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A Direct Demonstration of the Catalytic Action of Monodehydroascorbate Reductase by Pulse Radiolysis*

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To elucidate the catalytic mechanism of monodehydroascorbate (MDA) reductase from cucumber, its interaction with MDA radical was investigated by the use of pulse radiolysis. When approximately equimolar MDA radical to the fully reduced MDA reductase was generated, the fully reduced enzyme reacted first with MDA radical to form the red semiquinone, and the semiquinone further reacted with MDA radical to form the oxidized enzyme. At a low ratio (<20) of MDA radical to enzyme concentration, the fully reduced enzyme reacted quantitatively with MDA radical to form the semiquinone with a second-order rate constant of 2.6×10^8 M^{-1} s^{-1} at pH 7.4. At excess MDA radical to enzyme concentration, a similar rate constant was obtained from the decay of MDA radical. These results suggest that the reaction of the semiquinone with MDA radical occurs at the same rate or rate-limiting step of the oxidation of the fully reduced enzyme by MDA radical. The rate constants decreased with an increase in NaCl concentration, suggesting that the localization of cationic groups of amino acid residue near the active site may provide electrostatic guidance to the anionic substrate of MDA radical.

Ascorbate (AsA^-)[1] is critically involved in cellular defense against oxidative injury, serving as a reductant in scavenging reactive species of oxygen and radical species (1–5). In these processes, MDA radical is produced by univalent oxidation of AsA^- (6–9). Regeneration of AsA^- from MDA radical is indispensable to keep the defense activity of AsA^- against oxidative stress. Rapid removal of MDA radical is also necessary to protect cells from the putative damaging effects of MDA radicals.

In mammalian cells, enzymatic activity responsible for the NADH-dependent regeneration of AsA^- from MDA radical has been demonstrated (10–13). In plants, NAD(P)H-dependent reducing activity for MDA radical has been found not only in chloroplasts (14) but also in non-photosynthetic tissues (15, 16) and in algae (17, 18). This activity regenerates AsA^- from MDA radical in chloroplasts and other components for scavenging hydrogen peroxide by ascorbate peroxidase (14, 19). A peculiar enzyme with this activity, named MDA reductase, has been purified from cucumber fruit (20), soybean root nodules (21), and potato tubers (22). The enzyme from cucumber is a soluble monomeric enzyme, with a molecular mass of 47 kDa, that contains 1 molecule of FAD per enzyme molecule. Recently, cDNAs of the enzyme from cucumber seedlings (23) and pea (24) have been cloned and the amino acid sequences deduced. In addition, an overproduced system of MDA reductase of cucumber using cDNA of the enzyme was established in *Escherichia coli*, and the enzyme produced in *E. coli* was purified to a crystalline state and partially characterized (25).

Because of the instability of its substrate, MDA radical, the molecular activity of MDA reductase can be measured only by the rate of oxidation of NADH in the presence of AsA^--AsA^- oxidase. On the other hand, it is possible to measure the catalytic action of MDA reductase directly by using pulse radiolysis to generate MDA radical (26–29). By the use of this technique, we have directly observed the reaction of MDA radical with hepatic NADH-cytochrome b_5 reductase (29). The b_5 reductase has been shown to be a good electron donor for MDA radical in mammalian cells, and its reaction mechanism is supposed to be similar to that of MDA reductase (30). In this report, pulse radiolysis has been used to study the reaction mechanism of MDA reductase. We have confirmed that MDA radical reacts with the fully reduced form of MDA reductase with a diffusion-controlled rate.

MATERIALS AND METHODS

The recombinant MDA reductase was overproduced in *E. coli* transformed with pET-CMR, an expression plasmid possessing the cDNA of cucumber (cytosolic) MDA reductase (23). The enzyme was purified to a homogeneous state, as reported previously (25). All other reagents were commercially obtained as the analytical grade. The concentration of MDA reductase was determined on the basis of FAD bound to the enzyme, using a molar extinction coefficient of 9.63 mM^{-1} cm^{-1} at 450 nm (20). The concentration of MDA radical generated by pulse radiolysis was estimated from the absorbance at 360 nm, using a molar extinction coefficient of 3300 M^{-1} cm^{-1} (28).

