Molecular Characterization of Monodehydroascorbate Radical Reductase from Cucumber Highly Expressed in Escherichia coli*

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Monodehydroascorbate radical (MDA) reductase, an FAD-enzyme, is the first enzyme to be identified whose substrate is an organic radical and catalyzes the reduction of MDA to ascorbate by NAD(P)H. Its cDNA has been cloned from cucumber seedlings (Sano, S., and Asada, K. (1994) Plant Cell Physiol. 35, 425-437), and a plasmid was constructed in the present study that allowed a high level expression in Escherichia coli of the cDNA-encoding MDA reductase using the T7 RNA polymerase expression system. The recombinant MDA reductase was purified to a crystalline state, with a yield of over 20 mg/liter of culture, and it exhibited spectroscopic properties of the FAD similar to those of the enzyme purified from cucumber fruits during redox reactions with NADH and MDA. The red semiquinone of the FAD of MDA reductase was generated by photoreduction. p-Chloromercuribenzoate inhibited the reduction of the enzyme-FAD by NADH, and dicumarol suppressed electron transfer from the reduced enzyme to MDA. The specificity of electron acceptors of the recombinant enzyme appeared to be similar to that of MDA reductase, even though the amino acid sequence encoded by the cDNA was somewhat different from that of the enzyme purified from cucumber fruits. The Km values for NADH and NADPH of the recombinant enzyme indicated a high affinity of the enzyme for NADH. The reaction catalyzed by the enzyme did not exhibit saturation kinetics with MDA up to 3 μM. A second order rate constant for the reduction of the enzyme-FAD with NADH was 1.25 × 10^5 M^(-1) s^(-1), as determined by a stopped-flow method, and its value decreased with increases in ionic strength, an indication of the enhanced electrostatic guidance of NADH to the enzyme-FAD.

Since the first isolation of ascorbate (AsA) from Hungarian red pepper (Svirbery and Szent-Györgyi, 1933), it has been established that AsA functions as an antioxidant and protects cells from oxidative stress. The level of AsA in plant cells, as postulated from its initial isolation from plant tissues, is high as compared to that in mammalian cells, and an AsA-specific peroxidase scavenges hydrogen peroxide. In mammalian cells, by contrast, a selenium-containing glutathione peroxidase plays a major role (Asada, 1992). When AsA acts as an antioxidant in cells, in most cases, monodehydroascorbate radicals (MDA) are produced as the primary oxidation product. AsA peroxidase in plants generates MDA when it scavenges hydrogen peroxide (Hossain et al., 1984), as do guaiacol peroxidases such as horseradish peroxidase (Yamazaki and Piette, 1961). Superoxide and hydroxyl radicals oxidize AsA to MDA. Other organic oxidizing radicals, such as tocopherol chromanoxo, carbon-centered, aminoxy, peroxy, and phenoxy radicals, are generated under oxidative stress and generate MDA via their interactions with AsA (Bielski, 1982). The glutathione thiol radical is produced by the interaction of GSH with various radicals (Winterbourn, 1993), and it generates MDA as a result of its reaction with AsA (Forst et al., 1983). Thus, the MDA radical functions as a “sink” for radicals and active species of oxygen that are generated in cells under oxidative stress. In addition, MDA is generated via the autoxidation of AsA (Scarpa et al., 1983) and via the spontaneous oxidation of AsA by electron carriers such as cytochrome c, cytochrome b₅₆₁, and cytochrome b₅₆₅. Furthermore, MDA is produced during the enzymatic reactions catalyzed by AsA oxidase (Yamazaki and Piette, 1961), thyroid peroxidase (Nakamura and Ohtaki, 1993), and dopamine β-monooxygenase (Dharirwal et al., 1991). De-epoxidation of violaxanthine to zeaxanthine in chloroplasts seems to be associated with a photoprotective function, and MDA is very probably generated in this reaction since AsA is required for the de-epoxidation reaction (Yamamoto et al., 1972).

