Characterization of 1-Deoxy-D-xylulose 5-Phosphate Reductoisomerase, an Enzyme Involved in Isopentenyl Diphosphate Biosynthesis, and Identification of Its Catalytic Amino Acid Residues*

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1-Deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase, which simultaneously catalyzes the intramolecular rearrangement and reduction of DXP to form 2-C-methyl-D-erythritol 4-phosphate, constitutes a key enzyme of an alternative mevalonate-independent pathway for isopentenyl diphosphate biosynthesis. The dxr gene encoding this enzyme from Escherichia coli was overexpressed as a histidine-tagged protein and characterized in detail. DNA sequencing analysis of the dxr genes from 10 E. coli dxr-deficient mutants revealed base substitution mutations at four points: two nonsense mutations and two amino acid substitutions (Gly14 to Asp14 and Glu231 to Lys231). Diethyl pyrocarbonate treatment inactivated the DXP reductoisomerase, and subsequent hydroxylamine treatment restored the activity of the diethyl pyrocarbonate-treated enzyme. To characterize these defects, we overexpressed the mutant enzymes G14D, E231K, H153Q, H209Q, and H257Q. All of these mutant enzymes except for G14D were obtained as soluble proteins. Although the purified enzyme E231K had wild-type $K_m$ values for DXP and NADPH, the mutant enzyme had less than a 0.24% wild-type activity. $K_m$ values of H153Q, H209Q, and H257Q for DXP increased to 3.5-, 7.6-, and 19-fold, respectively. These results indicate that Glu231 of E. coli DXP reductoisomerase plays an important role(s) in the conversion of DXP to 2-C-methyl-D-erythritol 4-phosphate, and that His153, His209, and His257, in part, associate with DXP binding in the enzyme molecule.

Isoprenoids play important roles in all living organisms; they act as steroid hormones in mammals, carotenoids in plants, and ubiquinone or menaquinone in bacteria (1). Since the initial discovery of the mevalonate pathway, it was widely accepted that isopentenyl diphosphate, the fundamental unit in terpenoid biosynthesis, was only formed through the ubiquitous mevalonate pathway. However, it has been disclosed that many organisms, including several bacteria, green algae, and the chloroplasts of higher plants, use an alternative mevalonate-independent pathway (nonmevalonate pathway) for the formation of isopentenyl diphosphate (2). Because the nonmevalonate pathway is absent from animals, the enzymes involved in the pathway are considered to be good targets for the screening of antimicrobials.

The initial step of this pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by the condensation of pyruvate and glyceraldehyde 3-phosphate catalyzed by thiamine diphosphate-dependent DXP synthase (Fig. 1). The ddx gene homologs encoding the enzyme have been cloned from Escherichia coli (3, 4), peppermint (Mentha X piperita) (5), pepper (6), and Streptomyces sp. strain CL190 (7). In the second step, the intramolecular rearrangement of DXP was assumed to give a hypothetical rearrangement product, 2-C-methylerythrose 4-phosphate, which was then converted to 2-C-methyl-D-erythritol 4-phosphate (MEP) by an unspecified reduction process (8, 9). We have succeeded in the first cloning and overexpression of the gene (dxr, formerly yaeM) encoding the DXP reductoisomerase from E. coli and have shown that the recombinant enzyme catalyzed the formation of MEP from DXP in a single step in the presence of both NADPH and a divalent cation, such as Mn$^{2+}$, Mg$^{2+}$, or Co$^{2+}$ (10, 11). Additionally, we have demonstrated that the enzyme activity is strongly and specifically inhibited by fosmidomycin (FR-31564) (12), an antibiotic possessing the formyl and phosphate functions in the molecule (13–16) (Fig. 1). Recently, it has been reported that fosmidomycin and its derivative inhibited DXP reductoisomerase from Plasmodium falciparum and that these inhibitors cured mice infected with the rodent malaria parasite Plasmodium vinckei. Thus, DXP reductoisomerase has been shown to represent an effective target for the chemotherapy of malaria (17).

