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One-electron oxidation-reduction properties of ascorbic acid

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The one-electron oxidation-reduction properties of ascorbate were investigated by EPR. The oxidations of ascorbate by 2,6-dichlorophenolindophenol (2-equivalent oxidant) and by ferricyanide (1-equivalent oxidant) both proceeded via a one-electron transfer mechanism, yielding ascorbate free radical as an intermediate. For the reduction of both 2,6-dichlorophenolindophenol and ferricyanide, the ascorbate free radical was much more reactive than ascorbate itself. The ascorbate free radical could also act as an effective one-electron oxidant for microsomal NADH-cytochrome b_5 reductase, cytochrome b_5 and mitochondrial outer membrane cytochrome b_5. The results suggest that in biological systems the reduction of ascorbate free radical is operative in the regeneration of fully reduced ascorbate.

Introduction

Ascorbic acid, which plays an important role in biological systems, is a bivalent oxidation-reduction molecule, and can reduce a number of electron acceptors. The overall electron-transfer reaction from ascorbic acid (AsH_2) to a bivalent electron acceptor, B, is described by the following equation:

$$AsH_2 + B \rightarrow As + BH_2$$

At physiological pH, ascorbic acid exists as an ionic form (AsH^-). Nonenzymatic oxidation of ascorbate by oxidants, such as cytochrome *c* [1–4] and molecular oxygen [5,6] are reported to proceed in two successive one-electron steps. It means that ascorbate free radical generated during the reactions further donates an electron to electron acceptors [2]. The kinetics of its formation and decay in such nonenzymatic systems has not been studied in detail.

Yamazaki and Piette [7] demonstrated that ascorbate oxidase catalyzes a typical one-electron oxidation of ascorbate and also that the free radical formed decays only through dismutation without further transfer of its electron to the enzyme, although the free radical is capable of reducing many electron acceptors. It is also shown [8,9] that the ascorbate free radical is formed during the catalytic reaction of dopamine-β-hydroxylase. Another interesting observation is that the ascorbate free radical is reduced by NADH-cytochrome b_5 reductase [10] and cytochrome b_5 [11]. A peculiar enzyme, named semidehydroascorbate reductase, is found in animals [12] and plants [13].

Therefore, the physiological role of ascorbate would be explained not only by the reactivity of ascorbate toward electron acceptors but also by the oxidation-reduction properties of the ascorbate free radical. The objective of the present investigation was to clarify the one-electron oxidation-re-

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; OM-cytochrome b_5, cytochrome b_5-like hemoprotein of outer mitochondrial membrane.

duction properties of ascorbate. For this purpose, we have studied by using EPR: (1) the reaction of ascorbate with a two-equivalent electron acceptor, DCIP; (2) the reaction of ascorbate with a one-equivalent electron acceptor, ferircyanide; (3) the reaction of the ascorbate free radical with NADH-cytochrome b_5 reductase, cytochrome b_5 and OM-cytochrome b_5. The mechanism of these electron transfer reactions will be discussed on the basis of one-electron redox potentials.

Materials and Methods

NADH-cytochrome b_5 reductase and cytochrome b_5 were prepared from pig microsomes by the method of Iyanagi et al. [14]. OM-cytochrome b_5 was prepared from rat or pig mitochondria outer membrane by the method of Ito [15]. The concentrations of the NADH-cytochrome b_5 reductase and cytochrome b_5 were determined using $\varepsilon = 10.2\ mM^{-1} \cdot cm^{-1}$ (460 nm) [16] and $\Delta\varepsilon = 185\ mM^{-1} \cdot cm^{-1}$ (424–409 nm) [17], respectively. The molar extinction coefficients of OM-cytochrome b_5 was taken to be the same as that for cytochrome b_5. The following molar extinction coefficients were used: for ferricyanide, $\varepsilon = 1.02\ mM^{-1} \cdot cm^{-1}$ (420 nm); for ascorbate, $\varepsilon = 15\ mM^{-1} \cdot cm^{-1}$ (265 nm) at pH 6.0; for 2,6-dichlorophenolindophenol, $\varepsilon = 20.6\ mM^{-1} \cdot cm^{-1}$ (600 nm). The ascorbate free radical concentration was determined using an EPR signal generated from an ascorbate oxidase reaction under the standard condition [18].