To maintain the antioxidant activity of AsA, the regeneration of AsA from MDA is obviously indispensable. For example, in leaf cells, AsA is found at or above 10 mM in chloroplasts. However, from rates of the photoproduction of superoxide and hydrogen peroxide, it can be calculated that the AsA in chloroplasts is consumed within 80 s if no system in regeneration of AsA is operative in illuminated chloroplasts (Asada, 1994). Furthermore, several enzymes are inactivated by MDA (Davison et al., 1986, Harwood et al., 1986), and the toxicity of AsA to cells (Stich et al., 1976) might be attributable to MDA. Thus, it is essential to maintain a low steady state concentration of the MDA radical in cells.

The NADPH-dependent activity for regeneration of AsA from MDA has been found in mammalian tissue, but the enzyme that catalyzes the regeneration has not been yet purified. Cytochrome b₅₆₁ and cytochrome b₅₆₅ reductase can reduce MDA (Iyanagi and Yamazaki, 1969; Nishino and Ito, 1986; Njus and Kelly, 1993), but the reactivity of MDA with reduced cytochrome b₅₆₁ reductase is several orders of magnitude lower than that of plant MDA reductase (Kobayashi et al., 1991). The activity for the reduction of MDA, with NAD(P)H as the electron donor, has been found in plants, and it is not only in

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The abbreviations used are: AsA, ascorbate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); IPTG, isopropyl-1-thio-β-D-galactoside; MDA, monodehydroascorbate radical; pCMβ, p-chloromercuribenzoate; MES, 4-morpholinoethanesulfonic acid; bis-tris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)-propane-1,3-diol.
chloroplasts (Hossain et al., 1984) but also in nonphotosynthetic tissues (Arrigoni et al., 1981; Bowditch and Donaldson, 1990) and in algae (Shigeoka et al., 1987; Miyake et al., 1991). It has also been demonstrated that the photoreduced ferredoxin in photosystem I of chloroplast thylakoids can reduce it. It has also been demonstrated that the photoreduced ferredoxin in photosystem I of chloroplast thylakoids can reduce MDA at a high rate (approximately $10^{7} \text{m}^{-3} \text{s}^{-1}$), which would contribute to the regeneration of AsA in the thylakoidal system for scavenging of hydrogen peroxide (Miyake and Asada, 1994).

MDA reductase is the first known enzyme that uses an organic radical as the substrate, and it catalyzes the following reaction: $2\text{MDA} + \text{NAD(P)}{\text{H}} + \text{H}^{+} \rightarrow 2\text{AsA} + \text{NAD(P)}^{+}$.

The enzyme has been purified from cucumber fruits (Hossain and Asada, 1985), soybean root nodules (Dalton et al., 1992), and potato tubers (Borraccino et al., 1984). MDA reductase has also been purified from spinach (Sano and Asada, 1994). Thus, the unusual nature of MDA reductase and the absence of similar flavoenzymes in mammalian and fungal systems suggest that it might have a unique function in the thylakoidal system.

**Properties of Monodehydroascorbate Reductase**

**MATERIALS AND METHODS**

**Construction of the Expression Plasmid—**Plasmid pCMR31KS$^{+}$ was derived from pBluescript II KS$^{+}$ by insertion of the cDNA for cucumber MDA reductase, as described previously (Sano and Asada, 1994). The cDNA insert has two Ncol sites, one of which includes the first Met codon of the open reading frame. The plasmid was digested with Ncol and blunted-ended with T4 DNA polymerase and dNTPs. The resultant linear DNA (14 micrograms) was partially digested with 10 units of Ncol at 37°C for 15 min and was separated with agarose-gel electrophoresis to purify the 1.3-kilobase fragment of the cDNA digested only at Ncol site including the authentic initiation codon.

pET-8c (Studier et al., 1990), in which a unique Ncol site contains the first codon of the $\phi 10$ gene adjacent to a T7 promoter, was used as the plasmid vector. pET-8c was digested with BamHI and blunt-ended by treatment with T4 DNA polymerase and dNTPs. It was digested with Ncol and dephosphorylated with calf intestinal alkaline phosphatase.

