with the method as described previously (Nakashima *et al*., 2002a). The enzyme preparation used for this study was sufficiently stable between pH 6.5 and 9.0 for complete initial steady state analyses including product inhibition analyses.

Furthermore, the integrity of the enzyme preparation under the present experimental conditions was confirmed by the strong inhibitory effect of piericydine A to the enzyme activity (90% inhibition or higher) in the presence of 50-μM Q_1, 150-μM NADH, and 1-μM pierycidine A in the pH range between 5.5 and 9.0. The initial steady state rates depending on NADH as the varying substrate and DQ as the fixed substrate showed a parallel double reciprocal plot at pH 9.0 and an intersecting double reciprocal plot at pH 6.5, as shown in Fig. 1(A). The product inhibitions for all substrate–product combinations given in Fig. 1(B) indicate that only NAD^+ inhibition against NADH is competitive at both pH, consistently with the product inhibition pattern at pH 8.0 as reported (Hano *et al*., 2003). The apparent parallel reciprocal plots in the absence of product at pH 9.0 (Fig. 1(A)) do not indicate a ping-pong mechanism but a set of plots intersecting at a point located far apart from the original point. These results including the one at pH 8.0 reported in the previous paper (Hano *et al*., 2003) indicate the ordered sequential mechanism with NADH and NAD^+ as the initial substrate and the last product, respectively, between pH 9.0 and 6.5, as given in Scheme 1. The values of V_{max} and K_m obtained here are given in Tables I and II.



Determination of the Kinetic Parameters Independent of the Substrate Concentrations

The steady state rate equation for the ordered sequential mechanism as given in Scheme 1 is as follows.

$$e/v = k_2/k_1a[\mathrm{NADH}][\mathrm{DQ}] + 1/k_1[\mathrm{NADH}] + 1/a[\mathrm{DQ}] + 1/b \quad (1)$$

where e and v denote the total concentration of the enzyme in the reaction system and the initial steady state rate, and a and b are as follows:

$$a = k_3/\{1 + (1 + k_6/k_7)k_4/k_5\} \quad (2)$$

$$1/b = k_6/k_5k_7 + 1/k_5 + 1/k_7 + 1/k_9 \quad (3)$$

Thus the parameters, k_1, k_2, a, and b, are experimentally determinable. The slope and the intersect of the double reciprocal plot ($1/v$ - 1/[NADH]) at each DQ concentration plotted manually against the reciprocal of DQ concentration provided linear relations (not shown) which confirm the existence of the rate equation, as given in Eq. (1).

Similarly, plots for the slopes and intersects determined by fixing NADH concentration against the reciprocal concentrations of NADH were clearly linear (not shown). The four parameters as described above, determined manually from the intersects and the slopes of these plots were used for the initial values for the nonlinear regression analysis for Eq. (1). Similar kinetic analyses in the absence of the products were performed at several other pH points. The V_{max} and K_m values are given in Table III and the values of the four parameters calculated by the nonlinear regression analyses at each pH are given in Table IV.

As shown in Fig. 2, k_1 clearly decreases with pH increase from 6.5 to 7.5, while no significant pH-dependency of the parameter is detectable above pH 7.5. On the other hand, other kinetic parameters are essentially pH-independent in the whole pH range examined, as given in Table IV. No statistically significant deviation from zero value is detectable for k_2 except for the experimental value at pH 7.5 with the small standard error. The small standard error at pH 7.5 is likely to be obtained accidentally, since the experimental conditions at pH 7.5 are identical to those at other pH except for pH of the reaction medium. However, the averaged standard error calculated from the other k_2 values given in Table IV indicates that the deviation from zero is not significant. These results indicate that k_2 is negligibly small at any pH tested. The small k_2 value gives the intersecting point of the double reciprocal plots ($1/v$ - 1/[NADH]) far apart from the original point. If k_2 is negligibly small, a set of apparently parallel double

reciprocal plots is obtained as in the results above pH 7.0. Similar double reciprocal plots suggesting small k_2 value were also reported for the enzyme system including Q_2 instead of DQ (Hano *et al*., 2003).