Ascorbate oxidase was purchased from Toyobo Co., Ltd. Ascorbate, NADH, 2,6-dichlorophenolindophenol (DCIP), ferricyanide and ferrocyanide were purchased from Wako Co. Dehydroascorbate was purchased from ICN Pharmaceuticals Inc. All other materials were obtained from commercial sources at the highest available states of purity.

EPR spectrometer equipped with a flow apparatus was the same as those described in the previous paper [10]. Optical absorbance measurements were carried out with a Hitachi recording spectrophotometer, Model 124 or 200-10, equipped with a thermostatically controlled cell compartment at 25°C.

Results

DCIP, which undergoes two-electron reduction, is a good electron acceptor for ascorbate [19]. It is of interest to clarify whether ascorbate is oxidized by way of a one-electron or a two-electron transfer in the reaction with DCIP [20]. The overall reaction is given by the following equation:

$$AsH^- + DCIP + H^+ \xrightarrow{k_1} As + DCIPH_2 \quad (1)$$

Ascorbate free radical was, however, observed in the steady state during the oxidation of ascorbate by DCIP (Fig. 1). The hyperfine parameter of the EPR signal was the same as that obtained in the ascorbate oxidase system [18]. This result indicates that the ascorbate free radical ($As^{\bar{\cdot}}$) observed did not electronically interact with DCIP. The free radical of DCIP was not observed under the present experimental conditions. This is due to fast dismutation constant of the DCIP free radical, the rate constant being measured as $10^8\ M^{-1} \cdot s^{-1}$ by pulse radiolysis (Kobayashi, K. and Iyanagi, T., unpublished results). The ascorbate free radical we observed was different in the kinetic pattern from that generated by reverse dismutation of ascorbate and dehydroascorbate [21] and the following reaction scheme was assumed for the formation and decay of the ascorbate free radical.

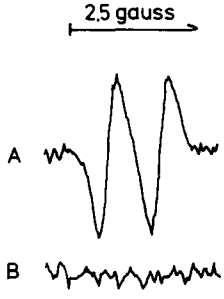

Fig. 1. The EPR spectra of the ascorbate free radical (A) formed in the steady state during the oxidation of ascorbate by DCIP. The solution of ascorbate was mixed with the solution of DCIP using continuous-flow method. Final concentrations: 200 μM ascorbate/50 μM DCIP/0.1 M potassium phosphate (pH 6.0). The same magnetic field was scanned in B soon after the flow stopped. The concentration of ascorbate free radical (A) was found to be 0.8 μM.

For formation:

$$AsH^- + DCIP \xrightarrow{k_2} As^\delta + DCIPH^\circ \quad (2)$$

$$AsH^- + DCIPH^\circ \xrightarrow{k_3} As^\delta + DCIPH_2 \quad (3)$$

For decay:

$$2As^\delta + H^+ \xrightarrow{k_4} AsH^- + As \quad (4)$$

$$As^\delta + DCIP + H^+ \xrightarrow{k_5} As + DCIPH^\circ \quad (5)$$

$$As^\delta + DCIPH^\circ + H^+ \xrightarrow{k_6} As + DCIPH_2 \quad (6)$$

If the ascorbate free radical is formed only by Eqn. 2 and decays only by dismutation (Eqn. 4), the steady state concentration of the free radical, $(As^\delta)s$ is given by the following Eqn. 7:

$$(As^\delta)s = \left(\frac{v}{k_4}\right)^{1/2} \quad (7)$$

where k_4 is a dismutation constant for the ascorbate free radical, and v is the initial rate of oxidation of ascorbate by DCIP. The curve calculated according to Eqn. 7 is shown in Fig. 2, curve A. However, the observed steady state concentration of ascorbate free radical was lower than that predicted from Eqn. 7, and deviation from the calculated curve became evident as the concentration of DCIP increased (Fig. 2, curve B). The results

