The mechanism of the quinone reductase reaction of pig heart lipoamide dehydrogenase

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The relationship between the NADH:lipoamide reductase and NADH:quinone reductase reactions of pig heart lipoamide dehydrogenase (EC 1.6.4.3) was investigated. At pH 7.0 the catalytic constant of the quinone reductase reaction ($k_{\text{cat.}}$) is 70 s^{-1} and the rate constant of the active-centre reduction by NADH ($k_{\text{cat.}}/K_{\text{m}}$) is $9.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. These constants are almost an order lower than those for the lipoamide reductase reaction. The maximal quinone reductase activity is observed at pH 6.0–5.5. The use of $[4(S)\text{-}^3\text{H}]\text{NADH}$ as substrate decreases $k_{\text{cat.}}/K_{\text{m}}$ for the lipoamide reductase reaction and both $k_{\text{cat.}}$ and $k_{\text{cat.}}/K_{\text{m}}$ for the quinone reductase reaction. The $k_{\text{cat.}}/K_{\text{m}}$ values for quinones in this case are decreased 1.85–3.0-fold. NAD^+ is a more effective inhibitor in the quinone reductase reaction than in the lipoamide reductase reaction. The pattern of inhibition reflects the shift of the reaction equilibrium. Various forms of the four-electron-reduced enzyme are believed to reduce quinones. Simple and ‘hybrid ping-pong’ mechanisms of this reaction are discussed. The logarithms of $k_{\text{cat.}}/K_{\text{m}}$ for quinones are hyperbolically dependent on their single-electron reduction potentials (E_1^0). A three-step mechanism for a mixed one-electron and two-electron reduction of quinones by lipoamide dehydrogenase is proposed.

INTRODUCTION

Lipoamide dehydrogenase (EC 1.6.4.3) catalyses the reversible oxidation of NADH by lipoamide and contains FAD and redox-active disulphide in the active centre (Massey *et al.*, 1960; Williams, 1976). During the reaction NADH reduces FAD, which further transfers the electrons to the disulphide, forming a charge-transfer complex (Massey *et al.*, 1960). The limiting step of catalysis is the oxidation of reduced disulphide by lipoamide (Matthews *et al.*, 1977). A four-electron reduction, prevented by NAD^+ and proceeding efficiently at low pH (Matthews & Williams, 1976), gives a form of the enzyme non-oxidizable by lipoamide. On the basis of lipoamide dehydrogenase amino acid sequence (Otulakowski & Robinson, 1987) and X-ray data (Takenaka *et al.*, 1988) its structural similarity to another disulphide-containing flavoprotein, namely glutathione reductase, as well as the spatial separation of the nicotinamide nucleotide-binding and lipoamide-binding domains may be deduced (Pai & Schulz, 1983).

Lipoamide dehydrogenase is characterized as a flavin-containing dehydrogenase transhydrogenase (Hemmerich & Massey, 1979) catalysing a single-step transfer of two redox equivalents in the course of the physiological reaction without the formation of intermediate semiquinone compounds. However, the enzyme is efficiently reduced by the single-electron reducer viologen radical (Simon *et al.*, 1985), is oxidized by ferricyanide (Tsai, 1980) and catalyses a mixed single-electron and two-electron reduction of quinones (Nakamura & Yamazaki, 1972). The last-mentioned reaction is of great interest owing to toxicity of quinones formed as the products of pollution and drugs, which is caused by their single-electron reduction by low-potential NAD(P)H-oxidizing flavin-containing dehydrogenases (Chesis *et al.*, 1984). Low-potential semiquinones, formed in this reaction, are rapidly oxidized by O_2 and H_2O_2 , forming superoxide and hydroxyl radicals (Peters *et al.*, 1986). An increased rate of generation of these radicals, i.e. oxidative stress, results in peroxidation of lipids and modification of nucleic acids.

The kinetic parameters of the quinone reductase reaction differ

from those of the lipoamide reductase reaction. Various modifications of the enzyme causing a decrease in physiological activity are often accompanied by an increase in quinone reductase activity (Searls *et al.*, 1961; Casola & Massey, 1966). Sometimes this increase is correlated with a diminished formation of the FAD–disulphide charge-transfer complex and an increased possibility for FAD to exist in a reduced state. It is considered that reduced FAD is involved in the reduction of quinones (Nakamura & Yamazaki, 1972).