Samples of MDA reductase for pulse radiolysis were prepared as follows. Solutions containing 10 mM potassium phosphate buffer (pH 7.4) and 5 mM AsA^- were bubbled with N_2O gas for 5 min. A concentrated solution of MDA reductase (420 μM) was added to the solution. Subsequently, 1 equivalent NADH to the enzyme was added to the solution to give a complex of reduced enzyme with NAD^+ that exhibits distinctive long-wavelength absorbance. A fresh enzyme solution was used for each pulse. Pulse radiolysis experiments were performed with an electron linear accelerator in the Institute of Scientific and Industrial Research (Osaka University) (29, 31, 32). The pulse width and energy were 8 ns and 27 MeV, respectively. Absorption changes were measured using a fast spectrophotometric system composed a Nikon monochromator, an R-928 photomutiplier, and Unisoku data analyzing system.

RESULTS

Hydrated electron (e^-_{aq}), $OH^\cdot$, and $H^\cdot$ are produced by pulse radiolysis of aqueous solutions. In the presence of 5 mM AsA^-,

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[1] The abbreviations used are: AsA^-, ascorbate; MDA, monodehydroascorbate; e^-_{aq}, hydrated electron; E-FAD, oxidized MDA reductase, $E\text{-}FAD^\cdot$, semiquinone enzyme, $E\text{-}FADH^-$, fully reduced enzyme.

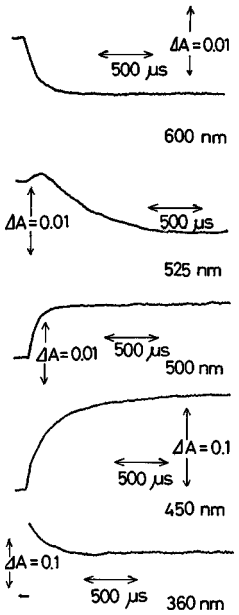

FIG. 1. **Absorption changes after pulse radiolysis of the fully reduced form of MDA reductase measured at 600, 525, 500, 450, and 360 nm in the presence of AsA^- and N_2O at pH 7.4.** The reaction mixture contained 28 μM MDA reductase, 30 μM NADH, 5 mM AsA^-, and 10 mM potassium phosphate buffer at pH 7.4.

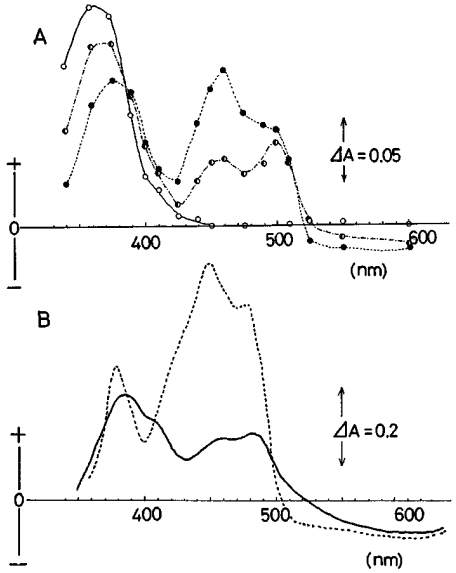

FIG. 2. **Kinetic difference spectra after pulse radiolysis of MDA reductase at pH 7.4 (*A*) and the difference spectra of the semiquinone form of MDA reductase (*straight line*) and the oxidized enzyme (*dashed line*) minus the fully reduced form of the enzyme in complex with NAD^+, respectively (*B*).** *A*, the spectra were taken at 1 μs (○), 100 μs (◐), and 800 μs (●) after pulse radiolysis. Experimental conditions are the same as in Fig. 1. *B*, enzyme concentration was 74 μM in 50 mM phosphate buffer (pH 7.4). The semiquinone was obtained by illumination for 20 min with a 1-kW tungsten lamp. The fully reduced form was obtained by the addition of 80 μM NADH.

