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Kinetic Behavior of the Monodehydroascorbate Radical Studied by Pulse Radiolysis

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ABSTRACT: The reactions of the monodehydroascorbate radical ($As^{\bullet -}$) with various biological molecules were investigated by pulse radiolysis. $As^{\bullet -}$ reacted with both fully reduced and semiquinone forms of hepatic NADH-cytochrome b_5 reductase with second-order rate constants of 4.3×10^6 and 3.7×10^5 M^{-1} s^{-1}, respectively, at pH 7.0. In contrast, no reaction of $As^{\bullet -}$ with ferrous cytochrome b_5 could be detected by pulse radiolysis, whereas the oxidation of cytochrome b_5 by $As^{\bullet -}$ was observed by ascorbate–ascorbate oxidase method. This suggests that the rate constant of $As^{\bullet -}$ with the ferrous cytochrome b_5 must be several orders in magnitude smaller than that of the disproportionation of $As^{\bullet -}$. On the other hand, $As^{\bullet -}$ reduced Fe^{3+}EDTA with a second-order rate constant of 4.0×10^6 M^{-1} s^{-1} but did not reduce ferric hemoproteins such as metmyoglobin, methemoglobin, and cytochrome b_5 by either the pulse radiolysis or the ascorbate–ascorbate oxidase method.

Ascorbate (AsH^-)[1] plays physiologically important roles in various metabolic reactions involving the biosyntheses of collagen (Prockop et al., 1976; Kivirikko & Myllyla, 1980; Myllyla et al., 1984) and norepinephrine (Rosenberg & Lovenberg, 1980). In addition, AsH^- is critically involved in cellular defense against oxidative injury, serving as a reductant in scavenging reactive oxygen and radical species (Packer et al., 1979; Galaris et al., 1989; Rose et al., 1990; Pietri et al., 1990). In these processes monodehydroascorbate radical ($As^{\bullet -}$) is produced by univalent oxidation of AsH^-. Its production has been shown in enzymatic and nonenzymatic reactions (Yamazaki & Piette, 1961; Skotland & Ljones, 1980;

[1] Abbreviations: AsH^-, ascorbate; $As^{\bullet -}$, monodehydroascorbate; As, dehydroascorbate; OM-cytochrome *b*, cytochrome *b* of the outer mitochondrial membrane; e_{aq}^-, hydrated electron; E-FAD, oxidized NADH-cytochrome b_5 reductase; E-$FAD^{\bullet -}$, semiquinone enzyme; E-$FADH^-$, fully reduced enzyme; EDTA, ethylenediaminetetraacetic acid.

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Diliberto & Allen, 1981; Cabelli & Bielski, 1983; Packer et al., 1979; Niki et al., 1984; Mukai et al., 1991). In the absence of a reactant for $As^{\bullet -}$, $As^{\bullet -}$ is spontaneously disproportionated to AsH^- and dehydroascorbate (As) (Bielski et al., 1971). At present, however, the kinetic behavior of $As^{\bullet -}$ in biological systems has not been elucidated. It seems likely that $As^{\bullet -}$ generated in cells is scavenged, since $As^{\bullet -}$ may attack various components of cells. In fact, it was reported that some enzyme reactions are inhibited by $As^{\bullet -}$ (Davison et al., 1986; Harwood et al., 1986).

It has been shown that $As^{\bullet -}$ is reduced to regenerate AsH^- by an NAD(P)H-dependent activity in many plants (Mathews, 1951; Arrigoni et al., 1981; Hossain et al., 1984; Yamauchi et al., 1984) and mammalian cells (Schneider & Staudinger, 1965). In plants, this activity participates in AsH^- regeneration in chloroplasts for scavenging hydrogen peroxide by ascorbate peroxidase (Hossain et al., 1984). Hossain and Asada first purified a peculiar enzyme, named monodehydroascorbate reductase, from cucumber fruit (1985). In mammalian cells, on the other hand, NADH-dependent $As^{\bullet -}$ reductase activity is localized in the outer membranes of mitochondria (Ito et al., 1981; Diliberto et al., 1982), microsomes (Schulze & Staudinger, 1971; Hara & Minakami, 1971), and the Golgi apparatus (Sun et al., 1983). Ito et al. have proposed that cytochrome *b* of the outer mitochondrial membranes (OM-cytochrome *b*) is associated with this activity (1981). In adrenal medulla, Diliberto et al. (1982) and Wakefield et al. (1986) demonstrated that mitochondrial NADH–$As^{\bullet -}$ reductase drives the re-reduction of $As^{\bullet -}$ generated inside the chromaffin granule by dopamine β-hydroxylase. On the other hand, the purified microsomal NADH–cytochrome b_5 reductase is a good electron donor for $As^{\bullet -}$, compared with cytochrome b_5 and OM-cytochrome *b* (Iyanagi et al., 1985).