The aim of the present work is to elucidate the mechanism of the quinone reductase reaction of pig heart lipoamide dehydrogenase by the use of quinones characterized by different single-electron and two-electron reduction energetics.

MATERIALS AND METHODS

Lipoamide dehydrogenase characterized by the A_{280}/A_{460} ratio 7.1 was isolated from pig heart (Massey *et al.*, 1960). The concentration of the enzyme was determined spectrophotometrically by the use of ϵ_{455} , $11.3 \text{ mm}^{-1} \cdot \text{cm}^{-1}$.

NADH, NAD^+ and mitomycin-C (Sigma Chemical Co.), cytochrome *c* and tetramethyl-1,4-benzoquinone (Serva), 2,6-dimethyl-1,4-benzoquinone (Aldrich Chemical Co.) and 5,8-dioxy-1,4-naphthoquinone (Fluka) were used as received, 1,4-Benzoquinone, 2-methyl-1,4-benzoquinone, 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone (Reachim, Shostkino, U.S.S.R.) were purified by sublimation *in vacuo* or recrystallized from benzene or ethanol. $\text{K}_3\text{Fe}(\text{CN})_6$ (Reachim) was recrystallized from water. DL-Lipoamide was synthesized at the All-Union Scientific Research Institute of Vitamins (Moscow, U.S.S.R.). $[4(S)\text{-}^3\text{H}]\text{NADH}$ was obtained by reduction of NAD^+ by lipoamide dehydrogenase in $^3\text{H}_2\text{O}$ (Oppenheimer *et al.*, 1971). The extent of deuteration, determined by n.m.r., was not less than 97%.

The reaction rate was determined by the decrease in the NADH absorption ($\Delta\epsilon_{340}$, $6.2 \text{ mm}^{-1} \cdot \text{cm}^{-1}$). A Hitachi 557 spectrophotometer was used. The concentration of NADH was

Abbreviations used: $k_{\text{cat.}}$, catalytic constant; $k_{\text{cat.}}/K_{\text{m}}$, bimolecular rate constant; E_1^0 , single-electron reduction potential; E_0^0 , standard potential.

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10–150 μM , that of lipoamide was 0.15–1.0 mM, that of electron acceptor was 10–200 μM and that of enzyme was 2–500 nM. Measurements were carried out under aerobic conditions at $25 \pm 0.1^\circ\text{C}$. The 0.1 M-phosphate (pH 6.0–8.0) and 0.1 M-phosphate/citrate (pH 5.0–6.0) buffer solutions containing 1 mM-EDTA were prepared from 0.1 M- KH_2PO_4 and H_3PO_4 or citric acid solutions. Before introduction into the assay system the enzyme was kept in 0.1 M-phosphate/citrate buffer, pH 7.0, at 4°C . The reaction rate was not affected by the buffer solution composition or the presence of 1% (v/v) acetonitrile when poorly soluble oxidizers were used. The benzosemiquinone-mediated reduction of cytochrome *c* was monitored at 550 nm ($\Delta\epsilon_{550}$ 20 $\text{mM}^{-1}\cdot\text{cm}^{-1}$). The concentration of cytochrome *c* was 35–45 μM , and the rate of NADH oxidation did not exceed 0.3 $\mu\text{M/s}$.

The steady-state kinetic parameters K_m and $V_{\text{max}}/[E]$ were determined by using the Lineweaver–Burk plot or by means of the direct linear plot (Eisenthal & Cornish-Bowden, 1974). The catalytic constants of the enzyme (k_{cat}) and bimolecular rate constants (k_{cat}/K_m) were determined from the intercepts and slopes of double-reciprocal plots of the dependence of $V_{\text{max}}/[E]$ on the substrate concentration. $V_{\text{max}}/[E]$ and k_{cat} are expressed as molecules of NADH oxidized by FAD/s and as s^{-1} respectively.