For a more effective drug design, it is important to identify the amino acid residues essential for catalyzing this reaction. We initially assumed the possibility that MEP might be synthesized from DXP by the same reaction mechanism as that by the ketol-acid reductoisomerase (EC 1.1.1.86), which is involved in the biosynthesis of amino acids with a branched chain, such as valine, isoleucine, and leucine (18). No significant similarity, however, was found between the total amino acid sequences of both reductoisomerases except for the NADPH binding motif (11). Because DXP reductoisomerase is a novel target for the systematic search of antimicrobials (12),

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* The abbreviations used are: DXP, 1-deoxy-D-xylulose 5-phosphate; DX, 1-deoxyxylulose; MEP, 2-C-methyl-D-erythritol 4-phosphate; PAGE, polyacrylamide gel electrophoresis.

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herbicides (19, 20), and antimalaria drugs (17), it is very significant to identify the catalytically important residues in the DXP reductoisomerase. In this study, we describe the characterization of the DXP reductoisomerase from E. coli and the first identification of its catalytic residues by analyzing the mutant DXP reductoisomerases, which were constructed from E. coli dxr-deficient mutants induced by N-nitro-N-nitrosoguanidine and by site-directed mutagenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials from commercial sources included NADPH (Sigma), nickel nitriolactate acid agarose resin (Qiagen), and high and low molecular weight electrophoresis calibration kits (Amersham Pharmacia Biotech). All other reagents were of the highest analytical grade available.

**Standard Assay of DXP Reductoisomerase**—The expression and purification of the recombinant DXP reductoisomerase were done as already described (10, 11). The standard assay system consisted of 100 mM Tris-HCl (pH 7.5), 1 mM MnCl2, 0.3 mM NADPH, and 0.3 mM enzymatically synthesized DXP (10) in a final volume of 200 μl. The reaction was initiated by adding the enzyme solution to the complete assay mixture. The oxidation of NADPH was monitored at 340 nm with a Pharmacia LKB model Uvicord S spectrophotometer. To determine the Km of the enzyme, various concentrations of DXP were used with the total DNA from the 10 mutants to amplify each mutant screening and the protocol of the supplier.

**Electrophoresis**—Proteins were separated by SDS-PAGE on 8–25% gels or native PAGE on 8–25% gels with the PhastSystem (Amersham Pharmacia Biotech).

**Site-directed Mutagenesis**—All in vitro mutations were generated using a Takara polymerase chain reaction in vitro mutagenesis kit (Takara Shuzo). The sequences of the mutagenic primers are as follows: H153Q, 5'-GGGGGATCCGCTTGCGAGA-3'; H209Q, 5'-TTCGGTTGAG-CGGCAGGCTTG-3'; and H257Q, 5'-TATTCAGTCAATGGTGCGC-3' (Amersham Pharmacia Biotech). The underlined letters represent the substituted nucleotides. Each mutant gene was sequenced to verify the presence of the desired mutation and the absence of the polymerase chain reaction-generated mutations. Construction of the plasmid for overexpression of these mutant dxr genes was done as already described.

**Results**

**Optimum pH and Temperature and Heat Stability of the DXP Reductoisomerase**—E. coli DXP reductoisomerase appeared to have a broad pH optimum, with more than 75% of its maximum activity observed between pH 7.0 and 8.5 in 100 mM Tris-HCl buffer. The effect of temperature on the enzyme activity was investigated over a range of 16–80 °C. The maximum activity was observed at 40–60 °C, and 70% of the maximum activity was obtained even at 80 °C (Fig. 2). The activation energy was estimated to be 75 kJ/mol based on an Arrhenius plot (Fig. 2, inset). The purified enzyme was not heat stable above 50 °C. For example, heating the enzyme at 55 or 60 °C for 10 min led to 70 or 100% losses in the activity, respectively. On the other hand, the enzyme retained more than 90% of the activity after storage at 4 °C in 100 mM Tris-HCl buffer (pH 7.5) for 1 month.