Some of the advantages of the pulse radiolysis technique for determining the spectral and kinetic behavior of $As^{\bullet -}$ have been demonstrated (Bielski & Allen, 1970; Bielski et al., 1971; Bielski, 1981; Cabelli & Bielski, 1983). The present paper describes the kinetic behavior of $As^{\bullet -}$ in biological systems by use of pulse radiolysis technique. We have confirmed that $As^{\bullet -}$ is reduced by both semiquinone and fully reduced forms of hepatic NADH–cytochrome b_5 reductase at appreciable rates. In contrast, we have been unable to detect reactions of $As^{\bullet -}$ with ferric hemoproteins such as metmyoglobin, methemoglobin, and cytochrome b_5, though $As^{\bullet -}$ was found to reduce Fe^{3+}EDTA.

Materials and Methods

Lysosome-solubilized NADH–cytochrome b_5 reductase was prepared from pig liver by the method of Iyanagi et al. (1984). Cytochrome b_5 prepared from pig microsomes was a generous gift from Dr. Iyanagi. Ascorbate oxidase, NADH, and NAD^+ were purchased from the Oriental Yeast Co. All other reagents were commercially obtained as the analytical grade. The concentrations of NADH–cytochrome b_5 reductase and cytochrome b_5 were determined with the use of $\epsilon = 10.2\ mM^{-1}\ cm^{-1}$ (460 nm) (Strittmatter & Velick, 1957) and $\Delta\epsilon = 185\ mM^{-1}\ cm^{-1}$ (424–409 nm) (Omura & Takesue, 1970), respectively. The concentration of $As^{\bullet -}$ generated by pulse radiolysis was estimated from the absorbance change at 360 nm (Bielski et al., 1971).

A fully reduced form of NADH–cytochrome b_5 reductase was prepared by the addition of 2 equiv of NADH to the oxidized enzyme anaerobically. A semiquinone form of the enzyme was prepared by the anaerobic addition of 0.5 equiv of NADH and 2 equiv of NAD^+ to the oxidized enzyme by the method of Iyanagi et al. (1984). A ferrous form of cytochrome b_5 was prepared by the addition of sodium dithionite to the ferric form, and the cytochrome thus formed was passed through a Sephadex G-25 column to eliminate sodium dithionite.