Two DNA fragments described above had one Ncol cohesive end and one blunt end each and ligated with T4 DNA ligase. After transformation of competent E. coli DH5$\alpha$ cells with the ligated DNA, plasmid DNA from ampicillin-resistant colonies was prepared and examined by restriction digestion to confirm that the construction was correct. The expression plasmid is referred to as pET-CMR (Fig. 1).

**Preparation of the Bacterial Extract for Analysis—**For analysis of whole cells, pelleted cells obtained by centrifugation from 1 ml of culture were suspended in 100 ml of the loading buffer for SDS-polyacrylamide gel electrophoresis (50 mm Tris-HCl, pH 6.8, 0.1 M diithiothreitol, 2% (w/v) SDS, 0.1% (w/v) bromphenol blue, and 10% (v/v) glycerol) and boiled for 3 min. For analysis of the soluble fraction, cells were suspended in one-tenth volume of extraction buffer (0.2 M HEPES-NaOH, pH 6.8, 10 mM 2-mercaptoethanol, and 0.5 mM EDTA) and sonicated twice by a Branson sonifier with a microtip at 3 Å for 5 s with 1-min intervals.
Properties of Monodehydroascorbate Reductase

Expression of Recombinant MDA Reductase in E. coli and Its Purification—The proteins from E. coli BL21(DE3) cells, cultured in LB medium, were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. A distinct band of protein with the molecular mass predicted from the open reading frame of the cDNA (47 kDa) was detected by Coomassie Brilliant Blue staining (data not shown) in the lanes that correspond to the cells that harbored pET-CMR and confirmed to be MDA reductase by Western blotting in the extracts from cultures incubated both in the presence and the absence of IPTG (Fig. 2). Thus, T7 RNA polymerase was expressed in a leaky manner without induction by IPTG, and it transcribed the cDNA for MDA reductase on the plasmid. Fragmentation of MDA reductase probably by proteases may have occurred in E. coli both in the presence and absence of IPTG when the cells were cultured for more than 2 h (Fig. 2).

A soluble fraction from the cells transformed with pET-CMR, prepared after incubation for various times in LB medium in the presence and absence of IPTG, catalyzed the NADH-dependent reduction of MDA, but that from cells without the plasmid did not. Although the amount of MDA reductase expressed in E. coli cells in response to IPTG was larger than that in noninduced cells, as determined by Western analysis, higher activity of MDA reductase was found in the soluble fraction when cells were incubated in LB medium for 12 h without the induction by IPTG than after induction by IPTG (data not shown). A larger fraction of the MDA reductase protein, expressed in the induced cells, might have formed insoluble inclusion bodies in the latter case. We observed the maximal activity of MDA reductase under the culture conditions described under "Materials and Methods."

A soluble extract of E. coli cells that harbored pET-CMR and had been cultured in LB medium at 37°C for 12 h without induction has a specific activity of about 40 units mg⁻¹ of protein⁻¹, which was about 80-fold higher than that in extracts of cucumber fruits. MDA reductase accounted for nearly 20% of the soluble protein in the E. coli cells. The enzyme was purified to homogeneity by a simple procedure with a yield of about 45% from 2 liters of culture, as summarized in Table I. The analysis by SDS-polyacrylamide gel electrophoresis of the purified enzyme gave a molecular mass of 47 kDa, as expected from the open reading frame of the cDNA (Sano and Asada, 1994), and the specific activity of the purified enzyme was similar to that of MDA reductase purified from cucumber fruits.
Flavoenzymes mediate, but static titration of the reduced enzyme did not allow us to show a spectrum of the semiquinone. Flavoproteins reduced by two electrons are oxidized by electron acceptors via a semiquinone form (Strittmatter, 1965). Most flavoenzymes form either a blue or a red semiquinone independently of the external pH with the exception of glucose oxidase (Massey and Palmer, 1966). The blue semiquinone reflects a hydrogen bond between an amino acid residue and N-5 of the flavin, and the red semiquinone reflects one between an amino acid residue and N(1)-C(2)-O of the flavin (Massey and Hemmerich, 1980).