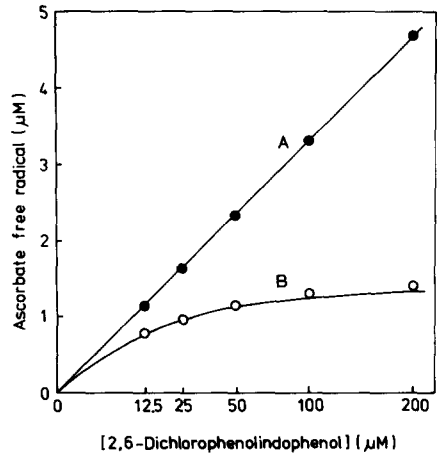

Fig. 2. Effect of the concentration of DCIP on the concentration of the ascorbate free radical at the steady state. The solution of ascorbate was mixed with various concentrations of DCIP. In curve A, the radical concentration was calculated from Eqn. 7, using $k_4 = 6 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ [3] and v, the initial velocity of ascorbate oxidation. Curve B shows the observed data (open circle), and the solid line drawn through the data points was calculated from Eqn. 8, using $k_4 = 6 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ and $k_5 = 9.5 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ (Table I). Final concentratoins: 400 μM ascorbate/various concentration of DCIP/0.1 M potassium phosphate (pH 6.0).

suggest that the ascorbate free radical generated by Eqn. 2 reacted further with DCIP and probably in part with DCIPH° (Eqns. 5 and 6). The reaction of ascorbate free radical with DCIP was directly demonstrated by the fact that the ascorbate free radical generated by the ascorbate-oxidase system decreased in the presence of DCIP (Table I). In

TABLE I

EFFECT OF DCIP UPON THE STEADY STATE CONCENTRATION OF ASCORBATE FREE RADICAL IN THE ASCORBATE OXIDASE REACTION

Final concentrations: 50 μM ascorbate/50 μM DCIP/0.05 μM ascorbate oxidase (the initial rate of ascorbate oxidation, 10 μM s^{-1})/0.1 M potassium phosphate (pH 6.0). The k_5 was calculated from Eqn. 8, using $k_4 = 6 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ [3] and $k_1 = 1.5 \cdot 10^3$ $M^{-1} \cdot s^{-1}$.

Ascorbate (μM)	DCIP (μM)	Ascorbate oxidase (μM)	Steady-state concentration of As^δ (μM)	$k_5 \cdot 10^{-5}$ ($M^{-1} \cdot s^{-1}$)
50	50	–	0.3	–
50	–	0.05	1.3	–
50	50	0.05	0.7	9.5

the present experiments the initial velocity of the reaction was analyzed and the reverse reaction could be neglected. As shown in Table I, k_5 was calculated to be about $9.5 \cdot 10^5\ M^{-1} \cdot s^{-1}$ from the following equation at steady state [10]:

$$\frac{d(As^{\delta})}{dt} = 2v - \left[2k_4(As^{\delta})_s^2 + k_5(As^{\delta})_s(DCIP)\right] = 0 \qquad (8)$$

The present studies gave a value of $1.5 \cdot 10^3\ M^{-1} \cdot s^{-1}$ for k_1. Therefore, the ascorbate free radical was much more reactive for DCIP than ascorbate itself. The data points in Fig. 2, curve B fitted to Eqn. 8, suggesting that at low ratios of DCIP to ascorbate the main reaction consisted of reactions 2 and 4 and at the high ratios reaction 5 as well became dominant (Fig. 2).

Ferricyanide, which undergoes one-electron reduction, was also used as an electron acceptor for ascorbate. The overall reaction is given by the following equation:

$$AsH^- + 2Fe(CN)_6^{3-} \xrightarrow{k_9} As + 2Fe(CN)_6^{4-} + H^+ \qquad (9)$$

In the reaction between ascorbate (AsH^-) and ferricyanide one-electron transfer reactions are compulsorily involved. The formation and decay of the ascorbate free radical is given by the following equation.
For formation:

$$AsH^- + Fe(CN)_6^{3-} \xrightarrow{k_{10}} As^{\delta} + Fe(CN)_6^{4-} + H^+ \qquad (10)$$

For decay:

$$As^{\delta} + Fe(CN)_6^{3-} \xrightarrow{k_{11}} As + Fe(CN)_6^{4-} \qquad (11)$$

$$2As^{\delta} + H^+ \xrightarrow{k_4} AsH^- + As \qquad (4)$$

When ascorbate and ferricyanide were mixed in the flow apparatus, the formation of ascorbate free radical was observed. As shown in Fig. 3, the steady state concentration of ascorbate free radical decreased with increasing the ferricyanide concentration. It is demonstrated in Fig. 4 that the ascorbate free radical directly reacted with ferricyanide. The steady state concentration of ascorbate free radical, generated during the ascorbate oxidase reaction was decreased when ferricyanide was present (Fig. 4), similarly as was seen when DCIP was present (Table I). The rate constant of