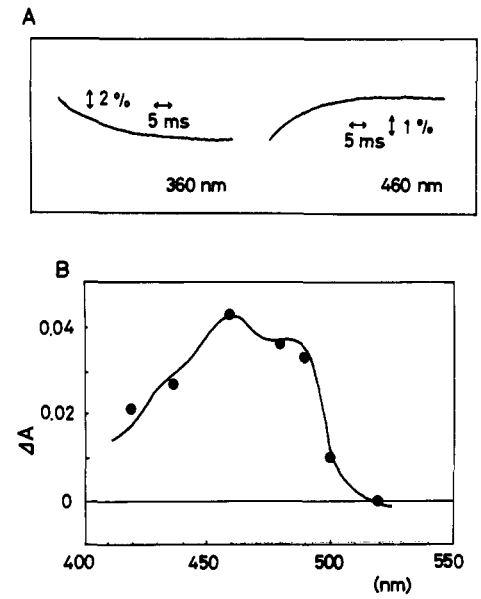

FIGURE 1: (A) Oscilloscope traces of the absorption change after pulse radiolysis of the fully reduced form of NADH–cytochrome b_5 reductase measured at 360 and 460 nm in the presence of AsH^- and N_2O. The reaction medium contained 22.3 μM fully reduced enzyme, 45 μM NADH, 5 mM sodium ascorbate, and 20 mM phosphate buffer at pH 7.0. (B) Kinetic difference spectrum at 20 ms after the pulse. Experimental points (●) were obtained from the absorbance changes after reaction by $As^{\bullet -}$. The line shows the difference spectrum of the semiquinone form of NADH–cytochrome b_5 reductase minus the fully reduced form. The spectrum was obtained by the titration of sodium dithionite to the oxidized enzyme in the presence of 5 mM NAD^+ and was normalized against the absorbance change at 460 nm after the pulse.

Samples for pulse radiolysis were prepared as follows. Solutions containing 20 mM phosphate buffer, 5 mM sodium ascorbate, and the reactants were deaerated by repeated evacuation and flushing with nitrogen, and then 1 atm of N_2O gas was introduced anaerobically. A fresh solution was used for each pulse.

Pulse radiolysis experiments were performed with an electron linear accelerator of the Institute of Scientific and Industrial Research, Osaka University (Kobayashi & Hayashi, 1984). The pulse width and energy were 7 ns and 27 MeV, respectively. Photolysis by the analyzed light was minimized by means of an optical shutter and selected filters.

Optical absorption spectra were measured by use of a Shimadzu MPS-2000 spectrophotometer.

Results

Reaction of NADH–Cytochrome b_5 Reductase. In the presence of 5 mM ascorbate (AsH^-), monodehydroascorbate ($As^{\bullet -}$) is produced via reactions 1 and 2 by pulse radiolysis

$$e_{aq}^- + N_2O + H_2O \rightarrow OH^\bullet + OH^- + N_2 \quad (1)$$

$$OH^\bullet + AsH^- \rightarrow H_2O + As^{\bullet -} \quad (2)$$

of N_2O saturated aqueous solution. A transient spectrum with an absorption maximum at 360 nm was observed at 100 ns after pulse radiolysis. This spectrum is identical with that obtained upon pulse radiolysis of free AsH^- in aqueous solution (Bielski et al., 1971). In the presence of the fully reduced form of NADH–cytochrome b_5 reductase, the decay of $As^{\bullet -}$ at 360

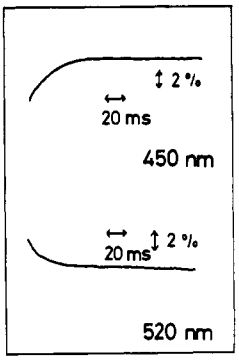

FIGURE 2: Oscilloscope traces of the absorption change after pulse radiolysis of the semiquinone form of b_5 reductase measured at 460 and 520 nm in the presence of AsH^- and N_2O at pH 7.0. The semiquinone form of the enzyme was prepared by the addition of 11 μM NADH and 44 μM NAD^+ to 22 μM oxidized enzyme.

nm was accompanied by an absorption increase at 460 nm, as shown in Figure 1A. Figure 1B shows the kinetic difference spectrum at 20 ms after the pulse. The figure also shows the difference spectrum of the semiquinone of the enzyme minus the reduced form. Here, the semiquinone and fully reduced forms were obtained by the titration of sodium dithionite to the oxidized enzyme in the presence of NAD^+, and the difference spectrum was normalized against the absorbance change observed at 460 nm after the pulse. These spectra are almost identical with each other, as shown in Figure 1B. It is, therefore, concluded that $As^{\bullet -}$ reacts with the fully reduced enzyme (E–$FADH^-$) to form the semiquinone form (E–$FAD^{\bullet -}$), as shown in reaction 3, where NAD^+ is bound to both

$$As^{\bullet -} + E\text{–}FADH^{-}\cdots NAD^+ \rightarrow AsH^- + E\text{–}FAD^{\bullet -}\cdots NAD^+ \quad (3)$$

fully reduced and semiquinone forms (Iyanagi et al., 1984; Kobayashi et al., 1988). The absorption change in reaction 3 obeyed the pseudo-first-order kinetics, where 2 μM $As^{\bullet -}$ is generated in 20–60 μM enzyme. The second-order rate constant for reaction 3 is calculated to be 4.2×10^6 M^{-1} s^{-1} at pH 7.0. The rate constant does not depend on pH in the range of 6–8.