DISCUSSION

The parameter, a becomes k_3 when k_5 is much larger than k_4 (Eq. (2)), otherwise a is smaller than k_3. In this enzyme system, the assumption, $k_5 \gg k_4$ is fully reasonable, since release of the large hydrophobic molecule, DQ, is likely to be much slower than the electron transfer within the ternary complex composed of the enzyme and the two substrates. As shown in Eq. (1), b is the maximal velocity per enzyme molecule at the saturating concentrations of the two substrates. If k_5 is much larger than k_6, b becomes a waited average of the rates of product formation and releases, as follows:

$$1/b = 1/k_5 + 1/k_7 + 1/k_9 \qquad (4)$$

If not, b is smaller than the waited average. The above assumption is reasonable, since stoichiometric reduction of DQ by NADH, which indicates no reverse reaction (k_6), has been confirmed experimentally under the present experimental conditions as described above.

The extensive structural studies suggest that the NADH-binding site is far apart from the coenzyme Q binding site. Thus, during the catalytic turnover, the oxidized Complex I is reduced by NADH and then the reduced

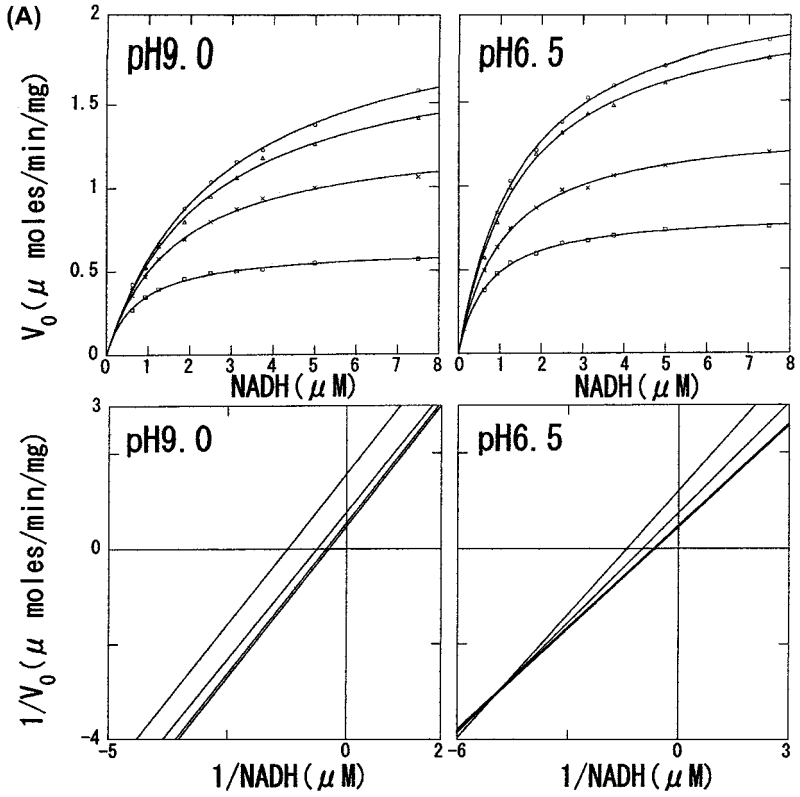


Fig. 1. Complete initial steady analysis of NADH: DQ oxidoreductase reaction at pH 9.0 and pH 6.5. (A) NADH-dependent oxidation rates at various fixed concentrations of DQ in the absense of products. The DQ concentrations were, 25 μM (square), 50 μM (cross), 100 μM (triangle), 150 μM (circle). The enzyme concentration was 3 μg/mL, in 0.1 M potassium phosphate buffer pH. 6.5 (right panels) or 0.1 M potassium pyrophosphate buffer pH. 9.0 (left panels) containing 0.2% dodecylmaltoside. Solid curves were obtained by fitting the data to the Michaelis–Menten equation. Bottom panels: 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 top panels. (B) Product inhibition for the NADH: DQ reductase reaction at pH 9.0 and pH 6.5. Only double reciprocal plots are given. Each plot was calculated from the experimental values of V_{max} and K_m given in Tables I and II.