MDA radical is produced via the following reactions by pulse radiolysis of N_2O-saturated aqueous solution.

$$e_{aq}^- + N_2O + H_2O \rightarrow OH\cdot + OH^- + N_2$$

REACTION 1

$$OH\cdot + AsA^- \rightarrow H_2O + MDA^{\cdot-}$$

REACTION 2

The participation of $H^\cdot$ in this system can be neglected, since the total yield of e_{aq}^- and $OH^\cdot$ is considerably larger than that of $H^\cdot$ at neutral pH (27). A transient spectrum of MDA radical with an absorption maximum at 360 nm was observed at 100 ns after pulse radiolysis. On pulse radiolysis of the fully NADH-reduced form of MDA reductase in the presence of AsA^-, the absorption changes due to the oxidation of $FADH^-$ of the enzyme were observed as shown in Fig. 1. Under the experimental conditions, 30 μM MDA radical was generated in 28 μM fully reduced form of the enzyme. The absorption at 600 nm, characteristic of a charge transfer band between reduced enzyme and NAD^+, decreased, but that at 500 nm increased. On the other hand, the absorption increase at 450 nm consisted of a fast and a slow phase. At 525 nm, only a slow absorption change was observed. This indicates that at least two distinct reactions are involved in the oxidation of the NADH-reduced MDA reductase by MDA radical.

The kinetic difference spectra at 1, 100, and 800 μs after the pulse are shown in Fig. 2*A*. The spectrum at 1 μs after the pulse with an absorption maximum at 360 nm corresponds to the MDA radical. In this stage, little oxidation of the enzyme-$FADH^-$-NAD^+ complex was observed. The spectrum at 100 μs has twin absorption peaks at 455 and 500 nm, which is similar to that of the difference spectrum of the semiquinone minus that of the E-$FADH^-$-NAD^+ complex in Fig. 2*B*. In this time range, no absorption change was observed at 525 nm, an isosbestic point between the semiquinone and the fully reduced enzyme (Fig. 2). On the other hand, the difference spectrum at 800 μs is not similar to that of the semiquinone minus E-$FADH^-$-NAD^+ but to that of the oxidized form minus E-$FADH^-$-NAD^+. No absorption change was observed at 500 nm, an isosbestic point between the oxidized and the semiquinone forms, on this time scale. Therefore, the slower phase seen in Fig. 1 at 525 and 450 nm is attributable to the oxidation of the semiquinone to the oxidized form of the enzyme. From these results, under the conditions where equimolar MDA radical and NADH-reduced MDA reductase were reacted, MDA radical reacted first with the fully reduced enzyme to form the semiquinone enzyme. Subsequently, the semiquinone thus formed further reacted with MDA radical to form the oxidized form of the enzyme. From the absorbance changes of Fig. 2*A*, 13 μM of the red semiquinone was formed initially in 28 μM of the fully reduced enzyme, and subsequently 10 μM of the semiquinone was further oxidized to the fully oxidized form of the enzyme.

When the similar experiment was performed in the presence of NADH (50–200 μM), further slow absorption change at 460 nm was observed with a half time of 2 ms (data not shown). This absorption change is attributable to the re-reduction of the oxidized enzyme with NADH, since a similar absorption change was not seen in the presence of 1 equivalent of NADH to the enzyme. The rate constant of this process depends on the concentration of NADH.