RESULTS

During the enzymic reduction of lipoamide by NADH variation of the concentration of NADH at fixed lipoamide concentrations gives a series of parallel lines in Lineweaver–Burk plots. At pH 7.0 k_{cat} is 420 s^{-1} , k_{cat}/K_m for NADH is $1.1 \times 10^7 \text{ M}^{-1}\cdot\text{s}^{-1}$ and k_{cat}/K_m for lipoamide is $2.8 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$. These values are close to those determined previously (Tsai, 1980). A lag period was observed in the reaction course at or below pH 6.0 when high concentrations of NADH were used. To prevent this happening the reactions were performed in the presence of 50–150 μM -NAD⁺ (Matthews & Williams, 1976), and the kinetic parameters were determined by extrapolating to zero NAD⁺ concentrations. The k_{cat}/K_m for NADH determined at low (10–20 μM) concentrations of NADH was close to the extrapolated value. The presence of NAD⁺ does not affect the k_{cat} of the reaction. The pH-dependence of the kinetic parameters is shown in Fig. 1.

When 1,4-benzoquinone was used as oxidizing substrate a series of parallel lines in Lineweaver–Burk plots was again observed. At pH 7.0 k_{cat} is 70 s^{-1} , k_{cat}/K_m for NADH is

$9.2 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$ and k_{cat}/K_m for 1,4-benzoquinone is $1.3 \times 10^6 \text{ M}^{-1}\cdot\text{s}^{-1}$. The values of the initial rates of reaction at a fixed [NADH]/[1,4-benzoquinone] ratio (1:1) also linearized in the Lineweaver–Burk plot, showing that k_{cat} was not affected. The reduction of 2-methyl-1,4-benzoquinone, ferricyanide and 5,8-dioxy-1,4-naphthoquinone proceeded according to kinetic patterns analogous to those for the reduction of 1,4-benzoquinone, and both k_{cat} and k_{cat}/K_m for NADH are almost identical with the values determined in the latter reaction (Fig. 1). However, 5,8-dioxy-1,4-naphthoquinone behaved as a weak competitive inhibitor for NADH (K_i 300 μM). For other electron acceptors the maximal rates of the reaction at fixed NADH concentration (50 μM) are almost identical. The bimolecular rate constants of electron acceptors are given in Table 1. A competitive inhibition of NADH with respect to electron acceptors is observed only at pH 5.5–5.0 (K_i 50–60 μM).

Fig. 1. pH-dependence of k_{cat} (a) and k_{cat}/K_m for NADH (b) for the NADH:lipoamide dehydrogenase (curves 1) and NADH:acceptor reductase (curves 2) reactions of lipoamide dehydrogenase

Electron acceptors: ○, 1,4-benzoquinone; △, 5,8-dioxy-1,4-naphthoquinone; □, ferricyanide.

Table 1. Rate constants (k_{cat}/K_m) and kinetic isotope effects ($k_1\text{H}/k_2\text{H}$) in the lipoamide dehydrogenase-catalysed oxidation by non-physiological electron acceptors at pH 7.0 and 25°C

The confidence coefficient is 95% and the relative standard deviation is 5%.

Oxidizing substrate	E_1° (V)*	E_2° (V)†	k_{cat}/K_m ($\text{M}^{-1}\cdot\text{s}^{-1}$)	$k_1\text{H}/k_2\text{H}$
1,4-Benzoquinone	0.09	0.29	1.3×10^6	2.15
2-Methyl-1,4-benzoquinone	0.01	0.21	1.0×10^6	2.25
5,8-Dioxy-1,4-naphthoquinone	−0.11	−0.06	7.8×10^5	2.5
2,6-Dimethyl-1,4-benzoquinone	−0.08	0.16	3.6×10^5	1.85
9,10-Phenanthrenequinone	−0.12	0.02	2.3×10^5	2.15
1,4-Naphthoquinone	−0.15	0.04	2.0×10^5	2.65
2-Methyl-1,4-naphthoquinone	−0.20	−0.03	3.0×10^4	2.4
Tetramethyl-1,4-benzoquinone	−0.26	0.04	7.0×10^3	3.0
Mitomycin-C	−0.31	—	2.0×10^3	—
Ferricyanide	0.41	—	2.6×10^5	1.35

* Taken from Butler & Hoey (1986), Land *et al.* (1983) and Swallow (1982).

† Taken from Moriconi *et al.* (1962).