The electron acceptor of MDA reductase is the MDA radical, which was continuously generated by the AsA-AsA oxidase system (Fig. 5). The reduced enzyme was completely oxidized when 2.18 mol of MDA radicals were generated per mol of MDA reductase. During the oxidation process, the absorption spectrum of the semiquinone form could not be detected, although a blue-shifted peak of the oxidized flavin around 370 nm was observed at an early stage of the oxidation.

Spectrum of the Flavosemiquinone Form—Flavoenzymes reduced by two electrons are oxidized by electron acceptors via a semiquinone form (Strittmatter, 1965). Most flavoenzymes form either a blue or a red semiquinone independently of the external pH with the exception of glucose oxidase (Massey and Palmer, 1966). The blue semiquinone reflects a hydrogen bond between an amino acid residue and N-5 of the flavin, and the red semiquinone reflects one between an amino acid residue and N(1)-C(2)-O of the flavin (Massey and Hemmerich, 1980).

The electron acceptor of MDA reductase is the MDA radical, and the NADH-reduced enzyme should be oxidized via two successive oxidations by two MDA radicals. Therefore, the semiquinone form of the enzyme should be formed as an intermediate, but static titration of the reduced enzyme did not allow us to show a spectrum of the semiquinone. Flavoproteins can be reduced by illumination under anaerobic conditions in the presence of EDTA as the electron donor (Massey and Palmer, 1966). The semiquinone form is stabilized by binding of NAD$^+$ as a catalytic intermediate, as shown for ferredoxin-NADP reductase (Keirns and Wang, 1972), adrenodoxin reductase (Kitagawa et al., 1982), and cytochrome b$_5$ reductase (Iyanagi, 1977). The spectrum of the NADH-reduced MDA reductase indicates the formation of a stable charge-transfer complex (Fig. 4), as in the case of cytochrome b$_5$ reductase (Iyanagi, 1977). Therefore, photooxidation of MDA reductase was performed in the presence of NAD$^+$ at a molar ratio of 1:1 with the enzyme under anaerobic conditions using EDTA as the electron donor. During illumination, a new spectrum with a peak at 370 nm and a flat absorption in the long-wavelength region was generated (Fig. 6). This spectrum is characteristic of NAD$^+$-bound, red semiquinone forms in flavoenzymes of the dehydrogenase-oxidase group (Massey and Hemmerich, 1980). Thus, the NAD$^+$ bound semiquinone, which can infer the spec-
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Thiol Groups—MDA reductase is inhibited by thiol-modifying reagents (Hossain and Asada, 1985; Borraccino et al., 1986, Dalton et al., 1992, Murthy and Zilinskas, 1994) and it contains two Cys residues, at positions 69 and 198, of the enzyme from cucumber and also from pea (Sano and Asada, 1994, Murthy and Zilinskas, 1994). The addition of DTNB to the recombinant MDA reductase caused rapid and slow increases in absorbance at 410 nm, which corresponded to 0.63 and 1.4 mol of thiol groups per mol of enzyme 10 min and 5 h after the addition of DTNB, respectively. Thus, one thiol group in the native enzyme reacted rapidly with DTNB, but the remaining one reacted only slowly. By contrast, when the enzyme was incubated with DTNB in 0.5% (w/v) SDS for 10 min, an increase in absorbance at 410 nm occurred that corresponded to 1.8 mol of thiol groups per mol of enzyme, as expected from the predicted sequence.

Thus, the two Cys residues predicted from the cDNA (Cys-69 and -198) do not form a disulfide bridge.

Preincubation of recombinant MDA reductase with 1.5 mol eq of DTNB for 10 min inhibited the reduction of FAD by NADH, as determined by the decrease in absorbance at 450 nm (Fig. 7), as is the case for the enzyme purified from cucumber fruits (Hossain and Asada, 1985). Thus, one Cys residue seems to participate in the reduction of the enzyme-FAD by NADH. Neither of the two Cys residues in MDA reductase is conserved in other flavin-containing oxidoreductases (Sano and Asada, 1994), and it is not known which Cys residue reacts rapidly with thiol reagents and participates in the transfer of electrons from NADH to the FAD. Cys-198 of the enzyme from cucumber is located near the putative NADH binding domain (Sano and Asada, 1994) and could participate in electron transfer between NADH and the enzyme-FAD.