For determination of the rate constant of the reduction of MDA radical with the fully reduced MDA reductase, 1–2 μM MDA radical is generated in a solution containing 28–50 μM MDA reductase. Typical examples are shown in Fig. 3*A*. Under the present conditions, MDA radical reacted quantitatively with the fully reduced enzyme to form the semiquinone, and no further slow absorption change was observed. The absorption changes at 460 and 600 nm obeyed pseudo-first-order kinetics. Fig. 3*B* shows the dependence of the apparent first-order rate constant on the concentration of MDA reductase, giving a straight line. From the slope of the line, the second-order rate constant of the reaction is estimated to be 2.8×10^8 M^{-1} s^{-1}. The effect of NaCl on the rate constant was also examined. As shown in Fig. 4, the rate constants decreased nearly to 0.5 ×

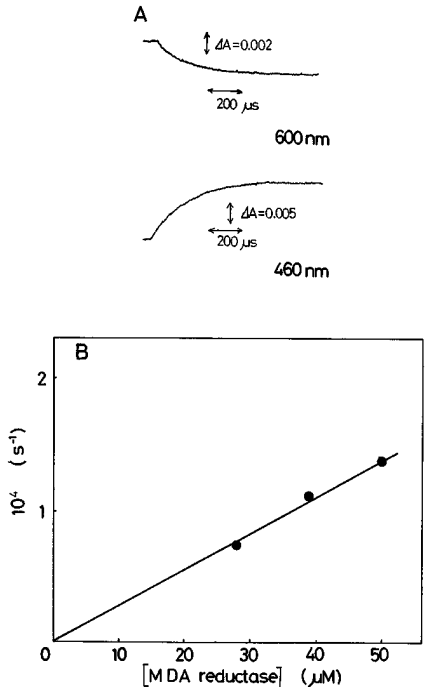

FIG. 3. **Absorption changes after pulse radiolysis of the fully reduced form of MDA reductase measured at 600 and 460 nm in the presence of AsA^- and N_2O (*A*) and concentration dependence of apparent rate constants of the oxidation of the fully reduced MDA reductase from the decrease of absorption at 600 nm and the increase of absorption at 460 nm.** The reaction mixture contained 38 μM MDA reductase, 40 μM NADH, 5 mM AsA^-, and 10 mM phosphate buffer pH 7.4 (*A*).

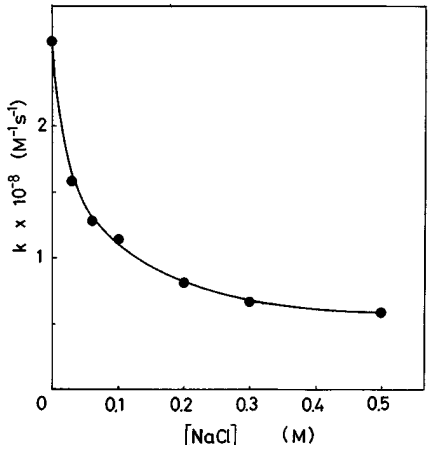

FIG. 4. **NaCl concentration dependence of the rate constant of the fully reduced form of MDA reductase with MDA radical at pH 7.4.** The reaction mixture contained 41.2 μM MDA reductase, 60 μM NADH, 10 mM phosphate buffer (pH 7.4), and 5 mM AsA^- and NaCl at indicated concentrations.

10^8 M^{-1} s^{-1} with an increase in NaCl concentration.

The rate constant for the reaction of MDA radical with the semiquinone form of the enzyme was not determined directly in the present experiment, since the semiquinone state for pulse radiolysis experiments could not be prepared as a stable form.

At excess MDA radical concentration (20–40 μM) to the fully reduced MDA reductase (1–3 μM), the decay of MDA radical was followed at 360 nm. Fig. 5*A* shows the absorption changes at 360 nm in the absence and presence of the E-$FADH^-$-NAD^+ complex. In the presence of 2.2 μM E-$FADH^-$-NAD^+ complex, an initial rapid decrease that obeyed pseudo-first-order kinetics was observed, whereas in the absence of the enzyme a similar initial decay was not seen on this time scale. Under the