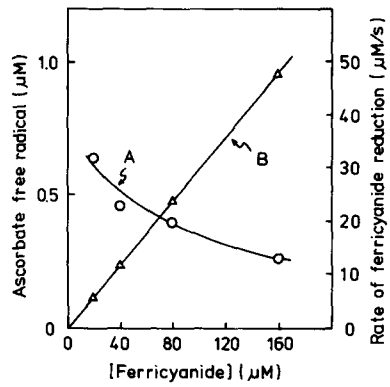

Fig. 3. Relationship between the concentrations of ferricyanide and ascorbate free radical at the steady state. The ascorbate solution was mixed with a various concentrations of ferricyanide. Curve A is free radical concentration observed at the steady state, and curve B is the initial velocity of ferricyanide reduction by ascorbate. Final concentrations: 1 mM ascorbate/various concentrations of ferricyanide/0.1 M potassium phosphate (pH 6.0).

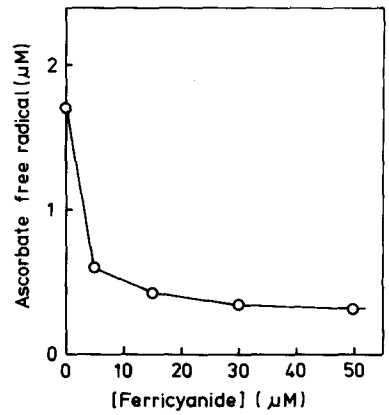

Fig. 4. Effect of ferricyanide concentration upon the steady-state concentration of ascorbate free radical formed in the ascorbate oxidase reaction. The free radical concentration was measured in the absence or presence of various concentrations of ferricyanide. The steady state was attained in 0.1 s. Final concentrations: 200 μM ascorbate/0.085 μM ascorbate oxidase (the initial rate of ascorbate oxidation, 17 $\mu M \cdot s^{-1}$)/various concentrations of ferricyanide/0.1 M potassium phosphate (pH 6.0).

k_{11} could be roughly calculated from the following equation at steady state [10]:

$$\frac{d(As^{\bar{\cdot}})}{dt} = 2v - \left[2k_4(As^{\bar{\cdot}})_s^2 + k_{11}(As^{\bar{\cdot}})_s\left(Fe(CN)_6^{3-}\right)\right] = 0 \quad (12)$$

Where v is the initial rate of oxidation of ascorbate by ferricyanide. The k_{11} value was estimated to be about $4 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ from the data of Fig. 3. The present studies gave a value of $5 \cdot 10^2$ $M^{-1} \cdot s^{-1}$ for k_{10} (Fig. 3). The results indicate that the ascorbate free radical form ($As^{\bar{\cdot}}$) is more active for ferricyanide than ascorbate itself.

The ascorbate free radical is directly reduced by NADH-cytochrome b_5 reductase [10] and cytochrome b_5 [11], which are concentrated in the endoplasmic reticulum. It has also been proposed that the cytochrome b_5 of the outer mitochondrial membrane is associated with semidehydroascorbate reductase activity [22].

Fig. 5 shows that the rate of NADH oxidation was closely correlated to the concentration of ascorbate free radical calculated from the equation of $(Ks)^{2/2}(As)^{1/2}(AsH^-)^{1/2}$, where Ks is the semi-

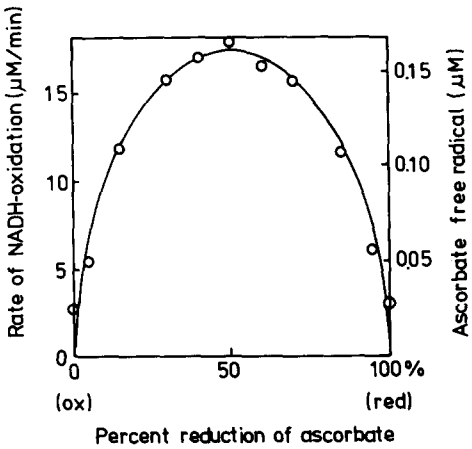

Fig. 5. The reduction of the ascorbate free radical by the NADH-cytochrome b_5 reductase. NADH was oxidized by the NADH-cytochrome b_5 reductase, which was reoxidized by the ascorbate free radical formed from comproportionating reaction of ascorbate and dehydroascorbate. Concentrations: 100 μM NADH/2 μM NADH-cytochrome b_5 reductase/various ratios of AsH^-/As ($AsH^- + As = 10$ mM)/0.1 M potassium phosphate (pH 7.0). The solid line drawn through the data points (open circle) was calculated from the equation $(Ks)^{1/2}(As)^{1/2}(AsH_2)^{1/2}$, using $Ks = 10^{-9}$ [23].