**1-Deoxyxylulose Is Not a Substrate for DXP Reductoisomerase**—When 1-deoxyxylulose (DX) in place of DXP was added to the reaction mixture over a concentration range of 0.05–2 mM, the oxidation of NADPH was not observed. Furthermore, the reaction of DXP reductoisomerase was not inhibited by the addition of 0.05–2 mM DX (data not shown).

**Kinetic Parameters for the Wild-type DXP Reductoisomerase**—Kinetic parameters for DXP reductoisomerase were determined in the presence of each divalent cation (Mn2+, Mg2+, or Co2+), as summarized in Table I. In each case, the addition of 1 mM divalent cation showed the highest enzyme activity. The DXP reductoisomerase yielded the lowest Km value for the substrate DXP when assayed in the reaction mixture containing Co2+. On the other hand, the lowest Km value for NADPH was calculated in the presence of Mn2+. Because the highest...
The cat value was also obtained in the assay mixture containing Mn2⁺, an additional standard assay for the mutant enzymes was carried out in the reaction mixture with 1 mM MnCl2.

**Determination of Mutational Points of E. coli DXP Reductoisomerase-deficient Mutants**—We had isolated the E. coli dxr-deficient mutants prepared by N-methyl-N⁹-nitro-N-nitrosoguanidine treatment. (10, 11). The deficiency could be a result of the amino acid substitutions caused by base substitution mutations introduced by this treatment. Because kinetic characterization of such mutant enzymes provides important information on the amino acid residues for catalysis, we decided to determine the mutational points of the dxr-deficient mutants. A DNA sequencing analysis of the dxr genes from the 10 dxr-deficient mutants revealed base substitution mutations at four points: two nonsense mutations (C472 to T472 and C757 to T757) and two amino acid substitutions (G41 to A41 and G691 to A691, corresponding to Gly14 to Asp14 and Glu231 to Lys231, respectively).

**Chemical Modification by Diethyl Pyrocarbonate**—E. coli DXP reductoisomerase has eight histidine residues per subunit. Of these histidine residues, His153, His209, and His257 are conserved among the DXP reductoisomerases from six genera of bacteria, two plants (23, 24), and a malaria parasite P. falciparum (17) (Fig. 3). A reaction of the wild-type enzyme with diethyl pyrocarbonate was accompanied by a time-dependent loss in activity. The subsequent addition of hydroxylamine restored approximately 50% of the initial activity (data not shown). This result suggested involvement of the histidine residue(s) in the catalytic site.

**Site-directed Mutagenesis of His153, His209, and His257 and Their Overexpression in E. coli**—To examine which histidine residues of His153, His209, and His257 in the DXP reductoisomerase are essential for the activity, we used site-directed mutagenesis in the E. coli enzyme. The codon for each histidine was altered to encode glutamine, a residue that has an amide nitrogen that can be isosteric with the imidazole nitrogen of histidine but that can serve neither as an acid nor as a base. The mutant enzymes, H153Q, H209Q, and H257Q, were then overexpressed as histidine-tagged proteins. They were ob-

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**TABLE 1**

| Enzyme | Divalent cation (1 mM) | Kₘ (µM) | kcat (10⁴ × min⁻¹) | kcat/Kₘ for DXP (µM⁻¹ × min⁻¹) |
|--------|------------------------|---------|---------------------|-----------------------------|
| Wild-type | MnCl₂  | 250 | 7.4 | 5900 | 24 |
|          | MgCl₂  | 99  | 18  | 1300 | 13 |
|          | CoCl₂  | 60  | 8.8 | 3100 | 51 |
| Mutant   | E231K  | 270 | 18  | 14  | 0.053 |
|          | H153Q  | 850 | 7.0 | 590  | 0.67 |
|          | H257Q  | 1900 | 8.6 | 8.6  | 0.0046 |