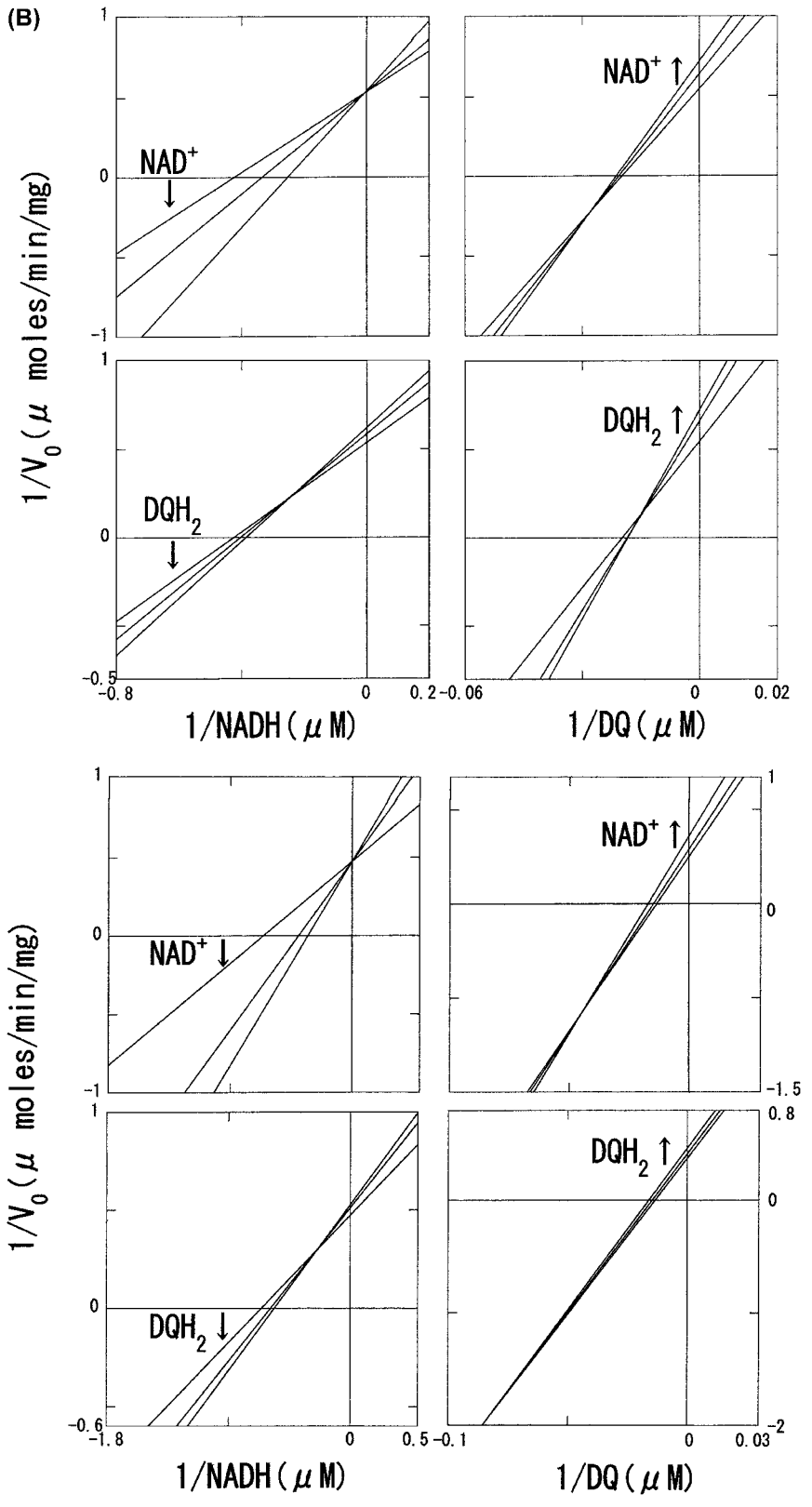


Fig. 1. Continued.