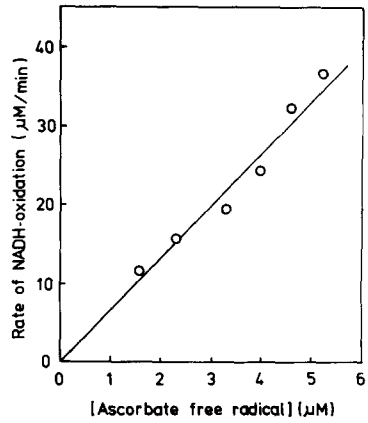

Fig. 6. Relationship between NADH oxidation and ascorbate free radical concentration at the steady state. The initial rate of NADH oxidation was measured by the addition of ascorbate and its oxidase in the NADH-cytochrome b_5 reductase system. Concentrations: 100 μM NADH/0.15 μM NADH-cytochrome b_5 reductase/0.1 M potassium phosphate (pH 7.0). The steady-state concentration of ascorbate free radical was changed by amount of ascorbate oxidase and calculated from Eqn. 7, using $k_4 = 6 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ [3] and v, the initial velocity of ascorbate oxidation.

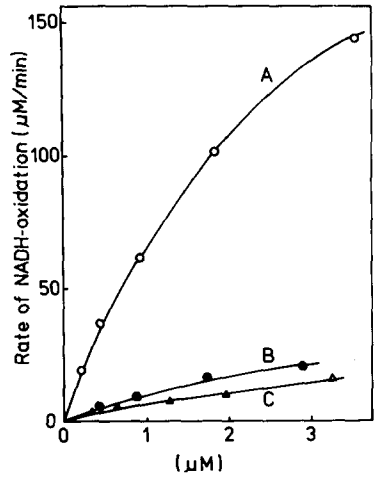

Fig. 7. Relationship between the rate of ascorbate free radical-induced oxidation of NADH and the concentrations of NAdH-cytochrome b_5 reductase, cytochrome b_5 or OM-cytochrome b_5. The initial rate of NADH oxidation was measured after the addition of ascorbate oxidase. Concentrations: (A) 100 μM NADH/various concentrations of NADH-cytochrome b_5 reductase; (B) 100 μM NADH/0.045 μM NADH-cytochrome b_5 reductase/various concentrations of OM-cytochrome b_5; (C) 100 μM NADH/0.045 μM NADH-cytochrome b_5 reductase/various concentrations of cytochrome b_5. In (A), (B) and (C), the initial rate of ascorbate oxidation by ascorbate oxidase was 168 μM/min, and the steady state concentration of ascorbate free radical was found to be 2.2 μM. In all systems, 0.1 M potassium phosphate (pH 7.0) was used.

quinone formation constant defined by Michaelis [24]. The rate of NADH oxidation was proportional to the steady-state concentration of ascorbate free radical (Fig. 6) generated from ascorbate oxidase reaction, and the rate constant for the reaction of the reductase with ascorbate free radical was estimated to be $1.5 \cdot 10^6\ M^{-1} \cdot s^{-1}$. Our previous conclusion [10] was thus confirmed by quantitative data. As shown in Fig. 7, NADH-cytochrome b_5 reductase was a good electron donor for the ascorbate free radical compared with cytochrome b_5 and OM-cytochrome b_5. At a high concentration of the NADH-cytochrome b_5 reductase, the ascorbate free radical formed by the ascorbate oxidase system was stoichiometrically reduced to ascorbate by the reductase (data not shown).