Fig. 2. Dependence of the maximal rate of lipoamide reductase (a) and quinone reductase (b) reactions of lipoamide dehydrogenase at pH 7.0 on the NADH (curves 1) and [4(S)-²H]NADH (curves 2) concentrations at saturating concentrations of oxidizing substrate

Fig. 3. Effect of NAD⁺ on the maximal rate of 1 mM-lipoamide reduction at infinite NADH concentrations (a) and k_{cat}/K_m for NADH under the same conditions (b) at pH 5.5 (curves 1), pH 7.0 (curves 2) and pH 8.0 (curves 3)

Since lipoamide dehydrogenase possesses a pro-S stereospecificity (Oppenheimer *et al.*, 1971) the use of [4(S)-²H]NADH as substrate decreases the reaction rate. The deuteration of NADH decreases k_{cat}/K_m in the lipoamide reductase reaction, but does not affect k_{cat} . However, both k_{cat}/K_m and k_{cat} are decreased about 2-fold in the quinone reductase reaction (Fig. 2). With the use of [4(S)-²H]NADH the k_{cat}/K_m value for lipoamide is decreased only 1.2-fold, but the decrease in k_{cat}/K_m for quinones

Fig. 4. NAD⁺ inhibition with respect to 1,4-benzoquinone in the NADH:quinone reductase reaction at pH 7.0

NAD⁺ concentrations: 0 μ M (curve 1); 10 μ M (curve 2); 35 μ M (curve 3); 70 μ M (curve 4); 100 μ M (curve 5); 150 μ M (curve 6); 250 μ M (curve 7). NADH concentration: 83 μ M.

Fig. 5. NAD⁺ inhibition with respect to NADH in the NADH:quinone reductase reaction at pH 7.0

NAD⁺ concentrations: 0 μ M (curve 1); 20 μ M (curve 2); 30 μ M (curve 3); 45 μ M (curve 4); 67 μ M (curve 5); 100 μ M (curve 6); 150 μ M (curve 7). 1,4-Benzoquinone concentration: 50 μ M.

is more considerable. The kinetic isotope effects (k_H/k_D) on the acceptor rate constants are presented in Table 1.

In the NADH:lipoamide reductase reaction NAD⁺ acts as a mixed-type inhibitor for NADH increasing slopes and intercepts in the double-reciprocal plots at fixed lipoamide concentrations (Fig. 3). At pH 5.5 the efficiency of inhibition is markedly decreased, especially the influence of NAD⁺ on the values of the intercepts.

In the NADH:quinone reductase reaction of lipoamide dehydrogenase NAD⁺ acts as a mixed-type inhibitor for 1,4-benzoquinone and NADH (Figs. 4 and 5). The efficiency of NAD⁺ as inhibitor in this reaction is about one order higher than that in the lipoamide reductase reaction. In the presence of NAD⁺ variation in NADH and 1,4-benzoquinone concentrations

Fig. 6. Dependence of the reaction rate on NADH and 1,4-benzoquinone concentrations at pH 7.0 and in the presence of 50 μM -NAD⁺

NADH concentrations: 85 μM (curve 1); 54 μM (curve 2); 30 μM (curve 3); 19 μM (curve 4); 12.5 μM (curve 5); 8.5 μM (curve 6).

Fig. 7. Dependence of k_{cat}/K_m for quinones (○) and their reduction kinetic isotope effects ($k_{\text{H}}/k_{\text{H}}$) (●) on their single-electron reduction potential (E_1^0)

Key: 1, 1,4-benzoquinone; 2, 2-methyl-1,4-benzoquinone; 3, 2,6-dimethyl-1,4-benzoquinone; 4, 5,8-dioxy-1,4-naphthoquinone; 5, 9,10-phenanthrenequinone; 6, 1,4-naphthoquinone; 7, 2-methyl-1,4-naphthoquinone; 8, tetramethyl-1,4-benzoquinone; 9, mitomycin-C.

gives a series of converging lines in Lineweaver-Burk plots (Fig. 6), and the k_{cat} of the reaction does not change. An analogous type of inhibition and its linearity up to 250 μM -NAD⁺ is observed in the pH range 6.0–8.0.

The efficiency of a single-electron reduction of quinones by lipoamide dehydrogenase is expressed as the ratio of the rate of cytochrome *c* reduction by benzosemiquinone, formed in the reaction, and the doubled rate of the enzymic NADH oxidation. This method is based on the fact that at or below pH 7.2 benzohydroquinone reduces cytochrome *c* at a negligible low rate, whereas the rate of its reduction by benzosemiquinone is high ($k = 1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$). At low enzymic reaction rates and high cytochrome *c* concentrations (30–40 μM) more than 90% of the semiquinone formed is trapped (Iyanagi & Yamazaki, 1970). It

was found that for lipoamide dehydrogenase a single-electron reduction of quinones at pH 6.5 makes up 22–24% of the total electron flux, which is close to previously published data (Nakamura & Yamazaki, 1972). The efficiency of the process does not change with the use of [4(*S*)-³H]NADH and in the presence of 50 μM -NAD⁺.