Kₘ Values for NADH and NADPH—The Kₘ values for the electron donors, NADH and NADPH, of the recombinant MDA reductase at 3 μM MDA were determined to be 4.4 μM and 210 μM, respectively, from plots of [S]₀ versus [S]₀ (Cornish-Bowden, 1979) (Table II). The Kₘ for NADPH of the recombinant enzyme is similar to that of the enzyme from cucumber fruits, but the Kₘ for NADPH is 9-fold higher than that of the cucumber enzyme (Hossain and Asada, 1985). The Kₘ for MDA radicals could not be estimated because the enzyme did not exhibit saturation kinetics below 3 μM MDA, which is the maximum concentration generated by AsA oxidase as a consequence of an increase in the rate of disproportionation of the radical. The molecular activities of the recombinant MDA reductase (Vₘₐₓ) were estimated to be 175 mol of NADH oxidized mol of enzyme⁻¹ s⁻¹ and 33 mol of NADPH mol of enzyme⁻¹ s⁻¹ when the concentration of MDA was 3 μM. Thus, the Vₙₐₓ of the recombinant enzyme also is lower when NADPH is the electron donor than when NADH is the donor.

The Kₘ values for NADH and NADPH of MDA reductases from various plants are summarized in Table II. These data allow us to divide MDA reductases into two groups. The enzyme from soybean root nodules and the recombinant enzyme from cucumber are characterized by a low specificity for NADPH. Other MDA reductases, purified from cucumber fruits, spinach, and potato tubers, gave only severalfold higher...
values of $K_m$ for NADPH than those for NADH. Although the amino acid sequence of cytosolic MDA reductase predicted from pea cDNA had a high degree of homology (78%) to that of cucumber, the fusion protein of MDA reductase of pea with a maltose-binding protein did not show high specificity for NADH (Murthy and Zilinskas, 1994). The domain of the maltose-binding protein might affect the interaction of electron donors with the fused pea enzyme.

Specificity for Electron Acceptors—The MDA reductase that we overexpressed in E. coli is a cytosolic isozyme of cucumber, and its deduced amino acid sequence differed by 16% from that of the purified enzyme from cucumber fruits (Sano and Asada, 1994). However, the recombinant enzyme had specificity for its electron acceptors similar to that of the purified enzyme (Hossain and Asada, 1985). In addition to the MDA radical, the recombinant MDA reductase was capable of catalyzing the reduction of ferricyanide and 2,6-dichlorophenolindophenol. However, cytochrome c, dehydroascorbate, methylene blue, GSSG, and ferredoxin were ineffective as the electron acceptors. Among quinones, p-benzoquinone could serve as an acceptor, but menadione and 2-naphthoquinone could not (Table III).

This specificity is similar not only to that of MDA reductase from cucumber fruits but also to that of the enzyme from soybean root nodules (Dalton et al., 1992). The high specificity for the MDA radical distinguishes the recombinant enzyme from the FAD-containing enzymes menadione reductase (Spitzberg and Cascia, 1982), DT diaphorase, glutathione reductase, and ferredoxin-NADP reductase. The extent of sequence homology between MDA reductase and the aforementioned flavoproteins is very low (Sano and Asada, 1994). Therefore, the high specificity of MDA reductase for the MDA radical is not unexpected. It is of interest to note that MDA reductase cannot reduce ferredoxin, and the extent of sequence homology between MDA reductase and ferredoxin-NADP reductase is low. The MDA reductase from cucumber has rather high sequence homology to several non-heme iron reductases from bacteria (Sano and Asada, 1994). The specificity for electron acceptors of the recombinant enzyme provides further evidence that the cDNA isolated by immunoscreening with antisera against MDA reductase from cucumber fruits encodes an isozyme of MDA reductase.