Table I. Effects of Products on Kinetic Parameters for NADH-DQ Reductase at pH 9.0

Varying substrate	Fixed substrate	(μM)	Product	(μM)	V_{max} (μmoles/min/mg) (SE)	K_m (μM) (SE)
NADH	DQ	25		0	0.629 (0.007)	0.761 (0.035)
		50		0	1.312 (0.019)	1.622 (0.065)
		100		0	1.860 (0.036)	2.354 (0.107)
		150		0	2.135 (0.037)	2.714 (0.104)
	DQ	100	NAD^+	0	1.860 (0.036)	2.354 (0.107)
				300	1.852 (0.025)	2.981 (0.087)
				600	1.838 (0.029)	3.987 (0.122)
	DQ	100	DQH_2	0	1.860 (0.036)	2.354 (0.107)
				50	1.706 (0.037)	2.478 (0.124)
				100	1.610 (0.017)	2.625 (0.063)
DQ	NADH	5	NAD^+	0	1.830 (0.043)	50.51 (3.12)
				300	1.561 (0.043)	48.51 (3.64)
				600	1.387 (0.050)	46.32 (4.56)
	NADH	5	DQH_2	0	1.830 (0.043)	50.51 (3.12)
				50	1.511 (0.045)	53.94 (4.22)
				100	1.383 (0.042)	54.46 (4.28)

enzyme is oxidized by DQ. Thus, the reductive phase starts after NADH binding and the oxidative phase starts after DQ binding. The reductive phase is likely to control the DQ-binding rate (k_3) and the oxidative phase determines the rate constant, k_5, in Eq. (1) and Scheme 1. The oxidative phase could also control the product release steps (k_7 and k_9). The rate of NADH binding (k_1) is not influenced by any electron transfer reaction inside the enzyme molecule.

As shown in Fig. 2, the significant pH dependence below pH 7.5 suggests that the NADH binding is coupled with a proton uptake possibly at a histidine residue. If the amino acid residue is located in the intermembrane surface of the enzyme the control site is active under the physiological conditions in the mitochondrial membrane, which is exposed to the acidic pH in the intermembrane phase. For complete reduction of DQ by NADH, one equivalent of protons must be taken up from the matrix space. Furthermore, the enzyme pumps protons from the matrix space to the intermembrane space. The control site active in the acidic pH, if located on the intermembrane surface, would stimulate (or trigger) the proton uptake from the matrix space for both purposes, via a long range interaction over the large protein complex. Long range interactions between the substrate-binding sites over the large protein complex have been shown by the ordered sequential mechanism (Nakashima *et al*., 2002a). Thus, the NADH binding could be coupled to proton uptake

Table II. Effects of Products on Kinetic Parameters for NADH-DQ Reductase at pH 6.5

Varying substrate	Fixed substrate	(μM)	Product	(μM)	V_{max} (μmoles/min/mg) (SE)	K_m (μM) (SE)
NADH	DQ	25		0	0.836 (0.007)	0.719 (0.026)
		50		0	1.356 (0.016)	1.057 (0.042)
		100		0	2.107 (0.037)	1.519 (0.076)
		150		0	2.243 (0.024)	1.543 (0.046)
	DQ	100	NAD^+	0	2.107 (0.037)	1.519 (0.076)
				300	2.101 (0.053)	2.512 (0.145)
				600	2.032 (0.019)	3.047 (0.061)
	DQ	100	DQH_2	0	2.107 (0.037)	1.519 (0.076)
				50	1.955 (0.021)	1.625 (0.048)
				100	1.895 (0.018)	1.779 (0.045)
DQ	NADH	5	NAD^+	0	2.671 (0.093)	74.07 (6.00)
				300	2.231 (0.037)	69.17 (2.71)
				600	1.862 (0.040)	58.46 (3.15)
	NADH	5	DQH_2	0	2.671 (0.093)	74.07 (6.00)
				50	2.413 (0.061)	68.60 (4.15)
				100	2.154 (0.061)	62.03 (4.32)