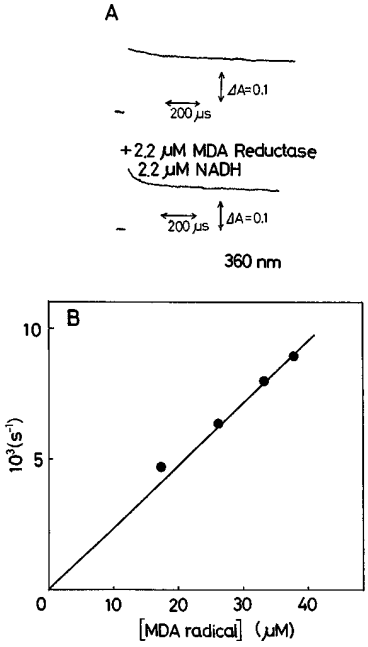

FIG. 5. **Absorption changes of MDA radical measured at 360 nm in the absence and presence of the fully reduced form of MDA reductase (*A*) and AsA^- concentration dependence of the initial decay at 360 nm (*B*).** The reaction mixture contained 2.2 μM MDA reductase, 2.2 μM NADH, 10 mM phosphate buffer (pH 7.4), and 5 mM AsA^-.

conditions, 4 μM MDA radical, which corresponds to approximately two equivalents of the enzyme, decayed within 200 μs. This indicates that the fully reduced MDA reductase is oxidized through two successive one-electron transfers to MDA radical. The decay of MDA radical remaining at the end of reaction with the enzyme occurs due to spontaneous disproportionation in the time range of 100 ms (data not shown). When the same experiments were performed in various doses to generate MDA radical at the indicated concentration, the apparent initial rate of the decay was linear to the concentration of MDA radical (Fig. 5*B*). From the slope of the figures, the second-order rate constant is calculated to be 2.6×10^8 M^{-1} s^{-1}.

DISCUSSION

The present experiments clearly show that MDA reductase is an effective scavenger of MDA radical. The reaction scheme for NADH-dependent MDA radical reduction by cucumber MDA reductase can be described as follows, which has been also supported by a ping-pong mechanism as evidenced by reaction kinetics (20).

$$\text{NADH} + \text{E-FAD} \xrightarrow[k_1]{} \text{E-}FADH^-\text{—}NAD^+$$

REACTION 3

$$MDA^{\cdot} + \text{E-}FADH^-\text{—}NAD^+ \xrightarrow[k_2]{} AsA^- + \text{E-}FAD^{\cdot}\text{—}NAD^+$$

REACTION 4

$$MDA^{\cdot} + \text{E-}FAD^{\cdot}\text{—}NAD^+ \xrightarrow[k_3]{H^+} AsA^- + \text{E-FAD} + NAD^+$$

REACTION 5

The enzyme FAD is reduced by NADH to form a charge-transfer complex, E-$FADH^-$—NAD^+ in Reaction 1 (20, 25). Then, the reduced enzyme donates the electron to MDA radical through two successive one-electron transfers, and the FAD semiquinone, E-FAD—NAD^+, is an intermediate (Reactions 2 and 3). We find that the charge-transfer complex is oxidized by

MDA radical to form the red semiquinone of the enzyme. The anionic red semiquinone form of the enzyme has also been shown by photochemical reduction of the enzyme in the presence of 1 mol equivalent NAD^+ to the enzyme (25). By contrast, the semiquinone cannot be obtained by the photochemical reduction in the absence of NAD^+. Furthermore, preliminary studies using pulse radiolysis showed that a blue semiquinone is transiently observed by the reaction of MDA radical with the fully dithionite-reduced enzyme in the absence of NAD^+.[2] From these results, the semiquinone form of MDA reductase is the blue species, and it is converted into the red species by the binding of NAD^+.