Inhibition by Dicumarol—Dicumarol very strongly inhibits menadione reductase, with a $K_i$ of the order of 10 nM. The inhibition is competitive with respect to NAD(P) but independent of the concentration of the electron acceptor (Ernst et al., 1962). The MDA reductase purified from cucumber fruits was not inhibited by dicumarol up to 0.5 mM (Hossain and Asada, 1985). The recombinant enzyme was, however, inhibited not only when the MDA radical was the electron acceptor but also when ferricyanide, 2,6-dichlorophenolindophenol, and p-benzoquinone were used. The MDA-reducing activity of MDA reductase was suppressed by 90% by 0.5 mM dicumarol (Table IV). When equimolar NADH was added to the oxidized enzyme in the presence of 0.5 mM dicumarol, the enzyme was fully reduced, as judged from the absorption spectrum (data not shown). Thus, dicumarol did not inhibit the reduction of the FAD of MDA reductase by NADH, unlike its action on menadione reductase. Dixon plots (Dixon, 1972) indicated that the inhibition by dicumarol of recombinant MDA reductase was competitive to the MDA radical, and the $K_i$ value is estimated to be 74.3 nM (Fig. 8). Since the electron acceptor, not the electron donor, competed with dicumarol and the $K_i$ values are different from each other, the mechanism of inhibition of the recombinant MDA reductase by dicumarol is different from that of menadione reductase.

Rate Constant for the Reduction of the Enzyme-FAD by NADH—The reaction kinetics of MDA reductase show that the reaction proceeds via a ping-pong mechanism, as follows (Hossain and Asada, 1985):

\[
E \text{-FAD} + \text{NADH} + H^+ \rightarrow E \text{-FADH}_2\text{-NAD}^+ \quad \text{(Reaction 1)}
\]

\[
E \text{-FADH}_2\text{-NAD}^+ + \text{MDA} \rightarrow E \text{-FADH}_2\text{-NAD}^+ \text{-AsA} + H^+ \quad \text{(Reaction 2)}
\]

\[
E \text{-FADH}_2\text{-NAD}^+ + \text{MDA} + H^+ \rightarrow E \text{-FADH}_2\text{-NAD}^+ \text{-AsA} + H^+ \quad \text{(Reaction 3)}
\]

The enzyme-bound FAD (E-FAD) is reduced by NADH, and a charge-transfer complex (E-FADH$_2$NAD$^+$) is formed (Fig. 4). The reduced enzyme donates electrons to MDA by two successive one-electron transfers, and a red semiquinone form (E-FAD-NAD$^+$) is thought to be the intermediate (Fig. 6). The second order rate constant for the reduction of the enzyme-bound FAD by NADH ($k_2$) was determined by a stopped-flow analysis, which was monitored at 452.9 nm after rapid mixing. When we tried to determine the rate under the pseudo-first order conditions (10 $\mu$M enzyme and 100 $\mu$M NADH), almost all of the enzyme-FAD was reduced within the dead time after the mixing of 450 $\mu$s of the instrument. This result corresponds to a rate above $10^8$ M$^{-1}$ s$^{-1}$, and so we could not determine the rate accurately. The reduction of the enzyme-FAD by NADH in a second order mode (10 $\mu$M enzyme and 10 $\mu$M NADH) allowed us to determine the rate (Fig. 9A). The reciprocal plot of the oxidized E-FAD against time after mixing (Fig. 9B) gives a straight line, and $k_2$ is estimated from its slope to be $1.25 \times 10^8$.

### Table II

| MDA reductase                              | $K_m$ (NADH) | $K_m$ (NADPH) |
|-------------------------------------------|--------------|--------------|
| Recombinant enzyme of cucumber (present work) | 4.4          | 210          |
| Soybean root nodule (Dalton et al., 1992)     | 5.6          | 150          |
| Cucumber fruit (Hossain and Asada, 1985)     | 4.6          | 23           |
| Spinach leaf (Hossain et al., 1984)          | 7            | 22           |
| Potato tuber (Borraccino et al., 1986)       | 7.7          | 30           |
| Fusion enzyme of pea with maltose-binding protein (Murthy and Zilinskas, 1994) | 5.3          | 22           |