The reaction of the semiquinone form of the enzyme with $As^{\bullet -}$ was performed. The semiquinone form was prepared by the addition of 0.5 equiv of NADH and 2 equiv of NAD^+ to the oxidized enzyme. Under our experimental conditions, however, 58.5, 20.7, and 20.7% of the sample are the semiquinone, the fully reduced, and the oxidized forms, respectively. The contents were estimated from the semiquinone formation constant of 8 at pH 7.0 (Iyanagi et al., 1984). Figure 2 shows the absorption change of the enzyme prepared as described above after the pulse. The absorption at 520 nm, an isosbestic point between the fully reduced and the semiquinone forms, decreased with the increase of absorption at 450 nm. The absorption change at 520 nm, therefore, is concluded to correspond to the reaction of $As^{\bullet -}$ with the semiquinone form. Therefore, it is concluded that $As^{\bullet -}$ also reacts with the semiquinone form to yield the oxidized form, as shown in reaction 4. Under these conditions, NAD^+ dissociates after

$$As^{\bullet -} + E\text{–}FAD^{\bullet -}\cdots NAD^+ \xrightarrow{H^+} AsH^- + E\text{–}FAD + NAD^+ \quad (4)$$

the reaction, since the affinity of NAD^+ toward the oxidized enzyme is low (Iyanagi, 1977).

The rate of the decay of $As^{\bullet -}$ is expressed by

$$d[As^{\bullet -}]/dt = \{k_3[FADH^-] + k_4[FAD^{\bullet -}]\}[As^{\bullet -}]$$

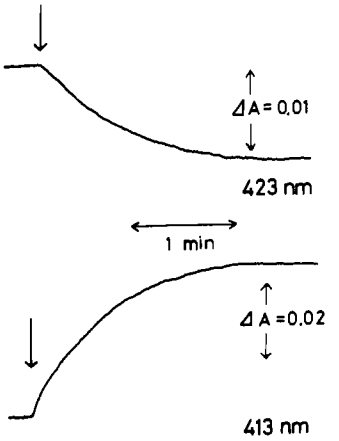

FIGURE 3: Oxidation of ferrous cytochrome b_5 by $As^{\bullet -}$ measured at 423 and 413 nm. The assay mixture contained (in 3 mL) 0.16 μM ferrous cytochrome b_5, 1 mM sodium ascorbate, 0.1 M phosphate buffer (pH 7.0), and 4 units of ascorbate oxidase. The arrows indicate the addition of ascorbate oxidase.

Table I: Rate Constants of $As^{\bullet -}$ with Various Molecules and Their Redox Potentials

reactant	second-order rate constants (M^{-1} s^{-1})	$E_{m,7}$ (mV)
	Oxidation	
cytochrome b_5 reductase		
fully reduced form	4.3×10^6	−147[a]
semiquinone form	3.7×10^5	−88[a]
ferrous cytochrome b_5	$<10^4$	0[b]
	Reduction	
Fe^{3+}EDTA	4.0×10^6	117[c]
$Fe(CN)_6^{3-}$	4.0×10^6[d]	425[e]
cytochrome *c*	6.6×10^3[f]	262
metmyoglobin	nd[i]	50[g]
methemoglobin	nd[i]	150[h]
ferric cytochrome b_5	nd[i]	0[a]

[a] Iyanagi et al. (1984). [b] Iyanagi (1977). [c] Ilan et al. (1977). [d] Iyanagi et al. (1985). [e] O'Reilly (1973). [f] Yamazaki (1962). [g] Brunori et al. (1971). [h] Antonini et al. (1964). [i] Not determined.

where k_3 and k_4 are the rate constants for the reactions of $As^{\bullet -}$ with the fully reduced and the semiquinone forms, respectively. The apparent second-order rate constant (k_{app}) determined at 360 nm can be expressed as

$$k_{app}[b_5 \text{ reductase}] = k_3[FADH^-] + k_4[FAD^{\bullet -}]$$

Since the values of k_{app} and k_3 are 1.1×10^6 M^{-1} s^{-1} and 4.2×10^6 M^{-1} s^{-1}, respectively, the k_4 value is calculated to be 3.7×10^5 M^{-1} s^{-1}. The value was in good agreement with that determined by the absorption change at 520 nm.