The second-order rate constant (k_2) between MDA radical and the fully reduced MDA reductase in Reaction 2 is 2.6×10^8 M^{-1} s^{-1}. On the other hand, the good agreement of the rate constants determined under both conditions $MDA^{\cdot} \gg$ MDA reductase and MDA reductase $\gg MDA^{\cdot}$ suggests that $k_2 \leq k_3$ and that formation of the semiquinone of the enzyme is principally rate limiting. Based on this estimate, a lower limit of the second-order rate constant of 2.6×10^8 M^{-1} s^{-1} is obtained for the reaction of MDA radical with the semiquinone of the enzyme (k_3). A similar reaction sequence has been proposed for NADH-cytochrome b_5 reductase. However, the rate constants of k_2 and k_3 in MDA reductase are about 50 times larger than those in b_5 reductase ($k_2 = 4.3 \times 10^6$ M^{-1} s^{-1}, $k_3 = 3.7 \times 10^5$ M^{-1} s^{-1}) (29). We have proposed that the electron transfer from b_5 reductase to MDA radical simply occurs at a flavin edge to the solvent through bimolecular collision (30). In contrast, the high rate constants of MDA reductase and a high specificity of MDA radical to the enzyme (25) cannot be explained by a similar mechanism. It seems likely that MDA reductase has an active site structure that accepts MDA radical. This may be reflected in the homology in amino acid sequence between MDA reductase and bovine b_5 reductase (23). However, from the kinetic analysis of both NADH oxidation in the steady state (25) and the reaction of MDA radical with the reduced species of the enzyme in the present study, we have no evidence of the complex formation of the reduced species of MDA reductase with MDA radical. The MDA reductase does not exhibit saturation kinetics in the range of 50 μM MDA radical. One of possible factors for the facilitated reaction is that the localization of cationic groups of amino acid residue near the active site provides electrostatic guidance to the anionic substrate, MDA radical, whose pK_a is -0.45 (28). This is supported by the result that the rate constant of MDA reductase with MDA radical decreases with an increase in NaCl concentration (Fig. 4). On the other hand, a second-order rate constant for the reduction of MDA reductase by NADH (k_1) was determined to be 1.25×10^8 M^{-1} s^{-1} by stopped flow analysis (25). This process has been shown to proceed at the similar rate to those of Reactions 2 and 3 with electrostatic guidance of NADH (25). This suggests that the same residues of MDA reductase seem to facilitate rapid interaction of both the electron donor and the acceptor with the enzyme-FAD. A similar ionic strength effect was obtained in the reaction of O_2^- with copper-zinc superoxide dismutase (33). Although the high rate constant of the enzyme reaction is governed by the specific geometry and redox potential of the redox center, it is clear that modulation of electrostatic effects near the active site affect the rate by as much as 1 order of magnitude.

If the concentration of NADH is more than several hundredfold of that of MDA radical, the rate-limiting step of the reaction catalyzed with MDA reductase is the oxidation of the reduced enzyme with MDA radical rather than the reduction of the enzyme with NADH. To keep the rapid enzymatic rate of regeneration of AsA^- and avoid the injury with the radical, the ratio of the concentration of NADH to MDA radical must be kept more than several hundred in cells where MDA reductase functions. In cells where concentrations of NADH are typically very high as compared with that of MDA radical, the reaction of MDA reductase with MDA radical is rate limiting for the overall process. In illuminated chloroplasts, MDA radical generated by AsA^- peroxidase is reduced in a reaction mediated by photoreduced ferredoxin (34), and $NADP^+$ is also reduced to NADPH in photosystem I, resulting in a high ratio of NADPH to MDA radical. Probably, in the stroma, the ratio of NADPH to MDA radical would be kept high so that the reaction catalyzed with chloroplastic MDA reductase is not limited by the reductive half-reaction, which would have a higher affinity for NADPH than cytosolic isozymes does.

In conclusion, MDA reductase is an effective scavenger of MDA radical. The second-order rate constant for MDA reductase is the greatest value measured for the reaction of MDA radical with biological molecules (29). In contrast to plant cells, such efficient scavenge system of MDA radical has not been yet realized in mammalian cells, though NADPH-dependent regenerating activity of AsA^- from MDA radical has been found. The presence or absence of the system is necessary to clarify.

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[2] K. Kobayashi, S. Tagawa, S. Sano, and K. Asada, unpublished results.

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