Table III. Effects of pH on the Kinetic Parameters for NADH-DQ Reductase in the Absence of Products (Varying Substrate Is NADH)

pH	Fixed substrate	(μM)	Product	(μM)	V_{max} (μmoles/min/mg) (SE)	K_m (μM) (SE)
6.5	DQ	25		0	0.836 (0.007)	0.719 (0.026)
		50		0	1.356 (0.016)	1.057 (0.042)
		100		0	2.107 (0.037)	1.519 (0.076)
		150		0	2.243 (0.024)	1.543 (0.046)
7.0	DQ	25		0	0.798 (0.010)	0.652 (0.036)
		50		0	1.332 (0.027)	1.281 (0.080)
		100		0	2.138 (0.041)	1.965 (0.096)
		150		0	2.354 (0.043)	2.058 (0.094)
7.5	DQ	25		0	0.776 (0.007)	0.590 (0.023)
		50		0	1.242 (0.020)	1.039 (0.056)
		100		0	2.063 (0.028)	2.017 (0.069)
		150		0	2.380 (0.067)	2.342 (0.130)
8.0	DQ	25		0	0.770 (0.005)	0.694 (0.022)
		50		0	1.370 (0.008)	1.338 (0.022)
		100		0	2.131 (0.077)	2.336 (0.142)
		150		0	2.328 (0.057)	2.398 (0.137)
8.5	DQ	25		0	0.690 (0.008)	0.690 (0.030)
		50		0	1.294 (0.024)	1.342 (0.075)
		100		0	1.971 (0.025)	2.210 (0.067)
		150		0	2.074 (0.045)	2.292 (0.122)
9.0	DQ	25		0	0.629 (0.007)	0.761 (0.035)
		50		0	1.312 (0.019)	1.622 (0.065)
		100		0	1.860 (0.036)	2.354 (0.107)
		150		0	2.135 (0.037)	2.714 (0.104)

either for proton pumping or for formation of one of the product, DQH_2.

An unexpected result is the negligibly small k_2 value over the pH range examined, suggesting a significant conformational change stabilizing both NADH and NAD^+. This enzyme is likely to receive electrons from NADH before DQ binding. Then, why does this enzyme trap NAD^+, until DQH_2 is released? Perhaps, only these extensive initial steady state kinetic analyses such as reported here could discover this intriguing aspect of this enzyme. X-ray structural analyses together with vibrational spectral studies are required for elucidating the mechanism of this aspect.

Even if the proton uptake from the matrix space for proton pump is coupled with the NADH binding to the enzyme as suggested above, the proton release for proton pumping to the intermembrane space must be coupled to the reductive or oxidative phase during the catalytic turnover. The redox active centers of this enzme, which seem to be buried in the interior of the large membrane protein, are likely to be insulated from pH of the aqueous phases. However, the pH-dependent EPR signals of the iron–sulfur clusters, the flavin semiquinone, and the ubisemiquinone of the enzyme have been well characterized in the pH range similar to that of the present study (Ingeldew and Ohnishi, 1980; Sled *et al.*, 1994; Yano *et al.*, 2000), indicating that the redox potentials of most of the redox-active sites of the enzyme are controlled by pH of the aqueous phases. Furthermore, the electron transfer step coupled with the proton release is likely to be pH-dependent, since the proton release for pumping is controlled by a redox-coupled pK_a change of an amino

Table IV. Effects of pH on the Kinetic Parameters Independent of the Substrare Concentrations

pH	k_1 (10^3 min^{-1} μM^{-1}) (SD)	k_2 (10^3 min^{-1}) (SD)	a(10^3 min^{-1} μM^{-1}) (SD)	b($10^3 min^{-1}$) (SD)
6.5	1.538 (0.088)	0.437 (0.242)	0.0454 (0.0023)	3.527 (0.167)
7.0	1.116 (0.045)	0.014 (0.120)	0.0402 (0.0017)	4.090 (0.212)
7.5	0.963 (0.023)	−0.260 (0.041)	0.0369 (0.0010)	4.365 (0.190)
8.0	0.929 (0.046)	−0.157 (0.132)	0.0417 (0.0023)	3.929 (0.252)
8.5	0.896 (0.056)	−0.090 (0.148)	0.0394 (0.0026)	3.438 (0.257)
9.0	0.802 (0.052)	0.031 (0.165)	0.0360 (0.0026)	3.671 (0.337)