Reaction of Ferrous Cytochrome b_5. The reaction of ferrous cytochrome b_5 with $As^{\bullet -}$ was performed. However, we could not detect any absorption changes corresponding to the oxidation of cytochrome b_5. Under our conditions, $As^{\bullet -}$ decayed by second-order kinetics. This suggests that $As^{\bullet -}$ hardly reacts with the ferrous cytochrome b_5 but rather reacts with itself.

In order to investigate whether $As^{\bullet -}$ reacts with the ferrous cytochrome b_5, the absorption change of cytochrome b_5 was measured upon the addition of ascorbate and ascorbate oxidase. As shown in Figure 3, the absorptions at 423 and 413 nm were decreased and increased, respectively, as soon as ascorbate oxidase was added. These absorption changes correspond to the oxidation of cytochrome b_5 by $As^{\bullet -}$, suggesting that $As^{\bullet -}$ can react with the ferrous cytochrome b_5.

Reaction of $As^{\bullet -}$ with Fe^{3+}EDTA. The reaction of Fe^{3+}-EDTA with $As^{\bullet -}$ was performed. In pulse radiolysis of Fe^{3+}EDTA, the resulting absorbance at 330 nm increased,

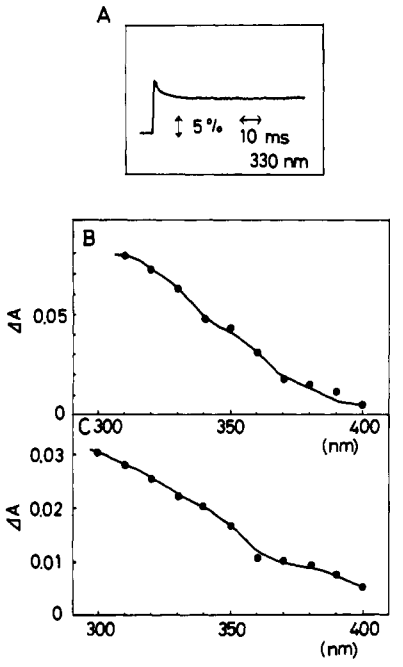

FIGURE 4: (A) Oscilloscope trace of the absorption change after pulse radiolysis of Fe^{3+}EDTA measured at 330 nm in the presence of ascorbate and N_2O. The reaction medium contained 100 μM Fe^{3+}EDTA, 5 mM sodium ascorbate, and 20 mM phosphate buffer at pH 7.0. (B) Kinetic difference spectrum at 20 ms after the reaction of Fe^{3+}EDTA with $As^{\bullet -}$. (C) Kinetic difference spectrum at 20 ms after the reaction of CO_2^- with Fe^{3+}EDTA.

after the decay of $As^{\bullet -}$ (Figure 4A). Figure 4B shows the difference spectrum of the reaction product. A similar spectrum was obtained in the reaction of Fe^{3+}EDTA with CO_2^- (Figure 4C). It is, therefore, concluded that $As^{\bullet -}$ reacts with Fe^{3+}EDTA to form Fe^{2+}EDTA, as shown in reaction 5. The

$$As^{\bullet -} + Fe^{3+}EDTA \rightarrow As + Fe^{2+}EDTA \quad (5)$$

second-order rate constant of reaction 5 is estimated to be 4.0×10^6 M^{-1} s^{-1} at pH 7.0. A similar rate constant was obtained in the reaction of $As^{\bullet -}$ with ferricyanide by the kinetic analysis at the steady state (4×10^6 M^{-1} s^{-1}) (Iyanagi et al., 1985). On the other hand, we could not detect the reactions of $As^{\bullet -}$ with the ferric hemoproteins such as metmyoglobin, methemoglobin, and cytochrome b_5 by either ascorbate–ascorbate oxidase or pulse radiolysis methods.