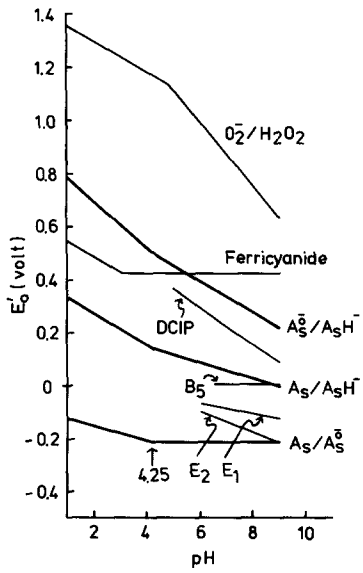

Fig. 8. pH dependence of the redox potentials for ascorbate and other couples. The one-electron redox potentials of ascorbate were calculated from the data; $E_{m,7} = 60$ mV [30], Ks = 10^{-9} (pH 7.0) [23] and $pK_a = 4.25$ [3]. The other redox potentials were cited from the following references: O_2^-/H_2O_2 [31]; ferricyanide/ferrocyanide [32]; DCIP [30]; cytochrome $b_5(B_5)$ [14]; cytochrome b_5 reductase, E_1 (E-FAD-NAD$^+$/E-FAD$^{\bar{\cdot}}$-NAD$^+$) and E_2 (E-FAD$^{\bar{\cdot}}$-NAD$^+$/E-FADH$^-$-NAD$^+$) [29].

Discussion

In the present study, we have demonstrated that the ascorbate free radical is formed during the oxidation of ascorbate by DCIP or ferricyanide. The rate of ascorbate oxidation depended on the concentrations of these electron acceptors, linearly up to the concentrations examined (data not shown). Applicability of Eqn. 8 for curve B in Fig. 2 suggests that ascorbate is oxidized by DCIP via two successive one-electron transfers. That is, the ascorbate free radical is formed through bimolecular collision as follows (25):

$$AsH^- + B \overset{slow}{\rightarrow} (AsH^- - B) \overset{fast}{\rightarrow} As^{\bar{\cdot}} + BH^{\circ}(B^{\bar{\cdot}} + H^+)$$

and decays through reaction 5 as well dismutation (reaction 4).

According to Marcus theory applied to outer sphere electron-transfer reactions [26], the difference in redox potentials between oxidant and reductant is a factor to control the rate of reaction. As shown in Fig. 8, the midpoint potential for couples of As/AsH$^-$, As$^{\bar{\cdot}}$/AsH$^-$ and As/As$^{\bar{\cdot}}$ couples are 90, 390 and −210 mV at pH 6.0, respectively. The midpoint potential for the ferricyanide/ferrocyanide couple is 425 mV at pH 6.0 [32]. Therefore, reactions 10 and 11 both are expected to be favourable and the latter is much faster. The midpoint potential for the DCIP/$DCIPH_2$ couple is 290 mV (pH 6.0), but that for the DCIP/DCIPH° couple is not known. At any rate, DCIP is reduced by the ascorbate free radical faster than by ascorbate.

Different reactivity of ascorbate with molecular oxygen (O_2) and superoxide (O_2^-) may also be explained by the one-electron redox potential of each molecule (Fig. 8). The redox couples, As$^{\bar{\cdot}}$/AsH$^-$ ($E_{m,7} = 330$ mV) and As/As$^{\bar{\cdot}}$ ($E_{m,7} = -210$ mV) are not operative in reducing oxygen to superoxide ($E_{m,7} = -270$ to -330 mV) [27,28], but are capable of reducing superoxide to hydrogen peroxide ($E_{m,7} = 870$ mV) with rate constants of $2.7 \cdot 10^5\ M^{-1} \cdot s^{-1}$ [5] and $2.3 \cdot 10^8\ M^{-1} \cdot s^{-1}$ [6], respectively.

The one-electron redox potentials, E_1 and E_2, of NADH-cytochrome b_5 reductase are shown in Fig. 8 [29]. Dehydroascorbate can not serve as an oxidant for the reductase (Fig. 5), but the ascorbate free radical was reduced by the reductase,

with a rate constant of $1.5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ (Fig. 6). The ascorbate free radical is reduced also by reduced forms of cytochrome b_5 and OM-cytochrome b_5, but cannot reduce the cytochromes (data not shown). The latter fact is contrary to our expectations, and the reaction is not at present unexplained. Cytochrome c ($E_{m,7} = 262$ mV) is reduced by the ascorbate free radical with a rate constant of $6.6 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ [2,27]. In general, the occurrence of electron transfer cannot be predicted simply by reference to the redox potentials. As discussed by Salemme [33] and Meyer et al. [34], we must consider another factor, such as an extent of heme exposure to solvent and a charge group in the vicinity of the electron-transfer site.

In conclusion, the ascorbate is oxidized by two-electron acceptors via free radical intermediates, and the free radicals serve not only as a one-electron reductant but also as a one-electron oxidant. A systematic analysis for the reactivity of ascorbate free radical will yield useful information on physiological roles of ascorbate.

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