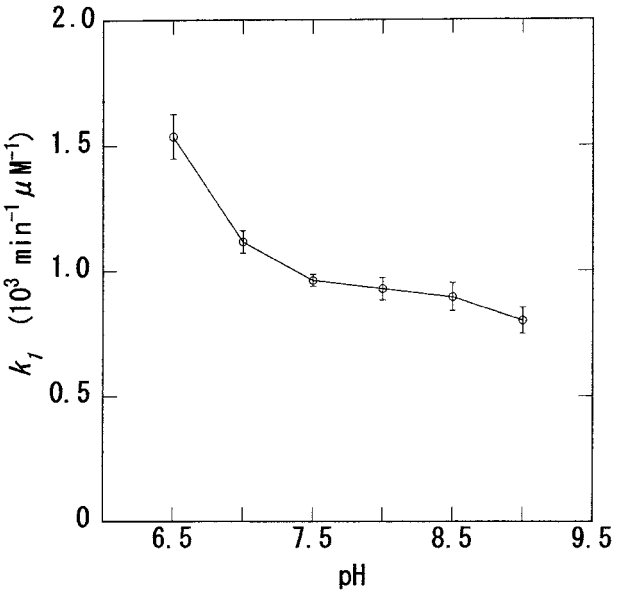


Fig. 2. Effect of pH on the rate of NADH binding to Complex I. Each k_1 value was calculated by fitting Eq. (1) to the kinetic data by nonlinear regression analysis. The error bars denote the standard errors.

acid residue. However, unexpectedly, no significant pH dependency is detectable for the parameters, *a* and *b*, which are likely to be controlled by the electron transfer events in the interior of the enzyme molecule, as described above. The experimental results are not inconsistent to the pH-sensitive redox potentials of the redox-active site, since the thermodynamic properties (redox potentials) do not always determine kinetic properties compulsory. For example, if the kinetic parameters, *a* and *b*, are dependent on the forward reactions of the electron transfers on the redox-active sites and independent of the reverse reactions, pH-insensitive electron transfers through a redox-active site with a pH-sensitive redox potential would be possible. Furthermore, pH-independent redox-driven proton release is possible, if proton release at a protonatable site is driven by a large redox-coupled pK_a shift of the proton-release site. For example, pK_a of acetic acid is 4.8 in water and 9.5 in methanol (Isaacs, 1995). Such a large pK_a shift in glutamate or aspartate is possible by a conformational change which involves migration of the carboxyl group from a protein surface, exposed to the aqueous phase, to the interior of the protein to be hydrrogen-bonded to alcoholic amino acids, serines, and threonines. If the interior of the protein has lower effective dielectric constant than that of methanol, the pK_a shift could be larger than in the above example. Then, the efficiency of the proton-release by the conformational change would not be influenced by the pH change between 6.5 and 9.0, as in this study. Thus, the pH-independencies in the parameters, *a* and *b*, suggest that the proton-pump by the enzyme is driven by a significantly large redox-coupled conformational change in the pumping element. The pH-insensitivity of the maximal velocity is unlikely to be due to instability of this large membrane protein since it integrity under the present assay conditions has been confirmed by piericidin A inhibition as described above. The pH-independency in the parameter *b*, is in contrast to the clear pH-dependencies in the maximal velocities of cytochrome *c* oxidase (Yonetani, 1961) and cytochrome bc_1 complex (Brandt and Okun, 1997) in the pH range similar to that of the present work. Extensive kinetic analyses for the partial reactions in the single turnover conditions with various spectral techniques as well as high-resolution three-dimensional analyses would reveal the structural and functional aspects giving the above pH-independencies.

Active and stable enzyme preparation of complex I is indispensable for the present pH effect experiments, since it takes at least 20 days for a set of the present kinetic analysis. It should be noted that such a complete kinetic analysis has not been performed for any other enzyme in the respiratory system.

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