Discussion

The reaction scheme in NADH-dependent $As^{\bullet -}$ reductase activity by b_5 reductase can be schematized as follows. The enzyme FAD is reduced by NADH (step i) to form a charge transfer complex, E–$FADH^-$···NAD^+. The reduced enzyme then donates the electrons to $As^{\bullet -}$ through two successive one-electron transfers, and the FAD semiquinone, E–$FAD^{\bullet -}$···NAD^+, is an intermediate (steps ii and iii). In this reaction scheme, it was confirmed directly by the use of the pulse radiolysis technique that $As^{\bullet -}$ was reduced by both fully reduced and semiquinone forms of b_5 reductase. The second-order rate constants of reactions 3 and 4 obtained with this technique ($k_3 = 4.3 \times 10^6$ M^{-1} s^{-1}, $k_4 = 3.7 \times 10^5$ M^{-1} s^{-1}) are good agreement with the value reported (1.5×10^6 M^{-1} s^{-1}) in which an ascorbate–ascorbate oxidase was employed (Iyanagi et al., 1985).

There was no spectral indication for the reaction of ferrous cytochrome b_5 with $As^{\bullet -}$ at any wavelength, though the reaction was observed by employing the ascorbate oxidase–ascorbate reaction. The difference can be explained by the slow reaction of $As^{\bullet -}$ with ferrous cytochrome b_5. If the reaction of $As^{\bullet -}$ with cytochrome b_5 occurs very slowly, then the reaction is suppressed by the disproportionation of $As^{\bullet -}$. Hence, the rate constant for the reaction must be several orders in magnitude smaller than that of the disproportionation of $As^{\bullet -}$ (2×10^5 M^{-1} s^{-1} at pH 7.4) (Bielski et al., 1971) and the rate constant may be smaller than 10^4 M^{-1} s^{-1}. This result is consistent with that calculated by the kinetic analysis at the steady state by employing the ascorbate–ascorbate oxidase reaction (10^4 M^{-1} s^{-1}) (Hara & Minakami, 1971). A similar discrepancy between pulse radiolysis and other methods was seen in the reaction of O_2^- with horseradish peroxidase A_2 (Shimizu et al., 1989).

Table I compares the rate constants for the reactions of $As^{\bullet -}$ with various reactants and their redox potentials. The redox couple $As^{\bullet -}/AsH^-$ ($E_{m,7}$ = 330 mV) (Iyanagi et al., 1985) is operative in the oxidation of the fully reduced ($E_{m,7}$ = −147 mV) and semiquinone forms ($E_{m,7}$ = −88 mV) of b_5 reductase (Iyanagi et al., 1984) and the ferrous cytochrome b_5 ($E_{m,7}$ = 0 mV) (Iyanagi, 1977). It is noted that the rate constants of these reactions increase with the decrease of the redox potentials of each molecule. In the reduction by $As^{\bullet -}$, on the other hand, the occurrence of electron transfer cannot be predicted simply by reference to the redox potentials. We could not detect the reaction of $As^{\bullet -}$ with ferric hemoproteins such as metmyoglobin ($E_{m,7}$ = 53 mV) (Brunori et al., 1971), methemoglobin ($E_{m,7}$ = 175 mV) (Antonini et al., 1964), and cytochrome b_5 ($E_{m,7}$ = 0 mV) (Iyanagi et al., 1977) even by the ascorbate–ascorbate oxidase method, though these reactions are energetically favorable to the redox couples of these hemoproteins and $As/As^{\bullet -}$ (−210 mV), as shown in Table I. In an exceptional case, cytochrome c ($E_{m,7}$ = 262 mV) is reduced by $As^{\bullet -}$ slowly (6.6×10^3 M^{-1} s^{-1}) (Yamazaki, 1962). In contrast, $As^{\bullet -}$ acts as an effective one-electron reductant for low molecular weight compounds such as Fe^{3+}EDTA, ferricyanide, and 2,6-dichlorophenolindophenol (Iyanagi et al., 1985). These results may be explained by the stability of $As^{\bullet -}$. The $As^{\bullet -}$ may decay prior to access to the heme buried in a hydrophobic pocket. A result similar to that reported here was obtained in the reaction of NAD$^\bullet$ with the higher oxidation state of peroxidase (Kobayashi, 1990).