kcat value was also obtained in the assay mixture containing Mn²⁺, an additional standard assay for the mutant enzymes was carried out in the reaction mixture with 1 mM MnCl₂.
wild-type enzyme, indicating the importance of His153, His209, and His257. These three mutant enzymes were much lower than those of the wild-type enzyme. In this study, the kinetic parameters of the enzyme in the presence of each divalent cation were determined. The highest kinetic parameter, the two dxr-deficient mutant enzymes, G14D and E231K, were overexpressed in E. coli as the histidine-tagged proteins. Although it was highly expressed, G14D was obtained as an insoluble product. E231K was overexpressed as a soluble enzyme and purified to homogeneity, as judged by SDS-PAGE (Fig. 4).

Comparison of Kinetic Parameters between the Wild-type and dxr-deficient Mutant Enzymes—As summarized in Table I, the kinetic parameters for the mutant DXP reductoisomerase were determined in the presence of MnCl2. To compare the kinetic parameters, the two dxr-deficient mutant enzymes, G14D and E231K, were overexpressed in E. coli as the histidine-tagged proteins. Although it was highly expressed, G14D was obtained as an insoluble product. E231K was overexpressed as a soluble enzyme and purified to homogeneity, as judged by SDS-PAGE (Fig. 4). Although the mutant enzyme E231K had normal $K_m$ values for DXP and NADPH of 270 and 18 M, respectively, the enzyme yielded a catalytic rate constant ($k_{cat}$) of $1.4 \times 10^3$ min$^{-1}$, representing a decrease of this value to 450-fold that of the wild-type.

Comparison of Kinetic Parameters between the Wild-type and His Mutant Enzymes—H153Q and H209Q yielded wild-type $K_m$ values for NADPH (Table I). However, these mutant enzymes gave 3.5- and 7.6-fold the wild-type $K_m$ value for DXP, respectively. H257Q yielded 20-fold the wild-type $K_m$ values for DXP and NADPH. $k_{cat}$ and the specificity constants ($k_{cat}/K_m$) of these three mutant enzymes were much lower than those of the wild-type enzyme, indicating the importance of His$^{153}$, His$^{209}$, and His$^{257}$.

DISCUSSION

The enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase simultaneously catalyzes the intramolecular rearrangement and reduction of 1-deoxy-D-xylulose 5-phosphate to yield 2-C-methyl-D-erythritol 4-phosphate (Fig. 1). Although DXP reductoisomerase has so far been cloned from E. coli, plants (23, 24), and a malaria parasite (17), its kinetic characterization has not fully been carried out. We describe here the biochemical characterization of the novel enzyme that constitutes the nonmevalonate pathway for isopentenyl dipiphosphate biosynthesis.

We have previously demonstrated that DXP reductoisomerase uses only NADPH as a cofactor (10, 11). In addition, we have shown that the enzyme also requires a divalent cation such as Mn$^{2+}$, Mg$^{2+}$, or Co$^{2+}$ (10, 11). Recently, Proteau et al. (25) and Radykewicz et al. (26) independently reported that the E. coli enzyme belonged to a class B dehydrogenase. In this study, the kinetic parameters of the enzyme in the presence of each divalent cation were determined. The highest $k_{cat}$ value was obtained when Mn$^{2+}$ was added to the reaction mixtures. The highest specificity constant ($k_{cat}/K_m$) was obtained when Co$^{2+}$ was added. In terms of the specificity constant, it is speculated that Co$^{2+}$ is an in vivo relevant cation. In addition, the enzyme activity in the presence of 0.1 mM of each cation was reduced to approximately 30% of the maximum activity obtained in the presence of a 1 mM concentration of each cation (data not shown), suggesting that the enzyme activity is significantly affected by the in vivo concentration of each cation. Furthermore, we investigated the possibility of producing 2-C-methylerythritol from DX using DXP reductoisomerase. When DX was used as the substrate in place of DXP, the oxidation of NADPH was not observed under the described conditions. This indicates that the DXP reductoisomerase cannot use DX as a substrate.