### Table III

| Electron acceptor | Recombinant enzyme | Cucumber fruits$^a$ |
|-------------------|--------------------|---------------------|
| 3 $\mu$M MDA radical | 100                | 100                 |
| 3 $\mu$M Monodehydroascorbate | 46                 | 50                  |
| 50 $\mu$M Ferri cyanide | 289               | 166                 |
| 50 $\mu$M 2,6-Dichlorophenolindophenol | 14              | 10                  |
| 100 $\mu$M p-Benzoquinone | 4.2               | 12                  |
| 100 $\mu$M Menadione | 0                  | 0                   |
| 100 $\mu$M ox-Naphthoquinone | 0               | ND$^b$            |
| 100 $\mu$M Methylene blue | 0               | 0                   |
| 100 $\mu$M Cytochrome c | 0.5               | 1                   |
| 5 $\mu$M Dehydroascorbate | 0                | 0                   |
| 5 $\mu$M GSSG | 0                  | 0                   |
| 100 $\mu$M Ferredoxin | 0                | 0                   |

$^a$ Hossain and Asada (1985).

$^b$ Not determined.

### Table IV

| Electron acceptor | % of activity |
|-------------------|--------------|
| 3 $\mu$M MDA radical | 9.2          |
| 100 $\mu$M p-Benzoquinone | 63          |
| 50 $\mu$M 2,6-Dichlorophenolindophenol | 12        |
| 50 $\mu$M Ferri cyanide | 16          |
The rate constant was not affected by buffers, when either 50 mM HEPES-KOH or 50 mM potassium phosphate at pH 7.8 was used. The effect of pH on the rate of reduction of E-FAD by NADH was determined (Fig. 10). Between pH 5.5 and 7, the rate was highest and constant, and the rate decreased gradually with increases in pH. It should be noted that the pKₐ of cysteine is around pH 8.5 and either Cys-69 or Cys-198 participates in the reduction of E-FAD by NADH, as discussed above. It appears, therefore, that the dissociation of either Cys residue lowers the interaction of the E-FAD with NADH. The pH optimum of the overall reaction is in a range from pH 7 to pH 9 (Hossain and Asada, 1985, Dalton et al., 1992), suggesting that the rates of reactions 2 and 3 are high above pH 7.

The maximal rate for bimolecular collisions is $8.4 \times 10^9$ M⁻¹ s⁻¹ in water at 30°C, and it is independent of their molecular sizes (Marshall, 1978). Assuming that MDA reductase is a sphere and the density of the enzyme is equal to that of hemoglobin, we can calculate that the ratio of the area of the isoalloxazine ring of FAD to the total surface of the enzyme is only 0.03%. Therefore, the maximal collision rate to NADH with E-FAD is $2.5 \times 10^9$ M⁻¹ s⁻¹. Thus, the observed value of $k_1$ is 44-fold higher than the estimated collision rate, and the interaction of NADH with E-FAD appears to be facilitated by a mechanism such as the electrostatic guidance of the electron donor to the FAD. At neutral pH, NADH is present mostly in an anionic form since its pKₐ is 3.9, and ionic interactions are assumed to participate in the guidance of NADH to E-FAD. To examine such ionic interactions, $k_1$ was determined at various concentrations of NaCl (Fig. 11). With increases in ionic strength, the rate of reduction of E-FAD by NADH fell to $8.5 \times 10^9$ M⁻¹ s⁻¹, with an inflection point at 0.74 (0.5 M NaCl), when the rate was plotted against ionic strength. At present, it is not known why the plot of $k_1$ against ionic strength shows an inflection at 0.5 M NaCl, but the conformation change of the enzyme by the salt is likely to affect the reduction rate by NADH. The rate constant was also lowered in phosphate buffer at its high concentrations (data not shown). The 1 M NaCl-suppressed rate was similar to the estimated rate of bimolecular collisions. The present results support the proposed enhanced electrostatic guidance of NADH to the isoalloxazine ring of the enzyme-FAD by positively charged amino acid residues. We have not identified the participating residues of the enzyme. However, three Lys residues (159, 161, and 165) and one Arg residue (183) are found around the putative NADH binding domain of NADH (Lys-162 to Leu-182 and Met-192 to Asp-195 with a loop between them) of MDA reductase (Sano and Asada, 1994).