The second-order rate constant for b_5 reductase is one of the greatest values measured for the reaction of $As^{\bullet -}$ with biological molecules. Recently, a similar rate constant (1.2×10^6 M^{-1} s^{-1}) was obtained in the oxidation of cytochrome b_{561} by $As^{\bullet -}$. This reaction is physiologically important for the passage of electrons from cytosolic AsH^- to intragranular $As^{\bullet -}$ in adrenal medullary chromaffin vesicles (Kelley et al., 1990). Similarly, reactions 3 and 4 appear to be physiologically significant. This raises the possibility that b_5 reductase operates in the regeneration of AsH^- from $As^{\bullet -}$. However, our kinetic result using the purified enzymes is apparently contrary to the studies of the cellular localization and the immunochemical method. Schulze et al. (1970) and Ponninghaus and Schulze (1972) have shown that the presence of the NADH-dependent $As^{\bullet -}$ reductase system is independent of cytochrome b_5 and b_5 reductase using an antibody against b_5 reductase. In addition, Ito et al. have demonstrated that NADH–$As^{\bullet -}$ reductase activity in rat liver is mainly attributed to OM-cytochrome b in mitochondria. Under the physiological condition, we must consider additional factors, such as the content of each system and the location of the redox site of each protein in membranes, other than the kinetic results.

A reaction mechanism similar to the reaction scheme given here was proposed in monodehydroascorbate reductase of

cucumber (Hossain & Asada, 1985). Here, we compare the kinetic property of b_5 reductase with that of monodehydroascorbate reductase. The rate of $As^{\bullet -}$ induced oxidation of NADH by b_5 reductase was measured at the steady state in a manner similar to a method previously described (Iyanagi et al., 1985; Hossain & Asada 1985). The rate of NADH oxidation by b_5 reductase (6 mol/enzyme s^{-1}) was about 30 times lower than that of monodehydroascorbate reductase (200 mol/enzyme s^{-1}) (Hossain & Asada, 1985), when the steady-state concentration of $As^{\bullet -}$ was 2 μM. This suggests that the rate constants of reactions 3 and 4 in monodehydroascorbate reductase should be 30 times larger than those of b_5 reductase, if we assume that NADH reduces the oxidized enzyme with similar rates (step i in the reaction scheme). The rate constant of monodehydroascorbate reductase, therefore, may be larger than 10^7 M^{-1} s^{-1}. Furthermore, the rate of NADH oxidation by b_5 reductase did not follow saturation kinetics with respect to the steady-state concentration of $As^{\bullet -}$ in the range of 6 μM, whereas the K_m value of $As^{\bullet -}$ in monodehydroascorbate reductase is 1.4 μM (Hossain & Asada, 1985). It seems likely that monodehydroascorbate reductase has an active site structure that accepts the physiological substrate $As^{\bullet -}$. In contrast, the electron transfer from b_5 reductase to $As^{\bullet -}$ may occur at a flavin edge exposed to the solvent through bimolecular collision. It is, therefore, of importance to know whether the reaction of $As^{\bullet -}$ in flavoproteins is characteristic of b_5 reductase. Further research is planned to investigate the reactions of $As^{\bullet -}$ with other flavoproteins.

In conclusion, the unique property of $As^{\bullet -}$ has now become evident. The $As^{\bullet -}$ serves not only as a one-electron reductant but also as a one-electron oxidant. In biological systems, on the other hand, $As^{\bullet -}$ is reduced to regenerate AsH^- by several systems, such as cytochrome b_5 reductase, but rarely serves as a reductant.

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Registry No. $As^{\bullet -}$, 6730-29-6; Fe^{3+}EDTA, 10058-42-1; $Fe(CN)_6^{3-}$, 13408-62-3; cytochrome b_5, 9035-39-6; cytochrome b_5 reductase, 9032-25-1; cytochrome *c*, 9007-43-6.