DISCUSSION

The data of this paper show significant differences between the lipoamide reductase and quinone reductase reactions of lipoamide dehydrogenase. First let us consider the lipoamide reductase reaction, which is described by a 'ping-pong' mechanism (Massey *et al.*, 1960; Cleland, 1963). To be more precise a 'hybrid ping-pong' mechanism, which is a characteristic of glutathione reductase (Northrop, 1969; Bulger & Brandt, 1971), seems to be possible in this case in view of great structural similarities between these two enzymes (Otulakowski & Robinson, 1987; Takenaka *et al.*, 1988). The character of the product inhibition (Fig. 3) is determined by the competition of NAD⁺ for an NADH-binding site in the oxidized enzyme (Williams, 1976) and the oxidation of the two-electron-reduced enzyme at high pH (Matthews *et al.*, 1979). The oxidation efficiency is markedly decreased at or below pH 5.9 (Matthews *et al.*, 1979). This determines the change in the character of the inhibition at pH 5.5 (Fig. 3). An analogous change in the inhibition mechanism is observed for glutathione reductase and is caused by the possibility for NADP⁺ to oxidize the two-electron-reduced enzyme at high pH (Rakauskienė *et al.*, 1989). Thus the product inhibition patterns of lipoamide dehydrogenase are analogous to those of glutathione reductase, which contains a single NAD(P)H-binding site (Pai & Schulz, 1983). Hence this mechanism does not require the participation of the second regulatory binding site for NAD⁺ (van Muiswinkel-Voetberg & Veege, 1973).

The data on the quinone reductase reaction also fit the 'ping-pong' mechanism (Rudolph & Fromm, 1979). The kinetic isotope effects with [4(*S*)-³H]NADH (Fig. 2) indicate the difference in the limiting steps of the lipoamide reductase and quinone reductase reactions. That the k_{cat} for the lipoamide reductase reaction is unaffected agrees with the fact that the oxidative half-reaction is rate-limiting (Matthews *et al.*, 1977), but the decrease in k_{cat} for the quinone reduction reaction points to the reductive half-reaction being the limiting step. Lower k_{cat}/K_m values for NADH as compared with those for the lipoamide reductase reaction and the increase in reaction rate at low pH (Fig. 1), where the super-reduction of enzyme is more effective (Matthews & Williams, 1976), favour the explanation that lipoamide dehydrogenase that has been reduced to a greater extent in the absence of lipoamide is responsible for reduction of quinones. This view can be supported by analysis of the data for the inhibition of the reaction.

As is seen from Figs. 4–6 the role of NAD⁺ as inhibitor is consistent with the oxidation of a reduced form of enzyme and, possibly, competition for an NADH-binding site in an oxidized form (Cleland, 1963). One can propose a minimal scheme for the quinone reductase reaction:

where E₀, E_r, ES and E₀P are the oxidized enzyme, reduced enzyme, enzyme-substrate complex and the complex of oxidized enzyme with NAD⁺, and S, P and Q are NADH, NAD⁺ and

electron acceptor respectively. The steady-state rate of the reaction may be expressed as:

$$\frac{[E]}{v} = \frac{1}{k_{+3}} + \frac{k_{-2} + k_{+3}}{k_{+2}k_{+3}[S]} \left(1 + \frac{k_{-1}[P]}{k_{+1}} \right) + \frac{1}{k_{+4}[Q]} \left(1 + \frac{k_{-3}[P]}{k_{+3}} \right) + \frac{k_{-2}k_{-3}[P]}{k_{+2}k_{+3}k_{+4}[S][Q]} \left(1 + \frac{k_{-1}[P]}{k_{+1}} \right) \quad (2)$$