**Fig. 8.** Dixon plot of the initial velocity of the reaction catalyzed by recombinant MDA reductase at various concentrations of dicumarol with 2 μM and 3 μM MDA. Assays were carried out under the standard conditions with the addition of dicumarol at indicated concentrations.

**Fig. 9.** Reduction of the FAD of MDA reductase by NADH after rapid mixing. MDA reductase (MDAR) and NADH at equimolar concentrations (10 μM) in 50 mM HEPES-NaOH, pH 7.8, were mixed rapidly in a stopped-flow apparatus, and the reduction of FAD was followed as the increase in transmittance at 452.9 nm. For improvement of the S/N ratio, the figure represents the average of four determinations (A). The reciprocal of the concentration of the oxidized enzyme is plotted against the time after the mixing (B).

**Fig. 10.** Effects of pH on the reaction rate constants ($k_1$) for the reduction of the FAD of MDA reductase by NADH. The assay conditions were the same as in Fig. 9, except that the following buffers were used: pH 5.5–6.0, 50 mM MES-KOH; pH 7–8, 50 mM HEPES-KOH; pH 9, bis-tris propane-KOH.

Isozymes of MDA Reductase from Cucumber—During the purification of MDA reductase from cucumber fruits, four isoforms were found in DEAE-Sephacel chromatography (Hossain
and Asada, 1985). Dalton et al. (1992) also reported the existence of two isozymes of MDA reductase in soybean root nodules. Intact chloroplasts contain MDA reductase (Hossain et al., 1984), in addition to dehydroascorbate reductase (Hossain and Asada, 1984), for the regeneration of AsA. Thus, MDA reductase is localized not only in chloroplasts but also in other cell compartments, and different isozymes might be localized in each compartment. The sequence of amino acid residues predicted from the cDNA used for high expression in E. coli does not have a transit peptide for targeting to cell organelles (Sano and Asada, 1994), and it does not agree completely to that of MDA reductase purified from cucumber fruits. Reflecting the differences between them, the two isozymes of MDA reductase are distinguishable by the specificity of their electron donors, differences between them, the two isozymes of MDA reductase purified from cucumber fruits. Reflecting the differences between them, the two isozymes of MDA reductase purified from cucumber fruits. Reflecting the differences between them, the two isozymes of MDA reductase purified from cucumber fruits. Reflecting the differences between them, the two isozymes of MDA reductase purified from cucumber fruits. Reflecting the differences between them, the two isozymes of MDA reductase purified from cucumber fruits.

Concluding Remarks—The present system of high expression for cytosolic isozyme of MDA reductase from cucumber in E. coli allowed us to purify and to crystallize the enzyme with simple purification steps. The recombinant enzyme prefers NADH as the electron donor to NADPH. The reduction of the enzyme-FAD by NADH (reaction 1) proceeds at a diffusion-controlled rate of \(1.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1}\) (Fig. 9), and either Cys-69 or Cys-198 participates (Fig. 7). This rapid reaction could not be accounted for by simple bimolecular collisions, and it is facilitated by enhanced electrostatic guidance of NADH by cationic amino acid residues. The steps in the oxidation of the reduced enzyme by MDA (reactions 2 and 3) also has been shown to proceed at rate similar to reaction 1 via the red semiquinone form (Fig. 6), with electrostatic guidance of the MDA anion radical to the reduced flavin. Thus, as in the case of Cu,Zn-superoxide dismutase, in which electrostatic guidance of the superoxide anion radical to the sulfur atom of the enzyme by the conserved cationic amino acid residues facilitates the diffusion-controlled disproportionation of superoxide (Getzoff et al., 1992), the cationic amino acid residues of MDA reductase seem to facilitate rapid interactions between both the electron donor and the electron acceptor with the enzyme-FAD. Actually, the overall cycle of the MDA reductase-catalyzed reaction is suppressed at higher ionic strength (Hossain and Asada, 1985).

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