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Investigation into the Source of Electron Transfer Asymmetry in Bacterial Reaction Centers†

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ABSTRACT: We have investigated the primary photochemistry of two symmetry-related mutants of *Rhodobacter sphaeroides* in which the histidine residues associated with the central Mg^{2+} ions of the two bacteriochlorophylls of the dimeric primary electron donor (His-L173 and His-M202) have been changed to leucine, affording bacteriochlorophyll (BChl)/bacteriopheophytin (BPh) heterodimers. Reaction centers (RCs) from the two mutants, (L)H173L and (M)H202L, have remarkably similar spectral and kinetic properties, although they are quite different from those of wild-type RCs. In both mutants, as in wild-type RCs, electron transfer to BPh_L and not to BPh_M is observed. These results suggest that asymmetry in the charge distribution of the excited BChl dimer (P*) in wild-type RCs (due to differing contributions of the two opposing intradimer charge-transfer states) contributes only modestly to the directionality of electron transfer. The results also suggest that differential orbital overlap of the two BChls of P with the chromophores on the L and M polypeptides does not contribute substantially to preferential electron transfer to BPh_L.

The three-dimensional structure of reaction centers (RCs) from both *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* reveals two possible electron-transfer pathways emanating from the bacteriochlorophyll (BChl) dimer (P) that serves as the primary electron donor (Deisenhofer et al., 1984; Allen et al., 1986; Chang et al., 1986). Much recent work has focused on determining the molecular factors responsible for the observed unidirectional charge separation via only one of these pathways, namely electron transfer from the excited primary donor (P*) to the bacteriopheophytin associated with the L polypeptide (BPh_L) and then to the primary quinone (Q_A) (Figure 1). Many structural and energetic factors have been considered for this directionality. This work addresses the extent to which charge separation exclusively via the L branch may be due to an asymmetric charge distribution in P*. Such charge asymmetry could be induced by differences in the structure and/or protein environment of the two BChl macrocycles of the dimer, which lead to unequal contributions of two intradimer charge-transfer (CT) configurations ($[BChl_{LP}^{+}BChl_{MP}^{-}]$ and $[BChl_{LP}^{-}BChl_{MP}^{+}]$). Net CT character in P* has received widespread attention from a number of experimental and theoretical studies (Meech et al., 1986; Parson & Warshel, 1987; Lockhart & Boxer, 1987; Lockhart et al., 1988; Warshel et al., 1988; Lösche et al., 1987; Scherer & Fischer, 1989; Boxer et al., 1989; Friesner & Won, 1989; Parson et al., 1990; DiMagno et al., 1990; McDowell et al., 1990; Thompson et al., 1991).

The importance of the intradimer CT configurations in defining the optical and photochemical properties of the excited primary donor has been explored in the (M)H200L mutant of *Rhodobacter capsulatus* in which the His residue coordinated to the central Mg^{2+} of $BChl_{MP}$ has been changed to Leu. This mutant RC contains a $BChl_{LP}BPh_{MP}$ heterodimer (D)[1]

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[1] In the original work on the primary photochemistry in *Rb. capsulatus* (M)H200L RCs, the nomenclature of D (for donor) was adopted to denote the heterodimer (Kirmaier et al., 1988). We will use D here in a generic sense and to simplify notation of states $D^{+}BPh_L^{-}$ and $D^{+}Q_A^{-}$ for *both* the M- and L-side *Rb. sphaeroides* heterodimers. However, for the M- and L-side mutants, D corresponds to $[BChl_{LP}BPh_{MP}]$ and $[BPh_{LP}BChl_{MP}]$, respectively, and D^{+} to the respective radical cations. In both cases the hole in the cation is expected (Bylina & Youvan, 1988; Kirmaier, et al., 1988) to reside largely on the BChl moiety due to its lower oxidation potential than BPh in vitro (Fajer et al., 1975). This has been demonstrated by recent ESR and ENDOR measurements (Bylina et al., 1990; Huber et al., 1990).

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