Calculations made using the rates in the absence of NAD^+ , the data from Figs. 4–6 and the results of analogous experiments on NAD^+ inhibition with respect to 1,4-benzoquinone at different fixed NADH concentrations revealed the corresponding rate and dissociation constants. At pH 7.0 $k_{+3} = k_{\text{cat.}} = 70.5 \pm 5 \text{ s}^{-1}$, $k_{+2}k_{+3}/(k_{-2} + k_{+3}) = 9.2 \times 10^5 \pm 0.4 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($k_{\text{cat.}}/K_m$ for NADH), $k_{+4} = 1.3 \times 10^6 \pm 0.07 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($k_{\text{cat.}}/K_m$ for 1,4-benzoquinone), $k_{-2}/k_{+2} = 1.65 \times 10^{-4} \pm 0.4 \times 10^{-4} \text{ M}$, $k_{+3}/k_{-3} = 5.05 \times 10^{-5} \pm 1.15 \times 10^{-5} \text{ M}$ and $k_{+1}/k_{-1} \geq 5.0 \times 10^{-4} \text{ M}$. The equilibrium constant of the reaction:

being equal to the ratio $k_{+2}k_{+3}/k_{-2}k_{-3}$, is calculated to be 0.31 ± 0.03 . The standard potential of the enzyme form estimated by the equation:

$$E_r^0 (\text{mV}) = -320 + 29 \log(k_{+2}k_{+3}/k_{-2}k_{-3}) \quad (4)$$

is calculated to be $-334 \pm 2 \text{ mV}$. This value is far more negative than the potential of the two-electron reduction of lipoamide dehydrogenase at pH 7.0, namely -280 mV , and is close to that of its four-electron reduction, namely -345 mV (Matthews & Williams, 1976). However, this does not necessarily imply that a free four-electron reduced enzyme is solely responsible for quinone reduction. One must note that an interdependent competitive inhibition of NADH and oxidizing substrate characteristic of a simple 'ping-pong' mechanism is observed only in particular cases (see the Results section). Therefore a 'hybrid ping-pong' mechanism involving separate oxidizing-substrate-binding and NAD(H)-binding sites cannot be excluded. In this case the oxidizing substrates may oxidize the free enzyme and its complexes with NAD^+/NADH at similar rates. It has been shown that quinoidal redox compounds occupy a separate binding site in glutathione reductase that is different from the $\text{NADP}^+/\text{NADPH}$ -binding and GSH-binding sites (Karplus *et al.*, 1989). These compounds can also oxidize glutathione reductase containing bound NADP^+ or NADPH (Čenas *et al.*, 1989a). Besides this the formation of a ternary complex has been proposed for the reduction of dichlorophenol-indophenol by lipoamide dehydrogenase (Tsai, 1980), although the reaction proceeds with kinetic parameters close to those reported in the present paper.

On analogy to the reaction of glutathione reductase (Čenas *et al.*, 1989a) the logarithms of $k_{\text{cat.}}/K_m$ for quinones hyperbolically depend on their single-electron-reduction potentials (E_r^0) (Fig. 7). The correlation between the reactivity and the standard potential is absent. Although this is formally consistent with the outer-spherical electron-transfer model (Marcus & Sutin, 1985), the parallel two-electron reduction of quinones and especially the kinetic isotope effect on $k_{\text{cat.}}/K_m$ for quinones when $[4(S)\text{-}^3\text{H}]\text{NADH}$ is used (Table 1) indicate a more complex mechanism. The latter indicates that a hydrogen atom is transferred from NADH to an oxidizing substrate, presumably via the N-5 position of an isoalloxazine ring of FAD (Powell *et al.*, 1982), in the case of a low exchange rate with the medium. Since an analogous kinetic isotope effect, reaching 4, is estimated in the two-electron reduction of quinones by diaphorase from *Clostridium kluyveri* (Čenas *et al.*, 1989b), it may be assumed that the hydrogen transfer proceeds via a two-electron reduction of quinones by the

enzyme, i.e. a net transfer of hydride ion occurs. A small kinetic isotope effect of ferricyanide (Table 1) can be regarded as being secondary (Gould & Farid, 1988).

Since during the reduction of quinones by lipoamide dehydrogenase a significant amount of free semiquinone is formed and the reaction rate increases with increasing E_r^0 of oxidizing substrates (Fig. 7), it can be suggested that the reaction pathway includes an initial single-electron transfer. An analogous three-step (electron–proton–electron) hydride transfer is proposed in the oxidation of dihydropyridines by quinones (Fukuzumi *et al.*, 1987). In our case the formation of free semiquinones may be a side reaction caused by dissociation of flavin–quinone ion–radical pairs.

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