We isolated several E. coli dxr-deficient mutants, which absolutely required for 2-C-methylerythritol, a free alcohol of MEP, for growth and survival (11). Thus, it is evident that the analyses of these mutants provide important information on critical amino acid residues in catalysis and/or structural integrity. DNA sequencing analysis revealed the point mutations of the amino acids, Gly$^{14}$ to Asp$^{14}$ and from Glu$^{231}$ to Lys$^{231}$ in the E. coli dxr-deficient mutants. These amino acid residues, Gly and Glu, were found in all the DXP reductoisomerase sequences from nine organisms (Fig. 3). In order to elucidate the enzymatic properties, we overexpressed the mutant enzymes G14D and E231K. The expression in E. coli of the mutant gene E231K yielded a high level of soluble protein, which was subsequently purified to homogeneity and characterized. E231K had less than a 0.24% wild-type $k_{cat}$ value, although the mutant enzyme had wild-type $K_m$ values for DXP and NADPH. These results strongly suggest that Glu$^{231}$ of the E. coli DXP reductoisomerase plays an important role(s) in the conversion of DXP to the reaction product, MEP, not only in vitro but also in vivo. Because the dxr-deficient mutant with Lys$^{231}$ requires 2-C-methylerythritol, a free alcohol of MEP, for growth, it is evident that the E231K enzyme does not in fact produce enough MEP for the growth. On the other hand, the expression in E. coli of the mutant gene G14D yielded an insoluble protein. Although glycine residue 14 is speculated to be a part of the NADPH binding motif, the residue may also contribute to maintaining the secondary or tertiary structure of the enzyme.

An amino acid sequence comparison of the DXP reductoisomerase from E. coli with those from bacteria and plants revealed three conserved histidine residues, His$^{153}$, His$^{209}$, and His$^{357}$ (Fig. 3). Diethyl pyrocarbonate treatment inactivated the wild-type enzyme, and subsequent hydroxylamine treatment restored the activity of the diethyl pyrocarbonate-treated enzyme. Thus, we employed site-directed mutagenesis of the E. coli DXP reductoisomerase to identify which histidine residues function during catalysis. The expression in E. coli of the mutant genes H153Q, H209Q, and H257Q yielded soluble proteins, which were subsequently purified to homogeneity and characterized in detail. H209Q and H257Q showed drastic decreases to 5200- and 27,000-fold the wild-type $k_{cat}/K_m$ value, respectively. Furthermore, the $k_{cat}/K_m$ values of H209Q and H257Q are much lower than that of E231K, which is obtained from a dxr-deficient mutant. Thus, it is suggested that H209Q and H257Q have no enzymatic function in vivo. Moreover, H153Q showed a decrease to 36-fold the wild-type $k_{cat}/K_m$ value, suggesting that His$^{153}$ also functions during catalysis.

We consider it unlikely that the low $k_{cat}$ values of the mutant enzymes, E231K, H153Q, and H209Q, are a consequence of the altered structural integrity, because these mutant enzymes exhibited $K_m$ values for NADPH not significantly different from that of the wild-type enzyme. Although H257Q was 20-fold the wild-type $K_m$ value, the mutant enzyme showed the same mobility in the native PAGE as did the wild-type enzyme.
(data not shown), suggesting that the mutant did not alter the quaternary structure.

Because the DXP reductoisomerase is a new target for antimicrobials (12), herbicides (18, 19), and antimalaria drugs (17), characterization of the enzymatic properties and identification of the catalytic amino acid residues are important. Although the crystal structure analysis of the DXP reductoisomerase is indispensable to determining the functional residues in detail, our study provides important information for the crystal structure analysis and the opportunity for modeling enzyme-drug interaction.

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